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Sans Ton secours et Ta présence, je n'y serai pas parvenue,
Tels les fils de Koré, c'est à Toi que je dédie cette œuvre

LISTE DES TRAVAUX

Publications issues du master

WABO PONE J., **FOSSI TANKOUA O.**, YONDO J., KOMTANGI M. C., MBIDA MPOAME & BILONG BILONG C. 2011. The *in vitro* effects of aqueous and ethanolic extracts of the leaves of *Ageratum conyzoides* (Asteraceae) on three life cycle stages of the parasitic nematode *Heligmosomoides bakeri* (Nematoda, Heligmosomatidae). *Veterinary Medecine International*. 14022011: 93.

WABO PONÉ J., YONDO J., **FOSSI TANKOUA O.**, KOMTANGI M. C., BILONG BILONG C. F AND MPOAME MBIDA. The *in vitro* effects of *Chenopodium ambrosioides* (Chenopodiaceae) extracts on the parasitic nematode *Heligmosomoides bakeri* (Nematoda, Heligmosomatidae).*Journal of Pharmacognosy and Phytotherapy* 3 (4): 56-62.

VANSTEELANDT M., KERZAON I., BLANCHET E., **FOSSI TANKOUA O.,** ROBIOU DU PONT T., JOUBERT Y., MONTEAU F., LE BIZEC B., FRISVAD J.C., POUCHUS Y. F., GROVEL O. Investigation of mycotoxin and secondary metabolite production by marine-derived Penicillium strains isolated from shellfish and their environment. *Fungal biology*. 2012 116 (9) : 954-61

Publications de la thèse

FOSSI TANKOUA O., AMIARD J.-C., AMIARD-TRIQUET C., BERTHET B., MOUNEYRAC C. & RAINBOW P.S., 2010. Towards a comprehensive methodology for assessing the health status of coastal and estuarine ecosystems (platform presentation). *In*: Intern Council for the Exploration of the Sea, Annual Science Conference, 20-24 september 2010, Nantes, France. e-paper: http://www.ices.dk/products/CMdocs/CM-2010/F/F1510.pdf

BUFFET P. E., **FOSSI TANKOUA O**., PAN J. F., BERHANU D., HERRENKNECHT C., POIRIER L., AMIARD-TRIQUET C., AMIARD J. C., BÉRARD J.B., RISSO C., GUIBBOLINI M., ROMÉO M., REIP P., VALSAMI-JONES E., MOUNEYRAC C., 2011. Behavioural and biochemical responses of two marine invertebrates *Scrobicularia plana* and *Hediste diversicolor* to copper oxide nanoparticles. *Chemosphere,* **84**, 166-174.

FOSSI TANKOUA O., BUFFET P.E., AMIARD J.C., AMIARD-TRIQUET C., MOUNEYRAC C., BERTHET B. 2011. Potential influence of confounding factors (size, salinity) on biomarker tools in the sentinel species *Scrobicularia plana* used in monitoring programmes of estuarine quality. *Environ. Sci. Pollut. Res*. **18**, 1253-1263.

FOSSI TANKOUA O., BUFFET P.E., AMIARD J.C., AMIARD-TRIQUET C., GILLET, P., MOUNEYRAC C., BERTHET B. 2011 Intersite variations of a battery of biomarkers at different levels of biological organisation in the estuarine endobenthic worm *Nereis diversicolor* (Polychaeta, Nereididae). *Aquatic Toxicology*, 2012 114-115 : 96-103.

FOSSI TANKOUA O., BUFFET P.E., AMIARD J.C., BERTHET B., MELEDER, V., MOUNEYRAC C., AMIARD-TRIQUET C. 2011 Comprehensive assessment of estuarine sediment quality by using a battery of biomarkers in the bivalve *Scrobicularia plana* and the characterization of natural and anthropogenic factors. **Soumise à *Aquatic Toxicology***.

FOSSI TANKOUA O., AMIARD-TRIQUET C., DENIS, F., MOUNEYRAC C., BERTHET B. 2011 Physiological status and intersex in the endobenthic bivalve *Scrobicularia plana* from thirteen estuaries in Northwest France. *Environmental Pollution***.** 2012 167 : 70-7

FOSSI TANKOUA O., BUDZINSKI H., AMIARD-TRIQUET C., BERTHET B., AÏT-AÏSSA S. 2011 Presence of endocrine disruptor compounds along a contamination gradient in twelve estuaries in Northwest of France: Is it a relation with occurrence of intersex in the endobenthic clam *Scrobicularia plana*? **En préparation**

Communications orales

AMIARD-TRIQUET C., BUFFET P.E., **FOSSI TANKOUA O**., KALMAN J., AMIARD J.C., DEDOURGE-GEFFARD O., MÉTAIS I. & MOUNEYRAC C., 2009. Biomarkers in the polychaete *Nereis diversicolor* as a sentinel species in estuaries. *In* : 14th International Symposoium on Toxicity Assessment. Metz, 30 August-4 September 2009.

FOSSI TANKOUA O., BUFFET P.E., AMIARD-TRIQUET C., BERTHET B. & MOUNEYRAC C., 2009. Perturbations du comportement et de la nutrition : des outils pour une évaluation précoce de la dégradation de la qualité de l'environnement estuarien et côtier. *In* : 25ème Forum des Jeunes Océanographes, La Rochelle, 28-29 octobre 2009.

BUFFET P.E., **FOSSI TANKOUA O.,** POIRIER L., AMIARD-TRIQUET C., HERRENKNECHT C. & MOUNEYRAC C., 2009. Evaluation du risque environnemental des nanoparticules de CuO sur deux organismes marins *Scrobicularia plana* et *Nereis diversicolor***.** *In* : 25ème Forum des Jeunes Océanographes, La Rochelle, 28-29 octobre 2009.

FOSSI TANKOUA O., BUFFET P.E., AMIARD J.C., BERTHET B., DEDOURGE-GEFFARD O., METAIS I., MOUNEYRAC C. & AMIARD-TRIQUET C., 2009. Cascading effects of chemical stress at different levels of biological organisation in endobenthic invertebrates. *In* : 20th Annual Meeting SETAC, Sevilla (Spain), 21-27 May 2010.

BUFFET P.E., **FOSSI TANKOUA O**., BERHANU D., HERRENKNECHT C., POIRIER L., AMIARD-TRIQUET C., AMIARD, J.-C. BÉRARD J.-B., RISSO C, GUIBBOLINI M., ROMÉO M., REIP P., VALSAMI-JONES E. & MOUNEYRAC C., 2010.. Behavioural and biochemical responses of two marine organisms Scrobicularia plana

and Nereis diversicolor to copper oxide nanoparticles. *In* : 20th Annual Meeting SETAC, Sevilla (Spain), 21-27 May 2010.

FOSSI TANKOUA O., AMIARD J.-C., AMIARD-TRIQUET C., BERTHET B., MOUNEYRAC C. & RAINBOW P.S., 2010. Towards a comprehensive methodology for assessing the health status of coastal and estuarine ecosystems (platform presentation). *In*: Intern Council for the Exploration of the Sea, Annual Science Conference, 20-24 september 2010, Nantes, France.

FOSSI TANKOUA O., PERISI F., AMIARD-TRIQUET C., BERTHET B., MAILLOT-MARECHAL E., AÏT-AÏSSA S., LEMENACH K. & BUDZINSKI H., 2011. Incidence of intersex along a contamination gradient in 13 estuaries of NW France: relation with endocrine disruptor compounds? *In* : 3rd CSTS and 5th SETAC Africa Joint Toxicology Conference. Buea, Cameroun, 31 May-3 June 2011.

FOSSI TANKOUA O., AMIARD J.C., BERTHET B., MOUNEYRAC C. & AMIARD-TRIQUET C., 2011. The use of ecologically relevant and cost effective biomarkers to assess the health status of estuarine and coastal waters. *In* : 3rd CSTS and 5th SETAC Africa Joint Toxicology Conference. Buea, Cameroun, 31 May-3 June 2011.

Communications par affiche

FOSSI TANKOUA O., PERISI F., AMIARD-TRIQUET C., BERTHET B., AÏT-AÏSSA S., LEMENACH K. & BUDZINSKI H., 2011. Incidence of intersex and reproductive status in the clam *Scrobicularia plana* from 13 sites in NW France (poster). *In* : 21st Annual Meeting SETAC, Milan (Italy), 15-19 May 2011.

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AChE : Acétylcholinestérase
ADN : Acide désoxyribonucléique
ADP : Adénosine diphosphate
AFNOR : Association française de la normalisation
Ag : Argent
ANOVA : *Analysis of variance*
AR: récepteur aux androgènes
ASE : *Accelerated Solvent Extraction*
BELQUAM: *Biological Effects Quality Assurance in Monitoring Programmes*
Cd: Cadmium
CEMP: *Coordinated Environmental Monitoring Programme*
CI : Ionisation Chimique
CIEM : Conseil international pour l'exploration de la mer
CPG : Chromatographie en Phase Gazeuse
Cu : Cuivre
CV : Coefficient de variation
DCE : Directive cadre sur l'eau (UE)
DDE : Dichloro-diphényl-dichloroéthylène
DDT : Dichloro-diphényl-trichloroéthane
DMSO : Dimethyl sulfoxide
E2 : 17β-œstradiol
EDM : Eau de mer
EI : Impact Electronique
EPOC : Environnements et Paléoenvironnements Océaniques et Continentaux
ER : récepteur aux oestrogènes
ERE : Element de réponse aux œtrogènes
EROD : Ethoxyrésorufine-o-dééthylase
GC/MS : Chromatographie en phase gazeuse associée au spectromètre de masse
GST : Glutathion-S-transférase
HAP : Hydrocarbures aromatiques polycycliques
INERIS : Institut National de l'Environnement Industriel et des Risques
IC : Indice de condition
IGS : Indice gonadosomatique
IHS: Indice hépatosomatique
IFREMER: Institut Français pour la Recherché et l'Exploitation de la Mer
IMS : indice de maturité sexuelle
ISO: *International Standards Organization*
IUPAC: *International Union of Pure and Appled Chemistry*
JAMP: *Joint Assessment Monitoring Programme*
L3: longueur des trios premiers segments
LPTC : Laboratoire de Toxico et Physico-Chimie de l'environnement
MDA : Malondialdéhyde
MT : Métallothioneine
Ni : Nickel
PBDE: Diphényl Ether PolyBromé
PCB: Polychlorobiphényle
PE: Perturbateur endocrinien

PENC: *Predicted no effect concentrations*

RNO: Réseau national d'observation du milieu marin

SPE : Extraction en phase solide

TBARS : Thiobarbituric acid reactive

TBT : Tributylétain

UE : union Européenne

UMA : Unité de Masse Atomique

V : Vanadium

V/V : Volume/Volume

Zn : Zinc

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CHAPITRE 1. INTRODUCTION GENERALE

1 CHAPITRE 1. INTRODUCTION GENERALE

Les écosystèmes marins et continentaux fournissent le quart de la production primaire mondiale, ainsi que 90 à 95% des poissons marins pêchés. Ils sont en plus à l'origine de 50% de la dénitrification, 80% de la production de carbonates, 90% de la minéralisation globale des sédiments (Tolba et al., 1992). De ces deux types d'écosystèmes, l'environnement côtier est le plus productif, il fait d'ailleurs partie des habitats naturels les plus productifs au monde (McLusky et Elliott, 2004). En effet, les estuaires et littoraux océaniques fournissent 50% de la production de pêche mondiale alors qu'ils ne représentent que 8% du milieu marin (Kennish, 1999). Les revenus générés par ces pêcheries sont estimés à au moins 34 milliards de dollars annuels (Dayton et al., 2005). La production primaire nette des estuaires serait de 1,5 $kg.m^{-2}.an^{-1}$ pour une biomasse moyenne de $1kg.m^{-2}$ (Dayton et al., 2005). Les estuaires participent au renouvellement des stocks de plusieurs espèces de poissons en servant de nourricerie pour leurs juvéniles, ils servent d'habitats et constituent un axe de passage pour certains poissons et oiseaux migrateurs. La présence de biomasses importantes de phytoplancton, d'espèces végétales, de mollusques et de crustacés en font des réservoirs de nourriture pour de nombreux oiseaux et poissons, ainsi que pour l'Homme. Ils jouent un rôle majeur dans l'épuration, le stockage, la transformation et la régulation des polluants chimiques et autres effluents apportés de l'amont, régulent les précipitations et l'humidité locale par le biais de l'évapotranspiration. Ce sont des espaces de choix pour le développement de l'aquaculture et la pêche, et d'autres activités humaines telles que les activités portuaires, l'agriculture, les sports de loisir, le tourisme, etc.

En 1994 la population mondiale vivant à moins de 100 km d'une côte était estimée à 37% (Cohen et al., 1997). En 1998 ce pourcentage s'élevait à 61% (Alongi, 1998) vivant à moins de 200 km des côtes, et il devrait atteindre 75% en 2025. Au sein de cette population vivant près des côtes, 71% seraient situés à moins de 50 km d'un estuaire, d'une mangrove ou d'un récif corallien (Dayton et al., 2005). Cet accroissement démographique, couplé à des activités anthropiques de plus en plus importantes (industrialisation, agriculture, aquaculture et pêche intensives, activités portuaires, construction de barrages etc.) exercent une forte pression sur l'environnement côtier, et les estuaires en particulier. En effet tous les polluants contenus dans les rejets issus entre autres d'activités domestiques, de stations d'épuration, de l'industrie et

l'agriculture finissent à plus ou moins long terme dans le milieu aquatique, et surtout les estuaires où ils s'accumulent dans le sédiment. Ces polluants présentent un risque sérieux pour la santé humaine, l'intégrité des chaînes trophiques et ont un coût économique assez conséquent. Parmi de nombreux exemples nous pouvons citer :

- L'intoxication de la baie de Minamata au Japon par le mercure produit par une industrie qui a fait au moins 1200 morts

- L'eutrophisation de nombreux cours d'eaux due aux rejets de composés organiques qui sont à l'origine de sérieux problèmes d'anoxie, influant sur la présence et la survie de certaines espèces

- La contamination de l'estuaire de la Gironde par du cadmium issu d'une mine de zinc abandonnée très en amont de l'estuaire. L'accumulation de ce métal (jusqu'à 30 $mg.kg^{-1}$) dans les huîtres a conduit à l'interdiction de toute activité conchylicole en Gironde et à des dépassements occasionnels de la valeur maximale admissible pour la consommation humaine (1 $mg.kg^{-1}$) dans les huîtres de Marennes Oléron.

- Les déversements accidentels de produits pétroliers (naufrage de l'ERIKA au large des côtes Françaises en 1999, explosion en 2010 dans le golfe du Mexique de la plateforme Deep Water Horizon exploitée par BP qui a libéré 779 000 m^3 de pétrole le long des côtes Américaines, etc.) ainsi que leur combustion libèrent des produits cancérogènes, les hydrocarbures aromatiques polycycliques dont la proportion dans les pétroles bruts varie de 15 à 30%.

- Un autre exemple est celui du tributylétain (TBT), un biocide utilisé jusqu'à son interdiction dans la fabrication des peintures antisalissure des bateaux par la convention internationale sur les systèmes antisalissure (convention AFS) adoptée le 5 Octobre 2001 par l'Organisation maritime internationale (OMI). Il a provoqué entre autres une hausse de la mortalité des larves d'huîtres dans le bassin d'Arcachon et des cas d'imposex et de stérilisation de certaines espèces de gastéropodes marins dont l'une des plus sensibles *Nucella lapillus* (RNO, 2009).

La prise de conscience de la fragilité de l'environnement marin et la nécessité de le préserver ont conduit à la limitation de l'usage de certains composés chimiques (Ex : TBT) mais aussi à l'élaboration et l'adoption de plusieurs mesures nationales et internationales telles que :

- La création d'un réseau national d'observation de la qualité du milieu marin (RNO, coordonné par l'IFREMER pour le compte du Ministère chargé de l'Environnement) en 1974 par l'état Français avec pour objectif l'évaluation des niveaux et des tendances des polluants et des paramètres généraux de la qualité du milieu marin. Durant les quatre premières années, les études faites ont essentiellement concerné les eaux marines, puis elles se sont étendues au vivant et au sédiment, ce qui a débouché en 1987 à l'établissement d'un nouvel objectif, celui de la surveillance des effets biologiques. Cette surveillance visait à évaluer la qualité du milieu marin par la mesure des perturbations biologiques de la flore ou de la faune. Les paramètres généraux de qualité des masses d'eaux portaient sur la température, la salinité, les sels nutritifs (nitrates, nitrites, ammonium, phosphates), la chlorophylle et les phéopigments et dans certains cas, l'oxygène dissous et les silicates.

- La signature de la convention des Nations Unies pour le droit de la mer en 1982 à Montego Bay. Cette convention délimite l'espace maritime appartenant à chaque état côtier, définit des principes généraux quant à l'exploitation et la préservation des ressources maritimes et crée un tribunal international du droit de la mer, charge ensuite à chaque état de prendre des mesures nécessaires à la protection de son espace maritime.

- La signature de la convention pour la protection du milieu marin de l'Atlantique du Nord-Est dite Convention OSPAR entrée en vigueur le 25 mars 1998 (http://www.ospar.org/). Elle remplace les Conventions d'Oslo sur les immersions et celle de Paris sur les pollutions d'origine tellurique et pétrolière et traite de l'aspect environnemental des activités pétrolières et gazières offshore dans l'Atlantique du Nord-Est. Elle définit en outre des modalités de coopération internationale pour la protection du milieu, définit les notions de « zone maritime », « eaux intérieures » et « pollution ». Elle engage toutes les parties (quinze gouvernements des côtes et îles occidentales d'Europe) qui l'ont ratifiée à mettre en œuvre tous les moyens

nécessaires afin de prévenir et supprimer la pollution, protéger la zone maritime contre les effets néfastes des activités anthropiques, et rétablir, dans la mesure du possible, les zones marines qui ont subi des effets préjudiciables, ceci dans le but ultime de sauvegarder la santé de l'homme et de préserver les écosystèmes marins. Cela a nécessité l'adoption commune ou individuelle de programmes et mesures, ainsi qu'une harmonisation des politiques et stratégies. Elle introduit aussi deux principes fondamentaux : i) le principe de précaution, selon lequel des mesures de prévention doivent être prises lorsqu'il existe des motifs raisonnables de s'inquiéter du fait que des substances ou de l'énergie introduites, directement ou indirectement, dans le milieu marin, puissent entraîner des risques pour la santé de l'homme, nuire aux ressources biologiques et aux écosystèmes marins, porter atteinte aux valeurs d'agrément ou entraver d'autres utilisations légitimes de la mer, même en l'absence de preuves concluantes d'un rapport de causalité entre les apports et les effets; ii) le principe « pollueur payeur », qui oblige le pollueur à supporter financièrement tous les frais engagés dans les mesures de prévention, de remédiation et de lutte contre la pollution. La convention OSPAR stipule en outre, une mise à jour régulière des programmes de surveillance de chacun des signataires en concordance avec les progrès techniques accessibles les plus récents, ainsi qu'une meilleure pratique environnementale. Les mesures mises en œuvre ne doivent pas augmenter la pollution de la mer en dehors de la zone maritime, ainsi que dans d'autres secteurs de l'environnement, ni générer une pollution transfrontalière. La commission OSPAR chargée de la mise en œuvre de la convention travaille en étroite collaboration avec plusieurs organismes internationaux dont le Conseil International pour l'Exploration de la Mer (CIEM) ayant pour mission principale : « de promouvoir et de coordonner la recherche scientifique dans les domaines de l'océanographie, de l'environnement marin, des écosystèmes marins et des ressources marines vivantes de l'Atlantique Nord ». Les avis scientifiques donnés par cet organisme font autorité auprès des gouvernements de l'Atlantique Nord-Est et sont pris en compte lors de l'élaboration de mesures visant à protéger l'environnement marin (http://www.ices.dk/aboutus/Press%20pack/ICES%20TRI%20FRENCH%20110308%20final.pdf).

- A la suite de la ratification de la convention de Montego Bay en 1994, l'Union Européenne (UE) a mis sur pied en 2000, une Directive Cadre sur l'Eau 2000/60/CE (DCE) avec pour objectifs principaux : la prévention de la détérioration de la qualité et la restauration des eaux souterraines et de surface, la réduction de l'émission de déchets et substances dangereuses et la préservation des zones protégées. Pour atteindre ces objectifs, l'UE a défini des méthodes de travail communes aux vingt-sept états membres qui reposent sur l'identification des problèmes à traiter, l'établissement d'objectifs environnementaux et d'actions à mener pour les atteindre, ainsi qu'un programme de surveillance de l'atteinte de ces objectifs. Ce programme nécessite entre autres i) la caractérisation des districts hydrographiques et leur typologie, l'identification des différentes masses d'eau (Article 5); ii) la définition d'un « bon état » par type de masse d'eau à partir duquel sera évalué l'évolution de l'état des masses d'eaux ; iii) le développement de méthodes de surveillance et de contrôle de la contamination chimique, de la qualité écologique des masses d'eaux et du risque encouru par la biocénose (Article 11).

- La DCE 2000/60/CE étant jugée insuffisante pour le milieu marin, le parlement de l'UE a mis sur pied en 2008, un nouveau cadre d'action communautaire dans le domaine de la politique pour le milieu marin : la directive cadre « stratégie pour le milieu marin » (2008/56/CE). Elle constitue le pilier environnemental de la future politique marine de l'UE. Son objectif est d'atteindre ou de maintenir le bon état écologique du milieu marin et la conservation de la biodiversité marine dans toute l'UE d'ici à 2020. Elle s'est appuyée sur des études du CIEM sur les régions marines de l'Union européenne pour établir des règlements basés sur une approche fondée sur les écosystèmes. Elle introduit la notion d'« eaux européennes », qui sont les « eaux situées au-delà de la ligne de base servant pour la mesure de la largeur des eaux territoriales et s'étendant jusqu'aux confins de la zone placée sous la souveraineté ou la juridiction des États membres, y compris le fond et le sous-sol de l'ensemble de ces eaux ». La motivation principale de la directive est de lutter contre les « nombreuses menaces qui pèsent sur le milieu marin, telles que l'appauvrissement ou la dégradation de la diversité biologique et les modifications de sa structure, la disparition des habitats, la contamination par les substances dangereuses et les substances nutritives,

et les répercussions du changement climatique », ce qui nécessite un cadre global pour coordonner les actions locales des états-membres.

Deux méthodes complémentaires sont actuellement utilisées pour la surveillance du milieu marin:

- La première consiste en l'analyse chimique des contaminants majeurs (trente trois substances prioritaires et huit dangereuses, incluant les polychlorobiphényles, métaux traces, pesticides organochlorés, hydrocarbures aromatiques polycycliques) dans différentes matrices environnementales (eau, air, sédiment, biota, etc.). Cette méthode a l'avantage de produire un nombre de données considérable, mais celles-ci sont souvent difficilement interprétables d'une part et fluctuent souvent au gré du temps et des conditions environnementales. Ces données toxicologiques permettent d'établir des seuils de toxicité (eg : concentration sans effets prédictible : PNEC) des polluants pris individuellement et qui ne tiennent pas compte des effets antagonistes ou synergiques potentiels en présence d'un cocktail de polluants, ce qui est généralement le cas dans l'environnement. D'autre part, les concentrations de polluants retrouvés dans la colonne d'eau ou le sédiment par exemple ne rendent pas compte de leur biodisponibilité, cette biodisponibilité étant fonction des caractéristiques physicochimiques du milieu et des organismes exposés. En effet, les polluants peuvent être dégradés ou transformés dans l'environnement par des phénomènes de photolyse, catalyse ou hydrolyse (exception faite des éléments non décomposables). De plus, leur absorption, distribution, bioaccumulation, métabolisation et leur élimination de l'organisme dépendent fortement de leur liposolubilité, et les biotransformations peuvent induire la formation de métabolites plus ou moins toxiques. Or la pertinence de l'évaluation du risque en écotoxicologie repose entre autre sur la capacité à prédire quelle fraction du polluant exercera un effet toxique sur l'individu exposé. Enfin, si la mesure des concentrations des polluants dans les organismes bioaccumulateurs rend compte de l'exposition à une ou des substances à caractère polluant, ces concentrations ne peuvent pas être directement reliées aux effets potentiellement néfastes sur les individus, leurs populations et leurs communautés.

- La deuxième méthode de surveillance repose sur l'identification des dysfonctionnements causés par les contaminants à différents niveaux d'intégration biologique dont: le niveau sub-organistique (biomarqueurs), le niveau de l'organisme (tests d'écotoxicologie *in vivo* et *in vitro*), le niveau de la population (bioindicateurs) et le niveau des écosystèmes (indicateurs écologiques) (Figure 1). Contrairement à la surveillance physico-chimique qui ne renseigne pas sur une perturbation passée, elle permet d'évaluer quels sont, pour les écosystèmes, les effets intégrés dans le temps de la présence de l'ensemble des contaminants et autres facteurs de stress dans le milieu naturel, à plus ou moins long terme (Amiard et al., 1998). Elle intègre notamment : i) les essais d'écotoxicité de matrices pollués (sédiments, effluents, sols pollués) ; ii) l'utilisation d'espèces sentinelles sensibles aux toxiques ; iii) l'utilisation d'indices de diversité biologique ou d'autres outils de diagnostic basés sur la connaissance de la biologie ou de l'écologie des groupes taxonomiques identifiés ainsi que iv) l'utilisation des biomarqueurs. Les biomarqueurs ont fait l'objet de nombreuses études au cours des deux dernières décennies. Ils sont définis par Depledge (1994) comme étant des changements observables et/ou mesurables à l'échelle moléculaire, biochimique, cellulaire, physiologique ou comportementale, qui révèlent l'exposition présente ou passée d'un organisme à un polluant ou groupe de polluants et ou des radiations. Ils donnent un signal d'alerte précoce de l'effet de la contamination sur les organismes exposés bien avant que des conséquences au niveau des populations et communautés ne soient atteintes (Amiard et Amiard-Triquet, 2008). La mesure de biomarqueurs renseigne sur la nature et le niveau de la contamination chimique et la santé des organismes vivants et des populations des écosystèmes aquatiques (Flammarion et al., 2000).

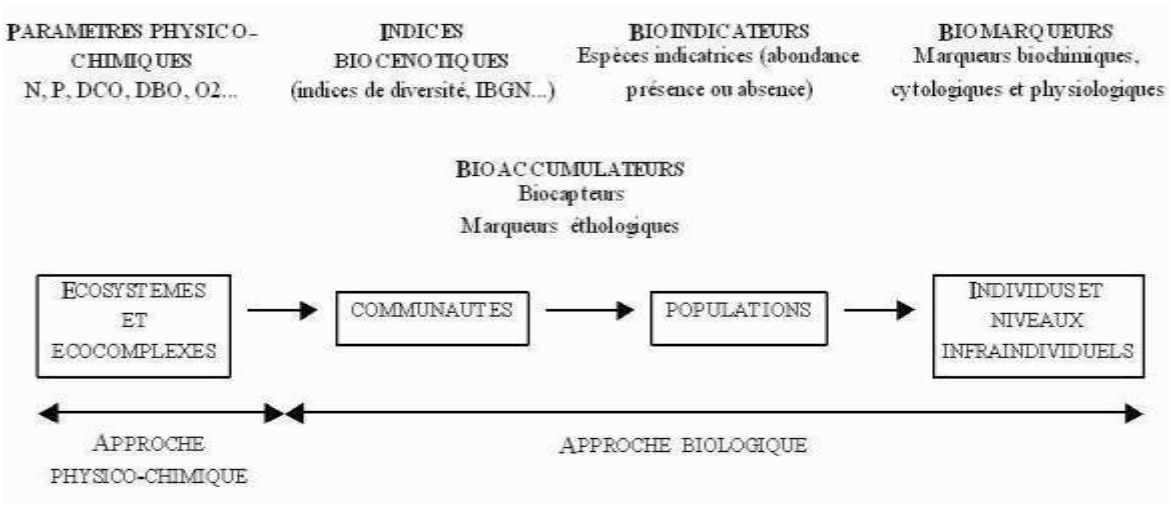


Figure 1. Diverses méthodologies utilisées à différents niveaux de l'organisation biologique pour l'évaluation de la qualité de l'environnement (Lagadic et al., 1997).

Malgré ces atouts indéniables, très peu de biomarqueurs sont validés comme outils pour la surveillance de l'environnement à part entière, surtout chez les invertébrés. Les raisons sont les suivantes : la grande variabilité des biomarqueurs au gré des conditions environnementales, l'absence de valeurs de références, ainsi qu'une significativité écologique encore limitée (les effets observés aux faibles niveaux de l'organisation biologique pouvant difficilement être reliés à des conséquences à des niveaux plus élevés).

C'est dans ce contexte que se situe cette thèse, dont le principal objectif est d'établir le lien entre les biomarqueurs mesurés à différents niveaux d'organisation biologique (du niveau sub-individuel au niveau des populations) chez deux espèces sentinelles : *Hediste diversicolor* et *Scrobicularia plana.*

Ce manuscrit se décline en cinq grandes parties :

- La première partie est consacrée à une synthèse bibliographique autour de la problématique des biomarqueurs, leur utilisation dans l'évaluation de la qualité de l'environnement ainsi qu'une description de la biologie de *S. plana* et de *H. diversicolor*.

- La deuxième partie présente les principaux sites d'études ainsi que la justification des choix méthodologiques réalisés pour la concrétisation de notre objectif de recherche

- La troisième partie s'articule autour de l'influence de la taille et de la salinité (facteurs de confusion) sur les biomarqueurs à différents niveaux de l'organisation biologique (biochimique, physiologique et comportementale) chez *S. plana*.

- Dans la quatrième partie sont présentées tour à tour les variations intersites d'une batterie de biomarqueurs mesurés à différents niveaux de l'organisation biologique chez chacune des espèces sentinelles. Nous y proposons ensuite une méthodologie complète pour évaluer l'état de santé des écosystèmes estuariens et côtiers.

- La cinquième partie est dédiée à la recherche de perturbations au niveau hormonal chez *S. plana*, en lien avec la présence de perturbateurs endocriniens dans le sédiment d'origine de l'animal.

- L sixième partie présente l'application des biomarqueurs utilisés dans notre étude à l'évaluation en conditions de laboratoire, de l'impact des nanoparticules métalliques chez *H. diversicolor* et *S. plana*.

- La dernière partie est dédiée aux conclusions générales des travaux effectués ainsi qu'aux perspectives qui en découlent

CHAPITRE 2. SYNTHESE BIBLIOGRAPHIQUE

2 CHAPITRE 2. SYNTHESE BIBLIOGRAPHIQUE

2.1 Les estuaires : Caractéristiques, contraintes physicochimiques et anthropiques

2.1.1 Description et contraintes physico-chimiques

Les estuaires sont généralement décrits comme des systèmes semi-fermés situés à l'interface entre la terre et l'océan, et où l'eau de mer est diluée de façon mesurable avec de l'eau douce venant de l'intérieur des terres (Hobbie, 2000). Bianchi (2006) apporte une définition plus complète en les qualifiant de systèmes semi-fermés d'eau côtières qui s'étendent à la limite effective de l'influence des marées, dans lesquels l'eau de mer entrant par une ou plusieurs connexions libres avec la mer ouverte, ou de tout autre système d'eau côtière saline, est sensiblement diluée par de l'eau douce en provenance des terres, et qui abrite des espèces biologiques euryhalines durant une partie ou la totalité de leur cycle de vie. Cette eau douce venue des terres apporte avec elle d'importantes quantités de sédiment, de matière organique, de nutriments inorganiques collectés dans les terres qui, s'ajoutant aux phénomènes de marées, aux conditions physicodynamiques et à la stratification des estuaires, en font des zones de si grande productivité (Hobbie, 2000). Ce mélange eau douce-eau salée de l'embouchure de la zone fluviale jusqu'à la zone marine conduit à un gradient de salinité qui s'étend de l'eau douce à de l'eau totalement marine (0,1‰ à 33‰) (Bianchi, 2006). Ce gradient de salinité, allié aux caractéristiques hydromorphologiques des estuaires permet de les compartimenter en trois grandes zones (figure 2A) :

- Une zone fluviale ou zone amont l'eau est douce, riche en sels nutritifs et en pigments chlorophylliens. Elle subit l'influence de la marée dynamique. Le sédiment à cet endroit est surtout d'origine fluviale

- Une zone de balancement des marées où l'eau douce et l'eau de mer se mélangent en proportion variable. Elle est fortement influencée par les processus biologiques (bioturbation, dépôts de fèces etc.) en raison de sa forte productivité. La figure 2B montre le lien entre les phénomènes physiques (courants intertidaux, apport des rivières

et eaux souterraines) et les processus biologiques (migration des poissons, transport larvaire) qui ont lieu dans l'estuaire

- Une zone marine ou zone aval, ou embouchure, dominée par les influences maritimes

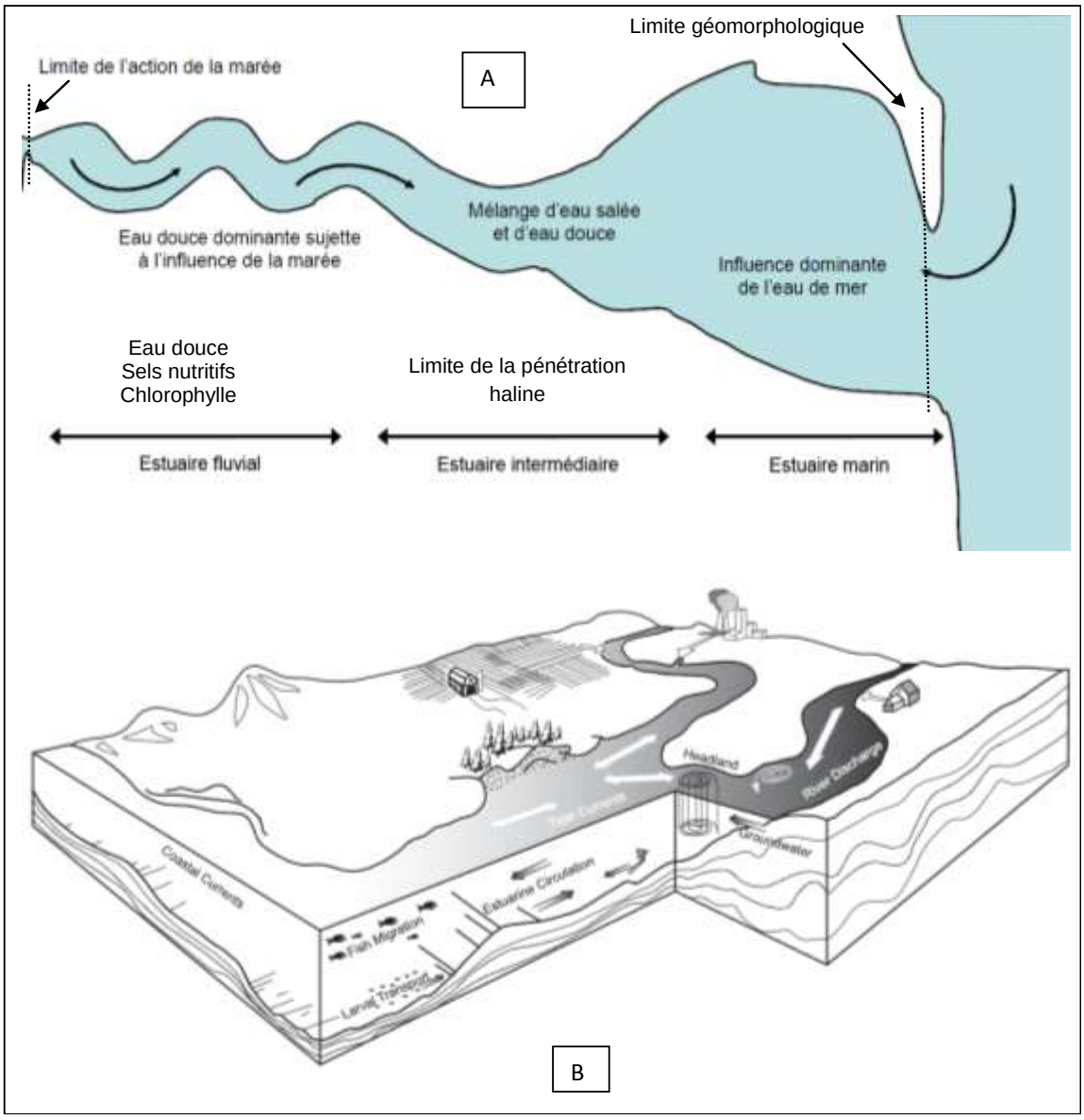


Figure 2. A : Différentes parties d'un estuaire modifié d'après Romaña, 1994 ; Cherkaoui, 2006); B: Lien entre les phénomènes physiques et les processus biologiques dans un estuaire (Bianchi, 2006).

Au gradient de salinité s'ajoute celui de la température, inhérent lui-aussi à la confrontation des masses d'eaux douces et salées. C'est le cas par exemple pour l'estuaire de la Seine où

l'amplitude thermique des eaux marines à proximité de l'embouchure peut varier de 5 à 20°C au cours de l'année, avec parfois l'installation d'une légère thermocline (Alizier et al., 2010). A ces variations à long terme, viennent se superposer des variations à plus court terme, liées à l'exondation des vasières intertidales qui sont alors soumises à la température de l'air Ces variations de salinité et de température font des estuaires un milieu de vie drastique que seules les espèces (d'eau douce ou marines) ayant développé de grandes capacités d'adaptation physiologique peuvent coloniser. Pour y survivre, les organismes doivent mettre en jeu des mécanismes d'osmorégulation (qui impliquent les organes tels les branchies, les téguments, le système digestif et excrétoire) ainsi que des mécanismes d'adaptation à la baisse d'oxygène à température égale, la concentration en oxygène étant plus élevée dans les eaux douces que dans les eaux salines (McLusky et Elliott, 2004, Cherkaoui, 2006). Ces processus nécessitent un apport énergétique d'autant plus élevé que la perturbation sera longue. De ce fait s'en suivront des répercussions directes sur les caractéristiques biologiques des individus et des populations (croissance et reproduction). Ceci explique probablement qu'en dépit de l'abondance des individus qu'on y retrouve, les estuaires n'abritent que peu d'espèces comparés aux eaux douces et marines; les espèces qui y vivent en permanence sont généralement euryhalines (McLusky et Elliott, 2004). Les organismes estuariens sont confrontés à d'autres difficultés que sont la nature du substrat vaseux (un substrat constitué de particules très fines peut obstruer certains organes délicats) et les conditions d'hypoxie pouvant régner périodiquement dans certaines zones (McLusky et Elliott, 2004). Ces conditions d'hypoxie résultent souvent de la présence d'un bouchon vaseux provoqué par l'accumulation en un point donné, de matières en suspension (issues des lessivages des sols en amont ou générées par l'eutrophisation) (Romaña, 1994). Le bouchon vaseux généralement situé au centre de l'estuaire, se déplace d'amont en aval, au gré des apports hydrologiques de l'amont et des marées. Sa présence cause de nombreux problèmes (Romaña, 1994, McLusky et Elliott, 2004, Cherkaoui, 2006) à savoir :

- Des phénomènes d'anoxie sévères de l'eau en été résultant de l'oxydation de matière organique particulaire biodégradable. Ces phénomènes sont récurrents dans l'estuaire de la Loire par exemple

- Une forte turbidité qui restreint la pénétration de la lumière et diminue de ce fait la production primaire

- Il est le siège de l'accumulation des polluants et bactéries d'origine animale ou anthropique

- Il constitue un obstacle pour la migration des poissons

La dynamique des sédiments dans un estuaire dépend de quatre facteurs principaux: l'érosion du sédiment superficiel, le transport par les courants ou la marée, le dépôt et les phénomènes de consolidation et d'agrégation des particules déposées (Nichols and Biggs, 1985 cités par Bianchi, 2007).

2.1.2 Contraintes anthropogéniques

Si l'apport de nutriments d'origine terrestre contribue à la richesse des estuaires, elle constitue aussi une sérieuse menace pour la biodiversité de ces derniers. En effet, les rivières reçoivent les épandages agricoles, les rejets industriels et urbains, riches en nitrates, ammonium, matière organique non traitée, polyphosphates sont à l'origine de sérieux problèmes d'eutrophisation. Les effluents industriels toxiques, de même que les microbes issus d'effluents urbains sont une source d'inquiétude pour la croissance et la reproduction des espèces d'élevage et pour la qualité sanitaire des produits de la mer. Les polluants présentant le plus de risques étant ceux qui sont bioamplifiés dans les chaînes alimentaires, en particulier (le méthylmercure et les PCB). Dans les zones industrielles, des activités de dragage sont régulièrement menées dans les estuaires afin d'y instaurer ou maintenir un niveau de bathymétrie pratique à la navigation. Ces activités de dragage sont d'autant plus préoccupantes que les sédiments dragués sont chargés de mélanges de nombreuses classes de contaminants.

L'encyclopédie d'Eisler sur les produits chimiques prioritaires dangereux pour l'environnement (Eisler, 2007) affiche 142 résultats lorsque l'on utilise les termes de recherche «estuaire» ou «estuarien». Bianchi (2007) donne aussi une revue de la littérature en rapport avec les nutriments et les contaminants qui sont introduits dans les estuaires dont: les métaux traces toxiques ou indispensables pour de nombreux organismes, les PCB très stables, de sorte qu'ils sont toujours présents malgré leur interdiction, les hydrocarbures aromatiques polycycliques (HAP). Les contaminants émergents de préoccupants dans les milieux côtiers et

estuariens ont été examinés par Hale et La Guardia (in Newman et al., 2002). Ils comprennent des retardateurs de flamme bromés, les œstrogènes naturels et synthétiques, les éthoxylates d'alkylphénol et produits de dégradation associés, les produits pharmaceutiques et de soins personnels. Les avancées récentes en nanotechnologie et l'utilisation croissante de nanomatériaux dans tous les secteurs de la société ont donné lieu à des incertitudes concernant leurs possibles impacts environnementaux. Les organismes estuariens benthiques sont de ce fait particulièrement à risque étant donné la probabilité de nanoparticules à s'agglomérer, s'agréger, et se déposer dans le sédiment (Klaine et al., 2008).

En réponse à ces activités anthropiques s'ensuit une dégradation de la qualité du milieu dans les estuaires et en particulier dans les régions urbanisées.

2.2 L'utilisation d'espèces sentinelles pour la biosurveillance des écosystèmes aquatiques

Certaines espèces sont plus sensibles que d'autres et nécessitent des conditions particulières pour se maintenir dans leur milieu naturel. Lorsque les conditions du milieu naturel sont altérées, par exemple dans le cas de contamination par des polluants, ces espèces peuvent être affectées de plusieurs façons reflétant ainsi la dégradation des conditions initiales du milieu naturel. De telles espèces sont appelées indicateurs biologiques (ou indicateurs écologiques) en raison de leur capacité à caractériser l'état d'un écosystème soumis à un stress environnemental, ainsi qu'à permettre de détecter ou prévoir des changements significatifs pouvant survenir à l'intérieur de ce même écosystème (Kaiser, 2001, Sammarco et al., 2007). Une espèce sentinelle est un indicateur biologique particulièrement sensible à un ou plusieurs polluants susceptibles de provoquer des perturbations ou des changements dans un milieu donné (Kaiser, 2001). Ces espèces, par leur sensibilité, ont la capacité de mettre en évidence des signes précurseurs d'altération du milieu naturel avant même que les effets soient ressentis à l'intérieur du milieu affecté. Ils agissent par conséquent comme une sorte de signal d'alarme pour l'ensemble de l'écosystème (Markert et al., 2003). De façon générale, une espèce sentinelle susceptible d'être utilisée à des fins d'évaluation des risques pour l'environnement devrait posséder les caractéristiques suivantes : 1) être relativement facile à capturer, 2) être présente dans le milieu en nombre suffisant, 3) posséder une aire de

répartition connue, 4) posséder une taille propice à la manipulation, 5) avoir déjà fait l'objet d'études confirmant ses qualités en tant que bioindicateur (Kaiser, 2001). Les annélides sont principalement représentées dans les écosystèmes marins par les vers de la classe des polychètes qui comptent souvent jusqu'à un tiers des espèces macrobenthiques rencontrées et jusqu'à 80% de l'abondance totale en plus de présenter de fortes valeurs de richesse, de diversité, de biomasse et de densité (Guerra-García et al., 2006, Licciano et al., 2007). Ils sont également connus comme étant de bons bioaccumulateurs de certains polluants comme les métaux lourds, les hydrocarbures aromatiques polycycliques (HAP) et les composés polychlorés issus de la pollution d'origine industrielle (Bocchetti et al., 2004). Ces caractéristiques, en plus de la tolérance aux polluants ainsi qu'aux différentes perturbations environnementales, sont autant de qualités recherchées chez un bioindicateur (Guerra-García et al., 2006).

La macrofaune benthique est très utilisée dans des programmes de biosurveillance du milieu aquatique, la malacofaune et les annélides en particulier. C'est le cas de la moule et de l'huître, bivalves clés utilisés dans le cadre du « Mussel Watch program » par exemple (Farrington et al., 1995) qui a été décliné en France au travers du RNO (Cf Chapitre 1). Les raisons de cet intérêt sont résumées ci après :

- Ce sont des organismes sédentaires, ubiquistes et généralement abondants, les polychètes étant le taxon le plus abondant de la faune benthique (Dean, 2008)

- Ils sont plus représentatifs du milieu dans lequel ils vivent que des espèces mobiles et/ou saisonnières

- Certaines espèces benthiques sont tolérantes aux changements de conditions environnementales (salinité, température, oxygène dissous, eutrophisation etc.)

- Leur récolte sur le terrain est généralement aisée et certaines espèces sont faciles à maintenir en élevage au laboratoire

- La plupart des bivalves et des annélides ont une durée de vie suffisamment longue (quelques mois à plusieurs années), ce qui permet d'étudier l'effet d'un polluant sur des individus de classes d'âge différentes

- Ces espèces sont de taille raisonnable, donc aisément manipulables tout en fournissant suffisamment de matériel biologique (tissus, fluides etc.) pour pouvoir effectuer de nombreuses analyses

- Beaucoup d'espèces de bivalves et d'annélides sont consommées par des consommateurs secondaires (oiseaux limicoles, poissons etc.) et/ou par l'homme et représentent de ce fait un risque direct et/ou indirect pour la santé humaine.

- Ces différentes caractéristiques font de la macrofaune benthique, un groupe particulièrement important pour évaluer l'état écologique des masses d'eau (comme cela est demandé par la DCE), même si dans les eaux de transition comme les estuaires, les différentes métriques disponibles (abondance, nombre de taxons et différents indices de diversité et de sensibilité) montrent une certaine inconsistence face au gradient de pression anthropique (Borja et al., 2011).

2.2.1 Présentation des espèces clés utilisées

En plus de répondre à tous les critères précédemment cités, les espèces utilisées dans la présente thèse, le bivalve *Scrobicularia plana* et l'annélide *Hediste diversicolor*, espèces endobenthiques (Figures 3 et 4), sont exposées doublement aux contaminants : à la fois ceux contenus dans la masse d'eau et ceux accumulés dans le sédiment (Dean, 2008). Les deux espèces jouent un rôle clé dans le fonctionnement des écosystèmes estuariens. En effet, elles contribuent au processus de bioturbation et jouent un rôle important dans le cycle biogéochimique des nutriments et contaminants (François et al., 1997, Banta et Andersen, 2003, Orvain, 2005, Lagauzère, 2008). Ces espèces constituent également une ressource importante pour les poissons plats et les limicoles dans les réseaux trophiques des vasières intertidales (Zwarts et Wanink, 1993, Zwarts et al., 1996, Zwarts et Ens, 1999, Rosa et al., 2008, Van Colen et al., 2009). Elles sont considérées comme bioindicatrices car par leur présence/abondance, elles sont significatives d'une ou de plusieurs propriétés de l'écosystème dont elles font partie. En raison de leur importance économique (appâts pour la pêche, aliment pour poissons en aquaculture pour *Hediste*), écologique (maillons clés de la chaîne alimentaire ; bioindicateurs), les cycles de vie de *S. plana* et *H. diversicolor* ont été très étudiés et sont bien décrits dans la littérature.

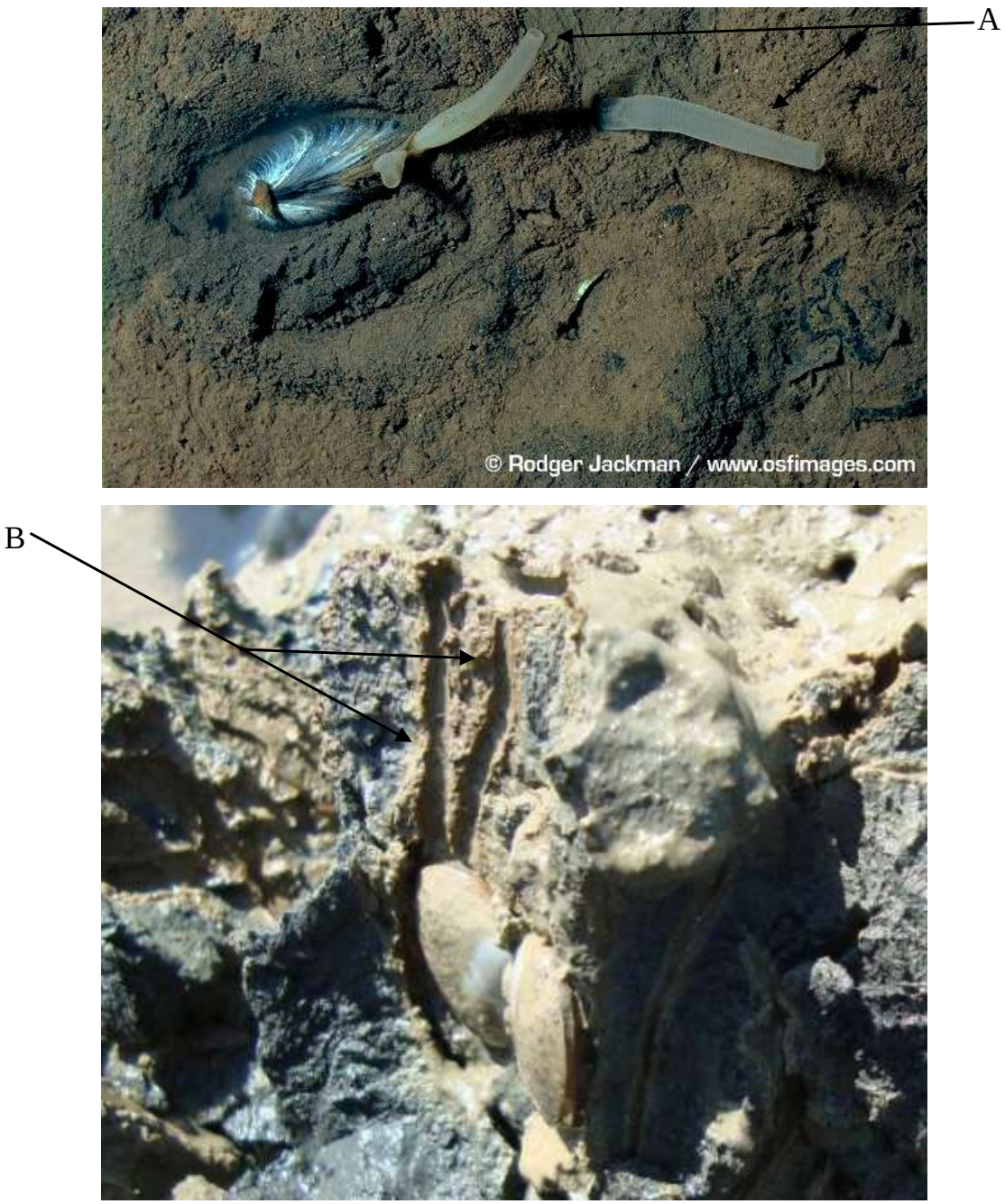


Figure 3. Photographies de *S. plana* dans le sédiment (A : Siphons, B : traces laissées par les siphons dans le sédiment)



Figure 4. ***Hediste diversicolor*** **(A : entrée d'un terrier ; B : terrier en forme de Y L'on distingue bien le sédiment anoxique gris-noir, du sédiment oxygéné plus clair)**

2.2.2 *Scrobicularia plana* (da Costa, 1778).

La scrobiculaire (nom commun : Lavignon poivré) est un bivalve de la sous-classe des Hétérodontes et de l'ordre des Vénéroïdes. Selon les auteurs, le nom de famille qui lui est attribué est celui de Scrobiculariidés (Poutiers, 1993, Gofas, 2011), mais plus souvent de Semelidés (Muséum National d'Histoire Naturelle, 2003-2010). Cette espèce est connue sous d'autres appellations dont celle de *Scrobicularia piperata* (Gmelin) qui est en lien avec le nom commun qui lui a été attribué (http://www.marinespecies.org/aphia.php?p=taxdetails&id=141424).

2.2.2.1 Répartition géographique et écologie

Scrobicularia plana est une espèce du médiolittoral, présente jusqu'à 30 mètres de profondeur. On la retrouve dans les zones sablo-vaseuses, argileuses ou vaseuses (estuaires, baies, lagunes). Elle affiche une préférence pour les sables fins et les zones de haute slikke (qui découvrent à marée basse). On la retrouve généralement dans les 20 premiers centimètres du sédiment, la profondeur d'enfouissement étant proportionnelle à la taille de la coquille (Hughes, 1970). *S. plana* est une espèce euryhaline très tolérante aux fluctuations des conditions environnementales (oxygène dissous, salinité, température) ce qui explique sa large répartition. Elle peut supporter des périodes de dessalures prolongées, ainsi qu'une salinité maximale de 34‰ (Guelorget et Mazoyer-Mayere, 1983, Kourradi, 2007). Cette espèce est présente sur la façade Atlantique Nord-Est, depuis les côtes de Norvège, de la mer Baltique jusqu'à celles du Sénégal (Poutiers, 1987), et dans la mer Méditerranée (Trebble, 1976). La densité de population de scrobiculaires varie de dizaines d'individus, à plusieurs centaines (Desprez et al., 1986) voire un ou plusieurs milliers d'individus au mètre carré (Sola, 1997), des densités de 1351 ind.m^{-2} ont été rapportées dans l'estuaire du Bou Regreg au Maroc (Cherkaoui, 2006). Elle fait partie des espèces constantes (fréquence d'apparition >51%) en secteur aval de l'estuaire de Loire (GIP Loire Estuaire, 2003). Sa durée de vie moyenne est de 5 ans (Sola, 1997, Verdelhos et al., 2005), mais des individus de 16 à 18 ans ont été retrouvés en estuaire de Gwendraeth en Irlande (Green, 1957).

Plusieurs études ont été menées sur le mode d'alimentation de *S. plana*. Il en ressort que la scrobiculaire a deux modes d'alimentation : à marée haute, elle filtre les particules présentes dans la colonne d'eau (suspensivore), et à marée basse, elle broute la surface du sédiment (déposivore) (Hughes, 1969, Riera et al., 1999). Elle se nourrit préférentiellement de diatomées benthiques et de phytoplancton marin (Riera et al., 1999).

2.2.2.2 Physiologie et biologie de la reproduction

Scrobicularia plana est une espèce gonochorique. De rares cas d'hermaphrodisme ont cependant été rapportés (Raleigh et Keegan, 2006). Il est possible que ces cas d'hermaphrodisme soient en réalité de l'intersexe. En effet des cas d'intersexe ont été décrits chez cette espèce en Angleterre et au Portugal (Chesman et Langston, 2006, Langston et al., 2007, Gomes et al., 2009). Son cycle gamétogénique varie selon un gradient latitudinal (Hughes, 1971, Santos et al., 2011a). On observe deux cas de figure :

- Dans les pays nordiques (Angleterre, Ecosse, Norvège), un cycle de reproduction court (3 à 6 mois) avec généralement une seule période de ponte au milieu de l'été (Hughes, 1971, Worrall et al., 1983, Zwarts, 1991), plus rarement deux (Frenkiel et Mouëza, 1979). Dans le Nord de la France la période de ponte peut avoir lieu de la fin du printemps au milieu de l'été (Mouneyrac et al., 2008).

- Plus au sud (Portugal, Espagne, Sud de la France), un cycle gamétogénique long (7 à 11 mois) avec deux périodes de ponte distinctes (Sola, 1997, Santos et al., 2011a) généralement en hiver et en été. Les pontes peuvent aussi avoir lieu tout le long de la période de maturité sexuelle (Guerreiro, 1991), comme dans l'estuaire du Guadalquivir, Espagne, où elle débute en février et s'achève en octobre (Rodríguez-Rúa et al., 2003).

Le déclenchement de la maturation des gamètes, ainsi que celui de la ponte sont intrinsèquement liés aux facteurs environnementaux tels que la température, la nourriture disponible (elle-même dépendante de l'intensité d'éclairement) (Worrall et al., 1983, Raleigh et Keegan, 2006, Santos et al., 2011a). La fécondation externe a lieu après émission des

gamètes dans l'eau. Les spermatozoïdes ont une tête en forme de faucille, mesurant environ 30µm (Frenkiel et Mouëza, 1979). L'ovocyte est entouré d'un épais chorion de diamètre compris entre 140 à 200 µm. Le diamètre ovocytaire (sans le chorion) varie selon la contrée : il est d'environ 42µm en Irlande (Raleigh et Keegan, 2006), 80 µm au Royaume-Uni (Ruiz et al., 1995), 75-80 µm au Pays de Galles (Frenkiel et Mouëza, 1979). L'éclosion a lieu 72 à 96h après la fécondation. Les larves véligères qui en sont issues mènent une vie planctonique pendant environ 3 semaines. Puis elles alternent entre phase nageuse et reptation grâce à la formation du pied, et une fois qu'elle ont trouvé des conditions et un substrat favorable, elles se sédentarisent et continuent leur développement (Frenkiel et Mouëza, 1979). Les scrobiculaires atteignent leur maturité sexuelle à partir de leur 2ème à 3ème année, à une longueur de coquille d'au moins 20 mm (Sola, 1997, Raleigh et Keegan, 2006). La figure 5 résume le cycle de vie de *S. plana*, de l'œuf à l'adulte.

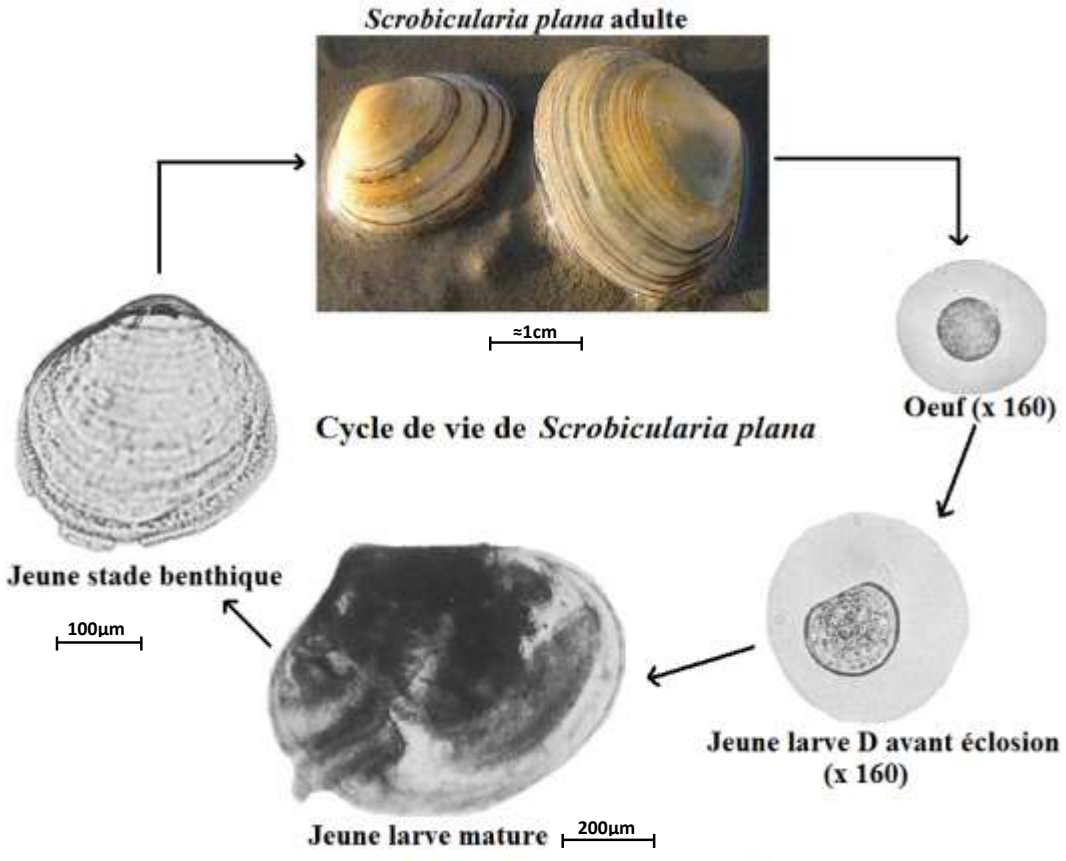


Figure 5. Cycle de vie de *S. plana* d'après Frenkiel et Mouëza (1979)

2.2.3 Hediste diversicolor (O. F. Müller, 1776)

Les polychètes font partie des invertébrés marins benthiques les plus abondants (Holthe, 1991). D'après le World Register of Marine Species (http://www.marinespecies.org/aphia.php?p=taxdetails&id=152302) *Hediste diversicolor* appartient à la famille des Nereididés, et au genre *Hediste*. Cette espèce est aussi connue sous le nom de *Nereis diversicolor* utilisé par de nombreux scientifiques bien que des études génétiques aient démontré son appartenance au genre *Hediste* (Fong et Garthwaite, 1994). Nous avons décidé de retenir l'appellation d'*Hediste diversicolor* pour ce document.

2.2.3.1 Répartition géographique et écologie

Cette espèce est eurytherme et euryhaline, ce qui explique en partie sa vaste répartition. Elle est cependant absente des eaux douces et des milieux très salés (Wolff, 1973). Elle peut survivre à des conditions d'hypoxie sévères, et est présente en abondance dans des milieux très eutrophisés. *Hediste diversicolor* est présente dans les zones marines et saumâtres de toutes les côtes Nord-Européennes jusqu'à la Mer Baltique, sur la partie atlantique de la côte Nord-Américaine ainsi qu'en Mers Noire et Caspienne où elle a été introduite (http://iobis.org/mapper/?taxon=Hediste%20diversicolor (Smith, 1977 *in* Scaps, 2002). Sa répartition et sa densité sont fonction de l'oxygénation du milieu et de la quantité de nourriture disponible (Obolewski et al., 2009). On la retrouve préférentiellement dans les estuaires vaseux à sablo-vaseux où sa densité varie de quelques individus à plusieurs milliers en eaux peu profondes et bien oxygénées (Scaps, 2002, Obolewski et al., 2009). Son abondance et son corps mou en font la proie de nombreuses espèces épibenthiques (poissons plats, crabes géants) et oiseaux limicoles dont la pression de prédation est si élevée (jusqu'à 90% de la population) qu'elle régule la population de *H. diversicolor* (Scaps, 2002, Rosa et al., 2008). Elle constitue ainsi un maillon clé de la chaîne alimentaire dont la disparition pourrait avoir des conséquences écologiques pour la survie de ses prédateurs (Rosa et al., 2008).

Cette espèce vit dans des terriers qu'elle creuse dans le sédiment. Les terriers peuvent mésurer plusieurs dizaines de centimètres de profondeur (Durou, 2006), ils sont en forme de U parfois

prolongés par un puits vertical (Y) qui sert de refuge en cas d'attaque (Loquet et Dupont, 1976). Chaque individu défend farouchement l'entrée de son terrier contre toute intrusion, mais une fois hors de celui-ci, ce territorialisme s'efface bien que les individus évitent tout contact entre eux lorsqu'ils se déplacent à la surface du sédiment (Marty, 1997, Scaps, 2002). De part son activité galéricole *H. diversicolor* joue un rôle majeur dans les processus de bioturbation. En effet, par son activité d'enfouissement, elle transporte de la matière organique des strates plus profondes et moins oxygénées à la surface du sédiment permettant ainsi la remise en suspension de particules et l'augmentation des surfaces de contact et d'échange eau-sédiment. L'irrigation du terrier permet son oxygénation, ce qui a pour conséquence l'augmentation des échanges de solutés à l'interface eau-sédiment et la stimulation de processus de minéralisation et de dégradation de la matière organique par les bactéries aérobies (Gilbert et al., 1997, Hansen et Kristensen, 1998, Banta et al., 1999, Scaps, 2002, Cardoso et al., 2009).

H. diversicolor est principalement omnivore (Fauchald et Jumars, 1979) et présente différents modes d'alimentation. Prédatrice occasionnelle, elle se nourrit aussi de diatomées, algues et détritus (Olivier et al., 1997), ainsi que de morceaux de plantes halophiles (Meziane et Thorin, 2000). Ses modes de nutrition qui varient en fonction du rythme nycthéméral, de la présence d'éventuels prédateurs, des conditions hydrodynamiques et trophiques du milieu (Fauchald et Jumars, 1979, Esnault et al., 1990, Riisgård, 1991, Marty, 1997) sont les suivants :

- Le « deposit-feeding » qui consiste en l'ingestion immédiate ou différée (dans son terrier) de particules de nourritures dont l'animal se saisit à la surface du sédiment. Ce mode d'alimentation est généralement utilisé à marée basse lorsque le sédiment est découvert (Esselink et Zwarts, 1989, Esnault et al., 1990).

- Le « filter-feeding » qui consiste en la sécrétion au travers de la galerie d'un épais filet de mucus, de part et d'autre de l'animal. Ce dernier effectue ensuite des mouvements ondulatoires qui génèrent un courant d'irrigation, lequel va entrainer les particules vers le mucus auquel elles vont adhérer (Esnault et al., 1990, Riisgård, 1991).

- Le troisième mode d'alimentation est presque semblable au second, à la seule différence que le filet de mucus est tendu à l'extérieur de la galerie ; une fois les particules

piégées, l'animal les roule en boule avec le mucus et les ramène à l'intérieur de son terrier (Esnault et al., 1990).

Des auteurs ont aussi démontré une activité bactériolytique chez cette espèce (Lucas et Bertru, 1997).

2.2.3.2 Physiologie et biologie de la reproduction

De nombreux travaux décrivent la physiologie et la biologie de la reproduction d'*Hediste diversicolor* (Cepede, 1914, Dales, 1950, Fauchald et Jumars, 1979, Porchet-Henneré et M'Berri, 1987, Esnault et al., 1990, Riisgård, 1991, Wanink et Zwarts, 1993, Batten et al., 1994, Luis et Passos, 1995, Marty, 1997, Abrantes et al., 1999, Meziane et Retiere, Duport et al., 2006, Durou, 2006, Fidalgo E Costa et al., 2006, Cuny et al., 2007, Engelsen et Pihl, 2008, Amiard-Triquet et al., 2009, Fernandes et al., 2009, Tairova et al., 2009). La reproduction fortement influencée par la température, le cycle lunaire et la quantité de nourriture disponible (Dales, 1950, Marty, 1997, Scaps, 2002). La saison et la durée de la reproduction sont aussi fonction de la localisation géographique et la température (Marty, 1997). Elle peut avoir lieu en hiver, au printemps, en automne, la durée variant de 1 mois à toute l'année selon la localisation, avec une ou plusieurs périodes de ponte (Dales, 1950, Olive et Garwood, 1981, Marty, 1997, Durou, 2006, Amiard-Triquet et al., 2009). Selon certains auteurs (Olive, 1995, Marty, 1997, García-Alonso et al., 2006), la gamétogénèse et la croissance de l'animal sont régulées par le système neuroendocrinien en sus des facteurs environnementaux (température, cycle lunaire, disponibilité alimentaire), ceci *via* la production d'une neurohormone qui présente en faible quantité permettrait le bon déroulement de la vitellogenèse, mais l'inhiberait à des proportions élevées. De ce fait, les ovocytes produiraient à leur tour des hormones qui réguleraient la sécrétion de la précédente. Mais d'après Lawrence et Soame (2009), le cycle de reproduction des Néréides serait régulé par des interactions « multi-hormonales » plus complexes tandis que la croissance somatique et la régénération des individus dépendrait surtout de l'action de l'hormone juvénile, sa sécrétion par le ganglion supraoesophagien diminuerait dès le début de l'oogenèse et la vitéllogenèse au profit de la dopamine ou la mélatonine. La sécrétion de 17β-œstradiol viendrait ensuite renforcer la

vitellogenèse en induisant production de vitellogénine par les éleocytes. La production de 17-β-œstradiol ou l'utilisation de vitelline serait à son tour sous le contrôle d'une deuxième neurohormone présente dans le ganglion de femelles matures et qui aurait une fonction gonadotrophique (Figure 6).

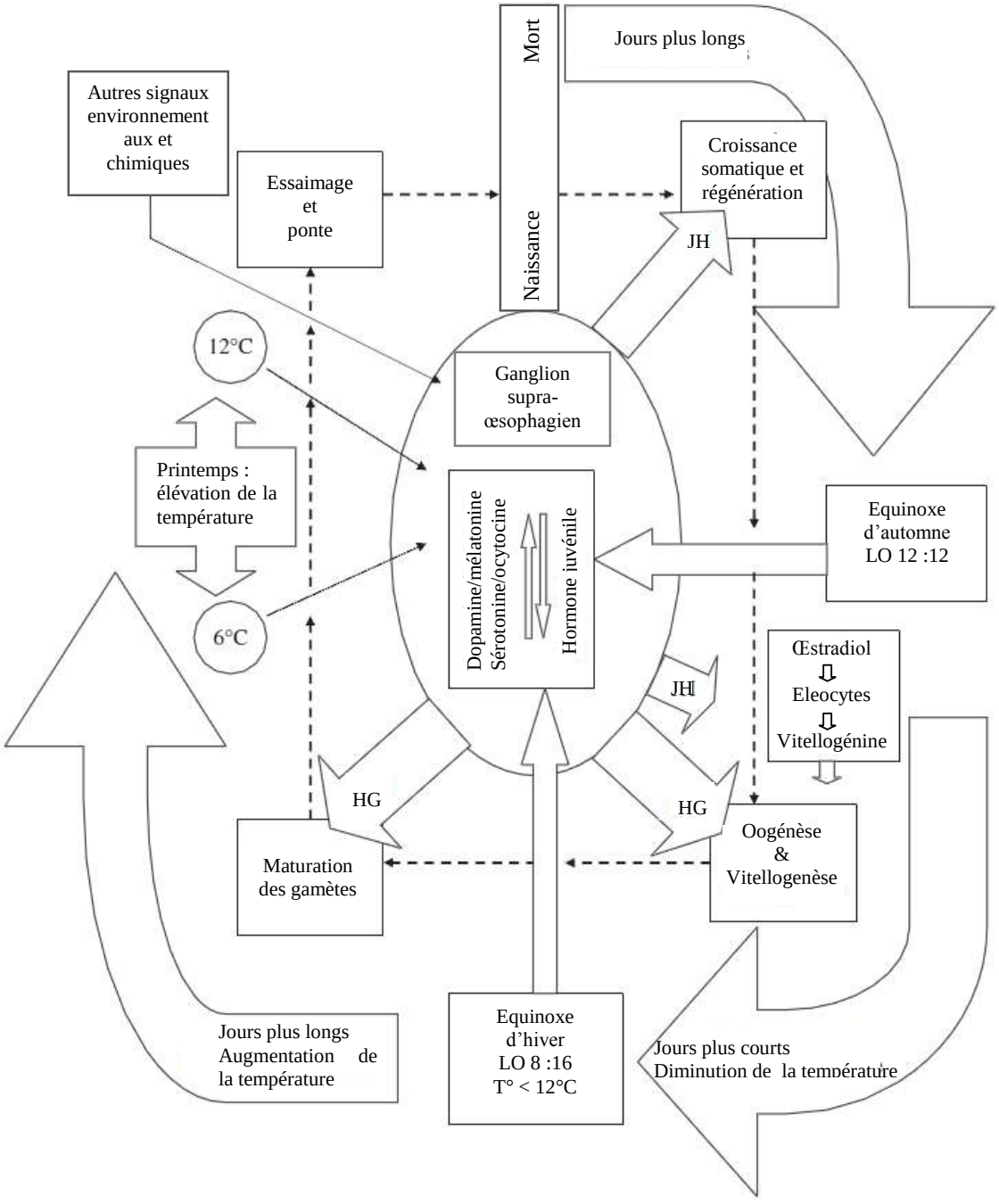


Figure 6. Modèle multi-hormonal présentant les interactions entre les facteurs environnementaux et endocriniens qui contrôlent le cycle de reproduction des *Nereidae*. HJ : hormone juvénile ; HG ; hormone gonadotrophique (Lawrence et Soame, 2009).

Contrairement à la plupart des Nereidés, *H. diversicolor* se reproduit à l'état atoque, c'est-à-dire qu'elle ne subit pas de changements morphologiques durant cette période, hormis un changement de couleur qui passe de l'orangé au vert (Dales, 1950, Marty, 1997). Cette couleur verte chez les adultes matures est due à la présence de biliverdine (formée par la dégradation de l'hémoglobine dans le sang) dans la paroi digestive, l'épiderme et les cellules cœlomiques. Chez les mâles, la masse blanche de sperme dans le cœlome lui donne une couleur plus claire vert-bouteille tandis que chez les femelles la teinte se rapproche du vert-olive (Dales, 1950, Dales et Kennedy, 1954). Chez les femelles matures, les ovocytes contenus dans la cavité cœlomique mesurent en moyenne 200µm (Dales, 1950). *H. diversicolor* est gonochorique, le sex-ratio, largement en faveur des femelles, oscille autour de 70 à 90% (Dales, 1950, Marty, 1997) et l'énergie allouée à l'effort de reproduction est conséquente (70%) (Olive, 1983, Grémare et Olive, 1986). Une fois la maturité sexuelle atteinte (entre 1 et 3 ans), chaque individu se reproduit une seule fois et meurt (Olive et Garwood, 1981). Guidé par des phéromones émis par la femelle, le mâle libère ses produits sexuels (par déchirure des téguments) à l'entrée du terrier et meurt peu de temps après (Marty, 1997). Chez la femelle la mort peut survenir plusieurs semaines après la ponte, le temps pour elle d'incuber ses œufs dans la galerie et d'en assurer la ventilation pour un bon développement de ces derniers (Marty, 1997). Les larves éclosent au stade trochophore 1 et continuent leur croissance dans le terrier maternel qu'elles quittent au stade de quatre sétigères, 11 à 13 jours après la fécondation. A leur sortie de la galerie maternelle, les larves ont un comportement benthique (Marty, 1997). Les larves libres se déplacent par nage-reptation et sont capables de s'enfouir dès le stade de six sétigères, dès lors qu'elles acquièrent des cirres tentaculaires postérieurs. Elles commencent alors à construire des galeries semblables à celles des adultes, se sédentarisent et adoptent le mode de nutrition des adultes (Marty, 1997).

2.2.4 *Scrobicularia plana* et *Hediste diversicolor* dans les études écotoxicologiques

Scrobicularia plana et *Hediste diversicolor* ont fait l'objet de nombreuses études écotoxicologiques au cours de cette dernière décade. Nous avons déjà mentionné qu'en tant qu'organismes endobenthiques ces deux espèces étant en étroit contact avec le sédiment qui constitue le principal réservoir de contaminants organiques et inorganiques dans les milieux aquatiques. La signification écotoxicologique de ces contaminants liés au sédiment a été une source de débats mais il est maintenant bien établi, qu'il ne s'agit pas d'un mécanisme d'exclusion et qu'une fraction de ces contaminants reste accessible aux organismes. Cette accessibilité est gouvernée par la nature des contaminants, les conditions physico-chimiques du milieu (pH, salinité, potentiel redox, teneur en matière organique, etc), l'activité microbienne et les capacités intrinsèques des espèces endogées (prise de sédiment par voie alimentaire, capacité digestive). De nombreux travaux portant sur la bioaccumulation des contaminants ont montré que ces deux espèces étaient de bons moniteurs de la biodisponibilité de plusieurs métaux (Poirier et al. 2006 et littérature citée) et contaminants organiques (Ruus et al., 2005).

Toutefois, les concentrations de métaux chez les deux espèces étaient différentes dans les sites étudiés par Solé et al. (2009). Ces différences étaient fortement amplifiées dans une zone fortement contaminée, comme Restronguet Creek (Bryan et al., 1985). Les concentrations ont étaient généralement plus élevées chez les bivalves que les polychètes, à l'exception du Cu qui était environ dix fois plus élevé chez *H. diversicolor* que chez *S. plana* originaires de la Restronguet Creek. La répartition des organismes sur le site de la Restronguet Creek suggère une meilleure tolérance de *H. diversicolor* par rapport à *S. plana* puisque cette dernière est absente des sites plus en amont (Bryan et al., 1987). La tolérance de *H. diversicolor* est bien documentée (Berthet et al., 2003, Mouneyrac et al., 2003). Sa base génétique a été établie (Grant et al., 1989, Hateley et al., 1989). On en sait moins sur la tolérance en métal du bivalve, mais alors que des concentrations de Zn aussi élevées que 3160 mg kg-1 ont été déterminées dans les échantillons vivant dans la Restronguet Creek (Bryan et al., 1985), les concentrations de près d'un ordre de grandeur inférieur (651 mg kg-1) étaient suffisamment élevées pour provoquer une létalité dans des échantillons provenant d'un site relativement

propre (Amiard, comm. pers.). Au contraire, dans l'estuaire de la Seine multi-pollué, le suivi de la macrofaune benthique sur deux décenies a montré une diminution des populations de *H. diversicolor* (Bessineton, 2009) alors que les populations de *S. plana* n'ont pas été touchées (Bessineton, Comm. Pers.)

Etant bioaccumulatrices et relativement résistantes à divers polluants, *H. diversicolor* et *S. plana*, dont nous avons souligné la place dans les réseaux trophiques estuariens, font typiquement partie des espèces à risque pour la contamination des réseaux trophiques (Amiard-Triquet et al., 2011). Le bivalve *S. plana* est peu consommé mais il est cependant pêché de façon artisanale (Poutiers, 1987). Ce rôle de vecteur de transfert du sédiment vers les niveaux trophiques supérieurs a été mis en évidence pour un contaminant particulièrement préoccupant en raison de sa capacité de bioamplification, le mercure (Coelho et al. 2008), pour lequel les deux espèces montrent une capacité de bioaccumulation (Cardoso et al., 2009).

Pour des contaminants organiques comme les HAP, la revue de Jørgensen et al. (2008) a établi que certains invertébrés, parmi lesquels des polychètes marins, étaient capables de les biotransformer en vue de les éliminer. Au cours de se processus, des métabolites intermédiaires toxiques (génotoxicité, carcinogénicité) peuvent se former et induire des dommages biologiques. Plusieurs études ont montré qu'il y avait alors un risque de transfert trophique de cette toxicité. La présence de HAP dans le contenu digestif de poissons se nourrissant sur le benthos a été corrélée avec une incidence élevée de tumeurs chez ces espèces (Chandler et al., 1997). Chez un poisson plat nourri de polychètes contaminés dans des sédiments naturellement contaminés ou dopés en HAP (benzo[a]pyrène) et composés organochlorés (pp'DDE, un produit de dégradation du DDT et Aroclor 1254 comme représentant des PCBs), la croissance a été affectée par rapport aux témoins nourris de polychètes non contaminés et des dommages à l'ADN (adduits) ont été mis en évidence dans le foie (Rice et al., 2002). De façon similaire, un transfert trophique de génotoxicité a été montré chez des rats nourris avec des bivalves contaminés en nature après l'accident d'un navire pétrolier (Lemière et al., 2005).

2.3 La problématique des biomarqueurs : avantages et inconvénients

Depuis plus de deux décennies, les recherches se sont intensifiées pour le développement et la validation de biomarqueurs en tant qu'outils fiables capables de révéler l'état de santé du milieu. Ils se sont révélés utiles pour évaluer non seulement l'exposition des individus aux toxiques présents dans l'environnement, mais aussi l'effet de ces derniers chez les organismes exposés. Selon la nature des réponses induites, les biomarqueurs ont d'abord été classés en trois grandes catégories (Lagadic et al., 1997) : i) les biomarqueurs d'exposition qui rendent compte d'une exposition récente ou passée d'un individu à au moins une substance à caractère polluant, indiquant qu'il y a eu une pénétration du ou des polluant(s) dans l'organisme ; ii) les biomarqueurs d'effet de l'exposition aux xénobiotiques ; iii) les biomarqueurs de sensibilité aux effets des xénobiotiques. Or les scientifiques ne s'accordent pas toujours quant aux définitions attribuées à ces différents types de biomarqueurs (De Lafontaine et al., 2000, Amiard et Amiard-Triquet, 2008). En effet, la notion de biomarqueurs d'exposition et d'effet semble indiquer qu'il existerait nécessairement un lien direct de cause à effet entre l'exposition à un xénobiotique et son effet, ce qui n'est pas toujours vérifié (De Lafontaine et al., 2000). De nos jours, les écotoxicologues s'accordent plutôt à les regrouper en deux catégories comme l'ont préconisé De Lafontaine et al. (2000) :

- Les biomarqueurs de défense (induction de la métallothionéine, augmentation des processus de biotransformation comme l'activité EthoxyRésofurine-O-Dééthylase, EROD etc.) qui regroupent les différents processus biochimiques mis en œuvre par les organismes exposés pour compenser l'action des polluants dans leur environnement. Ce sont des réponses de type adaptatif qui traduisent une « souffrance » de l'organisme exposé qui se bat pour faire face à la présence de ces xénobiotiques (Lagadic et al., 1997).

- Les biomarqueurs de dommage (génotoxicité, lésions cytoplasmiques, inhibition de l'acétylcholinestérase, AChE), péroxydation lipidique, vitellogénine, etc.) qui sont les marques laissées par le passage des polluants dans l'organisme. Ils révèlent une atteinte biologique chez les individus exposés, qui peut remettre en cause les capacités

normales dont résulte la fitness (métabolisme de base, croissance, reproduction) et dans des cas extrêmes peut causer la mort des individus.

Les réponses biologiques fournissent des informations sur des échelles temporelles variées (Figure 7), depuis les réponses infra-individuelles précoces à court terme jusqu'aux conséquences sur les écosystèmes à long terme (Adams et al., 1989). Les biomarqueurs sont mesurés à des niveaux individuels et sub-individuels, contrairement aux indicateurs biologiques utilisés en écologie qui intègrent plutôt les réponses allant des populations aux communautés. Or au contact d'un xénobiotique, les premiers niveaux d'organisation biologique (moléculaires, cellulaires, individuels) de part leur sensibilité, seront les premiers à exprimer des effets mesurables liés à cette exposition contrairement aux niveaux organisationnels les plus élevés (populations, communautés) chez qui les effets observables impliquent souvent qu'un point de non retour a été franchi. En plus de ce caractère d'alerte précoce qui est l'un des atouts principaux de l'utilisation des biomarqueurs, ce sont des outils sensibles qui prennent en compte non seulement les effets synergiques, antagonistes ou cumulatifs de tous les polluants présents dans le milieu, mais aussi leur biodisponibilité (Durou, 2006).

Malgré ces avantages indéniables, et leur utilisation dans de nombreux programme de surveillance (OSPAR, MEDPOL), les biomarqueurs peinent à être intégrés dans la législation du fait de quelques inconvénients, à savoir :

- Leur grande variabilité due aux facteurs naturels. En effet les réponses biologiques observées peuvent être causées non seulement par le stress chimique, mais aussi par les paramètres physicochimiques du milieu et les paramètres biologiques de l'individu. Ainsi, des changements de température, salinité et même l'âge, le sexe le degré de maturité sexuelle etc. peuvent ainsi agir comme des facteurs de confusion et rendre l'interprétation des réponses observées plus complexe ou même caduque si ces derniers ne sont pas pris en compte.

- La remise en question de leur spécificité. En effet, les effets observés sur les organismes exposés ne sont pas toujours directement imputables à une classe de contaminants bien précise, ce d'autant plus que dans l'environnement, le cocktail de polluants présent implique certainement des effets de synergie ou d'antagonisme.

- La difficulté de relier les réponses observées au niveau individuel ou sub-individuel à des effets à l'échelle des communautés (manque de pertinence écologique). La figure 7 montre que des réponses liées à la reproduction sont détectables à tous les niveaux d'organisations biologiques. Sans aller jusqu'à la position de Tannenbaum (2005) qui considère que déterminer tout paramètre toxicologique sans lien avec la reproduction est un effort perdu, il est évident que le succès de la reproduction, conditionné par toute une série de réponses biologiques en cascade, est une clé de la conservation des populations et de la biodiversité.

- La difficulté de trouver de nos jours « un référentiel témoin » non contaminé qui donnerait des valeurs de base que l'on pourrait ensuite comparer à celles des sites impactés.

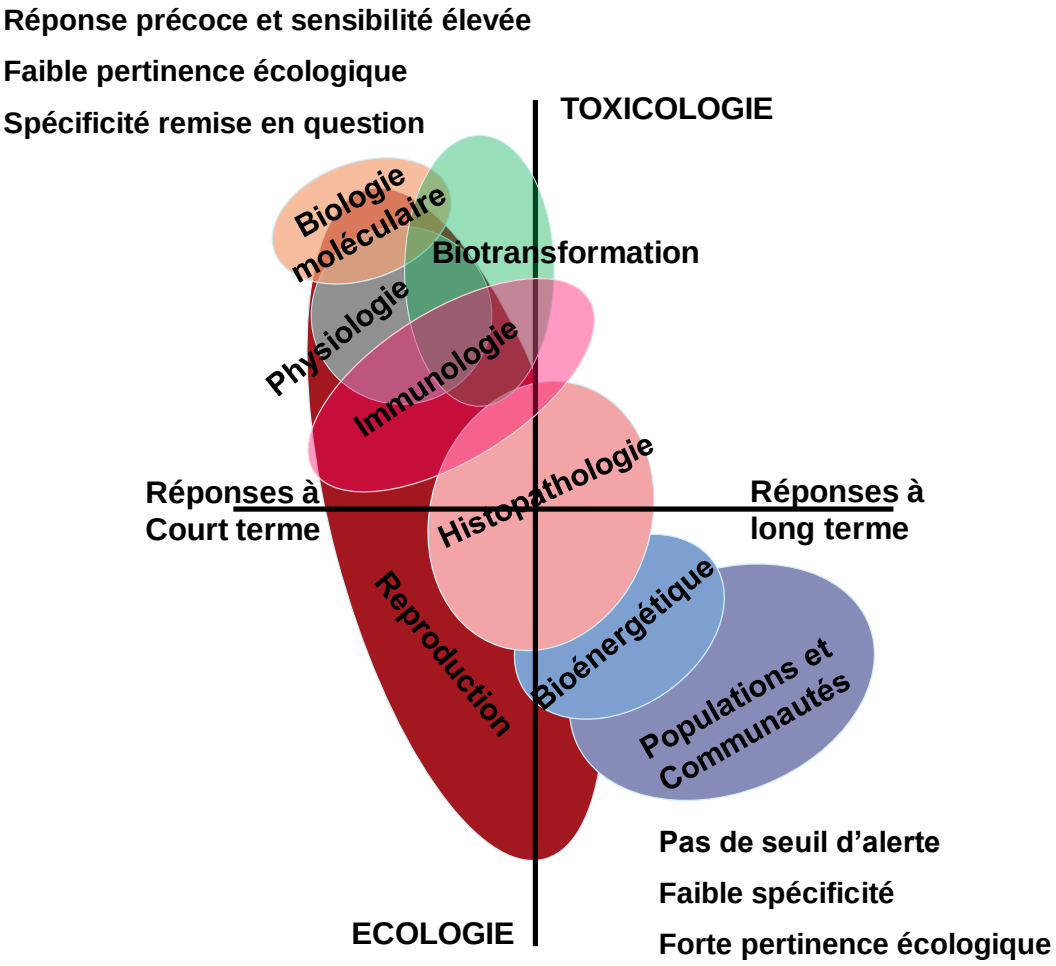


Figure 7. Organisation des propriétés des réponses biologiques au stress résultant de l'exposition aux polluants; (modifié d'après (Adams et al., 1989)

La biosurveillance a donc nécessité la mise au point d'outils permettant de : i) caractériser les composantes biotiques et abiotiques de l'écosystème perturbé par les contaminants; ii) d'en estimer les modifications structuro-fonctionnelles, iii) de mesurer les effets au niveau de l'écosystème, des contaminants persistants et en mélange complexe dans les sédiments; iv) d'identifier les effets sur les individus d'une communauté cible; v) de rechercher les causes des effets observés aux différents niveaux de l'organisation biologique (individu, population, communauté); vi) d'établir des ponts entre les différents paramètres mesurés grâce au traitement statistique des données; de mettre en place un modèle conceptuel qui permettra d'intégrer les résultats obtenus, d'estimer l'état d'un écosystème et d'identifier les causes de perturbations; de fournir des outils de gestion précis, grâce à la validation de biomarqueurs, indicateurs de qualité et révélateurs des caractéristiques des composantes de l'écosystème dans un but de diagnostic écotoxicologique de sites déjà perturbés.

2.3.1 Les biomarqueurs classiques ou « Core Biomarkers »

Certains biomarqueurs ont fait l'objet de nombreuses études et validation en laboratoire et sur le terrain, ce sont les « core biomarkers ». Ils sont couramment utilisés par de nombreux laboratoires dans des études écotoxicologiques. Dans ce groupe, on retrouve l'activité éthoxyrésorufine-o-dééthylase (EROD), l'inhibition de l'activité de l'acéthylcholinestérase (AChE), les métabolites fluorescents de la bile (FACs) ainsi que la stabilité de la membrane lysosomale (SML) (Roméo et Giambérini, 2008).

2.3.1.1 Les biomarqueurs de défense

Dans cette catégorie, on retrouve :

- **L'activité EROD** : c'est le premier biomarqueur normalisé AFNOR (NF T90-385). C'est une enzyme monoxygénase à fonction mixte et dépendante du cytochrome P450 impliquée dans les mécanismes de détoxication des xénobiotiques (HAP, PCB coplanaires, dioxines) dont elle favorise l'excrétion en les rendant plus hydrosolubles (Phase I). Ces composés dangereux seront ensuite conjugués (Phase II) puis excrétés

par voie rénale, biliaire ou branchiale (Roméo et Giambérini, 2008). L'activité EROD est cependant mesurée plus souvent chez les poissons que chez les invertébrés où l'existence de ce mécanisme de détoxication a été longtemps mise en doute. Toutefois la pertinence de ce biomarqueur semble à présent bien établie chez les bivalves (Ramos-Gómez et al., 2011a). Chez l'annélide *Arenicola marina*, les mêmes auteurs montrent que l'activité EROD est accrue dans des sédiments pollués mais ils ne démontrent pas un lien indiscutable avec la présence de contaminants connus pour l'induire (Ramos-Gómez et al., 2011b).

- **Les enzymes de phase II**: Ce sont des enzymes membranaires ou cytosoliques qui favorisent les réactions de conjugaison. L'augmentation de leur activité traduit un accroissement des biotransformations de phase II, généralement liées à la présence de polluants organiques, insecticides organochlorés et PCBs que l'organisme cherche à excréter. Les enzymes impliquées dans ces biotransformations sont généralement la glutathion-S-transférase (GST), l'UDP-glucuronosyl-transférase (UDPGT) ainsi que les sulphotransférases. L'interprétation de l'activité EROD étant encore un sujet de discussion chez les inverténrés, l'exposition aux polluants organiques est souvent détectée par la mesure du taux de GST (Roméo et Giambérini, 2008) et c'est cette stratégie qui sera privilégiée dans le présent travail de thèse.

- **Les enzymes de phase III** : Ce sont des transporteurs membranaires qui participent à l'élimination des xénobiotiques modifiés ou conjugués par les enzymes de phase I et II. Ils constitueraient la première ligne des défenses des organismes face au cumul de composés organiques d'origine anthropique ou naturelle. Les transporteurs ABC (pour ATP-binding cassette) sont responsables de l'efflux de nombreux contaminants de l'environnement tels que des métaux (Cd, Zn), des pesticides (Buss et Callaghan, 2008), des PCBs et PAHs, etc (revue par Damiens et Minier in Amiard-Triquet et al., 2011). Ce processus a été détecté chez de nombreux organismes aquatiques incluant des invertbrés (dont les annélides et les bivalves) et des vertébrés (Bard, 2000). Des séquences complètes ou partielles de gènes ABC ont été publiées pour des mollusques, des échinodermes, des poissons et des amphibiens (Damiens and Minier in Amiard-Triquet et al., 2011). En s'appuyant sur des études *in vitro* et *in vivo*, ces auteurs ont mis en evidence que l'activité ABC-like dépendait de la concentration de

contaminants du milieu, pouvant donc être utilisée comme biomarqueur d'exposition. Une étude menée sur 43 sites le ong des côtes françaises a clairement montré que l'expression des proteines ABC chez les bivalves était reliée à l'exposition aux xénobiotiques (Minier et al., 2006).

- **Les métallothionéines (MT) :** elles interviennent dans les mécanismes d'homéostasie des métaux essentiels et de détoxication des métaux essentiels en surcharge et non essentiels. En présence d'un stress métallique elles « séquestrent » les métaux (Ag, Cd, Cu, Hg, V, Zn, …) au niveau de leurs groupements thiols (Thirumoorthy et al., 2007) afin de limiter leur fixation sur les sites actifs d'autres protéines, limitant ainsi leur dénaturation ou leur inactivation... Leur induction se produit lorsqu'il y a un excès de ces métaux dans l'environnement de l'organisme (Viarengo et al., 1993, Klaassen et al., 1999, Amiard et al., 2006). De plus, les propriétes anti-oxydantes des MT sont fréquemment évoquées mais plusieurs études expérimentales contradictoires ont été publiées sur cette question (Moreau et al., 2008). Ces auteurs ont montré que différentes isoformes de MT, présentes dans différents taxons depuis la bactérie jusqu'au mammifère, avaient des propriétés différentes. Un ouvrage récent a été consacré à ces ligands dans de nombreux taxons incluant des invertébrés et des vertébrés des écosystèmes marins et d'eau douce (Sigel et al., 2009).

- **Les défenses antioxydantes enzymatiques et non-enzymatiques** : les espèces réactives de l'oxygène (ROS) sont généralement des sous-produits issus du métabolisme oxydatif cellulaire mais leur production est augmentée par les polluants chimiques tels que les quinones, les métaux de transition, les herbicides bipyridyles etc. Elles peuvent engendrer chez les organismes exposés dans leur milieu un stress oxydant responsable de l'oxydation des composés lipidiques, protéiques et même de l'ADN, et pouvant conduire à des phénomènes d'apoptose (auquel cas il peut être aussi être considéré comme un biomarqueur de dommage) (Roméo et Giambérini, 2008). Pour s'en défendre, les organismes mettent en place des systèmes de captage des formes actives de l'oxygène qui peuvent être enzymatiques (e.g. catalase, superoxyde dismutases) ou non enzymatiques (glutathion réduit, vitamines A et E, caroténoïdes). Ces mécanismes ont été récemment passés en revue par Regoli et al. (in Amiard-Triquet et al., 2011)

- **Les protéines de choc thermique** : Initialement décrites comme répondant au stress thermique (Heat Shock Proteins, HSP en anglais), ce sont plus généralement des protéines de stress, dites molécules « chaperonnes » car elles protègent les autres protéines de l'organisme, par exemple d'une dégradation enzymatique; réparent les protéines endommagées; empêchent ou stimulent l'expression de certains gènes, pouvant ainsi jouer un rôle majeur dans la prévention des mutations et l'apparition de certains cancers. Sous l'effet de la chaleur ainsi que différents stress physiques, chimiques ou métaboliques, elles sont surexprimées pour faire face au stress. Pour plus de détails, le lecteur peu se reporter à la revue de Mouneyrac et Roméo (in Amiard-Triquet et al., 2011).

2.3.1.2 Les biomarqueurs de dommage

- **L'inhibition de l'activité de l'acétylcholinestérase** : l'AChE appartient à la famille des cholinestérases dont l'inhibition a été longtemps utilisée chez l'homme pour diagnostiquer des intoxications aux insecticides organophosphorés (OPs) et aux carbamates (Cs). Chez les espèces aquatiques, l'inhibition de l'AChE est causée par une exposition à ces mêmes familles de composés mais des contaminants autres que les pesticides (métaux, détergents, toxines algales, hydrocarbures) peuvent inhiber l'activité de l'AChE qui est également influencée par des facteurs naturels tels que la température et la salinité (Burgeot et al., 2010). Leiniö et Lehtonen (2005) considèrent en conséquence que l'inhibition de l'activité de l'AChE peut être considérée comme un biomarqueur généraliste de l'état physiologique des organismes. Cette inhibition cause l'accumulation de l'acétylcholine dans la fente synaptique, maintenant ainsi une transmission permanente de l'influx nerveux, pouvant entraîner la mort de l'individu (Xuereb et al., 2009a). Nous verrons ci-après que l'inhibition de l'AChE peut également affecter le comportement des individus.

- **Les marqueurs de perturbation endocrinienne** :

Avant de présenter ces biomarqueurs, nous rappellerons brièvement ce que sont les perturbateurs endocriniens : des substances exogènes capables d'interférer avec la synthèse, la sécrétion, le transport, la liaison, l'action ou l'élimination des hormones naturelles (Kavlock et

al., 1996). Ces substances ont différents modes d'action (Figure 8) incluant soit i) un mimétisme de l'action d'hormones naturelles (e.g. œstrogènes, testostérone, hormones thyroïdiennes etc.) ; ii) le blocage des récepteurs cellulaires des hormones, empêchant ainsi leur action ; iii) une action sur la synthèse, le transport, le métabolisme et l'excrétion des hormones, modifiant ainsi les concentrations d'hormones naturelles.

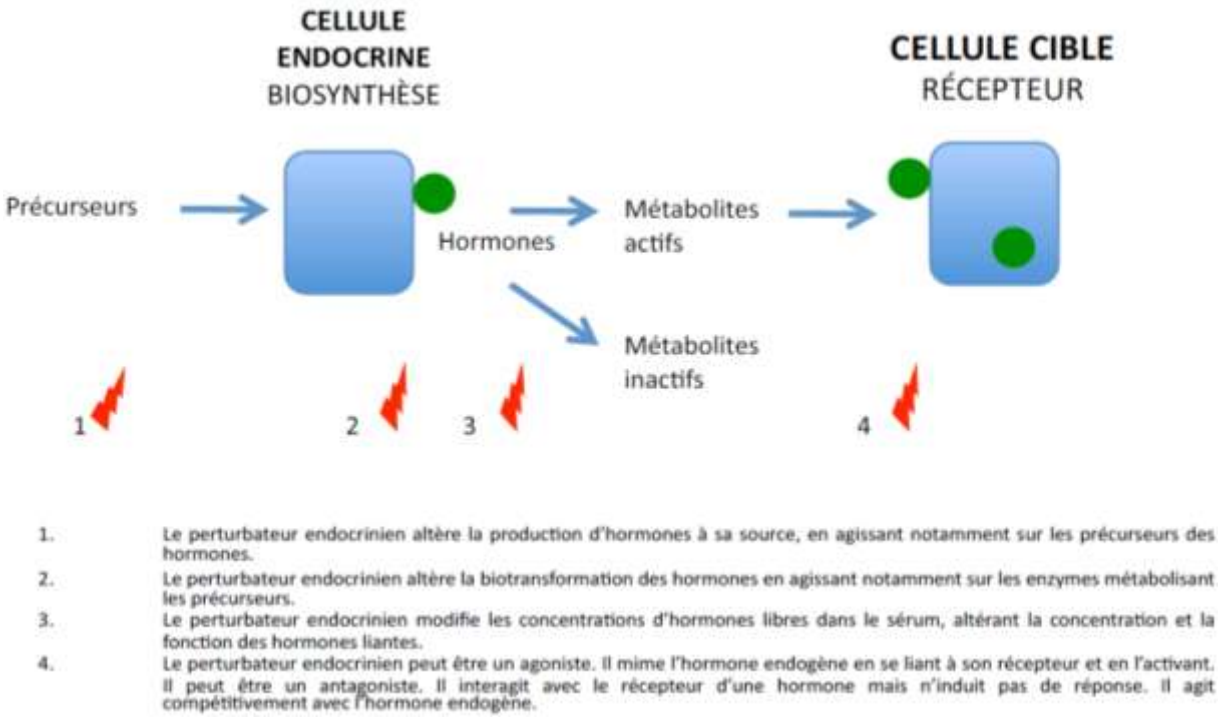


Figure 8. Mode d'action simplifié des perturbateurs endocriniens (Teixeira Alves, 2009)

- **La vitellogénine** : La vitellogénine (Vtg) est une protéine précurseur de la synthèse du vitellus (réserve énergétique utilisée au cours du développement embryonnaire chez les organismes ovipares). Elle est de ce fait fortement impliquée dans la reproduction. Sa présence chez les mâles et les femelles immatures est le signe d'une perturbation. La Vtg est induite chez les individus par la présence d'œstrogènes ou de substances qui miment l'action des œstrogènes tels que les phyto-estrogènes ou les hormones de synthèse (Aarab, 2004, Roméo et Giambérini, 2008). Selon Ferreira et al. (2009), l'induction de la Vtg chez les poisssons mâles est la méthodologie la plus largement utilisée pour détectée l'exposition aux perturbateurs endocriniens. Des protéines Vtg-

likes produites par les invertébrés comme l'huître sont très similaires à celles présentes chez les vertébrés (Matozzo et al., 2008). Les Vtg-like sont de ce fait des marqueurs utilisés pour la détection des perturbations endocriniennes chez des crustacés et des mollusques (Mills et al., 2001, Gagné et al., 2002, Lye et al., 2005, Matozzo et al., 2008, Xuereb et al., 2011), les approches impliquant l'expression génique ou le niveau de protéine.

- **L'imposex chez les gastéropodes :** L'une des conséquences de l'utilisation du TBT (tributylétain) dans les peintures anti-salissures a été le développement d'un pénis non fonctionnel chez de nombreux gastéropodes femelles, parmi lesquelles l'espèce la plus sensible est *Nucella lapillus* (Cf. Chapitre 1). Ce phénomène de masculinisation est spécifique au TBT et son intensité est proportionnelle au degré de contamination. Il est de ce fait utilisé comme biomarqueur spécifique du TBT notamment chez *N. lapillus* en raison de son extrême sensibilité, des concentrations de 0.3 $ng.L^{-1}$, difficilement détectables par des analyses chimiques étant capables d'induire les premiers signes d'imposex (Huet et al., 2009).

- **La stabilité de la membrane lysosomale** : Les lysosomes sont des organites cellulaires polymorphiques dont le rôle principal est la digestion des constituants intra- et extracellulaires, ainsi que d'organismes phagocytés. Ils contiennent de ce fait des enzymes hydrolytiques aussi nombreuses que variées qui en cas d'atteinte à la perméabilité de la membrane lysosomale, pourraient s'en échapper et causer des dégâts dans le cytoplasme. Or les lysosomes sont le réceptacle de nombreux polluants. L'accumulation de ces substances dans les cellules et leur prise en charge par les lysosomes donne lieu à une augmentation des activités enzymatiques de détoxication qui sont susceptibles d'entraîner une dégradation de la structure des polluants, leur bioactivation et une surproduction de radicaux libres susceptibles d'endommager la membrane lysosomale. L'évaluation de l'intégrité de la fonction physiologique de la paroi lysosomale constitue donc un outil de choix pour l'évaluation des effets des xénobiotiques chez les invertébrés (Dagnino et al., 2007) et les vertébrés (Broeg et al., 2005).

- **Les TBARS** : Lorsque les systèmes antioxydants enzymatiques et non enzymatiques évoqués ci-dessus ne parviennent pas à neutraliser les espèces réactives de l'oxygène, celles-

ci provoquent une dégradation oxydative des lipides. Les produits issus de cette dégradation sont de différente nature (radicaux lipidiques, alcoxyles, cétones, alcanes, aldhéhydes etc. Ces produits peuvent réagir avec l'acide thiobarbiturique et sont qualifiés de TBARS (substances réactives à l'acide thiobarbiturique) (Roméo et Giambérini, 2008). Le dosage des niveaux de TBARS dont le plus important est le malondialdéhyde (MDA) – représente donc une première approche de l'exploration du stress oxydant chez les vertébrés et invertébrés marins (Durou, 2006, Bergayou et al., 2009).

- **Les dommages à l'ADN** : Le maintien de l'intégrité de l'ADN est essentiel à la survie des cellules et des organismes. Sa structure peut cependant être endommagée par des xénobiotiques à caractère génotoxique. Ces dommages à l'ADN sont causés par les espèces réactives de l'oxygène que l'organisme n'a pas réussi à excréter et sont d'ordre chimique (adduits) ou physiques (cassure d'un ou des deux brins de l'ADN pouvant conduire à la formation de micronoyaux). Les adduits peuvent avoir des effets biologiques néfastes lors de la réplication de l'ADN et/ou lors de sa traduction et générer des modifications de l'information génétique (recombinaisons, mutations) si les systèmes de réparation cellulaires n'arrivent pas à les éliminer de manière efficace (Jawich, 2006). Les marqueurs de génotoxicité ont été recensés par Vasseur (2008) qui concluent que la mesure des adduits est certainement l'indicateur dosimétrique le plus reconnu mais que le test Comet constitue un indicateur de sensibilité équivalente sinon supérieure tout en étant plus facile à mettre en œuvre. Ces auteurs soulignent également l'intérêt des techniques nouvelles de biologie moléculaire, souvent plus praticable chez les poissons que chez les invertébrés en raison d'une meilleure connaissance du génome. Chez le bivalve d'intérêt dans la présente thèse, *Scrobicularia plana*, des données préliminaires sur la réponse des cellules sanguines exposées à des xénoœstrogènes suggèrent que le test Comet pourrait être efficace pour rechercher des effets génotoxiques (Petridis et al., 2009). Chez le second modèle biologique de cette thèse, *H. diversicolor*, le test Comet a été utilisé avec succès pour révéler les effets génotoxiques de l'argent sous forme ionique, de micro- ou de nano-particules (Cong et al., 2011).

Les biomarqueurs que nous venons de citer ne sont pas exhaustifs, il en existe de nombreux autres liés à la génotoxicité, aux perturbations du métabolisme énergétique, aux perturbations endocriniennes et comportementales qui sont en cours de développement et dont nous citerons des exemples dans la section « les biomarqueurs écologiquement significatifs).

2.3.2 Les biomarqueurs reconnus par la législation

Dans le cadre de l'élaboration et la mise en place des programmes de surveillance et d'évaluation, une stratégie pour un programme commun d'évaluation et de surveillance continue (JAMP) a vu le jour (OSPAR, 2006). Elle portait notamment sur l'élaboration : i) d'un programme coordonné de surveillance continue de l'environnement (CEMP) ; ii) d'un programme exhaustif de surveillance continue de l'atmosphère (CAMP) ; et enfin d'une étude exhaustive des apports fluviaux et des rejets indirects (RID). La mise en place du CEMP exige une collaboration entre tous les chercheurs et laboratoires de l'atlantique Nord Est impliqués dans les programmes de biomonitoring (http://www.ospar.org/content/content.asp?menu=30170301000000_000000_000000). Deux projets d'assurance ont ainsi été coordonnés par OSPAR-JAMP : BELQUAM (Biological Effects Quality Assurance in Monitoring Programmes, http://www.bequalm.org/eu_project.htm) pour les effets biologiques et QUASIMENE (Quality of Measurement in the Marine and Estuarine Environmment) dans le but de définir des critères standards de qualité communs à tous les laboratoires des pays membres d'OSPAR. Ceci implique la mise sur pied et la validation de protocoles relatifs aux méthodes biologiques et chimiques utilisées pour la surveillance de l'environnement marin, l'établissement de limites de variation acceptables pour chaque méthode développée, ainsi qu'un système de suivi de la conformité aux normes de qualité des laboratoires participants. Le programme des nations unies pour l'environnement (PNUE) a initié en 1995 un plan d'action pour la Méditerranée (MED POL : Programme coordonné de surveillance continue et de recherche en matière de pollution dans la Méditerranée) avec pour objectif d'assister les pays signataires de la convention de Barcelone dans la mise en place de programmes de surveillance de la pollution au travers du monitoring de la pollution marine, la vérification de la conformité aux normes ainsi que du monitoring des effets biologiques (http://www.unepmap.org/index.php?module=content2&catid=001001002). Dans le cadre de ce programme, des exercices d'intercalibration de dosage de certains biomarqueurs ont été effectués (EROD, MT, SML : stabilité de la membrane lysosomale) (Viarengo et al., 2000), et un manuel résumant différents protocoles de mesure des biomarqueurs recommandés pour la biosurveillance a été publié en 1999 par une action conjointe l'UNEP/RAMOGE (Programme de coopération entre la France, Monaco et l'Italie) (UNEP/RAMOGE, 1999). A la suite de ces

différentes études écotoxicologiques menées dans des sites ateliers sur des poissons, des moules et des gastéropodes, seul un nombre restreint de biomarqueurs a été validé et est pris en compte dans les réglementations nationales et internationales ; Il s'agit de ceux qui à l'issue de ces tests standardisés ce sont montrés parfaitement reproductibles quel que soit l'expérimentateur, dans des conditions expérimentales clairement définies. Ils sont à présent couramment utilisés par les réseaux de surveillance (OSPAR, MED) et sont recommandés pour la mise en ouvre de la Directive Cadre Stratégie pour le Milieu Marin (DCSMM).

Parmi ces biomarqueurs ceux validés par OSPAR chez les le poisson, les moules, ou les gastéropodes sont : l'activité EROD, les adduits à l'ADN, le taux de MT, la stabilité de la membrane lysosomale (SML), les métabolites de HAP dans la bile, la vitellogénine (Vtg), l'acide amino-lévulinique déshydratase (ALA-D), l'activité AChE, l'imposex, les pathologies externes et les pathologies du foie. Ceux validés par MED POL-RAMOGE chez la moule sont : la SML, la catalase, l'AChE, le glutathion réduit (GSH), la MT, le « Stress on stress », le « Scope for growth », les micronoyaux, les dommages à l'ADN (UNEP/RAMOGE, 1999, ICES, 2007, 2009).

2.4 Les biomarqueurs écologiquement significatifs

L'un des obstacles majeurs de l'intégration des biomarqueurs dans la législation relative au biomonitoring réside en la difficulté de lier des dysfonctionnements subcellulaires ou individuels à des conséquences à l'échelle de la population et des écosystèmes. De ce fait depuis quelques années, les scientifiques ont recherché et mis en exergue des « biomarqueurs à valeur écologiquement significative » dans le sens où ils permettraient de faire le lien entre les niveaux de l'organisation biologique, mais surtout de prédire les effets populationnels. Ces biomarqueurs sont basés sur tous les processus susceptibles d'affecter le succès de la reproduction directement (dommages à l'ADN, perturbations du système endocrinien) ou indirectement (perturbations du métabolisme énergétique et du comportement). Nous présentons ci-après, quelques uns de ces biomarqueurs.

2.4.1 Les biomarqueurs comportementaux

La détection des perturbations comportementales est un outil sensible qui renseigne sur l'impact des contaminants à des concentrations bien en dessous des niveaux léthaux. Ce sont des réponses au niveau individuel qui peuvent être reliées à des effets populationnels. Les effets en cascade depuis les altérations infra-individuelles jusqu'aux effets populationnels ou même au niveau de la communauté ont donné lieu à plusieurs synthèses (Weis et al., 2001, Amiard-Triquet, 2009, Weis et al., 2011). Les origines des perturbations du comportement sont variées, incluant le dysfonctionnement de la perception sensorielle (perception des phéromones, orientation, etc.), des perturbations du métabolisme énergétique retentissant sur l'activité locomotrice de déplacement, des perturbations hormonales (en particulier thyroïdiennes) affectant l'activité générale mais ce sont les liens entre les perturbations du comportement et des troubles neurologiques qui sont les mieux documentés. Par exemple, une inhibition de l'AChE, suivie d'une baisse de la capacité à s'alimenter et de la nage ont été observées chez des gammares exposés au chlorpyrifos (pesticide organochloré) ou au methomyl (carbamate) (Xuereb et al., 2009b). D'autres auteurs notent également une diminution des performances et/ou du type de nage, de la prise alimentaire et de la croissance chez des poissons présentant une inhibition de l'activité de cette enzyme (Kumar et Chapman, 1998, Tierney et al., 2007). Chez l'isopode terrestre *Porcellio dilatatus*, une exposition au dimethoane a conduit à l'inhibition de l'activité de l'AChE qui s'est ensuite traduite par une baisse de l'activité locomotrice (Engenheiro et al., 2005). De nombreux travaux ont ainsi montré le lien entre l'inhibition de l'activité des cholinestérases et l'apparition des troubles de la locomotion et ou de l'alimentation (Hopkins et al., 2005, DuRant et al., 2007, Amiard-Triquet et al., 2009), bien que chez certaines espèces telles que l'annélide polychète *Hediste diversicolor*, des troubles de l'alimentation aient été observés sans modification de l'activité de l'AChE (Moreira et al., 2006). Weis et al. (2001) constatent un lien entre la diminution du taux de sérotonine dans le cerveau et des troubles comportementaux (lenteur, difficulté à capturer des proies et à échapper aux prédateurs) chez un poisson (*Fundulus heteroclitus*) provenant d'un site pollué.

Des perturbations comportementales de différente nature susceptibles d'affecter la « fitness » ont été observées chez des poissons préalablement exposés an laboratoire ou *in situ* à des substances neurotoxiques. Ces perturbations comportementales étaient : la baisse de la prise

en charge et la protection des juvéniles (Scholz et al., 2000), la diminution de la vitesse et de la capacité de nage (Bretaud et al., 2001), la diminution de la capacité d'évitement des prédateurs (Scholz et al., 2000), une augmentation de la mortalité due à la prédation (Labenia et al., 2007) ainsi que la diminution du taux d'alimentation (Pavlov et al., 1992, Sandahl et al., 2005). Palanisamy et al. (2011) rapportent des troubles comportementaux (respiration, locomotion, sécrétion de mucus) chez le poisson *Mystus cavasius* exposé à des effluents de galvanoplastie. D'autres auteurs ont observé des troubles comportementaux (nage, activité) chez des poissons estuariens (*Pomatoschistus microps*) exposés aux métaux, à des doses bien en dessous des valeurs de CL_{50} (Vieira et al. 2009).

Les conséquences de ces troubles au niveau supra-individuel ont bien été mises en évidence dans les estuaires nord-américains étudiés par Weis et al. (2001, 2011). Chez le poisson *F. heteroclitus*, les chercheurs ont enregistré des effets sur la croissance et un indice de condition plus faible (liés à l'alimentation) ainsi qu'une diminution de l'espérance de vie (aussi causée par la diminution de la capacité d'évitement face à son prédateur le crabe bleu). La baisse de la pression de prédation sur sa proie principale *Palaemonetes pugio* a favorisé cette dernière, entraînant une modification de la structure de la communauté.

2.4.2 Les biomarqueurs liés au métabolisme énergétique

McEwen et Wingfield (2003) suggèrent que les facteurs de stress augmentent le besoin énergétique de l'organisme et, quand ce besoin dépasse l'énergie que l'organisme animal peut acquérir de l'environnement, les effets délétères du stress deviennent apparents. C'est le cas de la mise en œuvre de certains mécanismes de défense face au stress qui sont métaboliquement coûteux (Calow, 1991) et impliquent une diminution de l'attribution de l'énergie disponible aux processus liés au métabolisme basal, ainsi qu'à la croissance et la reproduction (Holloway et al., 1990). Ce coût métabolique peut également provenir de la diminution de l'efficacité d'assimilation de l'énergie (Baird et al., 1990) ainsi que de la difficulté d'accéder aux sources d'énergie dans l'environnement (Hopkins et al., 2004). Plusieurs biomarqueurs du métabolisme énergétique comme le taux métabolique standard (SMR, *Standard Metabolic Rate*, mesure de la consommation d'oxygène), les niveaux de réserves énergétiques (glycogène, lipides, protéines), les indices de croissance (SFG, « Scope

For Growth », différence entre l'apport énergétique et la quantité allouée au métabolisme), l'allocation cellulaire énergétique (CEA Cellular Energetic Allocation, qui fournit des mesures de répartition de l'énergie au niveau cellulaire) ont été récemment documentés (Mouneyrac et al., 2011). Des travaux de Durou et mouneyrac (2007) font état d'une diminution des réserves énergétiques chez *H. diversicolor* originaire de l'estuaire de Seine en comparaison avec ceux d'un site de référence (l'estuaire de l'Authie). Cette diminution des réserves énergétiques pourrait correspondre au coût de la tolérance vis-à-vis des contaminants présents dans cet estuaire contaminé de Seine et pourrait avoir entraîné des effets populationnels (Durou et al., 2007b), voir écosystémiques au travers de la perturbation des activités de bioturbation (Gillet et al., 2008). L'inhibition de l'activité digestive après une exposition à des substances toxiques peut également conduire à des perturbations métaboliques et pourrait être liée à des effets au niveau de l'organisme. Par exemple, chez *Daphnia magna*, De Coen et al. (2001) ont montré des corrélations positives entre un ensemble d'enzymes digestives et les valeurs de CE_{50} déterminées après l'exposition de l'animal à divers contaminants. Une approche de modélisation énergétique comme le modèle *Dynamic Energy Budget* (DEB) (Kooijman et al., 2009) permet en outre l'extrapolation des effets du niveau individuel au niveau de la population (Lopes et al., 2005, Jager et Klok, 2010).

2.4.3 Les biomarqueurs de perturbation de la reproduction

Le succès de la reproduction étant indispensable à la pérennité des espèces, les éléments qui le contrôlent constituent des sources potentielles de biomarqueurs écologiquement significatifs. Un certain nombre de biomarqueurs de perturbations de la reproduction ont ainsi été développés dont la vitellogénine et l'imposex chez *Nucella lapillus* (Cf sous sections biomarqueurs de dommage et biomarqueurs reconnus par la législation). D'autres atteintes à la reproduction liées à la perturbation de l'activité hormonale, telles que l'intersexualité ont été retrouvées chez des mollusques bivalves (*Scrobicularia plana*, *Saccostrea glomerata*) exposés en conditions de laboratoire au 17α-ethinylœstradiol (Langston et al., 2007, Andrew et al., 2010). D'autres cas ont été décrits chez des bivalves en milieu naturel (Chesman et Langston, 2006, Gomes et al., 2009, Ortiz-Zarragoitia et Cajaraville, 2010, Gagné et al., 2011). De nombreux auteurs rapportent aussi des cas d'intersexualité chez des poissons

exposés en condition de laboratoire ou dans le milieu naturel à des substances capables de perturber le fonctionnement hormonal (Matthiessen et Sumpter, 1998, Koger et al., 2000, Minier et al., 2000, Gercken et Sordyl, 2002, Bjerregaard et al., 2006, Woodling et al., 2006, Kidd et al., 2007, Tetreault et al., 2011).

La stérilisation des femelles du gastéropode *Nucella lapillus* qui a entraîné des extinctions locales de populations (Bryan et al., 1987, Gubbins et al., 2010), constitue un exemple palpable de conséquence écologique liée à la présence de perturbateurs endocriniens dans l'environnement. Par modélisation (Miller et al., 2007) ou à l'occasion d'expériences *in situ* (Kidd et al., 2007), il a été montré un lien probable entre les altérations du taux de Vtg avec des effets populationnels. Chez les oiseaux, l'exposition au DDT a provoqué une diminution des capacités de reproduction, en particulier en altérant la calcification des œufs, phénomène dépendant des œstrogènes et de la vitamine D (Colborn et al., 1993). Des troubles de la reproduction chez l'alligator en Floride incluant des anomalies des organes génitaux mâles (micro-pénis) et des concentrations anormalement basses de testostérone chez les crocodiles mâles du lac Apopka ont été relevés après une contamination massive, en 1980, par un pesticide qui contenait du dicofol et du DDT (Guillette Jr et Gunderson, 2001).

Pour terminer cette sous-section, nous ferons le lien entre métabolisme énergétique et fonctionnement hormonal. Chez les bivalves, les réserves d'énergie sont nécessaires au développement normal des gamètes. Chez les femelles, l'ovogénèse se fait grâce aux apports de lipides, protéines, glucides et autres molécules véhiculées par l'hémolymphe (Pellerin et al., 2004). Or, de nombreux stéroïdes et neurohormones interviennent dans la remobilisation de ces réserves (Pellerin et al., 2004). Une perturbation et/ou une réduction de ces dernières chez les invertébrés aquatiques pourrait fort bien impacter le processus de gamétogénèse et influer sur le nombre ou la qualité des gamètes produits avec des conséquences éventuelles sur l'importance et l'état de santé de la descendance.

2.4.4 Les biomarqueurs d'immunotoxicité

Les xénobiotiques peuvent modifier les paramètres immunologiques d'un organisme lorsqu'ils interagissent avec le système immunitaire. Ces modifications du système immunitaire peuvent ainsi être utilisées comme biomarqueurs d'immunotoxicité. Parmi ces

marqueurs, nous pouvons citer entre autres : l'examen des tissus lymphoïdes, l'activité des macrophages, la prolifération lymphocytaire, les activités antioxydantes, la résistance des organismes etc. (Zelikoff et al., 2002, Luengen et al., 2004, Galloway et Goven, 2006). Les perturbations du système immunitaire pourraient conduire à une diminution de la résistance de l'organisme et le rendre plus vulnérable aux maladies ou autres facteurs de stress. L'immunosuppression due aux contaminants a été considérée comme un facteur potentiel des mortalités importantes recensées chez des mammifères marins (Isobe et al., 2011) et littérature citée par ces auteurs). Toutefois plusieurs études récentes montrent des interactions complexes entre le stress chimique et le rôle des pathogènes et à ce stade, les conclusions établies ne sont pas univoques (Nacci et al., 2009, Paul-Pont et al., 2010a, Paul-Pont et al., 2010b, Prosser et al., 2011).

2.4.5 Les biomarqueurs de génotoxicité

De nombreux xénobiotiques libérés dans l'environnement ont un potentiel génotoxique vis-à-vis des organismes vivants et constituent des menaces pour le maintien l'intégrité du matériel génétique cellulaire des cellules. Selon l'implication du génome dans le métabolisme cellulaire, l'exposition à des composés génotoxiques peut entraver le développement, la reproduction et la survie des individus et des populations (Cf sous-section 2.3.1.2). Plusieurs études récentes (Lewis et Galloway, 2009, Lacaze et al., 2010, Devaux et al., 2011) se sont intéressées aux conséquences de l'exposition paternelle à des substances génotoxiques sur le succès de la reproduction dans différents groupes zoologiques (annélides, bivalves, crustacés, poissons). Des dommages à l'ADN ont été observés sur le sperme de toutes les espèces testées, sans toutefois que le succès de la fertilisation soit affecté mais les effets à plus long terme incluaient des anomalies sévères du développement et chez le poisson *Salmo trutta*, deux mois après le traitement, une mortalité accrue était enregistrée et après un an, le taux de malformation était accru par rapport aux témoins (Devaux et al., 2011). Dans le milieu naturel, suite à l'accident pétrolier de l'Exxon-Valdez en Alaska affectant l'aire de reproduction du hareng du Pacifique *Clupea pallasi*, des aberrations de la mitose et des micronoyaux ont été observés chez les larves de cette espèce, des anomalies larvaires, des oedèmes, une diminution de la croissance larvaire et une augmentation de la mortalité. Trois à quatre ans plus tard, une déplétion de 75% de la population s'est produite mais en l'absence

de suivi jusqu'à cette date tardive, la génotoxicité des hydrocarbures n'a pas pu être fermement établie et on a évoqué une origine multi-factorielle (Vasseur et al., 2008).

2.4.6 Les biomarqueurs de stabilité de la membrane lysosomale

Nous avons précédemment parlé des effets du stress oxydant sur la stabilité de la membrane lysosomale et des conséquences qui peuvent découler de sa fragilisation (Cf. sous-section 2.3.1.2). Chez les huîtres, la stabilité de la membrane lysosomale des géniteurs parait directement corrélée à la viabilité des larves (Ringwood et al., 2004). La corrélation des paramètres lysosomaux avec des paramètres physiologiques ayant trait à la croissance ("scope for growth") ou à la lutte contre les radicaux libres (TOSC pour Total Oxyradical Scavenging Capacity) suggère que les biomarqueurs lysosomaux pourraient être indicatifs d'effets néfastes à des niveaux d'organisation biologique plus élevés (Moore et al., 2006).

2.5 L'approche multimarqueurs

Etant donné que les polluants sont le plus souvent présents sous forme de cocktail dans l'environnement, baser la biosurveillance sur l'étude de quelques biomarqueurs isolés, donc ne répondant qu'à un certain type de composé chimique, donnerait des informations incomplètes. De ce fait une approche, qualifiée d' « approche multimarqueurs » a été développée (Figure 9). Elle a pour principe l'utilisation simultanée de plusieurs biomarqueurs à différents niveaux de l'organisation biologique chez des espèces animales ou végétales pour diagnostiquer les effets des xénobiotiques chez les organismes exposés (Lagadic et al., 1997, De Lafontaine et al., 2000, Flammarion et al., 2000, Wosniok et al., 2005, Yeom et Adams, 2007, Hagger et al., 2008).

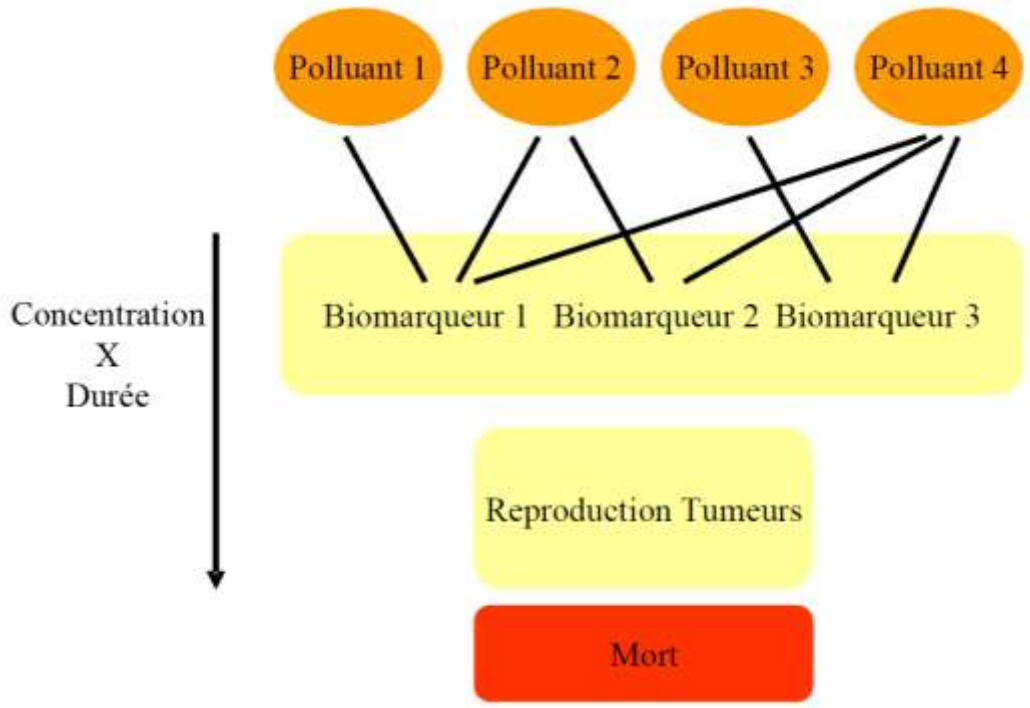


Figure 9. Caractère intégrateur de l'approche multimarqueurs (modifié d'après Flammarion, 2000)

Elle a pour objectif d'intégrer l'ensemble des effets du stress chimique subi par les organismes et est de plus en plus utilisée dans les programmes de surveillance. Elle se heurte cependant à des difficultés liées aux approches de terrain (Beliaeff et Burgeot, 2002) notamment la disponibilité des organismes qui peut limiter l'acquisition des données et rendre difficile l'utilisation des méthodes d'analyses statistiques multivariées. (Beliaeff et Burgeot, 2002) ont proposé un indice plus simple, la réponse biomarqueur intégrée (IBR : Integrated Biomarker Response), calculée sur la base de biomarqueurs analysés, qui permet de synthétiser les réponses obtenues, et d'évaluer ainsi le risque écologique. (Serafim et al., 2011) l'utilisent pour évaluer la variation temporelle de la qualité de deux estuaires portugais au travers de la mesure de biomarqueurs dans le foie du poisson *Pomatoschistus microps* et des contaminants dans le sédiment. L'IBR a permis d'identifier le printemps, l'été et l'automne comme étant les saisons où les biomarqueurs donnaient les réponses les plus fortes. Toutefois, l'utilisation de cet indice dépend des biomarqueurs choisis: en effet, lors de l'application de l'IBR pendant 3 années consécutives à des analyses de biomarqueurs (TBARs, CAT, GST, MT, AChE) et de contaminants bioaccumulés (Cd, Cu Zn, PCB, HAP) chez des moules transplantées sur des sites de degré de contamination différent, les auteurs se

sont rendus compte que l'IBR répondait surtout à la pollution en Cu et PCB, mais pas aux HAP. Il conviendrait donc de multiplier de nombre de biomarqueurs lorsque l'on utilise cet indice (Geret et al., 2003).

2.6 Les biomarqueurs utilisés dans cette étude

L'objectif principal de ce travail étant de relier les biomarqueurs à différents niveaux de l'organisation biologique, nous avons fait le choix d'utiliser certains des biomarqueurs écologiquement significatifs décrits précédemment. La figure 10 présente l'effet en cascade du stress à différents niveaux de l'organisation biologique. Nous sommes partis du postulat selon lequel lorsque des organismes sont soumis à un stress chimique, il peut s'ensuivre une perturbation de l'activité de l'acétylcholinestérase (action neurotoxique). La diminution de l'activité de cette enzyme peut engendrer une perturbation du comportement locomoteur (enfouissement) et/ou d'alimentation (les perturbations du comportement pourraient aussi être une réaction de fuite vis-à-vis d'un milieu contaminé). La baisse de l'activité locomotrice rend les organismes plus vulnérables face à leurs prédateurs, et une trop forte pression de ces derniers pourraient contribuer à l'extinction locale de l'espèce proie. Les perturbations de l'alimentation peuvent influer négativement sur le métabolisme énergétique et accroître ainsi des perturbations comportementales par un effet de feed-back. Le stress chimique peut aussi causer un dysfonctionnement de l'activité des enzymes digestives qui peut conduire à des problèmes d'assimilation des aliments ingérés et être à l'origine de perturbations du métabolisme énergétique. Le coût énergétique nécessaire à l'induction des mécanismes de défense pourra être minimisé ou au contraire exacerbé en fonction de la quantité de nourriture disponible dans le milieu. On s'attend donc à une modification de l'allocation énergétique au détriment de la croissance et/ou la reproduction, avec des effets potentiels sur la population (Calow, 1991) comme cela a été illustré en Seine pour l'annélide polychète *Hediste diversicolor* (Durou et al., 2007b). Notre ambition au cours de ce travail a été de valider le schéma conceptuel de la Figure 10 pour deux espèces d'invertébrés estuariens sélectionnés comme modèles selon les critères explicités dans les sous-sections 2.2.2 et 2.2.3. Dans une démarche d'évaluation écologique, il ne suffit pas de mesurer des biomarqueurs sensibles et qui soient en lien direct ou indirect avec le devenir des populations des organismes modèles. Cette approche sera d'autant plus pertinente que les signaux d'alerte fournis par les

biomarqueurs renseigneront sur le risque potentiel auquel se trouvent exposés des organismes importants pour la structure et le fonctionnement de l'écosystème.

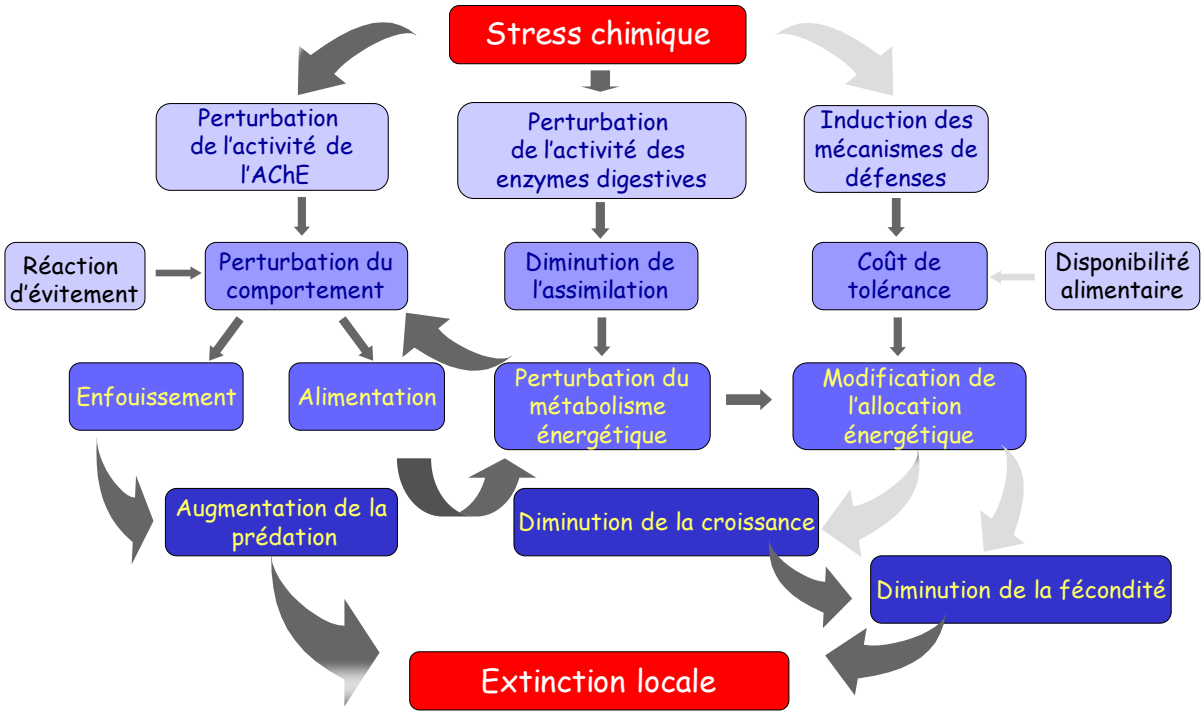


Figure 10. Effet en cascade du stress chimique à différents niveaux de l'organisation biologique

CHAPITRE 3. MATERIEL ET METHODES

3 CHAPITRE 3. MATERIEL ET METHODES

3.1 Présentation des sites d'étude

Dans cette étude, trois sites principaux et dix autres sites différant par leur degré de contamination ont été choisis sur les côtes nord-ouest de la France: la baie de Bourgneuf en tant que site de référence (46°56'23.08"N, 2°04'40.60"W), la pointe de l'Imperlay dans l'estuaire de la Loire (47°16'29.66"N, 2°07'59.76"W), l'estuaire de la Seine en tant que témoin positif (49°26'33.00"N, 0°16'03.18"E) et dix autres sites situés en Bretagne (les estuaires de la Vilaine, du Blavet, de la Laïta, de l'Odet, du Bélon, du Goyen, du Frémur, les rivières de Noyalo et de l'Hôpital Camfrout et la baie de Saint-Brieuc (Figure 11). Nous décrirons de façon succincte les dix estuaires Bretons et donnerons plus de détails sur les trois sites principaux.



Figure 11. Localisation des sites d'échantillonnage

3.1.1 La baie de Bourgneuf

La baie de Bourgneuf est située en limite côtière du Marais Breton qui est intégré dans le réseau Natura 2000. Cette zone constitue un site relativement exempt de pollution (Geffard, 2001, RNO, 2006), choisie comme référence dans cette étude. La baie de Bourgneuf se situe entre l'île de Noirmoutier et la pointe Saint Gildas (Figure 11). Cette baie est ouverte sur la mer, d'où la présence d'une influence océanique, mais en période de crue, le panache de la Loire peut y faire des intrusions. La turbidité et la salinité y sont élevées. La baie de Bourgneuf est peu industrialisée, les activités humaines qui y sont pratiquées sont principalement la pêche, l'agriculture extensive, la pisciculture, la conchyliculture, la saliculture et le tourisme. Les sources de pollutions possibles sont d'origine agricole et domestique. Cependant les eaux sont de qualité très acceptable selon le SAGE (2008) d'où le fait qu'il a été choisi comme site de référence pour cette étude.

3.1.2 L'estuaire de la Loire

La Loire est le fleuve le plus long de France. Elle traverse 10 régions et 31 départements et parcourt 1012 km sur un bassin versant de 118 000 km² représentant 1/5 du territoire national. L'estuaire de la Loire attire depuis longtemps les hommes en raison de sa biodiversité et sa proximité avec l'Océan Atlantique. La Loire est donc bordée de zones agricoles, de zones portuaires ainsi que d'agglomérations. Elle se retrouve ainsi chargée en nitrates et phosphates issus de l'agriculture, en molécules chimiques issues de l'industrie ou encore des rejets d'effluents urbains. Les aménagements urbains liés à la nécessité de desserte des pôles portuaires de Saint-Nazaire, ont rendu la partie nord fortement anthropisée. De plus, la rive nord est le siège d'un important pôle industriel (centrale électrique, terminal porte-conteneurs, chantiers navals, raffinerie…) contribuant à la dégradation du milieu. Elle a une fonction écologique très importante pour la faune et la flore : certaines espèces y vivent toute l'année, d'autre seulement pour une partie de leur cycle de vie. En effet, la Loire constitue un corridor migratoire, une zone de nourricerie, d'hivernage, de nidification, une aire d'alimentation ou encore un lieu de reproduction pour beaucoup d'espèces.

La pointe de l'Imperlay qui constitue le point de prélèvement en Loire suivi au cours de cette thèse est située en rive sud, face à la raffinerie de Donges. La rive sud, moins anthropisée que

la rive nord est bordée de vastes vasières intertidales abritant une faune endogée relativement abondante.

3.1.3 L'estuaire de la Seine

La Seine prend sa source sur le plateau de Langres (Champagne-Ardenne) et s'écoule sur 776 km jusqu'à son embouchure normande drainant un bassin versant de 79000 km². Elle compte environ 16 millions d'habitants sur son bassin versant, concentre 50 % du trafic fluvial, 40 % de l'activité économique et 30 % de l'activité agricole française. A l'exutoire de ce bassin versant, l'estuaire s'étend sur 160 kilomètres et draine un bassin versant de 11500 km². Il se situe entre le barrage de Poses en amont et la partie orientale de la baie de Seine en aval. L'estuaire de Seine reçoit des contaminants issus des deux ports maritimes d'importance internationale (les ports autonomes de Rouen et du Havre), de la population parisienne (10 millions d'habitants), celle de Rouen (400 000 habitants) et du Havre (250 000 habitants) (http://seine-aval.crihan.fr/web/attached_file/componentId/kmelia70/attachmentId/15133/lang/fr/name/Resume.pdf). L'estuaire comprend trois principales parties qui diffèrent par leurs caractéristiques physico-chimiques :

• L'estuaire amont ou fluvial, entre Poses (pk 202) et Vieux-Port (pk 325), dans lequel les eaux sont douces ;

• L'estuaire moyen, entre Vieux-Port et Honfleur, qui correspond à la zone du gradient de salinité (mélange des eaux douces et marines). C'est dans cette partie que se situe notre point de prélèvement en Seine, sur la vasière nord au niveau du pont de Normandie.

• L'estuaire aval ou marin, de Honfleur à la partie orientale de la baie de Seine, dans lequel les eaux sont de plus en plus franchement marines.

3.1.4 Les autres estuaires et baies de Bretagne

Neuf autres estuaires et une baie en plus des trois premiers sites précédemment décrits ont été échantillonnés dans le cadre de la recherche d'intersexe chez *S. plana*, et de perturbateurs endocriniens dans le sédiment de ces sites. Les dix sites (Figure 11) diffèrent les uns des autres, de part la surface du bassin versant, les activités pratiquées, le statut chimique et/ou écologique selon les données de la DCE (http://envlit.ifremer.fr/var/envlit/storage/documents/atlas_DCE/scripts/site/carte.php?map=LB). Le tableau 1 résume les principales caractéristiques de ces sites. Certains d'entre eux traversent de grandes villes (Seine, Loire), des villes de moyenne importance (Vilaine Noyalo, Blavet, Odet et baie de Saint-Brieuc) et de petites villes/et villages (Baie de Bourgneuf, rivière de l'Hôpital Camfrout, estuaires du Bélon, du Goyen et de Frémur). Mais l'un des points commun à tous ces sites est le fait qu'ils sont entourés de bassins versants de plus ou moins grande dimension, où sont pratiqués une agriculture plus ou moins intensive qui est source d'engrais et de pesticides, ces derniers étant soupconnés d'être des perturbateurs endocriniens. Etant donné la difficulté d'analyser toutes les classes de contaminants présents sur chaque site, nous nous sommes référés aux données du RNO et de la DCE pour estimer l'état de contamination global des sites (RNO, 2006).

Tableau 1. Caractéristiques des sites

Sites	Coordonnées GPS	Impact potentiel des cours d'eaux limitrophes	Impact potentiel des stations d'épuration	Densité des populations et impact	Importance de l'activité portuaire	Statut écologique de l'eau*	Statut chimique*
Baie de Bourgneuf	46° 56' 23.08" N, 2° 04' 40.60" O	Moyen	Moyen	Faible	Moyenne	Bon	Bon
Estuaire de la Loire	47° 16' 29.66" N, 2° 07' 59.76" O	Elevé	Elevé	Elevée	Elevée	Bon	Mauvais
Estuaire de la Vilaine	47° 29' 54.15" N, 2° 26' 22.22" O	Moyen	faible	Faible	Moyenne	Bon	Bon
Rivière de Noyalo	47° 37' 02.18" N, 2° 41' 07.9" O	Elevé	Moyen	Moyenne	Faible	Bon	Bon
Estuaire du Blavet	47° 46' 23.22" N, 3° 18' 1.67" O	Elevé	Elevé	Moyenne	Elevée	Moyen	Bon
Estuaire de la Laïta	47° 48' 13" N, 3° 31' 37" O	Moyen	Faible	Moyenne	Faible	Bon	Bon
Estuaire du Bélon	47° 49' 17.89" N, 3° 39' 04.47" O	Moyen	Faible	Faible	Faible	Bon	Bon
Estuaire de l'Odet	47° 58' 11" N, 4° 05' 48" W	Moyen	Moyen	Elevée	Moyenne	Moyen	Bon
Estuaire du Goyen	48° 02' 17"N, 4° 29' 16" O	Faible	Moyen	Faible	Faible	Bon	Mauvais
Rivière de l'Hôpital-Camfrout	48° 19' 15.36" N, 4° 15' 59.55" O	Elevé	Elevé	Faible	Elevée	Bon	Bon
Baie de Saint-Brieuc	48° 31' 35.3" N, 2° 37' 50,6" O	Moyen	Elevé	Elevée	Elevée	Médiocre	Bon
Estuaire du Frémur	48° 36' 18.1" N, 2° 07' 25" O	Moyen	Elevé	Moyenne	Faible	Médiocre	Bon

Sources : http://envlit.ifremer.fr/var/envlit/storage/documents/atlas_DCE/scripts/site/carte.php?map=LB

3.2 L'échantillonnage

Les animaux étaient récoltés précautionneusement, à la main à marée basse dans les 20 premiers centimètres du sédiment. A chaque sortie, du sédiment superficiel (≈ 0.5 cm) était prélevé en vue soit de l'analyse des contaminants, soit de la chlorophylle et des phéopigments utilisés comme « proxis » de la nourriture disponible. Lorsque des tests comportementaux étaient programmés, du sédiment (≈ 20 cm sous la surface) situé à la profondeur à laquelle se trouvaient les organismes était aussi prélevé pour l'analyse granulométrique. La salinité des flaques d'eau résiduelles à la surface des vasières à marée basse était mesurée comme préconisé par Amiard-Triquet et Dauvin (2009) (Amiard-Triquet et Dauvin, 2009).

Les organismes étaient transportés au laboratoire dans un conteneur isotherme afin d'éviter tout choc thermique. Le nombre d'individus prélevé était de 10/espèce pour l'analyse de chaque type de biomarqueurs. Dans le cas de l'analyse d'enzymes digestives chez la scrobiculaire, ce nombre passait à 12, car les organes (stylet cristallins et glande digestive) étaient groupés par 3. Pour les biomarqueurs comportementaux, le nombre de spécimens de chaque espèce était de 20 dans chaque condition testée comme recommandé par Bonnard et al. (2009). Les animaux destinés à l'analyse des biomarqueurs étaient choisis de taille comparable sur chaque site afin d'éviter toute influence due à la taille sur la réponse des biomarqueurs. Pour l'obtention des indices biométriques, les cinquante premiers individus étaient récoltés au hasard afin d'avoir un échantillon représentatif de toutes les classe de taille de la population. Les animaux destinés aux analyses de métaux étaient mis à dépurer pendant 48h afin d'éliminer toute contribution du sédiment ingéré sur le poids des animaux et le niveau de contaminants réellement incorporés.

Dans le cadre des prélèvements de scrobiculaires effectués pour la recherche d'intersexe, le nombre d'animaux récolté était de 30 bivalves adultes (taille de la coquille supérieure à 20mm) par site. Des travaux antérieurs menés par Langston et al. (2007) sur un échantillon de 150 individus ont en effet permis de vérifier qu'aucune information (sex ratio, indices biométriques, intersexe etc.) n'était significativement biaisée lorsque la taille de l'échantillon était restreinte à 30.

3.3 Aperçu des méthodologies mises en œuvre

La présente section n'a pas pour objet de détailler toutes les méthodes et techniques utilisées au cours de cette thèse mais de faire ressortir la pertinence des choix méthodologiques qui ont été réalisés pour concrétiser notre objectif de recherche tel qu'il apparaît au moins pour partie dans le schéma conceptuel présenté précédemment (Figure 10). Dans le cadre de notre démarche pluri-disciplinaire, ces méthodes et techniques sont nombreuses et diverses et pour plus de détails, il convient de se référer aux différentes publications.

3.3.1 Analyses chimiques

Les analyses de contaminants ont été menées pour caractériser le degré de contamination des sites d'études en complémentarité avec les données disponibles auprès des organismes officiels (RNO piloté par IFREMER, rapports des Agences de l'Eau dans le contexte de la Directive Cadre sur l'Eau).

3.3.1.1 Analyses de contaminants

Elles ont comporté des analyses classiques de métaux dans les tissus des organismes, en soutien à l'interprétation des données du biomarqueur métallothionéine. Elles ont été menées avec toutes les précautions nécessaires pour éviter les contaminations secondaires et ont été soumises à des contrôles de qualité internes (standard reference materials, NRC Canada) et externes (Exercice d'intercalibration entre laboratoires dédiés à l'environnement marin d'éléments traces et de méthylmercure chez la coquille Saint-Jacques : AIEA-452).

Des analyses des 16 HAP de l'US EPA, d'hydrocarbures totaux, de nickel et de vanadium ont été effectuées sur les sédiments de Loire et de Baie de Bourgneuf en vue de caractériser l'impact éventuel d'un petit déversement pétrolier intervenu au début des prélèvements exploités dans cette thèse. Les pétroles contiennent pour beaucoup d'entre eux des concentrations élevées en vanadium et en nickel. La détermination des teneurs en ces éléments peut nous renseigner sur une exposition aux pétroles comme l'ont démontré Amiard et al. (2004) suite à l'accident de l'Erika. Ces analyses ont été effectuées par l'Institut

Départemental d'Analyses et de Conseil (IDAC, Nantes)

Sur le sédiment estuarien superficiel récolté en parrallèle des scrobiculaires pour les travaux relatifs à la recherche de perturbations de la reproduction, des analyses fines des contaminants organiques (HAP, PCB, OCP, PBDE ont été effectuées (Laboratoire de Physico Toxico Chimie LPTC, Université de Bordeaux I) en vue de faire le lien avec les tests cellulaires et les effets sur la physiologie et la reproduction des bivalves *Scrobicularia plana*.

3.3.2 Caractérisation des sédiments

Les concentrations globales de contaminants au sein des sédiments sont fortement influencées par certaines caractéristiques connues du sédiment comme la charge en matière organique ou la granulométrie. Dans la présente thèse, l'analyse du carbone organique total (COT) et l'analyse granulométrique ont été externalisées dans des laboratoires aux compétences bien établies (IDAC, Nantes et LPTC, Bordeaux). La granulométrie et la charge en matière organique étaient des paramètres d'intérêt non seulement pour l'interprétation du degré de contamination des sédiments mais aussi parce qu'il a été montré qu'elles influencent le comportement d'enfouissement (Bonnard et al., 2009).

Nous avons mentionné précédemment (Figure 10) que la disponibilité de la nourriture était un facteur important pour moduler le coût énergétique de la tolérance lié à la mise en œuvre des mécanismes de défense en présence de contaminants chimiques. Pour les espèces d'intérêt dans la présente thèse, le COT peut être utilisé comme partiellement représentatif des disponibilités alimentaires dans les sédiments superficiels et nous avons également pris en considération le microphytobenthos évalué à l'aide des analyses de chlorophylle *a* et de phéopigments.

3.3.3 Recherche de perturbation d'activité endocrine sur extraits de sédiment

Cette recherche a été menée grâce à des tests *in vitro* sur lignées cellulaires visant à mettre en évidence la présence de composés PE dans des extraits de sédiments.

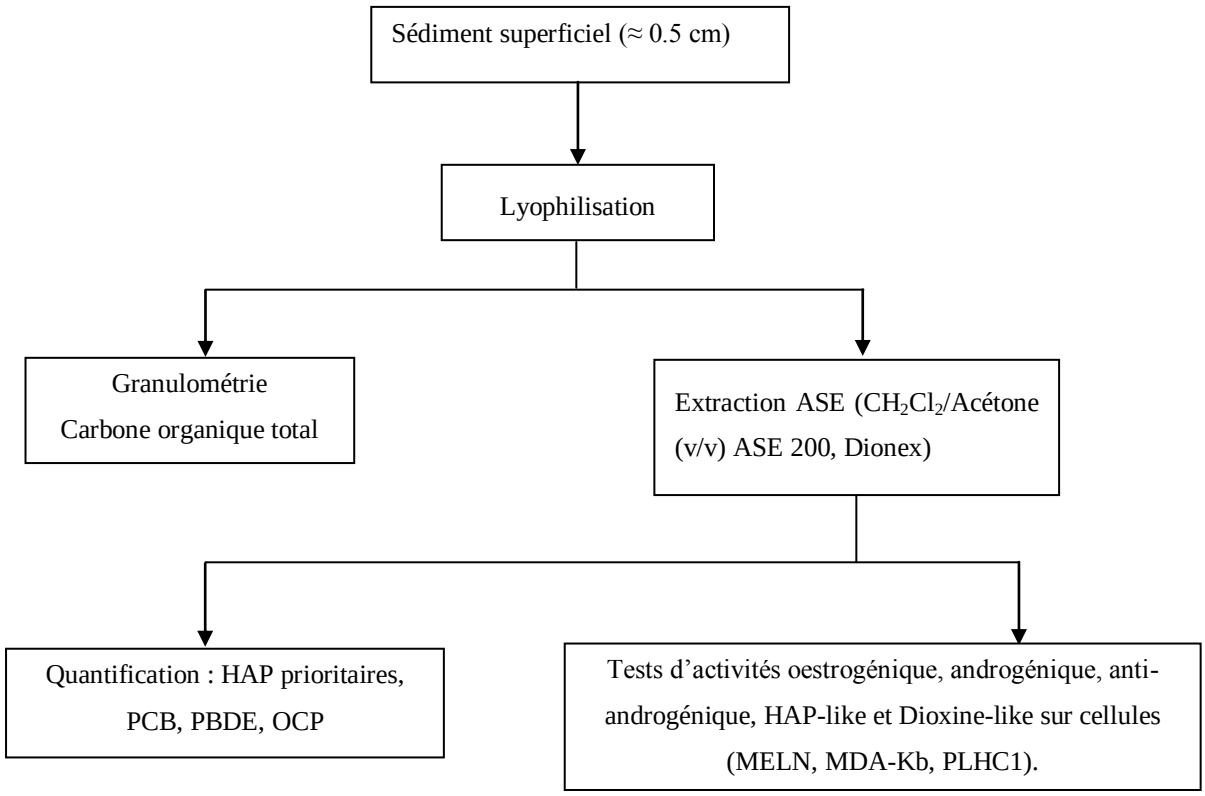


Figure 12. Schéma général des analyses chimiques biodirigées effectuées sur les sédiments dans le cadre de la recherche de perturbateurs endocriniens

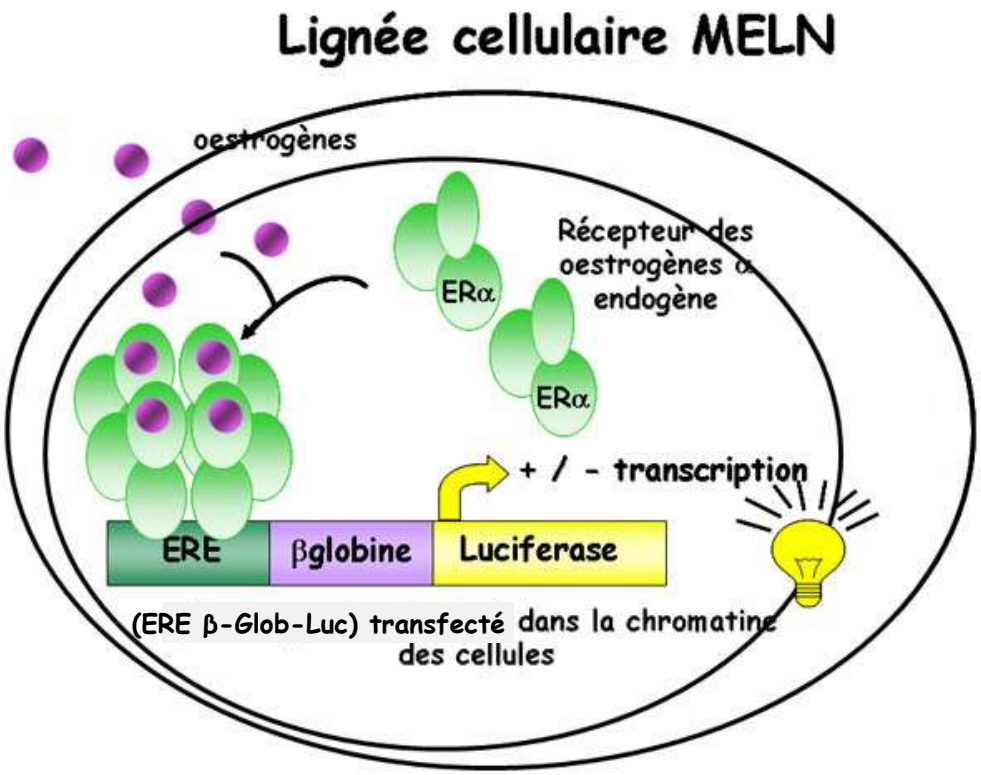
Les tests sur lignée cellulaire reposent sur la capacité d'un composé à se lier au récepteur, et à activer la réponse du gène rapporteur associé à ce récepteur. Les différents récepteurs cibles concernés par cette étude sont : les récepteurs des œstrogènes (ERα et ERβ), des androgènes (AR) et de la dioxine (AhR : aryl-hydrocarbone). Ce sont en effet les voies les plus classiques de la perturbation endocrinienne (Pillon et al., 2005). Les ligands (molécules d'origine endogène ou exogène) se lient à ces récepteurs, ce qui les activent et entraîne une cascade d'événements cellulaires (Pillon et al., 2005). Les molécules environnementales qui se lient aux ER sont d'origine naturelle (humaine, animale ou végétale), pharmaceutique, industrielle ou agricole (Kuiper et al., 1997). Celle se liant aux AhR sont essentiellement d'origine urbaine ou industrielle (Denison et Nagy, 2003). Ces deux récepteurs se lient sur les promoteurs de gènes qui contiennent leur séquence de reconnaissance respective (ERE pour "élément de réponse aux estrogènes" et XRE pour "élément de réponse aux xénobiotiques"). Dans certains cas, les récepteurs ER et AhR s'associent en un hétérodimère capable d'induire l'expression de gènes estrogénodépendants après activation par un ligand d'AhR (Ohtake et al., 2003). Le rôle du récepteur aux androgènes chez les organismes mâles est très similaire à

celui des récepteurs aux estrogènes chez les organismes femelles (Bursztyka, 2008).

3.3.3.1 Principe des tests cellulaires et lignées utilisées

Des lignées cellulaires bioluminescentes (MELN et MDA-kb2 (ATCC, #CRL-2713)) établies à partir de cellules issues d'adénocarcinome humain du sein (MCF-7 et MDA-MB-453 respectivement) ont été utilisées pour mesurer respectivement l'activité œstrogénique et les activités androgéniques et antiandrogéniques des extraits de sédiment. Les cellules MCF-7 qui expriment naturellement le récepteur aux œstrogènes α (ERα) ont été transfectées de façon stable avec un plasmide ERE-β-Glob-Luc (Balaguer et al., 1999). Le gène rapporteur luciférase (Luc), lié à des séquences estrogen response element (ERE) est sous le contrôle d'un promoteur β-globuline. Après fixation dans le complexe œstrogènes-recepteurs sur les séquences ERE, le gène rapporteur luciférase est transcrit et permet de détecter par luminescence l'activation dose dépendante des récepteurs œstrogéniques. La lignée cellulaire MDA-MB-453 qui exprime le récepteur aux androgènes (AR) a quant à elle été transfectée de façon stable avec un plasmide contenant le gène de la luciférase contrôlé par gène promoteur AR et MMTV (Mouse mamary tumor virus) (Wilson et al., 2002). Les activités dioxin-like ont été mesurées sur une lignée cellulaire PLHC-1 (ATCC, #CRL-2406) provenant d'un adénocarcinome hépatique de poisson (*Poeciliopis lucida*) (Ryan et Hightower, 1994). En présence de molécules activatrices, l'activation dose-dépendante du gène est quantifiée par luminescence (luciférase dans les cellules MELN et MDA-kb2) ou par fluorescence (activité EROD du CYP1A dépendante du récepteur Ah en cellules PLHC-1). Elle est proportionnelle à la concentration en ligands de ces récepteurs dans le milieu de culture. Les activités induites par les extraits sont comparées à celles de témoins positifs connus que sont :

- Le 17β-estradiol (E2) pour la mesure de l'activité œstrogénique
- Le dihydrotestostérone (DHT) pour la mesure de l'activité androgénique
- La flutamide pour la mesure de l'activité antiandrogénique
- Le benzo[a]pyrène (BaP) pour la mesure de l'activite HAP-like
- Le 2,3,7,8-tétrachlorodibenzo-p-dioxine (TCDD) pour la mesure de l'activité dioxine-like



ERE : Estrogen response element ; B-Glob : promoteur ; Luc : gène rapporteur

Figure 13. Mode d'action des œstrogènes et des dioxines

3.3.4 Biomarqueurs biochimiques

La métallothionéine a été utilisée comme biomarqueur de défense à l'égard des métaux uniquement chez le bivalve *S. plana*. En effet, si cette protéine existe bien chez *Hediste diversicolor* (Berthet et al., 2003), sa concentration ne s'accroit pas en présence de métaux (Poirier et al., 2006) son rôle de détoxication étant associé à un turn-over accru (Ng et al., 2008).

L'activité des enzymes digestives est utilisée comme indicateur précoce de perturbations potentielles du métabolisme énergétique. La technique utilisée au cours de ce travail a été l'objet d'un transfert de compétence entre le laboratoire d'Eco-Toxicologie de l'Université de Reims et notre laboratoire. La technique mise au point par (Dedourge-Geffard et al., 2009) pour les crustacés a été adaptée à l'annélide polychète *H. diversicolor* (Kalman et al., 2009) et

au bivalve *S. plana* (Boldina-Cosqueric et al., 2010).

Pour les biomarqueurs classiques (core biomarkers) les techniques utilisées sont établies de longue date et pour certains (LDH, GST, MT), nous avons bénéficié de la contribution de l'Institut de Biologie et Ecologie Appliquée (IBEA, UCO, Angers).

3.3.5 Biomarqueurs physiologiques

Les perturbations du métabolisme énergétique ont un rôle clé dans le succès de la reproduction. L'évaluation des réserves énergétiques était donc pertinente dans le contexte des effets en cascade représentés dans la figure 10. Les réserves énergétiques sous forme de glycogène, lipides et protéines ont été établies à l'IBEA.

Leurs altérations peuvent se traduire par des modifications de paramètres biométriques liés à l'état général de l'organisme, à la croissance ou à la reproduction. Chez les bivalves, les travaux de physiologie menés en lien avec les recherches en aquaculture ont permis de dégager des indices classiques qui ont été largement utilisés dans nos études (indices de condition, hépato-somatique et gonado-somatique). De manière comparable, il a été montré que chez les annélides, la relation longueur-poids pouvait renseigner sur le statut physiologique des organismes en lien avec la qualité du milieu (Durou et al., 2008). D'après les résultats de ces auteurs, les paramètres biométriques retenus ont été la longueur L3 (prostomium, peristomium et 1er sétigère) et le poids frais de l'organisme entier.

Pour l'étude du statut reproducteur et de l'intersexe des bivalves *S. plana* issus de sites du nord-ouest de la France, deux séries de prélèvements ont été réalisées pour se situer soit en début de phase de maturité sexuelle, soit au pic de la maturité sexuelle en se basant sur des études antérieures menées dans la région (Mouneyrac et al., 2008). Pour la première série d'échantillons, les gonades ont été examinées sur des coupes histologiques classiques qui ont permis la reconnaissance des sexes et de l'intersexe, et les stades de maturité sexuelle. Au pic de maturité sexuelle, cette technique n'est pas applicable et la reconnaissance des sexes et du degré d'intersexe a alors été réalisée sur des frottis selon la stratégie mise en œuvre par (Langston et al., 2007).

3.3.6 Biomarqueurs comportementaux

Les biomarqueurs comportementaux mis en œuvre dans cette thèse concernent l'enfouissement qui contribue à la défense des organismes contre leurs prédateurs et l'alimentation, source de toute l'énergie utilisée pour la maintenance, la croissance et al reproduction (Figure 10). La méthodologie utilisée pour étudier l'enfouissement chez nos deux espèces d'intérêt est dérivée de celle définie par (Bonnard et al., 2009). Par rapport à cette stratégie de base, nous avons proposé une amélioration qui consiste à séparer réponse à court terme (quelques minutes pour *H. diversicolor*, et une heure pour *S. plana*) évaluée par vitesse d'enfouissement et réponse à moyen terme (chez *S. plana* seulement, 7h) basée sur le nombre d'animaux enfouis ou non.

Les tests d'alimentation ont été élaborés pour des études *in situ* et en laboratoire par (Moreira et al., 2006) (ICBAS, CIMAR, Porto) et notre laboratoire a ensuite bénéficié d'un transfert de compétence (Kalman et al., 2009).

CHAPITRE 4. LES FACTEURS DE CONFUSION

4 CHAPITRE 4. LES FACTEURS DE CONFUSION

4.1 Contexte de l'étude

La limite que constituent les fluctuations des réponses biologiques utiles comme biomarqueurs de pollution à l'égard des facteurs naturels a été discutée précédemment. Avant toute étude d'envergure basée sur l'utilisation des biomarqueurs, il est donc indispensable d'évaluer l'importance relative des facteurs de confusion et en premier lieu, dans des milieux estuariens dont nous avons souligné la grande complexité de fonctionnement, l'influence de la salinité.

La taille apparaît *a priori* comme un facteur aisé à contrôler. Toutefois, en fonction du niveau de trophie de l'écosystème, de la pression des stress environnementaux anthropiques ou naturels, de l'absence de recrutement qui peut se produire certaines années (Santos et al., 2011b), il est quelquefois ardu de trouver des individus de taille similaire dans deux sites différents. De plus, une approche multi-biomarqueurs nécessite un échantillonnage important pour avoir une valeur statistiquement exploitable. L'expérience antérieure de l'équipe MMS a montré que pour les paramètres biochimiques, physiologiques et de contamination, il était indispensable d'avoir un effectif d'au moins 10 spécimens par paramètre (Durou, 2006), 20 spécimens étant nécessaires en ce qui concernent les tests de comportement (Bonnard et al., 2009).

La température est en mesure d'influencer les réponses biologiques et nous avons vu que ce facteur enregistrait des fluctuations à différentes échelles de temps en milieu estuarien. En nature, plusieurs auteurs ont abordé cette question en suivant les variations saisonnières des biomarqueurs (voir références citées dans la publication ci-après) et nous avons aussi développé cette stratégie (Cf. Chapitre 5). Toutefois, la variabilité saisonnière ne résulte que pour partie de la température car elle intègre aussi la luminosité, la ressource alimentaire qui en découle pour les espèces d'intérêt de cette thèse en terme de production primaire, et les stades de reproduction (Mouneyrac et al., 2008, Mouneyrac et al., 2010).

Chez la scrobiculaire, le sex-ratio est normalement de 1:1 (Hughes, 1971, Sola, 1997, Guerreiro, 1998, Rodríguez-Rúa et al., 2003, Raleigh et Keegan, 2006, Gomes et al., 2009). L'influence du sexe a été clairement reconnue chez *S. plana* pour certains facteurs

physiologiques comme les réserves énergétiques (Mouneyrac et al., 2008). Pour d'autres biomarqueurs comme la métallothionéine, l'influence du sexe a été examinée de façon détaillée. Chez le bivalve *Ruditapes decussatus*, le taux de MT était significativement corrélé aux taux de métaux chez les mâles et les femelles (Hamza-Chaffai et al., 2003). Toutefois, la probabilité était légèrement supérieure chez les mâles dont les taux de MT étaient moins affectés par la taille et les variations temporelles (statut reproducteur). D'un point de vue de faisabilité, il n'était toutefois pas possible de prendre ce facteur systématiquement en compte et le sexage n'a été effectué que pour les études focalisées sur la reproduction (Chapitre 5).

Des études récentes chez les bivalves d'eau douce *Dreissena polymorpha* (Minguez et al., 2009) et marins *Cerastoderma edule, Ruditapes philippinarum* (Paul-Pont et al., 2010a, Paul-Pont et al., 2010b) ont montré un certain nombre d'interférences entre la présence de pathogènes et des biomarqueurs comme : i) des changements de structure du système lysosomal et l'accumulation de lipides neutres chez la moule d'eau douce, ii) l'accumulation du cadmium, l'expression des gènes et les réponses immunes chez les bivalves marins. Une étude préliminaire a été menée en Baie de Saint Brieuc chez la scrobiculaire pour rechercher six espèces : le protozoaire cilié *Trichodina* sp. (100% de prévalence dans l'eau intervalvaire), les plathelminthes *Paravortex scrobiculariae* (100%, dans l'intestin), *Himasthla leptosoma* (100% sous forme de kyste dans le pied), *Meiogymnophallus minutus* (0%), *Proctoeces maculatus* (20,5% dans les branchies) et l'arthropode *Mytillicola intestinalis* (0,01% dans l'intestin) (De Rosa et Le Floch, UCO Bretagne Nord, Comm. Pers.). La stratégie n'était pas assez avancée pour être incluse dans les travaux de la présente thèse mais ultérieurement, il peut être recommandé de prendre ne considération ce facteur de confusion potentiel.

Pour l'annélide *Hediste diversicolor*, des études antérieures ont été menées pour évaluer l'importance des facteurs de confusion (taille, salinité, fluctuations temporelles) sur tous les biomarqueurs qui vont être utilisés dans le chapitre 5 d'après (Kalman et al., 2010).

En ce qui concerne un effet potentiel du sexe, il n'a pas été pris en compte ni dans les travaux antérieurs de l'équipe MMS ni dans la présente thèse, considérant que le sex-ratio environ 80%) était très largement en faveur des femelles (Dales, 1950, Olive et Garwood, 1981, Mettam et al., 1982).

L'un des challenges de l'application des biomarqueurs pour la surveillance des estuaires réside principalement dans la différenciation entre les réponses causées par la pollution de

celles résultant des facteurs de stress naturel.

Tableau 2. Réponses des différentes classes de biomarqueurs à la contamination et aux facteurs de confusion chez *H. diversicolor*. ND: non documenté; NS: pas de différence significative (Kalman et al., 2010)

	Contamination	Temperature	Weight class	Salinity
Water content	ND[1]	No seasonal pattern[1]	NS[1]	NS[2]
Burrowing	Decreased speed[3,4]	ND	NS[5]	NS[5]
Egestion rate	Decreased[3]	ND	> in larger worms[5]	> at higher S‰[5]
Post-feeding rate	Decreased[5,6,7]	Strong effect[6]	NS[5]	Moderate effect[6]
Glycogen	Decreased[8]	No seasonal pattern[8]	Generally higher in large specimens[8]	NS[9]
Lipids	Decreased[8]	No seasonal pattern[8]	Generally higher in large specimens[8]	NS[9]
Proteins	Decreased[8]	No seasonal pattern[8]	No consistent trend[8]	NS[9] or> at low S‰[5]
LDH	Increased[7]	ND	NS[5]	> at higher S‰[5]
GST	Increased[7,8,10,11]	No seasonal pattern[8,12] Limited effect[13]	NS[5]	NS[5]
AChE	Decreased[3,8,10,11]	No seasonal pattern[8]	≥ in small specimens[8]	NS[5] or transient effect[13]
CMCase	Decreased[3]	ND	NS[5]	> at higher S‰[5]
Amylase	Decreased[3]	ND	NS[5]	NS[5]

[1]Durou et al. 2008; [2]Poirier et al. Pers. Comm; [3]Kalman et al. 2009; [4]Mouneyrac et al. 2010; [5]Kalman et al. 2010; [6]Moreira et al. 2005; [7]Moreira et al. 2006; [8]Durou et al. 2007a; [9]Durou et al. 2007b; [10]Pérez et al. 2004 ; [11]Solé et al. 2009; [12]No seasonal pattern in *Laeonereis acuta* after Geracitano et al., 2004;[13]Scaps and Borot, 2000.

4.2 Publication 1

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Confounding factors (size, sainity) influencing biomarker responses in the estuarine tellinid clam *Scrobicularia plana*.

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RESEARCH ARTICLE

Potential influence of confounding factors (size, salinity) on biomarkers in the sentinel species *Scrobicularia plana* used in programmes monitoring estuarine quality

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Abstract
Introduction To use biomarkers in monitoring programmes, potential confounding factors must be considered. In the clam *Scrobicularia plana*, the influence of size and salinity on biomarkers at different levels of biological organisation has been examined.
Material and methods Biochemical (glutathione-S-transferase, lactate dehydrogenase, acetylcholinesterase, digestive enzymes, metallothionein), physiological (energy reserves) and behavioural (burrowing) responses were compared (a) in specimens of different sizes from the Loire estuary; (b) in specimens from the Belon estuary at two sites with salinities of 30.1 or 11.5.
Results Amongst the biomarkers able to reveal pollution effects, several are influenced by the size of the clams (Ag, Cu, Ni and glycogen concentrations, GST and AChE activities, condition indices). Salinity differences induced variations of the same order of magnitude (GST, AChE) or even higher (lactate dehydrogenase, digestive enzymes in the crystalline style) than contamination-induced variations. In burrowing tests, the number of burrowed specimens was similar at both salinities after an experiment time <3 h.
Conclusions Size is a factor necessarily but easily controlled. Because the weight may be different in clams of identical size, correction factors may be used to minimise the influence of weight changes on biomarkers. A correction factor taking into account salinity levels can also be used. The protein concentrations in the clams did not differ with salinity, a very favourable outcome since all enzyme activities are classically expressed by reference to total protein concentrations. For burrowing tests, the number of burrowed specimens at a particular time is an endpoint that is preferable to measures of burrowing speed.

Keywords Biomonitoring · Estuaries · Sentinel species · Biomarkers · Enzyme activities · Energy reserves · Burrowing tests · Confounding factors

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1 Introduction

Estuaries and adjoining coastal areas are important in the life cycles of many birds, fish and invertebrates, including commercially important species. Paradoxically, estuaries also represent habitats at risk through toxic anthropogenic effluents transported by rivers from remote and nearby conurbations and industrial and agricultural concerns. These areas are exposed to mixtures of well-known (mainly trace metals, PCBs, PAHs, pesticides) and emerging (such as PBDEs, pharmaceuticals, alkylphenols) contaminants. Chemical analysis may provide data on pollutant concentrations, although some can be measured only by expensive analytical techniques. Furthermore, total contaminant con-

centrations in sediment or water have poor ecotoxicological value since only a fraction of the total present will be bioavailable. Analyses based on biomonitor species or passive samplers can give access to contaminant bioavailabilities but again only a fraction of bioaccumulated chemicals can exert noxious effects on biota because of detoxification mechanisms in the organisms. Thus, a number of sensitive and early warning signals (biomarkers), providing information on the toxicity of pollutants on organisms have been proposed for use in environmental assessment and evaluation of the deleterious effects of contaminants on biota (Lagadic et al. 2000; Garrigues et al. 2001; Allan et al. 2006; Amiard and Amiard-Triquet 2008; Amiard-Triquet and Rainbow 2009). Despite a large number of scientific papers and monitoring programmes, the use of biomarkers in assessing environmental quality is still limited and even ignored in the Water Framework Directive (EC 2000). Several reasons can explain this, among which is the existence of natural fluctuations of biomarker measures that may concealed those changes attributable to differences in chemical stress. This spatial and temporal variability may be attributed to biotic and abiotic factors, called confounding factors, which include for example size of organisms, season, temperature, salinity (Handy et al. 2003; Hagger et al. 2006).

In general, parallel sampling in similar control or reference sites, sharing most natural factors with the impacted sites of interest, will help in the interpretation of monitoring data (Martínez-Gómez et al. 2010). Today, it is strictly no longer possible to find sites completely free of pollution, and even the location of sites only weakly impacted is problematic. Reference sites must be sought in zones remote from cities and from industrial concerns, leading, therefore, in the case of estuarine studies to choose estuaries of small size, the characteristics of which may differ strikingly from those of the more typically impacted larger estuaries, particularly in the case of salinity ranges. To validate the use of biomarkers, it is nevertheless indispensable to verify the importance of natural intersite differences in parameters that could interact as confounding factors.

Sediments are the main reservoirs for most contaminants; yet, they also provide habitat, feeding and breeding areas for a number of organisms. Nevertheless, in biomonitoring programmes, endobenthic species have not as yet been so widely used as organisms living in the water column. Therefore, in the present study, we have used the estuarine infaunal tellinid *Scrobicularia plana* to investigate the influence of salinity and organism size on biomarkers at different levels of biological organisation. This mollusc plays a key role in the structure and functioning of estuarine and coastal mudflats. It is a dominant species that plays a major role in the biogeochemical cycle of nutrients and contaminants as a consequence of its bioturbation activity. It is also an important link in estuarine foodwebs (Leitão et al. 2006; Lopes et al. 2006) contributing to the trophic transfer of contaminants (Coelho et al. 2008). Its biology and ecology are well known (Hughes 1969; Rodríguez-Rúa et al. 2003; Mouneyrac et al. 2008), and it is widely used in ecotoxicological studies (Byrne and O'Halloran 2001; Mazorra et al. 2002; Cheggour et al. 2005; Langston et al. 2007; Romero-Ruiz et al. 2008; Solé et al. 2009; Boldina-Cosqueric et al. 2010; Coelho et al. 2010). These features make it a relevant species in the study of confounding factors influencing biomarker responses.

The biomarkers studied have been chosen for their potential ecological value and their representativeness of different levels of biological organisation: biochemical (metallothionein concentration and activities of glutathione-S-transferase, lactate dehydrogenase, acetylcholinesterase, digestive enzymes), physiological (energy reserves as glycogen, lipids and proteins) and behavioural (burrowing) responses.

2 Material and methods

Clams used for the study of the influence of size were collected from the Loire estuary (France) in July 2008 (Precise location: 2°07′59.76″ W, 47°16′29.66″ N). The "small" and "big" specimens had very contrasted sizes and weights (Table 1).

Clams used for the study of the influence of salinity were collected from the Belon estuary (South Brittany, France) at two sites, near the mouth of the river (Moëlan Port: 3°42′03″ W, 47°18′34″ N) or upstream (Guilly: 3°39′03″ W, 47°49′17″ N). When collecting clams (May 2009), salinity was measured in the water remaining at the surface of the mudflat at low tide and was respectively 30.1 and 11.5. At each site, 50 clams were collected at random to determine the condition index and water content. Further clams, the shell length of which was between 2.9 and 3.8 cm, were collected for the measurement of biochemical, physiological and behavioural parameters.

Table 1 Biometric measurements of clams from the Loire estuary, France, used to study the influence of size on biomarkers

	Small specimens	Big specimens	Ratio big/small
Shell length (mm)	20 (2)*	31 (2)*	1.6
Total weight (g)	0.9 (0.3)*	3.8 (0.6)*	4.0
Soft tissue weight (g, ww)	0.25 (0.09)*	0.8 (0.2)*	3.2
Condition index	27 (5)*	21 (3)*	0.8
Gonado-somatic index GSI	9 (3)	10 (4)	1.1

Means and standard deviation in brackets (n=50 for each size class except n=10 for GSI)

*$p<0.05$ significant difference according to size

Sediment characteristics were determined in sediment samples from Guilly and Moëlan (about 20 cm deep, in agreement with the distribution of clams) by an official laboratory (Institut Départemental d'Analyse et de Conseil IDAC) working under Good Laboratory Practices (Table 2). Mixed samples (800 cm^3) were obtained by homogenising sediments collected at three different places within the immediate area of collection of the clams. Dry matter was determined by gravimetry according to the European standard method NF EN 12880 (X33-005), total organic carbon (TOC) by dry burning (NF EN 13137 (X30-404)). Grain size was determined by gravimetry according to the French standard method NFX31-107.

2.1 Biometric indices

The condition index (CI) of clams was determined according to the recommendation of the French Regulatory Agency (AFNOR 1985):

$$CI = (\text{wet weight of soft tissues/total weight}) \times 100 \quad (1)$$

The gonado-somatic index (GSI) was calculated using the following equation:

$$GSI = (\text{gonad weight/wet weight of soft tissues}) \times 100 \quad (2)$$

Then, the soft tissues were oven-dried at 100°C until constant weight. The water content was calculated using the following equation:

$$\text{Water content} = (\text{wet weight - dry weight/wet weight}) \times 100 \quad (3)$$

2.2 Metallothionein and metals

In order to eliminate their gut contents, clams (n=10 for each class size or each salinity) were allowed to depurate for 24 h at the temperature measured in sediment at the time of collection, in filtered seawater adjusted to the salinity of the site of origin. Soft tissues were homogenised and pellets (P1) and cytosol (C1) were recovered by centrifugation. Then metallothionein (MT) concentrations were determined by differential pulse polarography in the heated cytosol of the soft tissues according to the methods routinely used in the laboratory (Boldina-Cosqueric et al. 2010). Again according to these authors, metals were determined in C1 and P1 after digestion with suprapure concentrated nitric acid by flame atomic absorption spectrophotometry (Cu, Zn) or electrothermal atomic absorption spectrophotometry (Ag, Cd, Ni and V) with the Zeeman effect and the standard addition method in an iso-medium was used for calibration. The total concentration was recalculated from the concentrations in C1 and P1. The analytical methods have been validated through internal quality controls by using a standard reference material provided by NRC Canada (TORT-2, lobster hepatopancreas).

Table 2 Characterization of sediments from the two sites in the Belon estuary

	Guilly	Moëlan
Salinity	11.5	30.1
% Dry matter	35.5	51.6
Grain size distribution (g kg^{-1} d.w.)		
<2 (μm)	386	191
2–20 (μm)	331	136
20–50 (μm)	216	162
50–200 (μm)	72	424
200–2,000 (μm)	3	88
TOC g kg^{-1} C, d.w.	47.1	20.3

2.3 Determination of enzyme activities

Clams destined for enzyme activity analyses were dissected as soon as possible after sampling. The soft tissues were washed with filtered seawater to eliminate sediment and then kept at −80°C until analysis. For lactate dehydrogenase (LDH), GST and AChE, 10 clams from each size group or each condition of salinity were tested and determinations were carried out on the whole soft tissues. For digestive enzymes, 12 clams were dissected and digestive glands and crystalline styles were pooled (three organs for each sample), this was impossible in the case of the smaller clams, and so, the influence of size on digestive enzyme activity was based on analyses of the whole soft tissues (n=10).

The procedure for homogenization adapted for each enzyme and the recovery of supernatant have been described in detail by Boldina-Cosqueric et al. (2010). Enzyme activities were analysed in triplicate using a microplate reader as described by Galgani and Bocquené (1991) for AChE/ChE, Diamantino et al. (2001) for LDH, Frasco and Guilhermino (2002) for GST. The α amylase and cellulase activities were determined by the method of Bernfeld (1955) adapted by Boldina-Cosqueric et al. (2010). All the enzyme activities were calibrated to total protein concentrations which were measured in the soft tissue's supernatant by the method of Bradford (1976) adapted for microplate use with bovine γ-globulin as standard protein.

2.4 Glycogen and lipid quantification

After removal of the shells, the digestive glands and gonads of the clams were quickly dissected, frozen on dry ice and kept at −80°C until glycogen and lipid analysis. Glycogen and lipids were quantified in each organ from clams in each size group (n=10 per size group) or each condition of

salinity (n=10 per salinity level). After homogenization in liquid nitrogen, the powder obtained was then homogenised with 1.5 mL citrate buffer at pH 5.0. Total lipids were determined by a sulpho-phosphovanillin reaction according to the method of Frings et al. (1972). Glycogen concentrations were estimated in two aliquots of the homogenate, one of which was submitted to enzymatic digestion by amyloglucosidase according to Carr and Neff (1984). Olive oil and glycogen from oyster (Sigma, Type III) were used as standards for each method, respectively.

2.5 Behavioural tests

Behavioural tests were set up in a dark, temperature-controlled room (16°C) and conducted within 1 week of collection. Burrowing experiments were carried out as described by Bonnard et al. (2009). Briefly, 20 individuals were tested in two replicate experimental units which consisted of plastic containers filled with 5 cm of sediment and topped with 2 L of filtered aerated seawater. Animals were tested in the presence of the sediment of their site of origin. In addition, for specimens originating from two areas differing in salinity in the Belon estuary, cross tests were performed using the sediment of the other area.

2.6 Statistical analysis

Statistical analyses were performed using XLSTAT-Pro 2010. Normality of data was tested using Shapiro–Wilk's W test. Levene's test was used to check the homogeneity of variance. When the validity conditions were satisfied, comparisons of means were performed with a Student's t test (in the case of biometric indices) whereas the non-parametric Mann–Whitney test was used in all other cases (metals and biochemical markers). For behavioural tests, the burrowing kinetic curves were ln-transformed to linearize the data. These data were then compared by using analysis of covariance (ANCOVA) between regression coefficients of the least-square best-fit regression lines as recommended by several authors (De la Huz et al. 2002; Bonnard et al. 2009; Mouneyrac et al. 2011).The normality test was then performed on residuals, allowing the validation of the results obtained with ANCOVA. In all cases, the level of significance was set to 0.05.

3 Results

3.1 Biometric indices

The condition index was significantly higher in small specimens than in big ones (Table 1). The GSI did not differ between clams of different sizes (Table 1).

Water content was significantly higher for the bivalves collected at random (n=50 at each place) in the brackish area (85%, SD=1%) than for those originating from the marine area (83%, SD=1%) but the difference was only 2%. The mean condition index was not significantly different depending on salinity at the site of collection: considering specimens collected at random (n=50), means were 31 and 32 (SD=5 in both cases) respectively at salinities of 11.5 and 30.1; for specimens of selected size used for biomarker determinations (n=53), the means were, respectively, 31 and 32 (standard deviations 6 and 2). The mean GSI was also identical (n=22) at both salinities (GSI=11 with SD=5 and 3 at salinities of 11.5 and 30.1, respectively).

3.2 Energy reserves

Concentrations of energy reserves in the form of lipids and proteins were not affected by size differences whereas glycogen concentrations were much higher in both gonad and digestive gland of the smaller specimens (Table 3). No statistical differences were recorded between concentrations of energy reserves in clams from the two salinity regimes (Table 3).

3.3 Biochemical markers and metals

A significant influence of size was shown for GST and AChE activities, the former being lower in big specimens whereas the reverse was observed for AChE (Table 3). No significant differences depending on size were shown for LDH, digestive enzymes (CMCase and amylase) and MT concentrations. Metals analysed as explanatory factors for MT changes showed some significant differences according to size, Ag and Ni concentrations being higher in small specimens whereas Cu concentrations were higher in big ones (Table 4).

Many enzyme activities (LDH, GST, CMCase and amylase) were significantly higher in specimens from marine compared to brackish areas whereas the reverse was observed for AChE activity and MT concentration (Table 3). Three metals showed significantly different concentrations according to salinity, Ag and Zn concentrations being lower in marine specimens whereas V concentrations were enhanced (Table 4).

3.4 Behavioural tests

For each condition (clams in their sediment of origin or cross tests), the curves representing the percentages of organisms remaining unburrowed at each time of observation are depicted in Fig. 1. When clams were allowed to burrow in their sediment of origin, nearly all the individuals were burrowed within 3 h. However, burrowing was

Table 3 Influence of size (clams from the Loire estuary, Fr.) and salinity (clams of the Belon estuary, Fr.) on bioenergetics and biochemical markers

	Size		Salinity	
	Small	Big	11.5	30.1
Energy reserves				
Glycogen mg g^{-1} ww				
Gonad	38 (21)*	13 (8)*	41.8 (16.9)	43.7 (20.7)
Digestive gland	13 (8)*	2 (1)*	9.1 (2.8)	9.4 (4.6)
Lipids mg g^{-1} ww				
Gonad	7.99 (3.31)	7.55 (3.67)	10.1 (2.8)	10.8 (4.4)
Digestive gland	8.66 (3.3)	9.45 (2.33)	9.1 (2.2)	10.8 (2.9)
Proteins mg mL^{-1}	10.77 (1.17)	10.78 (2.17)	7.67 (1.81)	6.34 (1.18)
Biochemical markers				
MTLP µg g^{-1} ww	2800 (660)	2948 (208)	2156 (468)*	1757 (250)*
LDH				
nM min^{-1} mg $prot^{-1}$	19.16 (7.24)	16.75 (5.49)	10.6 (3.7)*	24.7 (6.6)*
GST				
nM min^{-1} mg $prot^{-1}$	89 (33)*	57 (17)*	30.6 (10.4)*	53.9 (13.4)*
AChE				
nM min^{-1} mg $prot^{-1}$	3.0 (0.7)*	4.2 (0.7)*	14.3 (4.1)*	9.3 (2.5)*
CMCase				
nM maltose min^{-1} mg $prot^{-1}$	0.004 (0.001)[a]	0.003 (0.002) [a]		
Digestive gland			0.004 (0.001)	0.003 (0.001)
Crystalline style			0.002 (0.000)*	0.004 (0.001)*
Amylase				
nM maltose min^{-1} mg $prot^{-1}$	0.011 (0.007) [a]	0.020 (0.007) [a]		
Digestive gland			0.012 (0.003)	0.010 (0.001)
Crystalline style			0.006 (0.003)*	0.014 (0.007)*

N=10 for each sample set for each parameter. Mean (standard deviation)

[a]Determination in the whole soft tissues

*p<0.05 significantly different either between size (columns 2 and 3) or between salinities (columns 4 and 5)

significantly faster at the highest salinity. In cross tests, both the burrowing speed and the final number of burrowed clams were significantly reduced in sediment of the other salinity (either 11.5 or 30.1) compared to the sediment of origin. In addition, burrowing speed was higher at the

Table 4 Influence of size (clams from the Loire estuary, Fr.) and salinity (clams of the Belon estuary, Fr.) on metal concentrations (mg kg^{-1} wet weight)

	Size		Salinity	
	Small	Big	11.5	30.1
Ag	0.69 (0.27)*	0.26 (0.06)*	0.08 (0.01)*	0.04 (0.01)*
Cd	0.03 (0.01)	0.03 (0.02)	0.04 (0.01)	0.04 (0.01)
Cu	7 (5)*	10 (4)*	5 (2)	6 (2)
Ni	7.9 (3.7)*	3.3 (1.3)*	0.6 (0.3)	0.8 (0. 2)
V	0.32 (0.04)	0.30 (0.10)	0.14 (0.06)*	0.78 (0.05)*
Zn	60 (56)	57 (19)	57 (19)*	30 (10)*

N=10 for each sample set for each parameter. Mean (standard deviation)

*p<0.05 significantly different either between size (columns 2 and 3) or between salinities (columns 4 and 5)

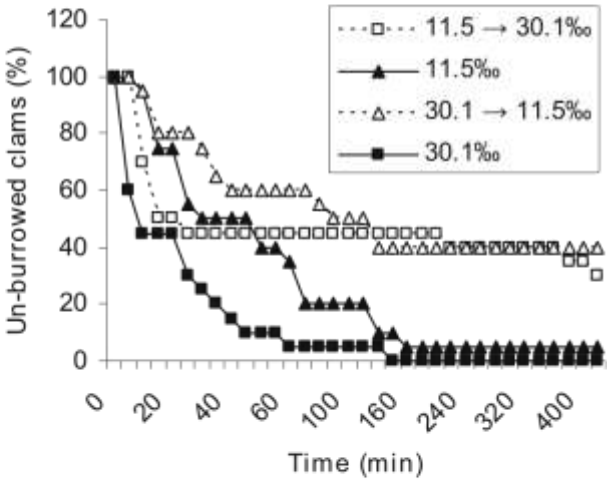


Fig. 1 Influence of salinity on burrowing behaviour. Clams (n=20 for each test) from two sites in the Belon estuary differing by salinity allowed to burrow in their sediment of origin (filled symbols black square Moëlan 30.1; black Triangle Guilly 11.5) or in the sediment from the other site (opened symbols, white square clams from Guilly 11.5, transferred into sediment from Moëlan 30.1; white triangle clams from Moëlan 30.1, transferred into sediment from Guilly 11.5)

higher salinity in cross tests (11.5→30.5) as it was the case in the sediments of origin.

4 Discussion

For clarity, it must be emphasised that the two parts of this study (influence of size or salinity on biomarkers) were carried out in specimens collected at different periods (July 2008 and May 2009, respectively) in estuaries submitted to contrasted anthropogenic impacts (French "Mussel Watch" biomonitoring programme http://wwz.ifremer.fr/envlit/). Thus, it is not surprising that differences occurred for many biological responses observed in the two sets of samples, which were not collected with a view to an intersite comparison.

During growth, we observed that the shell thickness (thus, its weight) increased proportionally more than the weight of soft tissues. Consequently, the significantly higher condition index observed in small specimens must not be interpreted as solely representative of a better physiological condition. The gonado-somatic index did not differ according to the size of specimens, because even the clams of the small class had a size (≥20 mm) at which they were already able to reproduce (Hughes 1971).

In clams of similar sizes collected from areas with different salinities, no differences were observed for either the condition index or the gonado-somatic index. Osmoregulation represents a key physiological response in estuarine organisms. It is brought about with or without water content changes depending on the potential of a species as an osmoregulator. In the case of *S. plana*, the water content differed significantly according to salinity determined in their place of origin, but by only 2%, and the total protein concentrations did not differ significantly. This is very important since all the enzyme activities are classically expressed by reference to total protein concentrations as in the present study.

According to the theory that there is a metabolic cost of coping with toxicants, energy reserves may be impacted (Mouneyrac et al. 2011). Thus, biomarkers linked with bioenergetic metabolism, such as glycogen, lipid and protein concentrations, may be valuable biomarkers in monitoring programmes. These parameters were not affected by either the size of clams or the salinity of the site of origin with the exception of significantly higher concentrations of glycogen in both gonads and digestive glands of the smaller specimens collected in the Loire estuary. In juvenile clams (*Ruditapes decussatus*), Laing and Child (1996) reported that carbohydrates represent the main form of energy easily mobilised. Thus, carbohydrates could supply the increased energy demand due to growth. This is probably a mechanism for protecting against the loss of protein and lipids and, therefore, the structural component of the animal (Lane 1986).

In bivalves, metal detoxification through MT induction is well documented (Amiard et al. 2006). MT in bivalves is a core biomarker determined for instance in mussels in the framework of the Mediterranean Action Plan of UNEP for the Barcelona Convention. In *S. plana*, significantly higher concentrations of MT were determined in specimens from contaminated estuaries (Loire and Seine, Fr.) compared to a relatively clean estuary (Goyen, Fr.), all the three sites showing similar salinities (11–15) (Boldina-Cosqueric et al. 2010). On the other hand, Solé et al. (2009) did not show any significant relationship between MT concentration and a large set of different metal concentrations, probably as a consequence of a restricted gradient of metal contamination (particularly Cd and Hg) in the studied environment, a marsh ecosystem in SW Spain.

In the present study, some significant differences have been shown between metal (Ag, Cu, Ni) concentrations among size classes of *S. plana*. The reasons have been documented for decades and have resulted in recommendations about size selection in "Mussel Watch" programmes based on the chemical analysis of bioaccumulators (NAS 1980). Despite the differences seen in these accumulated metal concentrations, MT concentrations were not significantly different among size classes, probably because Zn—a primary inducer of MT (Roesijadi 1996)—had the highest concentration of all metals, and no differences occurred according to size.

Conflicting salinity effects have been reported for MT concentrations analysed in different bivalve species (Romero-Ruiz et al. 2008). Simulating a metal accident under estuarine conditions, García-Luque et al. (2004) examined MT concentrations in the soft tissues of S. plana maintained in the laboratory for 15 days at eight different salinities (17.81 to 31.93). No simple trend was shown with the minimum MT concentration determined at a salinity of 22.38 and the maximum at 28.96. In the present study, in the absence of any known source of anthopogenic metals in the Belon estuary, MT concentrations were significantly higher in clams from the brackish area, in agreement with significantly higher concentrations of zinc in their tissues. Such an increase would be expected from changes in free zinc ion availabilities resulting from the physicochemical effect of reduced salinity on dissolved zinc speciation as explained by Rainbow and Black (2002) and exemplified in the study by García-Luque et al. (2004). A similar pattern was observed for silver with significantly higher concentrations in clams from the brackish area, in agreement again with increased chloride complexation (Wood et al. 2004). As mentioned above, Zn had the highest concentration of all metals and bioavailable Zn appears to play a central role. Despite a salinity effect, Romero-Ruiz et al. (2008) showed that in the Quadalquivir estuary, MT in *S. plana* was an efficient biomarker, able to reveal exposure to metals.

LDH activity, as the terminal enzyme of anaerobic glycolysis, plays an important function in anaerobic metabolism (Gagnon and Holdway 1999; Diamantino et al. 2001). Clams (*S. plana*) are able to maintain a working anaerobic metabolism under anoxia and hydrogen sulphide exposure (Oeschger and Pedersen 1994). Since anaerobic metabolism in bivalves can result in the accumulation of amino acids, a possible contribution of anaerobic pathways to the osmotic adjustment of cells during hyperosmotic stress has been investigated (Zurburg and De Zwaan 1981). Thus, in the present study, it is not surprising that bivalves with the highest LDH activities originated from the site exhibiting the highest salinity.

In order to avoid all modulating factors in biomarker responses, Solé et al. (2009) and Boldina-Cosqueric et al. (2010) carried out their samplings at the same time of the year, when temperature conditions, salinity and the physiological status of the clams *S. plana* were similar, using the specimens of similar size. In both studies, AChE proved to be a clearly discriminating factor between sites whereas GST activity was significantly induced only in the large contaminated French estuaries studied by Boldina-Cosqueric et al. (2010). On the other hand, in the marsh ecosystem studied by Solé et al. (2009), yearly fluctuations of GST activity were larger than within stations. More widely, both of these biomarkers have been shown to provide evidence of exposure to contaminants in a number of species and field situations, a reason why the confounding role of salinity has given rise to studies with other bivalves (e.g. Pfeifer et al. 2005), annelids (e.g. Kalman et al. 2010), and crustaceans (e.g. Menezes et al. 2006; Cailleaud et al. 2007). Even if the innermost mechanisms responsible for differences due to salinity are not established, it appears that the influence of this factor is linked to the ecological preference of each species.

The importance of digestive enzymes in energy acquisition in organisms living in polluted areas has been well established (Dedourge et al. In Amiard and Amiard-Triquet 2008), and the depletion of these enzyme activities in contaminated sites has been shown in crustaceans (Dedourge-Geffard et al. 2009) and annelids (Kalman et al. 2009). On the contrary, digestive enzymes of *S. plana*—originating from the same sites as these annelids—did not respond clearly to contamination (Boldina-Cosqueric et al. 2010). In another bivalve, the freshwater mussel *Dreissena polymorpha*, Bourgeault et al. (2010) did not show clear relationship between micro-pollution and amylase and cellulase activities and evoked the possible role of trophic status. In addition to this limited reactivity to pollutants in clams, the present study showed that digestive enzyme activities in the crystalline style were significantly influenced by salinity, thus needing a precautionary approach when monitoring estuarine versus coastal areas.

Burrowing speed may be used as a rapid sublethal assessment tool for determining the "attractiveness" of sediments (Byrne and O'Halloran 2001). By using the clam *S. plana*, these authors have shown a clear contrast between sediments from a reference site and from the contaminated Dublin Bay, in agreement with behavioural disturbances resulting from metal exposure shown in experiments by others (Akberali and Black 1980; Akberali et al. 1981). However, because Akberali (1978) recorded an influence of salinity on valve movements, it remains necessary to assess the value of behavioural tools in areas with changing salinity. In agreement with this previous work, the behaviour of the clams (both burrowing speed and number of burrowed individuals) was disturbed by salinity shocks, when animals were tested at a salinity differing from that of their original sediment. Even when animals were allowed to burrow in their sediment of origin, burrowing was significantly faster at the highest salinity tested (30.1 compared to 11.5). However, after a reasonable length of time (3 h), similar numbers of individuals kept at their salinity of origin had burrowed.

In the case of burrowing tests, the quality of sediments may act as a confounding factor. However, the optimum range of sediment grain size for burrowing activity of S. plana is much larger (medium sand to silt and mud) than the difference observed between the two sites sampled in the Belon estuary (Table 2) and more generally among mudflats harbouring *S. plana* (Alexander et al. 1993). Bonnard et al. (2009) have shown that burrowing of S. plana was favoured in a natural mud with more organic matter and finer grain size compared to an artificial sediment (mean grain size 0.25 mm). Thus, in the Belon estuary, the sediment from the brackish area which was finer and had higher concentrations of total organic carbon (Table 2) should be preferred to the sediment of the more haline area. Since the reverse was observed, it may be concluded that salinity was the more important driving parameter affecting burrowing.

In the present study, we have not assessed the influence of animal size on burrowing efficiency because the recent work by Bonnard et al. (2009) has demonstrated that *S. plana* with small shells (length <30 mm) burrowed more quickly than those with larger shells. However, when the experiment was extended to 7 to 8 h, the percentage of burrowed clams was identical whatever their size.

Temperature is commonly cited among the major factors influencing biological processes and thus complicating the interpretation of biomarkers (Handy et al. 2003). It is well established that, in particular, temperature can change the activity of the enzymes by changing the protein conformation and their catalytic efficiency or binding capacity. This is of special interest since many

biomarkers proposed for assessing environmental health are based on enzymatic activities. Most of the existing information on the variability in biomarker responses has been collected as part of field survey covering temporal variations even if it is known that seasonal variations include changes in sexual status, food availability, etc. in addition to temperature. The seasonal and/or temperature effect is particularly well studied in the case of AChE (Scaps and Borot 2000; Pfeifer et al. 2005; Menezes et al. 2006; Cailleaud et al. 2007; Burgeot et al. 2010) but other biochemical markers (MT, GST, LDH, catalase, Thiobarbituric acid reactive substances) have also examined from this point of view (e.g. Geffard et al. 2005; Ivanković et al. 2005; Menezes et al. 2006; Cailleaud et al. 2007; Durou et al. 2007). In order to complete the picture of the influence of confounding factors on the responses of the clam *S. plana* which can be used as biomarkers, the temporal variations (including temperature) of the whole set of biomarkers examined in the present study have been determined in specimens originating from a contaminated estuary and a comparatively clean site (paper in preparation).

5 Conclusion

S. plana is an interesting and relevant sentinel species presenting biomarkers that are able to reveal a number of disturbances in contaminated areas compared to comparatively clean sites (Table 5): biochemical (MT, GST, AChE), physiological (glycogen in gonads) and behavioural (burrowing) markers. Although among these biomarkers, several are influenced by size (Table 5) in most cases, this factor is easily controlled, provided that the same size classes are present in the different sites of interest. However, size per se is not the whole story, since at identical size, the weight may be different as shown by a higher condition index in small specimens (Table 5). Andral et al. (2004) have proposed a correction factor to minimise the influence of weight changes on metal levels in mussels translocated from a reference site to metal-contaminated sites and differing by trophic status. It may be commuted for use with other biological parameters (e.g. Mouneyrac et al. 2008).

Salinity influences several biomarkers of interest. The salinity must be carefully determined for each sampling

Table 5 Responsiveness of different classes of biomarkers, determined in *Scrobicularia plana*, to estuarine contamination and to confounding factors

	Contamination	Size class	Salinity
Physiological markers			
Condition index	NS[a]	> in small specimens	NS
Gonado-somatic index	NS[a]	NS	NS
Water content	NS[a]	ND	2% > at lower S‰
Glycogen			
Gonad	77% decrease in gonads[a]	192% > in small specimens	NS
Digestive gland	NS[a]	550% > in small specimens	NS
Lipids	NS[a]	NS	NS
Proteins	NS[a]	NS	NS
Biochemical markers			
MT	Increase[a, b] (46–68% in the French estuaries)	NS	23% > at lower S‰
LDH	Unclear[a]	NS	133% > at higher S‰
GST	25–94% increase[a]	56% > in small specimens	76% > at higher S‰
AChE	Decrease[a, c] (38–53% in the French estuaries)	40% > in big specimens	53% > at lower S‰
CMCase		NS[e]	
Digestive gland	Unclear[a]		NS
Crystalline style	Unclear[a]		100% > at higher S‰
Amylase		NS[e]	
Digestive gland	Unclear[a]		NS
Crystalline style	Unclear[a]		133% > at higher S‰
Behavioural markers			
Burrowing speed	Decreased burrowing speed[a, d]	> In small specimens[f]	> At higher S‰

ND not documented, NS no significant difference

[a] Boldina-Cosqueric et al. 2010

[b] Romero-Ruiz et al. 2008

[c] Solé et al. 2009

[d] Byrne and O'Halloran 2001

[e] Determination in the whole soft tissues

[f] Bonnard et al. 2009

collection, as must also be several parameters determining the quality of sediments (grain size, organic matter content). The salinity difference considered in the present study is important (11.5 to 30.1) for it has induced variations which are of the same order of magnitude (GST, AChE) or even higher (LDH, digestive enzymes in the crystalline style) than those observed in contaminated specimens (Table 5). In this case again, the use of a correction factor can be proposed to take into account the effect of salinity differences. The significance of digestive enzyme activities in bivalves seems questionable, but the small number of studies available does not allow the total condamnation at this stage, of their use as biomarkers. If needed, digestive enzyme activities can be determine in the digestive gland which is not as responsive as the crystalline style to the influence of salinity. The situation is much better in the case of MT which is only enhanced by 23% at the lower salinity whereas 46% to 68% increases have been observed in the Loire and Seine estuaries, France, and attributed to contamination effects. For burrowing tests, salinity shocks (in cross tests) reduced strongly both burrowing speed and the number of specimens able to burrow. From an operational point of view, the number of burrowed specimens is an endpoint which must be preferred to burrowing speed since after a reasonable experimental time (<3 h), it gave similar responses at both high and low salinities.

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References

AFNOR (1985) Huîtres creuses—Dénominations et classification, NF V45-056, Septembre 1985

Akberali HB (1978) Behaviour of *Scrobicularia plana* (Da Costa) in water of various salinities. J Exp Mar Biol Ecol 33:237–249

Akberali HB, Black JE (1980) Behavioural responses of the bivalve *Scrobicularia plana* (da Costa) subjected to short-term copper (Cu II) concentrations. Mar Environ Res 4:97–107

Akberali HB, Wong TM, Trueman ER (1981) Behavioural and siphonal tissue responses of *Scrobicularia plana* (bivalvia) to zinc. Mar Environ Res 5:251–264

Alexander RR, Stanton RJ Jr, Dodd JR (1993) Influence of sediment grain size on the burrowing of bivalves: correlation with distribution and stratigraphic persistence of selected neogene clams. Palaios 8:289–303

Allan IJ, Vrana B, Greenwood R, Mills GA, Roig B, Gonzalez C (2006) A "toolbox" for biological and chemical monitoring requirements for the European Union's water framework directive. Talanta 69:302–322

Amiard JC, Amiard-Triquet C (2008) Les biomarqueurs dans l'évaluation de l'état écologique des milieux aquatiques. Lavoisier, Paris

Amiard JC, Amiard-Triquet C, Barka S, Pellerin J, Rainbow PS (2006) Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat Toxicol 76:160–202

Amiard-Triquet C, Rainbow PS (2009) Environmental assessment of estuarine ecosystems a case study. CRC, Boca Raton

Andral B, Stanisière JY, Sauzade D, Damier E, Thébault H, Galgani F, Boissery P (2004) Monitoring chemical contamination levels in the Mediterranean based on the use of mussel caging. Mar Pollut Bull 49:704–712

Bernfeld P (1955) Amylases, α and β. In: Colowick SP, Kaplan NO (eds) Methods enzymol. Vol 1. Academic, New York, pp 149–158

Boldina-Cosqueric I, Amiard JC, Amiard-Triquet C, Dedourge-Geffard O, Métais I, Mouneyrac C, Moutel B, Berthet B (2010) Biochemical, physiological and behavioural markers in the endobenthic bivalve *Scrobicularia plana* as tools for the assessment of estuarine sediment quality. Ecotoxicol Environ Saf 73:1733–1741

Bonnard M, Roméo M, Amiard-Triquet C (2009) Effects of copper on the burrowing behavior of estuarine and coastal invertebrates, the polychaete Nereis diversicolor and the bivalve *Scrobicularia plana*. Hum Ecol Risk Assess 15:11–26

Bourgeault A, Gourlay-France C, Vincent-Hubert F, Palais F, Geffard A, Biagianti-Risbourg S, Pain-Devin S, Tusseau-Vuillemin MH (2010) Lessons from a transplantation of zebra mussels into a small urban river: an integrated ecotoxicological assessment. Environ Toxicol 25:468–478

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Burgeot T, Gagné F, Forget-Leray J, Bocquené G (2010) Acetylcholinesterase: methodology development of a biomarker and challenges of its application for biomonitoring. ICES CM 2010/F: 25

Byrne PA, O'Halloran J (2001) The role of bivalve molluscs as tools in estuarine sediment toxicity testing: a review. Hydrobiologia 465:209–217

Cailleaud K, Maillet G, Budzinski H, Souissi S, Forget-Leray J (2007) Effects of salinity and temperature on the expression of enzymatic biomarkers in Eurytemora affinis (Calanoida, Copepoda). Comp Biochem Physiol 147A:841–849

Carr RS, Neff JM (1984) Quantitative semi-automated enzymatic assay for tissue glycogen. Comp Biochem Physiol 77B:447–449

Cheggour M, Chafik A, Fisher NS, Benbrahim S (2005) Metal concentrations in sediments and clams in four Moroccan estuaries. Mar Environ Res 59:119–137

Coelho JP, Nunes M, Dolbeth M, Pereira ME, Duarte AC, Pardal MA (2008) The role of two sediment-dwelling invertebrates on the mercury transfer from sediments to the estuarine trophic web. Estuar Coast Shelf Sci 78:505–512

Coelho JP, Lillebø AI, Pacheco M, Pereira ME, Pardal MA, Duarte AC (2010) Biota analysis as a source of information on the state of aquatic environments. In: Namieśnik J, Szefer P (eds) Analytical measurements in aquatic environments. CRC, Boca Raton, pp 103–120

De la Huz R, Lastra M, López J (2002) The influence of sediment grain size on burrowing, growth and metabolism of *Donax trunculus* L. (Bivalvia: Donacidae). J Sea Res 47:85–95

Dedourge-Geffard O, Palais F, Biagianti-Risbourg S, Geffard O, Geffard A (2009) Effects of metals on feeding rate and digestive enzymes in *Gammarus fossarum*: an in situ experiment. Chemosphere 77:1569–1576

Diamantino TC, Almeida E, Soares AMVM, Guilhermino L (2001) Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* Straus. Chemosphere 45:553–560

Durou C, Poirier L, Amiard JC, Budzinski H, Gnassia-Barelli M, Lemenach K, Peluhet L, Mouneyrac C, Romeo M, Amiard-Triquet C (2007) Biomonitoring in a clean and a multi-contaminated estuary based on biomarkers and chemical analyses in the endobenthic worm *Nereis diversicolor*. Environ Pollut 148:445–458

EC (2000) Directive 2000/60/EC of the European Parliament and Council of 23 October 2000 establishing a framework for policy in the field of water (JO L 327 of 22 December 2000). European Commission, Brussels

Frasco MF, Guilhermino L (2002) Effects of dimethoate and beta-naphthoflavone on selected biomarkers of *Poecilia reticulata*. Fish Physiol Biochem 26:149–156

Frings CS, Fendley TW, Dunn RT, Queen CA (1972) Improved determination of total serum lipids by the sulpho-phospho-vanilin reaction. Clin Chem 18:673–674

Gagnon MM, Holdway DA (1999) Metabolic enzyme activities in fish gills as biomarkers of exposure to petroleum hydrocarbons. Ecotoxicol Environ Saf 44:92–99

Galgani F, Bocquené G (1991) Semi-automated colorimetric and enzymatic assays for aquatic organisms using microplate readers. Water Res 25:147–150

García-Luque E, DelValls TA, Casado-Martínez C, Forja JM, Gómez-Parra A (2004) Simulating a heavy metal spill under estuarine conditions: effects on the clam *Scrobicularia plana*. Mar Environ Res 58:671–674

Garrigues P, Barth H, Walker CH, Narbonne JF (2001) Biomarkers in marine organisms: a practical approach. Elsevier, Amsterdam

Geffard A, Amiard-Triquet C, Amiard JC (2005) Do seasonal changes interfere with metallothionein induction by metals in mussels *Mytilus edulis*? Ecotoxicol Environ Saf 61:209–220

Hagger JA, Jones MB, Leonard DRP, Owen R, Galloway TS (2006) Biomarkers and integrated environmental risk assessment: are there more questions than answers? Int Environ Assess Manag 2:312–329

Handy RD, Galloway TS, Depledge MH (2003) A proposal for the use of biomarkers for the assessment of chronic pollution and in regulatory toxicology. Ecotoxicology 12:331–343

Hughes RN (1969) A study of feeding in *Scrobicularia plana*. J Mar Biol Assoc UK 49:805–823

Hughes RN (1971) Reproduction of Scrobicularia plana Da Costa (Pelecypoda: Semelidae) in North Wales. Veliger 14:77–81

Ivanković D, Pavičić J, Erk M, Filipović-Marijić V, Raspor B (2005) Evaluation of the *Mytilus galloprovincialis* Lam. digestive gland metallothionein as a biomarker in a long-term field study: seasonal and spatial variability. Mar Pollut Bull 50:1303–1313

Kalman J, Palais F, Amiard JC, Mouneyrac C, Muntz A, Blasco J, Riba I, Amiard-Triquet C (2009) Assessment of the health status of populations of the ragworm *Nereis diversicolor* using biomarkers at different levels of biological organisation. Mar Ecol Prog Ser 393:55–67

Kalman J, Buffet PE, Amiard JC, Denis F, Mouneyrac C, Amiard-Triquet C (2010) Assessment of the influence of confounding factors (weight, salinity) on the response of biomarkers in the estuarine polychaete *Nereis diversicolor*. Biomarkers 15:461–469

Lagadic L, Caquet T, Amiard JC, Ramade F (2000) Use of biomarkers for environmental quality assessment. Science, Enfield

Laing I, Child AR (1996) Comparative tolerance of small palourdes (*Tapes decussatus* L.) and Manila clams (*Tapes philippinarum* Adams & Reeve) to low temperature. J Exp Mar Biol Ecol 195:267–285

Lane JM (1986) Allometric and biochemical studies on starved and unstarved clams, *Rangia cuneata* (Sowerby, 1831). J Exp Mar Biol Ecol 95:131–143

Langston WG, Burt GR, Chesman GS (2007) Feminisation of male clams Scrobicularia plana from estuaries in Southwest UK and its induction by endocrine-disrupting chemicals. Mar Ecol Prog Ser 333:173–184

Leitão R, Martinho F, Neto JM, Cabral H, Marques JC, Pardal MA (2006) Feeding ecology, population structure and distribution of Pomatoschistus microps (Krøyer, 1838) and *Pomatoschistus minutes* (Pallas, 1770) in a temperate estuary, Portugal. Estuar Coast Shelf Sci 66:231–239

Lopes RJ, Pardal MA, Múrias T, Cabral JA, Marques JC (2006) Influence of macroalgal mats on abundance and distribution of dunlin *Calidris alpina* in estuaries: a long-term approach. Mar Ecol Prog Ser 323:11–20

Martínez-Gómez C, Vethaak AD, Hylland K, Burgeot T, Köhler A, Lyons BP, Thain J, Gubbins MJ, Davies IM (2010) A guide to toxicity assessment and monitoring effects at lower levels of biological organization following marine oil spills in European waters. ICES J Mar Sci 67:1105–1118

Mazorra MT, Rubio JA, Blasco J (2002) Acid and alkaline phosphatase activities in the clam *Scrobicularia plana*: kinetic characteristics and effects of heavy metals. Comp Biochem Physiol 131B:241–249

Menezes S, Soares AMVM, Guilhermino LR, Peck M (2006) Biomarker responses of the estuarine brown shrimp Crangon crangon L. to non-toxic stressors: temperature, salinity and handling stress effects. J Exp Mar Biol Ecol 335:114–122

Mouneyrac C, Linot S, Amiard JC, Amiard-Triquet C, Métais I, Durou C, Minier C, Pellerin J (2008) Biological indices, energy reserves, steroid hormones and sexual maturity in the infaunal bivalve *Scrobicularia plana* from three sites differing by their level of contamination. Gen Comp Endocrinol 157:133–141

Mouneyrac C, Leung PTY, Leung KMY (2011) Cost of tolerance. In: Amiard-Triquet C, Rainbow PS, Roméo M (eds) Tolerance to environmental contaminants. CRC, Boca Raton, pp 265–297

NAS (1980) The International Mussel Watch. Report of a workshop sponsored by the environment studies board. Vol. Natural Resources Commission National Research Council National Academy of Sciences, Washington, DC

Oeschger R, Pedersen TF (1994) Influence of anoxia and hydrogen sulphide on the energy metabolism of *Scrobicularia plana* (da Costa) (Bivalvia). J Exp Mar Biol Ecol 184:255–268

Pfeifer S, Schiedek D, Dippner JW (2005) Effect of temperature and salinity on acetylcholinesterase activity, a common pollution biomarker, in Mytilus sp. from the south-western Baltic Sea. J Exp Mar Biol Ecol 320:93–103

Rainbow PS, Black WH (2002) Effects of changes in salinity and osmolality on the rate of uptake of zinc by three crabs of different ecologies. Mar Ecol Prog Ser 244:205–217

Rodríguez-Rúa A, Prado MA, Romero ZM, Bruzón M (2003) The gametogenic cycle of *Scrobicularia plana* (da Costa, 1778) (Mollusc: Bivalve) in Guadalquivir estuary (Cádiz, SW Spain). Aquaculture 217:157–166

Roesijadi G (1996) Metallothionein and its role in toxic metal regulation. Comp Biochem Physiol 113C:117–123

Romero-Ruiz A, Alhama J, Blasco J, Gomez-Ariza JL, Lopez-Barea J (2008) New metallothionein assay in *Scrobicularia plana*: heating effect and correlation with other biomarkers. Environ Pollut 156:1340–1347

Scaps P, Borot O (2000) Acetycholinesterase activity of the polyhaete *Nereis diversicolor*: effects of temperature and salinity. Comp Biochem Physiol 125C:377–383

Solé M, Kopecka-Pilarczyk J, Blasco J (2009) Pollution biomarkers in two estuarine invertebrates, *Nereis diversicolor* and *Scrobicularia plana*, from a Marsh ecosystem in SW Spain. Environ Int 35:523–531

Wood CM, Danielle McDonald M, Walker P, Grosell M, Barimo JF, Playle RC, Walsh PJ (2004) Bioavailability of silver and its relationship to ionoregulation and silver speciation across a range of salinities in the gulf toadfish (*Opsanus beta*). Aquat Toxicol 70:137–157

Zurburg W, De Zwaan A (1981) The role of amino acids in anaerobiosis and osmoregulation in bivalves. J Exp Zool 215:315–325

4.3 Conclusions

Bien que des outils d'évaluation soient indispensables pour révéler les changements des réponses biologiques susceptibles d'affecter les performances physiologiques des populations estuariennes, ceci est souvent un but difficile à atteindre. Idéalement, de tels outils doivent permettre de distinguer entre perturbations naturelles ou anthropogéniques.

En ce qui concerne l'effet de la taille, on remarque dans le tableau 2 pour *H. diversicolor* et dans le tableau 5 de la publication ci-dessus pour *S. plana* qu'il se manifeste sur un nombre limité de biomarqueurs. De plus, il est souvent possible de sélectionner des specimens de taille équivalente sur différents sites d'études. Même lorsque cette stratégie s'avère inapplicable, des facteurs de corrections peuvent être utilisés en s'inspirant des travaux de (Andral et al., 2004) sur la bioaccumulation des métaux par le bivalve *Mytilus galloprovincialis*; de Poirier et al. (2006) sur la bioaccumulation des métaux par *H. diversicolor*; de Mouneyrac et al. (2009) sur les concentrations de glycogène, lipides et hormones stéroïdiennes chez *S. plana*.

Pour la salinité, les mêmes tableaux montrent que de nombreux biomarqueurs échappent à l'influence de ce facteur. De nouveau, on peut utiliser des facteurs de correction pour contrebalancer cette influence pourvu que l'on dispose de données sur les différentes valeurs prises par les biomarqueurs d'intérêt au long d'un gradient de salinité. Il en ensuite nécessaire de disposer d'une valeur de salinité représentative de celle à laquelle sont soumis les organismes échantillonnés. Pour ce faire, il ne parait pas pertinent de se référer à l'eau interstitielle car la structure du terrier avec ses parois consolidées par le mucus isole les organismes du sédiment environnant. C'est pourquoi, il a paru préférable depuis les travaux menés en Seine (Amiard-Triquet et Dauvin, 2009) de mesurer la salinité dans les flaques d'eau résiduelles à marée basse, de qualité voisine de celle circulant dans les terriers, pourvu que le prélèvement ait lieu au plus tôt après l'émersion afin d'éviter les phénomènes de concentration (chaleur, vent) ou de dilution (précipitations).

Que ce soit par rapport à l'effet taille ou à l'effet salinité, *H. diversicolor* semble moins influencée par les facteurs de confusion (Tableau 2 et Tableau 5 de la publication ci-dessus).

En ce qui concerne la température, elle sera abordée pour *S. plana* dans le prochain chapitre au travers des variations temporelles. Chez *H. diversicolor*, le tableau 2 montre que de

nombreux biomarqueurs ne sont pas affectés par les variations saisonnières. En laboratoire, Scaps et Borot (2000) montraient seulement un effet limité sur l'activité acétylcholine estérasique. Par contre, un effet très marqué a été signalé en ce qui concerne le comportement alimentaire (Moreira et al., 2005).

L'importance de la qualité du sédiment est bien établie à un niveau très général en ce qui concerne l'habitat des espèces endogées, beaucoup montrant une préférence pour un type de sédiment particulier (granulométrie, riches en matière organique) comme cela a bien été montré pour les bivalves (Alexander et al. 1993). Plus spécifiquement dans le domaine du comportement, la qualité du sédiment a été reconnue comme un facteur affectant l'enfouissement de nos deux espèces modèles (Bonnard et al., 2009).

Enfin, il faut prendre en compte le fait que les effets des contaminants chimiques sur les perturbations des différentes réponses biologiques de l'organisme peuvent être modulés par la disponibilité de la nourriture. Selon Heugens (2001), les effets de la pollution seront plus marqués en condition de ressources alimentaires limitées et moins apparents quand la nourriture est abondante.

En conclusion, une évaluation précautionneuse des effets biologiques des polluants dans les organismes de l'environnement doit prendre en compte un maximum de facteurs non polluants susceptibles d'interférer avec le devenir et les effets des contaminants chimiques. Certains de ces facteurs qui ne peuvent être contrôlés (salinité, granulométrie, teneur en matière organique, disponibilité alimentaire…) doivent être mesurés en parallèle avec les déterminations écotoxicologiques.

CHAPITRE 5. MISE EN ŒUVRE DES BIOMARQUEURS POUR L'EVALUATION DE L'ETAT DE LA QUALITE DES SEDIMENTS

5 CHAPITRE 5. MISE EN ŒUVRE DES BIOMARQUEURS POUR L'EVALUATION DE L'ETAT DE LA QUALITE DES SEDIMENTS

5.1 Contexte de l'étude

Les trois publications présentées dans ce chapitre s'inscrivent dans une démarche compréhensive visant à proposer une stratégie pour l'évaluation environnementale des écosystèmes estuariens à l'aide de biomarqueurs, en combinant des biomarqueurs classiques et des biomarqueurs à vocation écologique. Rappelons que nous entendons par biomarqueurs classiques des réponses biologiques, généralement biochimiques, qui présentent une certaine spécificité (e.g. la métallothionéine qui permet de révéler la présence de métaux même si elle peut être répondre, mais généralement avec moins d'ampleur à d'autres composés et à des facteurs naturels). Les biomarqueurs à vocation écologique recouvrent par ailleurs des réponses à différents niveaux d'organisation biologique qui révèlent des dysfonctionnements généralement en lien direct ou indirect avec la reproduction, qui ont vocation à entraîner des effets au niveau des populations, voire – si on les détecte dans des espèces clés pour la structure et le fonctionnement des écosystèmes – au niveau des communautés ou des écosystèmes. L'ensemble des biomarqueurs examinés dans ces études sont impliqués dans les effets en cascade susceptibles de mener de l'existence de stress chimiques dans un milieu donné à l'extinction de populations de certaines espèces selon le schéma conceptuel proposé dans la figure 4 de la publication 2.

Nous avions conclu dans le chapitre précédent que l'annélide polychète *Hediste diversicolor* semblait moins influencée par les facteurs de confusion que le bivalves *Scrobicularia plana,* que ce soit par rapport à l'effet taille ou à l'effet salinité. Etant donné l'importance de l'investissement en temps et en moyens, il était tentant de se limiter à *H. diversicolor* comme espèce modèle. Toutefois, les travaux mentionnés précédemment sur l'écotoxicologie des deux espèces (sous-section 2.2.4.) faisaient apparaître des différences interspécifiques de la tolérance aux métaux et aux pollutions estuariennes multiples (cas de la Seine). Les concentrations métalliques dans les deux espèces étaient différentes dans les sites moyennement contaminés étudiés par (Solé et al., 2009). Ces différences étaient fortement

accrues dans une zone extrêmement contaminée par des activités minières historiques (Bryan et al., 1985). Cette variabilité interspécifique a été retrouvée lors d'études en laboratoire de l'écotoxicité des nanoparticules d'oxyde de cuivre (voir Chapitre 8). Elle a été attribuée par Solé et al. (2009) à des différences d'exposition aux contaminants entre l'annélide polychète inféodée au sédiment déposé pour son alimentation et le bivalve considéré comme filtreur. Toutefois, nous avons montré dans la présentation des espèces (sous-sections 2.2.2.1 et 2.2.3.1) que les régimes alimentaires n'étaient pas aussi contrastés. Selon Hughes (1969), *S. plana* est principalement un déposivore, qui obtient seulement une partie de sa nourriture par filtration de la matière en suspension dans la colonne d'eau tandis que *H. diversicolor* est une espèce opportuniste, capable de satisfaire ses besoins énergétiques en utilisant différents régimes (Olivier et al., 1995).

En accord avec les recommandations formulées en conclusion du chapitre 4, nous nous sommes attachés à déterminer la plupart des facteurs autres que la pollution qui pouvaient influencer les variations des biomarqueurs. Ainsi, en plus des contaminants, le carbone organique total et la granulométrie qui influencent leur géochimie, ont été déterminés dans les sédiments superficiels. En effet, l'alternance des phases de dépôt et de resuspension du sédiment sont très importantes, en particulier dans des sites estuariens où l'hydrodynamique est forte, et elle contrôle les concentrations de tous les contaminants dans les sédiments superficiels (Amiard et al., 2009) utilisés par nos espèces déposivores.

Le carbone organique total est partiellement représentatif de la matière organique particulaire inerte qui participe à la diète de nos espèces sentinelles. Le microphytobenthos en est une autre composante importante. C'est pourquoi dans les sédiments superficiels ont été également réalisées des mesures de chlorophylle a et de phéopigments.

Par rapport aux biomarqueurs comportementaux, la qualité du sédiment qui peut influencer l'enfouissement a été caractérisée sur une colonne sédimentaire correspondant à la profondeur des terriers, soit environ 20 cm.

En lien avec les résultats du chapitre précédent, nous avons collectés des animaux de taille sélectionnée. La salinité a été mesurée comme recommandé dans les flaques résiduelles à marée basse (Amiard-Triquet et Dauvin, 2009) et les données recueillies précédemment ont été utilisées pour évaluer la part respective de ce facteur naturel par rapport au facteur de pollution entre les organismes issus de l'estuaire contaminé de la Loire (eaux saumâtres, 15 à

25,5 selon les mois) et ceux du site de référence (baie de Bourgneuf, 29, 1 à 32,5). Les prélèvements ont été répétés sept fois sur chaque site en vue i) d'étudier l'évolution de la contamination et de ses effets après qu'un petit déversement pétrolier se soit produit en Loire, s'additionnant à la pollution chronique de ce grand estuaire très anthropisé; ii) d'intégrer les variations temporelles liées tant aux conditions climatiques (température, éclairement) qu'au statut de maturité sexuelle des organismes dont le cycle biologique a été décrit localement pour nos deux espèces (Gillet et Torresani, 2003, Mouneyrac et al., 2008, Mouneyrac et al., 2010).

5.2 Publication 2

E-paper http://www.ices.dk/products/CMdocs/CM-2010/CM2010.pdf

Towards a comprehensive methodology to assess the health status of coastal and estuarine ecosystems

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TOWARDS A COMPREHENSIVE METHODOLOGY TO ASSESS THE HEALTH STATUS OF COASTAL AND ESTUARINE ECOSYSTEMS

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Abstract— The methodology of biomarkers has been developed since the early nineties and core biomarkers have been validated for use in biomonitoring programmes. On the one hand, their apparent lack of ecological relevance is frequently criticised, yet, on the other hand, their ability to reveal environmental disturbance well before changes in benthic communities occur is of considerable advantage. The first goal of this presentation is to provide elements of responses to the following question: Can impairments at the infra-individual or individual levels allow an assessment of effects at higher levels of biological organization? Processes which can affect reproductive success, with associated probable consequences at the population level, provide biomarkers with added ecological value. Particular attention will be devoted to i) energy metabolism, ii) behaviour, iii) reproductive disturbance. Case studies with key species for intertidal mudflat ecological functioning (the endobenthic worm *Nereis diversicolor* and bivalve *Scrobicularia plana*) will be used to demonstrate the cascade of biological events leading from chemical stress to local extinction of the species. In conclusion a three-step comprehensive methodology to assess the health status of coastal and estuarine ecosystems will be proposed. A first step would consist of seeking evidence for impairments revealed by the so-called biomarkers with ecological value. When such impairments have been demonstrated, it is necessary then to identify the main classes of contaminants which may be responsible by using a multi-biomarker approach based on "core biomarkers". Thirdly, the quantification and characterisation (specific fractions, metabolites) of chemicals in water, sediments and biota may allow the correlative validation of hypotheses based on the biomarker studies.

Key-words—Estuaries, biomarkers, behaviour, energy, reproduction, sentinel species

INTRODUCTION

Once incorporated into biota, chemicals resulting from anthropogenic activities can exert many different effects such as lethal or sublethal, acute or chronic responses, at different levels of biological organization, from macromolecules to populations or communities. Since the early nineties, such responses have been used as biomarkers. A biomarker as defined by Depledge (1993) is "A biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical pollutants (and/or radiations)." The well-known figure (Fig. 1) derived from Adams et al. (1989), clearly poses the controversy which has developed between ecotoxicologists and ecologists. On the one hand, the ability of biomarkers at a low level of biological organization to reveal environmental disturbance well before changes in benthic communities occur is of considerable advantage. On the other hand, their lack of ecological relevance is frequently highlighted (Forbes et al. 2006).

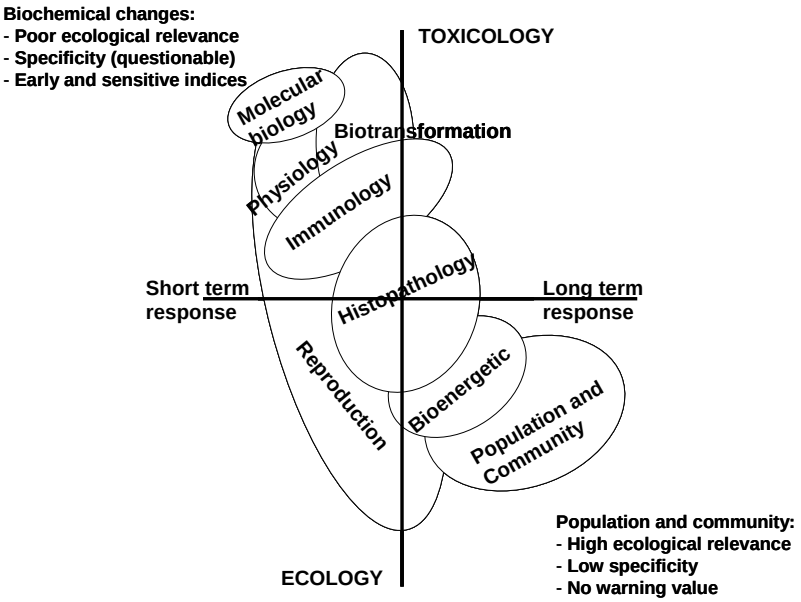


Fig. 1. Responses at infra- to supra-individual levels of biological organization in ecosystems exposed to chemicals introduced in the environment by human activities (After Adams et al. 1989)

Fig. 1 shows that a number of responses involved in reproduction may be observed at different levels of biological organization. According to Tannenbaum (2005) "pursuing toxicological endpoints other than reproduction is likely a wasted effort". Anyway, because the success of reproduction is the basis of the fate of populations (conservation, decrease or extinction), it is an important aspect which needs to be carefully assessed. The impairments at individual and infra-individual levels which are likely linked to effects at the supra-individual levels are shown in Fig. 2 and have been reviewed in Amiard and Amiard-Triquet (eds, 2008).

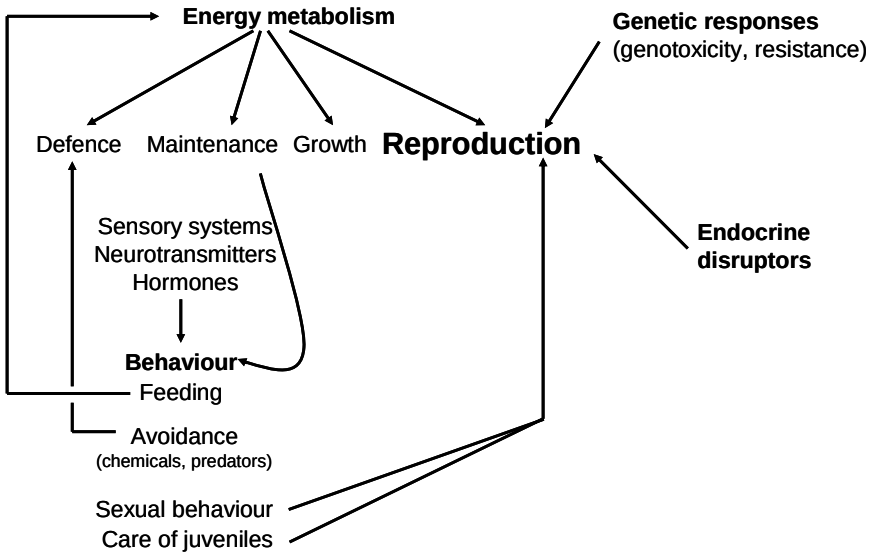


Fig. 2. Reproductive success depends on processes at different levels (biochemical, physiological, individual) of biological organization (After Amiard-Triquet 2009).

CAN IMPAIRMENTS AT THE INFRA-INDIVIDUAL OR INDIVIDUAL LEVELS ALLOW AN ASSESSMENT OF EFFECTS AT HIGHER LEVELS OF BIOLOGICAL ORGANIZATION?

The biomarkers "with added ecological value" include endocrine disruptions, behavioural disturbances, changes in bioenergetics and allocation of energy towards defence, maintenance, growth and reproduction, and genetic responses to chemical stress, either deleterious or adaptive. Convincing examples have been published. Kidd et al. (2007) have demonstrated the collapse of a fish population after exposure to a synthetic oestrogen which had led to a feminization of males through the production of vitellogenin mRNA and protein, impacts on gonadal development as demonstrated by intersex in males and altered oogenesis in females. De Coen and Janssen (2003) have shown that short-term changes in the energy budget, as measured by the cellular energy allocation (CEA) methodology, were correlated with long-term effects on growth and reproduction of the water flea *Daphnia magna* exposed to different model toxicants either inorganic or organic. These authors proposed that the "missing biomarker link" must be searched for in terms of energy metabolism impairment. Weis et al. (2001) have put together their different publications dealing with biochemical, behavioural and ecological studies with the mummichog *Fundulus heteroclitus* and, in doing so, they have clearly established connections between effects of contaminants cascading from the sub-organismal (neurotransmitter and thyroid hormone alterations) to the population (reduced growth and condition) and community (decreased predation pressure on prey species) levels.

A comprehensive approach has been applied to the assessment of the relative toxicity of estuarine sediments (Caeiro et al. 2005; Cunha et al. 2007). A battery of biomarkers (activities of liver ethoxyresorufin-O-deethylase, liver and gill glutathione S-transferases, muscle lactate dehydrogenase, and brain acetylcholinesterase) was examined in the fish *Spara aurata* exposed for 10 days to sediments collected from different sites in the Sado estuary (Portugal). For all the enzymes assayed, significant differences were found between sites, allowing discrimination between different types or levels of contamination, or both. The sediment ranking based upon these biomarkers was in good agreement with the one provided by a parallel study including chemical analysis of sediments, macrobenthic community analysis, amphipod mortality toxicity tests, and sea urchin abnormality embryo assays. Similarly, the assessment of the chronic toxicity of estuarine sediments at different levels of biological organization to the amphipod *Gammarus locusta* has shown a high consistency between chemical (bioaccumulation) and biochemical (metallothionein induction, DNA strand breakage, lipid peroxidation) responses and survival, growth and reproduction (Costa et al. 2005; Neuparth et al. 2005). A similar design was used in a freshwater system to investigate effluent impacts using a standard (*Daphnia magna*) and an indigenous (*Gammarus pulex*) test species (Maltby et al. 2000). *In situ* bioassays carried out downstream of the discharge showed a reduction in *D. magna* survival, in *G. pulex* survival and feeding rate, and in detritus processing, consistent with biotic indices based upon macroinvertebrate community structure.

CASE STUDIES WITH KEY SPECIES FOR INTERTIDAL MUDFLAT FUNCTIONING (THE ENDOBENTHIC WORM *NEREIS DIVERSICOLOR* AND BIVALVE *SCROBICULARIA PLANA*)

Criteria for the choice of sentinel species

The criteria for the choice of sentinel species have been recently reviewed (Galloway et al. 2006; Berthet 2008). Special attention is given to the representativeness of the species in its environment and to its role in the structure and the functioning of the ecosystem.



Fig. 3. Species of interest for estuarine mudflats in Europe: the ragworm *Nereis diversicolor* and the tellinid clam *Scrobicularia plana.*

For studies in the water column, filter-feeders (mussels, oysters) are particularly relevant and are very popular biological models in biomonitoring programmes such as the well-known "Mussel Watch" (NAS 1980) and its French version the Réseau National d'Observation managed by IFREMER (now ROCCH, Réseau d'Observation de la Contamination Chimique du milieu marin at http://wwz.ifremer.fr/envlit/). However, because on the whole aquatic environment sediments are the major compartment for the storage of most of the different classes of chemical contaminants, endobenthic invertebrates, among which the ragworm *Nereis diversicolor* and the tellinid clam *Scrobicularia plana* (Fig. 3) have received particular attention as bioaccumulators able to reveal environmental contamination (Bryan et al. 1985; Ruus et al. 2005; Poirier et al. 2006) and more recently as biological models for the determination of a number of biochemical, physiological and behavioural biomarkers (Scaps and Borot 2000; Mazorra et al. 2002; Pérez et al. 2004; Moreira et al. 2005, 2006; Romero-Ruiz et al. 2008; Solé et al. 2009).

Both the ragworm and the clam show high biomasses and population densities in estuarine mudflats. For *N. diversicolor*, values as high as 35 g m^{-2} and 4500 ind. m^{-2} were determined at a reference site in France, the Authie estuary (Gillet et al. 2008). In the case of *S. plana*, the maximum density reported for natural populations is 2000 ind. m^{-2} (Orvain 2005). Both species are common dietary items for predatory species like crabs, flatfish and wading birds. So a reduction of their populations would have consequences for food availability to their predators. It is also the reason why their role in transferring contaminants from sediment to higher trophic levels has become a topic of concern (Coelho et al. 2008; Cardoso et al. 2009). The ragworm and the clam are among the main macrofaunal species responsible for most of the bioturbation observable in estuaries such as the Tamar in England (Davey 1993). Due to bioturbation, these organisms are known to modify sediment properties and influence the flux of materials (nutrients, contaminants) to the water column (e.g. Banta and Andersen 2003; Orvain 2005; Soares and Sobral 2009). In addition, their influence on oxygen diffusion via their burrows has cascading effects on the distributions of other species (Bouchet et al. 2009).

From the above considerations, it is clear that both the ragworm and the clam are key species for the ecosystem structure and the functioning of estuarine mudflats. Because of their close contact with the reservoir of contaminants stored in sediments, they are also particularly at risk and correspondingly are relevant models for ecotoxicological studies, particularly

sediment toxicity assessment. Both are widely distributed in European estuaries and related organisms such as *Nereis virens* and *Mya arenaria* (see for instance the review by Gagné et al. 2009) are available for such studies in other areas of the world.

From chemical stress to local extinction

Cascading effects may be assumed from impairments of biological responses at the infra-individual (as early and sensitive indices), to individual and supra-individual (for their high ecological relevance) levels (Fig. 4).

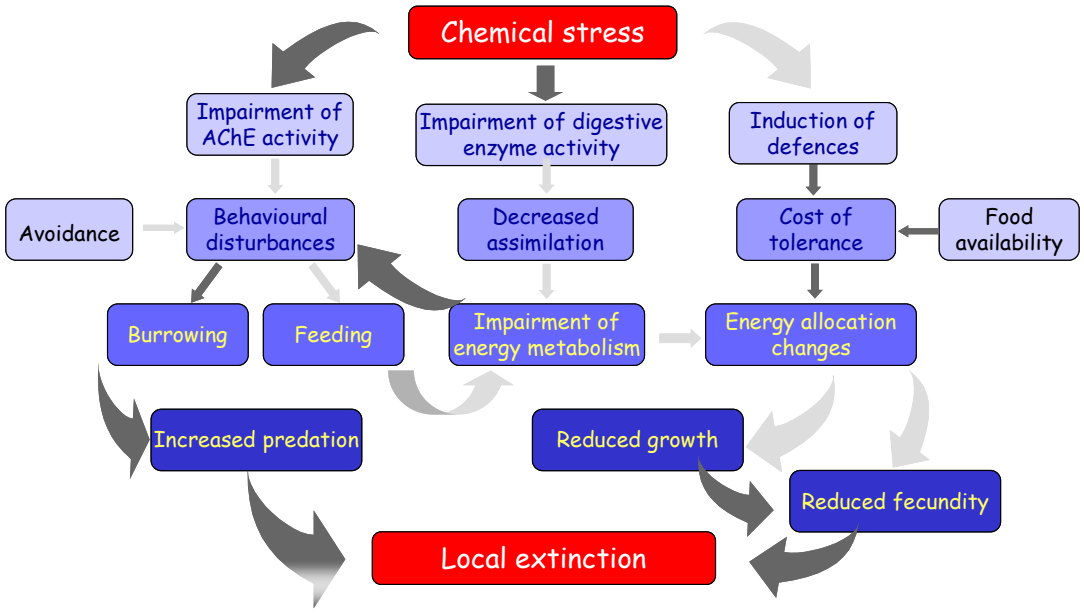


Fig. 4. Cascading effects of chemical stress at different levels of biological organization

Reduced feeding and impairment of digestive enzymes can lead to reduced energy intake. In addition to this impairment of energy metabolism, activation of defences (metallothionein induction, increased activity of glutathione-S-transferase…) can result in a cost of tolerance, leading to changes in energy allocation with implications for growth and reproduction and potential effects at the population level. Bioenergetics may also be affected by food availability. In a context where prey species are sensitive to chemical stress, resulting in a depletion of food resources, the cost of tolerance may be particularly harsh. On the contrary, if sentinel invertebrate species are sensitive to stresses which do not affect their food species, the cost of tolerance can be mitigated.

In parallel, neurotoxic effects may be revealed by determining levels of AChE activity (Fig. 4). Due to the large number of studies dealing with pesticides, the relationships between AChE inhibition (used as a biomarker of exposure to organophosphorus and carbamate pesticides) and behavioural impairments are well-documented for aquatic biota (Amiard-Triquet 2009). Energy depletion can interfere with behaviour since it is often based on active movements, needing energy expenditure (Amiard-Triquet 2009). On the other hand, behavioural changes can reflect an avoidance response since the capability to avoid high concentrations of metals and organic compounds is well known in both invertebrates and vertebrates. This leads to a reduced exposure with limited effect on long term survival when the animal leaves the contaminated area, but in a given ecosystem, the result is similar to a

local extinction. In addition to feeding which can contribute to changes in energy budgets as explained above, burrowing of endobenthic invertebrates is also a behaviour of interest, controlling the relationship between these organisms and their predators.

The studies carried out in our laboratory for eight years with *N. diversicolor* and *S. plana* were designed to inform the conceptual model described above.

Biochemical stress, physiological and behavioural impairments in ragworms and clams along a gradient of pollution

The choice of the sites (Fig. 5) was guided by the data available from the French "Mussel Watch" Programme (RNO 2006), since chemical contamination has been well documented for more than 30 years with quarterly samples analysed all along the French coast. Chemical analyses of mussels, including metals (Cd, Cu, Hg, Pb, Zn and more recently Ag, Cr, Ni and V), ΣDDT (DDT+DDD+DDE), γHCH, αHCH, PCBs and PAHs, clearly highlight the pollution status of the estuaries of interest. With a population of 16 million inhabitants, 80% of whom live in urban areas, the Seine basin (78,650km^2) concentrates 40% of the economic activity of France and 50% of the river traffic; 60% of the Seine catchment area consists of arable land. Thus pollutant inputs are very important in the Seine estuary. The Loire estuary also receives a cocktail of pollutants originating from urban, industrial and agricultural activities but the concentrations of chemicals are not so high as in the Seine. The Bay of Bourgneuf, the Authie estuary and the Goyen estuary are less impacted by human activity or at least (Bay of Bourgneuf) by more environmentally friendly activities, such as oyster culture. Because over the eight year period during which ecotoxicological studies with endobenthic invertebrates have been carried out, the situation has changed in these sites (topographic evolution, occasional eutrophication), we were obliged to move between the three possible reference sites. In addition, chemical surveys in biota and sediments were carried out in parallel with ecotoxicological studies (Amiard et al. in Amiard-Triquet & Rainbow 2009).

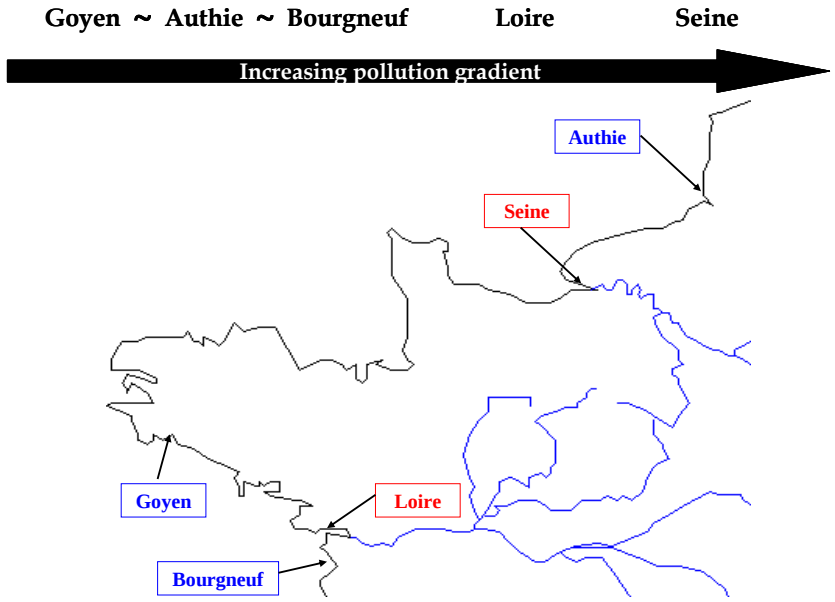


Fig. 5. Sampling sites in the Northwest of France

In France, as in other developed countries, pristine sites no longer exist and even relatively clean sites are scarce, being generally restricted to small estuaries, more likely to be spared from urbanization and industrialization. As a consequence, sites which are potentially available for use as controls have a number of natural characteristics which may differ from those of bigger estuaries. Thus for the present studies, particular attention has been paid to the selection of collection sites as similar as possible in terms of salinity and sediment characteristics in each estuary. Because a perfect similarity is impossible, particularly because salinity is highly fluctuating in estuaries, specific studies were devoted to the assessment of the potential effects of confounding factors such as salinity, season and individual size (Durou et al. 2007a, b; Kalman et al. 2010; Fossi Tankoua et al. in prep.).

Biochemical markers

Three classical biomarkers –glutathione S-transferase (GST) and acetylcholinesterase (AChE) activities, metallothionein-like protein (MTLP) concentrations – have been determined in both ragworms *Nereis diversicolor* (Poirier et al. 2006; Durou et al. 2007a; Kalman et al. 2010) and bivalves *Scrobicularia plana* from the Loire and Seine estuaries (Boldina-Cosqueric et al., in press and Fig. 6).

For GST, a phase II enzyme involved in the metabolism of lipophilic organic contaminants (van der Oost et al. 2005), the activity was consistently lower in the polychaete than in the bivalve, in the cases of invertebrates originating from the Loire estuary (Fig. 6) and several sites from SW Spain (Solé et al. 2009). For both species in both studies, significant intersite differences were rare but considering the whole sampling period, a clear trend was observed for a higher enzyme activity in bivalves from the Loire compared to the reference site (Fig. 6). In the Seine estuary, the global contamination of which is higher than in the Loire, GST activity was induced not only in the bivalves but also in the ragworms as shown by Durou et al. (2007a).

AChE activity, inhibited by some neurotoxic pesticides, can also respond to the presence of metals, detergents and algal toxins in marine invertebrates. AChE activity was consistently lower in the bivalves than in the polychaetes. In the former, significant intersite differences were scarce whereas the polychaete was much more responsive with significantly lower activities in the worms from the more contaminated sites (Loire, Fig. 6; Seine, Durou et al. 2007a; Spain, Solé et al. 2009).

For MTLPs, a family of proteins involved in metal homeostasis and detoxification (Amiard et al. 2006), no relationship was found in *S. plana* between any bioaccumulated metal and MT concentrations in the study by Solé et al. (2009). The authors proposed that this lack of response can be explained by the small metal gradient represented across their sites. In the case of *N. diversicolor*, even in highly contaminated sites in France and the UK or in experiments, no increased concentrations of MTLPs were observed despite these molecules representing a relatively important store for metals (Berthet et al. 2003; Poirier et al. 2006). It remains likely that MTLPs intervene in detoxification via an increased turnover (Ng et al. 2008) since it has been proposed that after the degradation of the proteins, the associated metals may become stored in lysosomal residual bodies or granules which are abundant in this species (Mouneyrac et al. 2003). For *S. plana*, in the multi-polluted Loire and Seine estuaries, concentrations of MTLPs were generally significantly different from those determined in specimens from the reference site (Boldina-Cosqueric et al., in press).

Toxicant-induced inhibition of digestive activity can result in metabolic disruption as explained above (Fig. 4). *N. diversicolor*, as an opportunistic species, is considered able to fulfil its energy needs using different feeding strategies: carnivory, scavenging, filter- and deposit-feeding. Its diet may be (partly) composed of phytoplankton, microphytobenthos, and/or detritus of halophytic plants, i.e. food materials especially rich in storage (e.g. starch) and structural (e.g. cellulose) carbohydrates. *S. plana* as an alternative deposit/filter-feeder also feeds on both phytoplankton and microphytobenthos in addition to deposited detritus. Thus to assay any assimilation impairment, digestive amylolytic and cellulolytic activities of amylase and cellulase enzymes have been measured. In the Loire estuary, the responses of the bivalve and the polychaete were contrasted. Significant and consistent intersite differences

were depicted in the case of *N. diversicolor* (Fig. 6). For amylase, one month after a small oil spill which occurred in the Loire estuary in March 2008, the activity was 65 times lower in local worms than in worms from a reference site. The ratio decreased to 13 and 20 for the two following months, then stabilized at 3 to 4 after October 2008. Anyway, the conditions chronically prevailing in the Loire estuary are sufficient to induce an intersite difference. The pattern for carboxymethylcellulase (CMCase) activity is typically the same, even if intersite differences of activities in *N. diversicolor* are not so large as in the case of amylase (Fig. 6). In the case of *S. plana*, the pattern was not so consistent since in the Loire estuary only one significant difference was shown (April 2008), with higher activities in this contaminated site compared to control (Fig. 6). In the Seine estuary, occasional determinations (April 2008) indicated significant decreases of both digestive enzymes in *N. diversicolor* and in *S. plana* (Kalman, Pers. Comm.; Boldina-Cosqueric et al. in press).

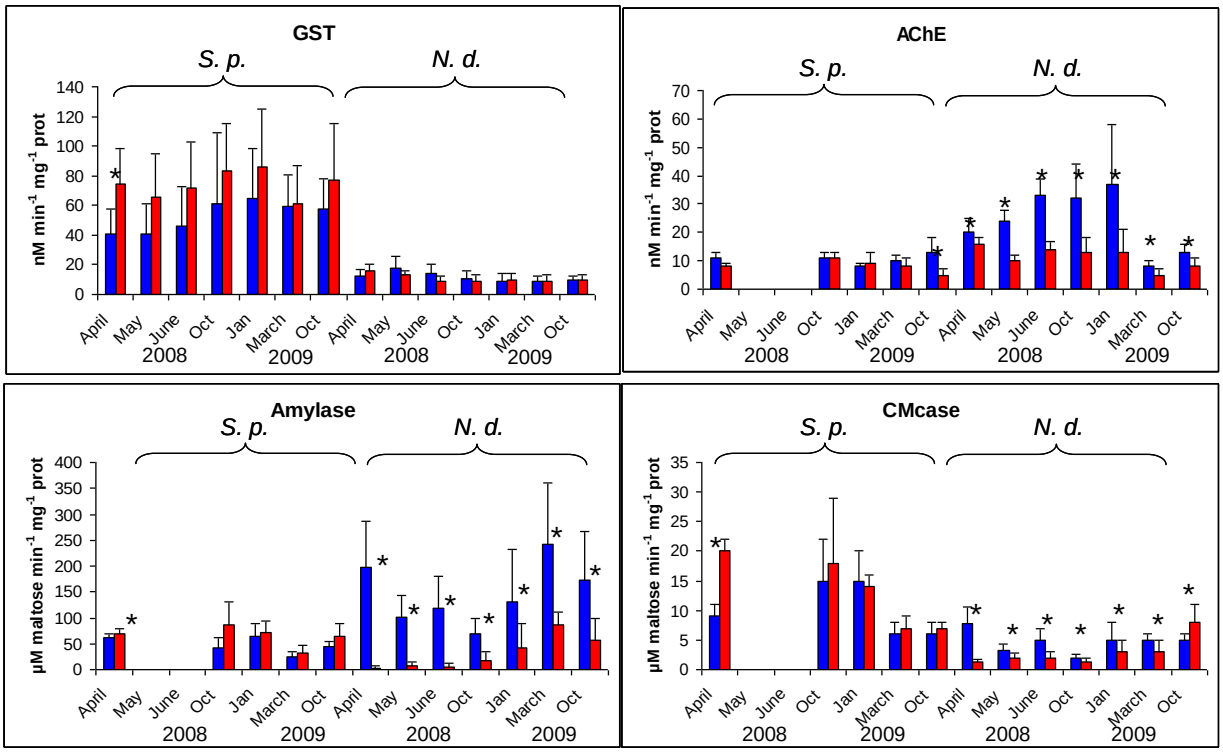


Fig. 6. Biomarker responses in *Scrobicularia plana* (S. p.) and *Nereis diversicolor* (N.d.) originating from the Bay of Bourgneuf, France (reference site, blue bars) and the multi-polluted Loire estuary, France (red bars). Asterisks indicate significant intersite differences (at least $p<0.05$). Data provided by I. Boldina-Cosqueric, P.E. Buffet, O. Fossi Tankoua, J. Kalman, PhD students.

Behavioural biomarkers

Burrowing behaviour is also a response which is different between these two model species. In parallel with biochemical measurements shown in Fig. 6 in the multi-polluted Loire estuary and the comparatively clean site of the Bay of Bourgneuf, the burrowing kinetics of worms and clams in their sediments of origin were examined (Fig. 7). For worms of both origins, nearly all the specimens had burrowed within a quarter of an hour, whereas in the case of the bivalves, some specimens were still at the surface of the sediment after one hour. After the small oil spill mentioned above, the behaviour of *S. plana* was disturbed for two months (Fig. 7), the burrowing kinetics being then restored, whereas no impairment occurred for *N. diversicolor* (five additional data –not shown– from June 2008 to October 2009). This difference between bivalves (*Mercenaria mercenaria*, Olla et al. 1983; *Protothaca staminea*, Pearson et al. 1981) and polychaetes (*Nereis virens*, Olla et al. 1984) has also been observed in the presence of oiled sediment. The responsiveness of burrowing behaviour in *S. plana* was also observed in the multi-polluted Seine estuary (Boldina-Cosqueric et al. in press) and in Irish sites mainly impacted with metals (Byrne and O'Halloran 1999). However, when the pollution gradient is important, differences in burrowing speed of the affected populations of *N. diversicolor* have also been observed (Mouneyrac et al. 2010).

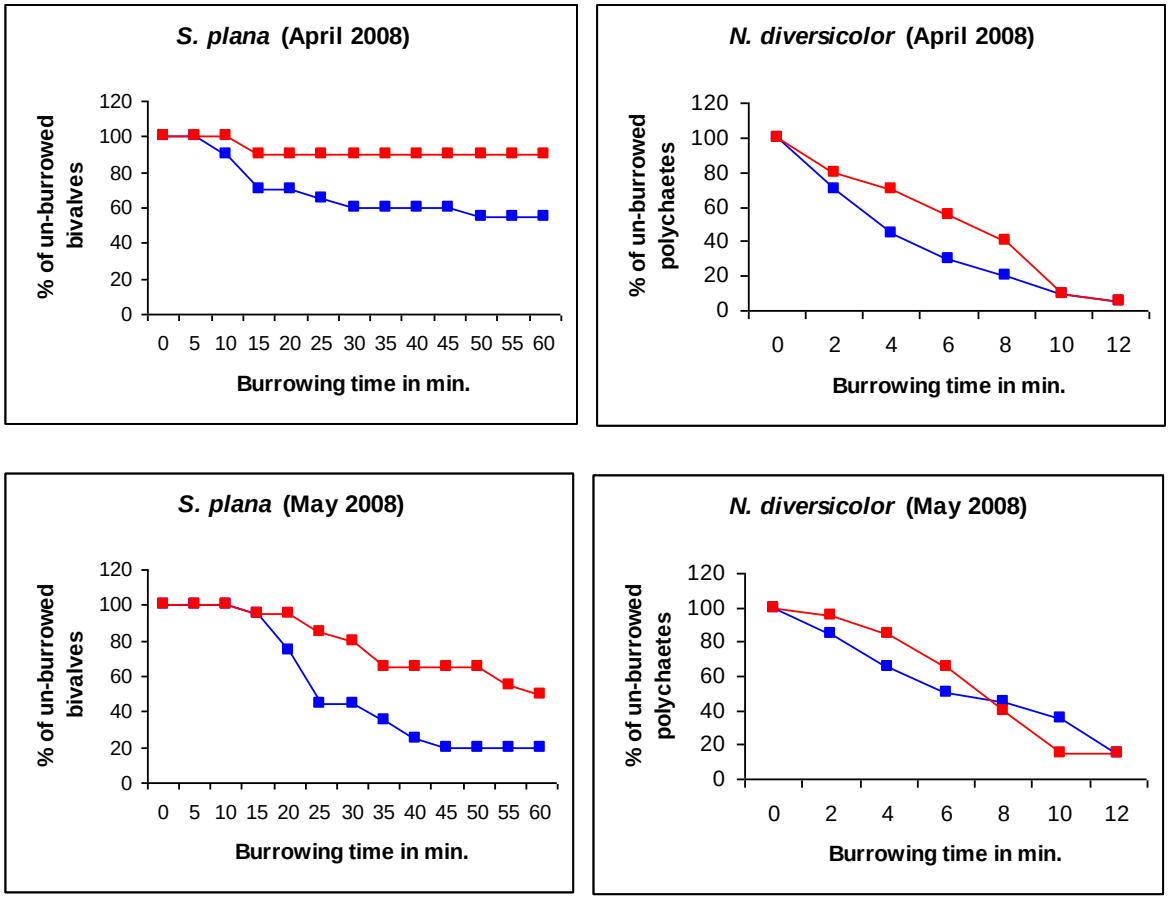


Fig. 7. Burrowing kinetics of *Scrobicularia plana* and *Nereis diversicolor* from the Bay of Bourgneuf, France (reference site in blue) and the multi-polluted Loire estuary, France (red symbols). After Boldina-Cosqueric et al. (in press) and Kalman et al. 2009.

Wilding and Maltby (2006) have discussed the origins of impairments of feeding behaviour in the freshwater crustacean *Gammarus pulex*. They have shown that disturbances could result from either physiological damage or an avoidance response. We have extrapolated their approach to the burrowing behaviour of *S. plana* and *N. diversicolor* by using cross tests with biota and sediments from the multi-polluted Seine estuary and the comparatively clean Goyen estuary (Mouneyrac et al. 2010; Boldina-Cosqueric et al. in press). The reduced burrowing speed of clams and ragworms from the clean site exposed to contaminated sediment may be interpreted as an avoidance response. On the other hand, in the presence of comparatively clean sediment, clams from the Seine did not show a significant change in their burrowing behaviour, an observation which indicates that physiological damage is most probably at the origin of their hypoactivity. On the other hand, ragworms from the Seine showed a significant increase of their burrowing speed in cleaner sediment; thus avoidance rather physiological damage is most probably at the origin of their hypoactivity in the presence of their sediment of origin.

Prey capture is another behaviour which has been demonstrated as a very sensitive response in organisms exposed to contaminants, as in the cases of the grass shrimp *Palaemonetes pugio* (Wallace et al. 2000), the mummichog *Fundulus heteroclitus* (Weis et al. 2001) and also *N. diversicolor* (Moreira et al. 2006).

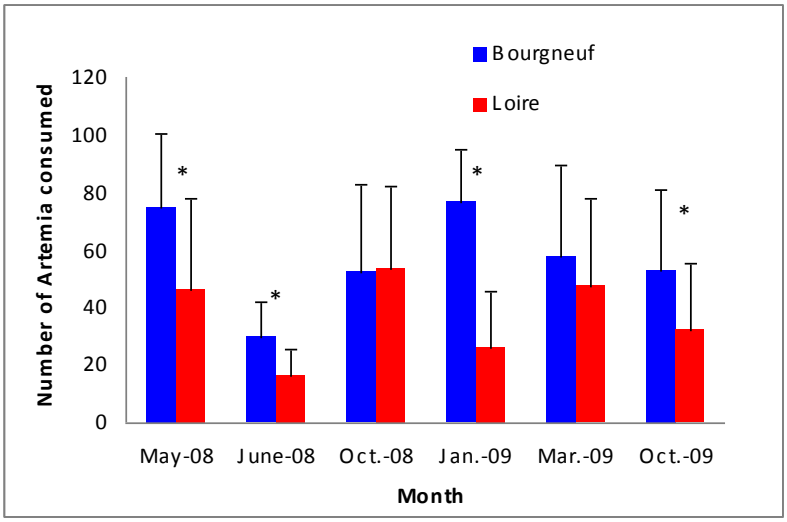


Fig. 8. Post-feeding rate in *Nereis diversicolor* from the multi-polluted Loire estuary and the comparatively clean site Bay of Bourgneuf. *Significant differences between sites.

The results obtained from feeding-rate experiments with *N. diversicolor* from the Loire and the Bay of Bourgneuf are depicted in Fig. 8. Significant intersite differences were generally recorded except in October 2008 and March 2009.

Physiological biomarkers

Energy reserves. For *N. diversicolor*, energy reserves were relatively depleted in the multi-polluted Seine estuary (Durou et al. 2007a). On the other hand, when comparing the specimens from the less contaminated Loire estuary and the Bay of Bourgneuf (reference), an inverse relationship was generally observed (Table 1; Buffet, Pers. Comm.). This inversion was not confirmed considering energy reserves in the digestive gland and gonads of the bivalve *S. plana* (Buffet, Pers. Comm.; Boldina-Cosqueric et al. in press). As mentioned earlier (Fig. 4), energy reserves are not influenced only by the presence of toxicants but also by food availability which can compensate for deleterious effects of chemicals. Indeed, in the

Loire estuary, the consumption pressure (established from chlorophyll a and phaeopigment balance) in superficial sediments was more important than in the Bay of Bourgneuf (Meleder et al. in prep.).

It is perhaps too simplistic to envisage that energy reserves such as concentrations of glycogen, total lipids and proteins are alone able to reveal energy metabolism impairment in moderately contaminated sites. In larvae and young stages of fish, energy storage is mainly in the form of triacylglycerols (TAG). Studies have shown that ratio of these to structural lipids such as phospholipids (TAG / PL) and especially sterols (TAG / ST) were good predictors of the animal's physiological status (Fraser 1989; Häkanson 1989; Suthers et al. 1992; Amara & Galois 2004; Amara et al. 2007). In estuarine situations, the ratio of TAG / ST determined in sole (*Solea solea*) is respectively 2.21, 1.55, 1.70, 0.33 and 0.27 in the Seine, Vilaine, Loire, Perthuis Breton Bourgneuf (Robert Galois, Univ. La Rochelle Pers. Comm.). Shellfish contain non-negligible amounts of triacylglycerols. It is possible then to measure changes in the relative TAG / ST in bivalves exposed to sharply contrasting pollution levels in their environment. If this index is relevant, it could be applied to natural populations of bivalves in relatively clean and polluted estuaries.

Table 1. Contrasted responses of two endobenthic invertebrates (the clam *Scrobicularia plana* and the ragworm *Nereis diversicolor*) to environmental conditions in French estuaries, the Seine (highly polluted) and the Loire (moderately polluted) estuaries. The response is increased ⇗ or decreased ⇘ by comparison with a reference site as clean as possible. NS not significant. ⊘ Not determined

	Scrobicularia plana		*Nereis diversicolor*	
	Loire	Seine	Loire	Seine
Biochemical markers	.			
Metallothionein-like proteins	⇗	⇗	⊘	⊘
Glutathione S-transferase	⇗	⇗	NS	⇗
Acetylcholine esterase	⇘	⇘	⇘	⇘
Digestive enzymes	NS	⇘	⇘	⇘
Behavioural markers				
Burrowing rate	⇘	⇘	NS	⇘
Prey capture	⊘	⊘	⇘	⊘
Physiological markers				
Energy reserves	See text		⇗	⇘
Condition index	See text		⇘	⇘
Population effects				
Density	Comparable to other sites worldwide	No temporal changes over 20 years	No temporal changes compared to 1998-2000	Population depletion over 20 years

Condition. In ragworms, measurement of the animals' condition was based on the relationship between wet weight and the length of the first three segments L3 (Durou et al. 2008). In bivalves, the condition index (CI) was determined according to the recommendation of the French Regulatory Agency after 24 hours depuration (AFNOR 1985):

CI = (wet weight of soft tissues/total weight)*100.

In ragworms, condition was generally depleted in contaminated estuaries (Loire and moreover Seine) compared to reference sites (Durou et al. 2008; Kalman et al. 2009). For an example, see below Fig. 11A. Additional determinations, carried out over more than one year, have confirmed the intersite difference between the Loire and the Bay of Bourgneuf. Considering this parameter, the bivalve *S. plana* does not appear as responsive as the ragworm since intersite differences were not consistent (Mouneyrac et al. 2008; Boldina-Cosqueric et al. in press; personal observations in the Loire and the Bay of Bourgneuf, Fig. 9).

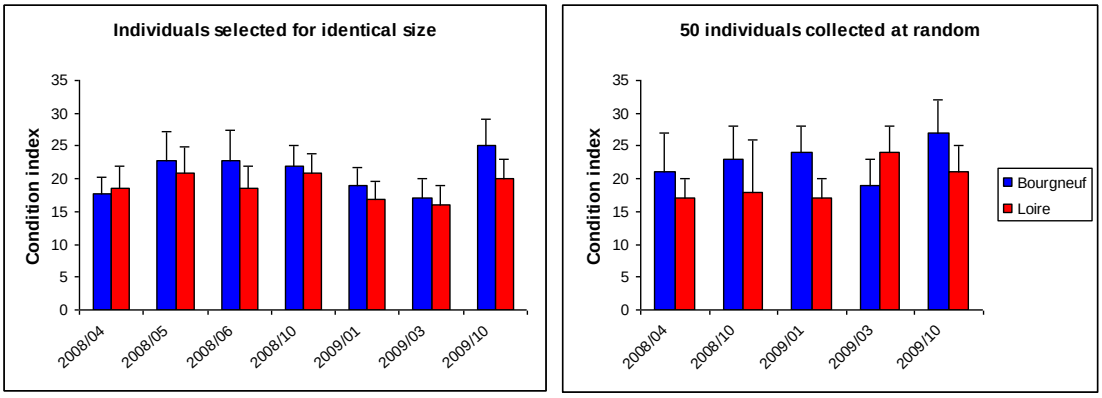


Fig. 9. Condition index of *Scrobicularia plana* from the Loire estuary and the Bay of Bourgneuf.

Gonado-somatic index (GSI). The GSI is the ratio between the weight of gonad and the total weight of soft tissues. It is a first approach allowing an assessment of the reproductive status in many animal species, particularly in bivalves. It may be used to follow the seasonal variation of the maturation of sexual products and tentatively to reveal intersite differences (Mouneyrac et al. 2010; Boldina-Cosqueric et al. in press). In the Loire estuary and the Bay of Bourgneuf (Fig. 10), no intersite differences were depicted, either at the beginning of sexual maturation (28 April to 26 May) or in specimens which have already spent sexual products (3 August). At the peak of maturity, the GSI was consistently higher in clams from the reference site (Bourgneuf). However, this tool is only useful when series of sample collection are carried out frequently throughout the maturation period (Fig. 10). When occasional sample collections are carried out at only one date on different sites, it can lead to misinterpretation since the peak of sexual maturity does not appear exactly at the same moment at different sites and many factors may be responsible for this situation. Differences in temperature are most probably an explanation, particularly when sites are at a certain distance (e.g. French coast of the Channel *versus* Bay of Biscay) but other factors such as those controlling food availability (light, available nutrients) are also involved and are not as easy to determine as temperature.

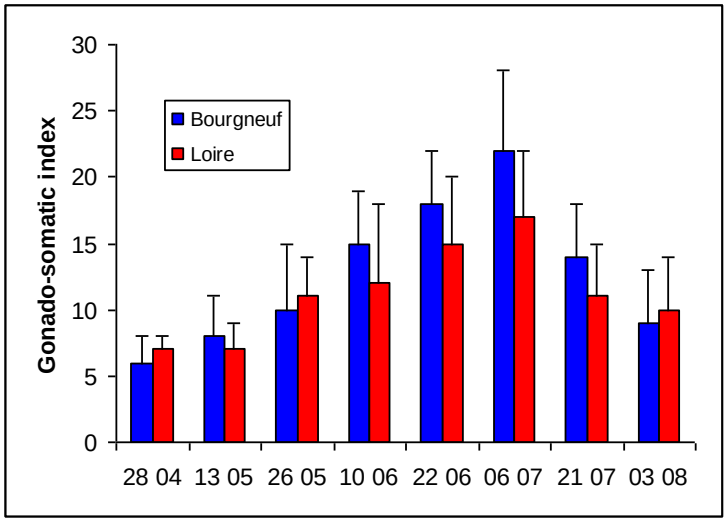


Fig. 10. Gonado-somatic index of *Scrobicularia plana* from the Loire estuary and the Bay of Bourgneuf during the maturation period in spring and summer 2009.

Sexual maturity. Delayed gametogenesis was observed in soft-shell clams (*Mya arenaria*) in relation to *in situ* contamination in the Saguenay fjord and the St Lawrence River (Canada) (Gauthier-Clerc et al. 2002; Siah et al. 2003). Similarly, recent results on development stages of the gonads of *S. plana* in the Loire estuary and the Bay of Bourgneuf have shown that maturation and spawning started earlier in the reference site (unpublished). Considering these results from an ecotoxicological point of view, it seems that the potentially contrasting timing of sexual maturity – which can be quantified by using a sexual maturity index (Siah et al. 2003) – is relevant in comparing sites with different environmental quality. However, the fact that maturation is delayed cannot be interpreted in term of reproductive success since it does not imply that the number and quality of gametes are impaired. In addition, from an operational point of view of any monitoring, the histology needed to study gametogenesis is relatively time-consuming.

In *N. diversicolor*, since sexual products are freely suspended in the body cavity, the procedure to monitor sexual development is simpler (Durou and Mouneyrac 2008). The estimation of size at maturity as a fitness component (Olive et al., 2000) reveals differences according to the site of origin, with worms breeding at smaller size (L3 measurement) in higher stress environments (Seine and Loire estuaries). Moreover, a lower proportion of reference worms (Authie estuary, bay of Bourgneuf) were undergoing gametogenesis for the whole sampling periods. These data seem to indicate that worms from reference sites were growing more slowly and reproducing later compared with worms from both contaminated sites (Durou and Mouneyrac 2008; Mouneyrac et al. 2010).

In species like *N. diversicolor* in which oocytes are freely suspended in the body cavity, it is well known that total fecundity (total production of oocytes by females) depends strongly upon body volume (Olive 1983). Moreover, in two species from the genus *Perinereis* similar to *Nereis*, a linear and strong relationship was found between oocyte number and length of jaws or wet weight (Cassai and Prevedelli 1998). Our results are in accordance with this finding since females from the Seine had smaller length and weight, thus a smaller body volume. Considering total fecundity, Seine females produced fewer oocytes than Authie ones, the difference being statistically significant for 3out of 6 dates of sampling over more than one annual cycle (for an example, see Fig. 11B).

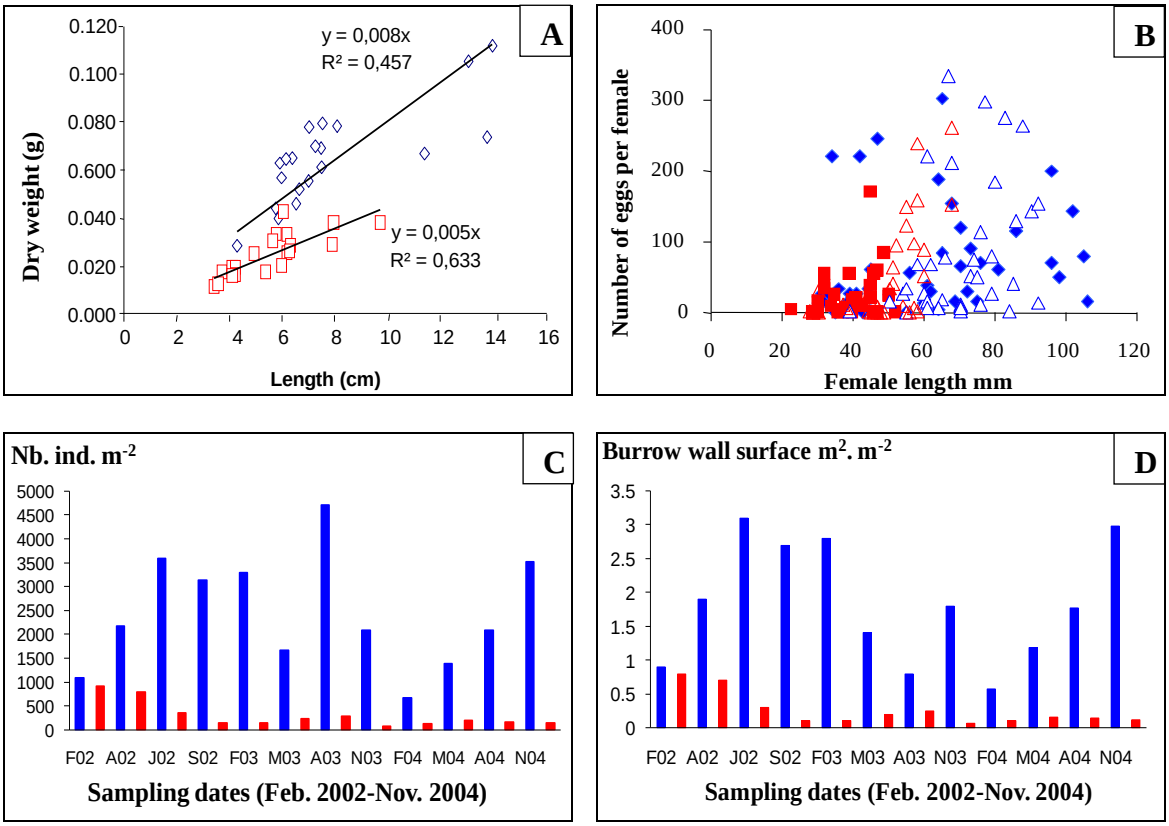


Fig. 11. From physiological impairments (A. Reduced growth; B. Reduced fecundity) to effects at supra-individual levels (C. Reduced density; D. Reduced bioturbation) in *Nereis diversicolor* from the multi-polluted Seine estuary (red) and the comparatively clean Authie estuary (blue).

Effects at higher levels of biological organization

When considering the different disturbances experienced by *N. diversicolor* in the Seine estuary (Table 1), as well as reduced growth and reduced fecundity (Fig. 11A, B), it is not surprising that populations of ragworms were depleted in this estuary. Reduction of densities was observed at the particular site where the comprehensive study, including assessment of many biochemical and physiological biomarkers, was carried out (Fig. 11C). But more generally, a field survey carried out in the whole estuary also showed a decrease of ragworm population densities (Bessineton, in Amiard-Triquet and Rainbow 2009) which can be influenced by several abiotic factors (sediment granulometry, river flow) as well as food bioavailability in the form of diatoms, foraminiferans and nematodes, at least in spring and summer (in Amiard-Triquet and Rainbow 2009). On the other hand, in the less contaminated Loire estuary, densities of ragworms were not consistently modified in the recent years (2008-2009) compared to evaluations carried out in 1998-2000 (Gillet and Torresani 2003). We have underlined above the ecosystem structure and functioning role of *N. diversicolor* associated with it bioturbation activity. Bioturbation was seriously impaired in the Seine estuary, considering the burrowing wall surface per m^2 of mudflat (Fig. 11D) as well as other factors representative of bioturbation such as the quantity of sediment reworked and the pumping rate (Gillet et al. 2008).

On the other hand, the multi-year survey of benthic fauna in the Seine estuary did not reveal a depletion of the *S. plana* populations (Bessineton, Pers. Comm.). In the Loire estuary, densities determined in 2008-2009 varied from 408 ± 124 to 1083 ± 237 ind. m^{-2} (unpublished). They are in the same order of magnitude as those encountered in different sites

in France and the U.K. (Tamar estuary, Spooner & Moore 1940; Gwendraeth estuary, South Wales, Green 1957; coast of Charentes, France, Bouchet et al. 2005), or even higher than those determined in regions further south (Tagus estuary, Cabral & Murta 2004; Ichkeul lake, Tunisia, Casagranda & Boudouresque 2005). However, the presence of intersex in this species has been observed in many estuaries from Portugal and the U.K. (Langston et al. 2007; Gomes et al. 2009). Our recent work has provided the first evidence of intersex in *S. plana* in estuaries from Britanny, France. Thus, potential consequences on reproduction success must be carefully monitored.

A THREE-STEP COMPREHENSIVE METHODOLOGY

As documented and discussed in Amiard-Triquet and Rainbow (2009), there is clearly value in analysing biomarkers. Biomarkers are direct manifestations of the exposure in the field of an organism to a toxicant or other form of stress. Evidence of field exposure is absolutely vital, and biomarkers are relatively effective in revealing overall toxicities of complex mixtures, particularly those which are at a high level of biological organization such as physiological biomarkers relating to the growth or reproduction of individual organisms.

Clearly the choice of biomarkers will involve the utilisation of particular organisms in which the use of these biomarkers has been established, as is the case for the above named invertebrates for one or more of the relevant biomarkers. A large range of natural factors, both biological (size, sex, position in the intertidal zone, seasonal changes in physiology) and abiotic, can influence living organisms, their health status and even their presence in ecosystems. This is particularly true for estuarine ecosystems which are sites associated with significant physical fluctuations (salinity, turbidity, hypoxia…) over different time scales (tidal rhythm, seasonal changes, inter-annual fluctuations, climate change). A number of these factors, which can interfere with the response of biota to anthropogenic stress, particularly chemical stress, may be easily controlled for in monitoring programmes as has been described previously in the case of biomonitoring based on chemical analysis in bioaccumulators (NAS 1980).

Those factors which cannot be controlled for (salinity, granulometry, hydrodynamics, food availability…) must at least be measured in parallel with ecotoxicological determinations. For some of these, it may be possible to determine their part in overall fluctuations by designing special studies that allow the measurement of variations of a biological response of interest along a gradient of salinity, temperature…, as well as body size, aiming at the establishment of correction factors (Poirier et al. 2006; Mouneyrac et al. 2008; Kalman et al. 2010).

Impairments revealed by biomarkers with ecological value

As described above for two model species of invertebrate, the ragworm *Nereis diversicolor* and the tellinid clam *Scrobicularia plana*, the first step could consist of investigation of impairments (bioenergetics, behaviour, endocrine disruption, as well as lysosomal parameters reviewed by Moore et al. 2006, genotoxicity reviewed by Vasseur et al. 2008, and immunotoxicity reviewed by Fournier et al. 2005) which are most likely to have consequences at the population level, even at the community level, simultaneously in contaminated and comparatively clean estuarine and coastal sites.

Multi-biomarker approach based on "core biomarkers"

When such impairments are revealed, it is necessary then to identify the main classes of contaminants which may be responsible for them. At this stage, a multi-biomarker approach based on so-called "core biomarkers" is appropriate. Both biomarkers of defence (e. g. SOD, HSP, Catalase, MT, P-gp...) and damage (e. g. AChE and TBARS) can be determined (Van

der Oost et al. 2005), considering either gene expression and induction or changes in protein concentrations and enzyme activities, or both. Then the data can be interpreted by using PCA (principal component analysis, for example see Durou et al. 2007a). According to Guerlet (2007), PCA presents several advantages: the possibility of bringing together the biological and physico-chemical data without the latter influencing the profile of the PCA (illustrative variables); possible application without any *a priori* information on the gradient of stress; reduced effect on discriminative power in the case of addition of redundant parameters.

Data obtained with batteries of biomarkers can also be incorporated into integrative indices (Beliaeff and Burgeot 2002; Wosniok 2005; Damiens et al. 2007; Yeom and Adams 2007; Hagger et al. 2008) or expert systems (Dagnino et al. 2007). Figure 12 provides a synthetic comparison of three tools of integration. With the objective of the avoidance of ecological disasters, as have occurred in the past, it is important to balance the correctness of any diagnosis with the simplicity of its implementation.

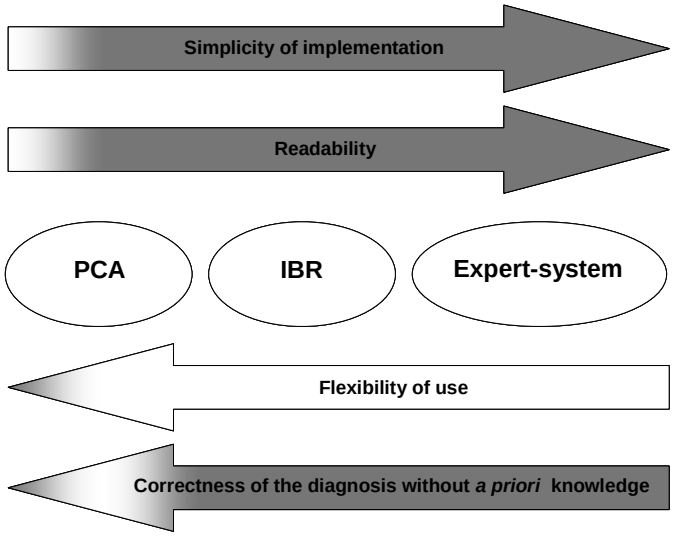


Fig. 12. Synthetic comparison of three tools of integration of the responses of the battery of biomarkers in aquatic organisms (adapted from Guerlet 2007 in Amiard-Triquet and Rainbow 2009). PCA: principal component analysis; IBR: integrated biomarker response.

Validation of the hypotheses provided by biomarkers by using analytical chemistry

The measurement of chemicals in physical components of the environment and in biota (either total concentrations or more interestingly concentrations in specific fractions and metabolites) will permit the validation of hypotheses based on biomarker studies. The analysis of selected biomonitors where present is a biologically and ecotoxicologically defensible procedure given that biomonitors provide a measure of the fraction of total contaminant in the environment that is bioavaialble (and therefore of potential ecotoxicological significance) to the organism chosen. The use of biomonitors, specifically in this case for contaminant metals, is discussed in detail in Luoma and Rainbow (2008).

However, in estuaries, complex mixtures of contaminants are present, including many classes of compounds (many persistent organic pollutants) which are not yet accessible to analysis or are extremely expensive to analyse. In some cases, it will remain impossible to establish a clear link between observed biological responses and specific contaminant doses. In such cases, techniques based on passive samplers (Zhang & Davison 1995; ITRC 2005; Stuer-Lauridsen 2005; Mills et al. 2010) allow the quantification of a larger number of contaminants and, in addition, give access to some of the characteristics which govern their environmental fate and their bioavailability. Among devices that rely on diffusion and sorption to accumulate analytes in the sampler, Semi-Permeable Membrane Devices (SPMDs)

are relevant for non-polar contaminants such as Polycyclic Aromatic Hydrocarbons (PAHs) whereas Polar Organic Chemical Integrative Samplers (POCIS) are relevant for polar compounds (detergents including alkykphenols, pharmaceuticals, pesticides). For trace metals, Diffusive Equilibration in Thin films (DET) and Diffusive Gradients in Thin films (DGT) allow the determination of free ions, labile organic or inorganic complexes. From an operational point of view, passive samplers have significant advantages over spot sampling techniques, allowing the measurement of time-weighted average concentrations. They also include a purification of the global sample and a pre-concentration of the chemicals of interest with represent an analytical benefit, reducing matrix effects and lowering the detection limit. Some of these sampling technologies are relatively mature and accepted for appropriate applications by regulators in some regions and states. For instance, The Virginia Department of Environmental Quality (DEQ 2009) formed a Research Advisory Committee to guide investigation after fish kills in the Shenandoah River and the Upper James River. One hypothesis was that chemical pollutants present in the water during the spring were responsible for causing the fish kills through direct toxicity or indirectly through suppressing immune system responses. To begin investigating this hypothesis, passive samplers were deployed in fish kill affected areas and control areas. Extracts from passive samplers were analyzed for nearly 200 polar and non-polar organic chemicals. Nonetheless, passive samplers are still considered to be innovative technologies and few specific policies governing their use have been written into official regulations.

CONCLUSION

Such a comprehensive approach would allow decisions on protective procedures well before the situation has reached a point at which remediation is nearly impossible. By focussing on key species, it is assumed that impairments of their responses used as biomarkers, will reveal a risk of cascading deleterious effects right through to the ecosystem level. Disturbances at the supra-individual levels affecting these organisms may result in ecological disorders such as changes in biogeochemical cycles and trophic relationships which govern nutrient/food availability (both as energy resource and risk of contaminant transfer) in the vital ecosystems that are estuarine (and other) coastal zones.

The state of knowledge attained would allow considerable improvement of present risk assessment and monitoring procedures. However, the integration of scientific advances into environmental regulations (perhaps inevitably) needs decade(s). The reason for this is often the higher technical sophistication and associated higher cost of new tools. Is society ready to pay for better conservation of the coastal environment?

References

Adams S.M., Shepard K.L., Greeley Jr M.S., Jimenez B.D.n Ryon M.G., Shugart L.R., McCarthy J.F., 1989. The use of bioindicators for assessing the effects of pollutant stress on fish. Mar. Environ. Res. 28: 459-464.

AFNOR. 1985. Huîtres creuses – Dénominations et classification, NF V45-056, Septembre 1985.

Amara R., Galois R., 2004. Nutritional condition of metamorphosing sole: spatial and temporal analyses. J. Fish Biol. 64: 72-88.

Amara R., Meziane T, Gilliers C., Hermel G., Laffargue P., 2007. Growth and condition indices in juvenile sole *Solea solea* measured to assess the quality of essential fish habitat. Mar. Ecol. Prog. Ser. 351: 201-208.

Amiard J.C., Amiard-Triquet C., eds., 2008. Les biomarqueurs dans l'évaluation de l'état écologique des milieux aquatiques. Lavoisier TEC & DOC, Paris, 375 p.

Amiard J.C., Amiard-Triquet C., Barka S., Pellerin J., Rainbow P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76: 160-202.

Amiard-Triquet C., 2009. Behavioral disturbances: the missing link between sub-organismal and supra-organismal responses to stress? Prospects based on aquatic research. Hum. Ecol. Risk Assess., Special issue "Behavioral ecotoxicology" 15: 87-110.

Amiard-Triquet C. & Rainbow P.S., eds., 2009. *Environmental assessment of estuarine ecosystems – A case study*, CRC Press, Boca Raton, Fl., USA, 355 p.

Banta G.T., Andersen O., 2003. Bioturbation and the fate of sediment pollutants. Vie Milieu 53: 233-248.

Beliaeff B., Burgeot T., 2002. Integrated biomarker response: a useful tool for ecological risk assessment. Environ. Toxicol. Chem. 21: 1316-1322.
Berthet B., Mouneyrac C., Amiard J.C., Amiard-Triquet C., Berthelot Y., Le Hen A., Mastain O., Rainbow P.S. & Smith B., 2003. Accumulation and soluble binding of Cd, Cu and Zn in the polychaete *Hediste diversicolor* from coastal sites with different trace metal bioavailabilities. Arch. Environ. Contam. Toxicol. 45 : 468-478.
Berthet B., 2008. Les espèces sentinelles. In *Les biomarqueurs dans l'évaluation de l'état écologique des milieux aquatiques*, Amiard & Amiard-Triquet, eds., Lavoisier TEC & Doc, Paris, 121- 148.
Boldina-Cosqueric I., Amiard J.C., Amiard-Triquet C., Dedourge-Geffard O., Métais I., Mouneyrac C., Moutel B., Berthet B. Biochemical, physiological and behavioural markers in the endobenthic bivalve *Scrobicularia plana* as tools for the assessment of estuarine sediment quality. Ecotox. Environ. Saf., in press.
Bouchet V., Degre D., Sauriau P.G., 2005. Population dynamic factors of *Scrobicularia plana* (Da Costa 1778) of Aiguillon Cove (Charente-Maritime, Vendee) under influence of predation by wintering limicoles. In: Richard, G. (Ed.), Proceedings, Third International Congress of European Societies of Malacology: Univ. La Rochelle, Laboratoire de Biologie et Environnement Marins (LBEM), 221-225.
Bouchet V.M.P., Sauriau P.G., Debenay J.P., Mermillod-Blondin F., Schmidt S., Amiard J.C., Dupas B., 2009. Influence of the mode of macrofauna-mediated bioturbation on the vertical distribution of living benthic foraminifera: first insight from axial tomodensitometry. J. Exp. Mar. Biol. Ecol. 371: 20-33.
Bryan G.W., Langston W.J., Hummerstone L.G., Burt G.R., 1985. A guide to the assessment of heavy-metal contamination in estuaries. Occ. Publ. Mar. Biol. Ass. UK 4: 1-92.
Byrne P.A., O'Halloran, J., 1999. Aspects of assaying sediment toxicity in Irish estuarine ecosystems. Mar. Pollut. Bull. 39: 97-105.
Cabral H.N., Murta A.G., 2004. Effect of sampling design on abundance estimates of benthic invertebrates in environmental monitoring studies. Mar. Ecol. Prog. Ser. 276: 19-24.
Caeiro S., Costa M.H., Ramos T.B., Fernandes F., Silveira N., Coimbra A., Medeiros G., Painho M., 2005. Assessing heavy metal contamination in Sado Estuary sediment: An index analysis approach. Ecol. Indic. 5: 151-169.
Cardoso P.G., Lillebø, A.I., Pereira, E., Duarte, A.C., Pardal, M.A., 2009. Different mercury bioaccumulation kinetics by two macrobenthic species: the bivalve Scrobicularia plana and the polychaete *Hediste diversicolor*. Mar. Environ. Res., doi: 10.1016/j.marenvres.2009.03.006
Casagranda C., Boudouresque C.F., 2005. Abundance, population structure and production of *Scrobicularia plana* and *Abra tenuis* (Bivalvia: Scrobicularidae) in a Mediterranean Brackish Lagoon, Lake Ichkeul, Tunisia. Internat. Rev. Hydrobiol. 90: 376-391.
Cassai C., Prevedelli P., 1998. Reproduction effort, fecundity and energy metabolism in two species of the genus *Perinereis* (Polychaeta : Neredidae). Invertebrate Reproduction and Development 34: 125-131.
Coelho J.P., Nunes M., Dolbeth M., Pereira M.E., Duarte A.C., Pardal M.A., 2008. The role of two sediment-dwelling invertebrates on the mercury transfer from sediments to the estuarine trophic web. Estuar. Coast. Shelf Sci. 78: 505-512.
Costa F.O., Neuparth T., Correia A.D., Costa M.H., 2005. Multi-level assessment of chronic toxicity of estuarine sediments with the amphipod *Gammarus locusta*: II. Organism and population-level endpoints. Mar. Environ. Res. 60: 93-110.
Cunha I., Neuparth T., Caeiro S., Costa M.H., Guilhermino L., 2007. Toxicoty ranking of estuarine sedimens on the basis of *Sparus aurata* biomarkers. Environ. Toxicol. Chem. 26: 444-453.
Dagnino A., Allen J.I., Moore M.N., Broeg K., Canesi L., Viarengo A., 2007. Development of an expert system for the integration of biomarker responses in mussels into an animal health index. Biomarkers 12 : 155-172.
Damiens G., Gnassia-Barelli M., Loquès F., Roméo M., Salbert V., 2007. Integrated biomarker response index as a useful tool for environmental assessment evaluated using transplanted mussels. Chemosphere 66 : 574-583.
Davey J.T., 1993. Macrofaunal community bioturbation along an estuarine gradient. Neth. J. Aquat. Ecol. 27: 147-153.
De Coen W.M., Janssen C.R., 2003. The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ. Toxicol. Chem. 22: 1632-1641.
Depledge M.H., 1993. The rational basis for the use of biomarkers as ecotoxicological tools. In: *Nondestructive biomarkers in vertebrates*, Fossi M.C. & Leonzio, C., eds., Lewis Publishers, Boca Raton, 261-285.
DEQ (Virginia Department of Environmental Quality), 2009. Shenandoah and James River Basin Fish Kills: 2007 Passive Sampler Results Summary. http://www.deq.state.va.us/export/sites/default/info/pdf/2007_DEQ_SPMD_Report_Final.pdf
Durou C., Mouneyrac C., 2007. Linking steroid hormone levels to sexual maturity index and energy reserves in *Nereis diversicolor* from clean and polluted estuaries. Gen. Comp. Endocrinol. 150: 106-113.
Durou C., Mouneyrac C., Amiard-Triquet C., 2008. Environmental quality assessment in estuarine ecosystems: Use of biometric measurements and fecundity of the ragworm *Nereis diversicolor* (Polychaeta, Nereididae). Water Res. 42:2157-2165.
Durou C., Poirier L., Amiard J.C., Budzinski H., Gnassia-Barelli M., Lemenach K., Peluhet L., Mouneyrac C., Romeo M.. Amiard-Triquet C., 2007a. Biomonitoring in a clean and a multi-contaminated estuary based on biomarkers and chemical analyses in the endobenthic worm *Nereis diversicolor*. Environ. Pollut. 148: 445-458.
Durou C., Smith B.D., Romeo M., Rainbow P.S., Mouneyrac C., Mouloud M., Gnassia-Barelli M., Gillet P., Deutch B., Amiard-Triquet C., 2007b. From biomarkers to population responses in *Nereis diversicolor*: assessment of stress in estuarine ecosystems. Ecotox. Environ. Saf. 66: 402-411.
Forbes V.E., Plamqvist A., Bach L., 2006. The use and misuse of biomarkers in ecotoxicology. Environ. Toxicol. Chem. 25 : 272-280.
Fournier M., Gauthier-Clerc S., Pillet S., Brousseau P., 2005. Biomarqueurs immunologiques appliqués à l'écotoxicologie. Bull. Soc. Zool. Fr. 130: 333-351.
Fraser A.J., 1989. Triacylglycerol content as a condition index for fish, bivalve and crustacean larva. Can. J. Fish. Aquat. Sci. 46: 1868-1873.
Gagné, F., Blaise C., Pellerin J., Fournier M., 2009. Etudes de biomarqueurs chez la mye commune (*Mya arenaria*) du fjord du Saguenay : bilan de recherches (1997 à 2006). Rev. Sci. Eau 22: 253-269.
Galloway T.S., Brown R.J., Browne M.A., Dissanayake A., Lowe D., Depledge M.H., Jones M.B., 2006. The ECOMAN project : – a novel approach to defining sustainable ecosystem function. Mar. Pollut. Bull. 53: 186-194.
Gauthier-Clerc S., Pellerin J., Blaise C., Gagné F., 2002. Delayed gametogenesis of *Mya arenaria* in the Saguenay fjord (Canada): a consequence of endocrine disruptors? Comp. Biochem. Physiol. 131C: 457-467.
Gillet P., Mouloud M., Durou C., Deutsch B., 2008. Response of Nereis diversicolor population (Polychaeta, Nereididae) to the pollution impact – Authie and Seine estuaries (France). Estuar. Coast. Shelf Sci. 76: 201-210.
Gillet P., Torresani S., 2003. Structure of the Population and Secondary Production of *Hediste diversicolor* (O.F. Müller, 1776),

(Polychaeta, Nereidae) in the Loire estuary, Atlantic Coast, France. Estuar. Coast. Shelf Sci. 56:621-628.
Gomes T., Gonzalez-Rey M., Bebianno M.G., 2009. Incidence of intersex in male clams *Scrobicularia plana* in the Guadiana Estuary (Portugal). Ecotoxicol. DOI 10.1007/s10646-009-0359-5.
Green J. 1957. The growth of *Scrobicularia plana* (Da Costa) in the Gwendraeth estuary. J. Mar. Biol. Ass. U.K. 36:41-47.
Guerlet E., 2007. *Utilisation de biomarqueurs cellulaires chez plusieurs espèces d'invertébrés pour l'évaluation de la contamination des milieux dulçaquicoles*. Thèse de Doctorat, Université de Metz.
Hagger J.A., Jones M.B., Lowe D., Paul Leonard D.R., Owen R., Galloway T.S., 2008. Application of biomarkers for improving risk assessments of chemicals under the Water Framework Directive: A case study. Mar. Pollut. Bull. 56: 1111-1118.
Häkanson J.L., 1989. Condition of larval anchovy (*Engraulis mordax*) in the Southern California Bight, as measured through lipid analysis. Mar. Biol. 102: 1531-59.
ITRC (Interstate Technology & Regulatory Council). 2005. Technology Overview of Passive Sampler Technologies. DSP-4. Washington, D.C.: Interstate Technology & Regulatory Council, Authoring Team. www.itrcweb.org.
Kalman J., Buffet P.E., Amiard J.C., Denis F., Mouneyrac C., Amiard-Triquet C., 2010. Assessment of the influence of confounding factors (weight, salinity) on the response of biomarkers in the estuarine polychaete *Nereis diversicolor*. Biomarkers 15: 461-469.
Kalman J., Palais F., Amiard J.-C., Mouneyrac C., Muntz A., Blasco J., Riba I. & Amiard-Triquet C., 2009. Assessment of the health status of populations of the ragworm *Nereis diversicolor* using biomarkers at different levels of biological organisation.. Mar. Ecol Pogr. Ser.: 393, 55-67.
Kidd K.A., Blanchfield P.J., Mills K.H., Palace V.P., Evans R.E., Lazorchak J.M., Flick R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Nat. Acad. Sci. 104: 8897-8901.
Langston W.G., Burt G.R., Chesman G.S., 2007. Feminisation of male clams *Scrobicularia plana* from estuaries in Southwest UK and its induction by endocrine-disrupting chemicals. Mar. Ecol. Prog. Ser. 333: 173-184.
Maltby, L., Clayton S.A., Yu H., McLoughlin N., Wood R.W., Yin D., 2000. Using single-species toxicity tests, community-level responses, and toxicity identification evaluations to investigate effluent impacts. Environ. Toxicol. Chem. 19: 151-157.
Mazorra M.T., Rubio J.A., Blasco J., 2002. Acid and alkaline phosphatase activities in the clam *Scrobicularia plana*: kinetic characteristics and effects of heavy metals. Comp. Biochem. Physiol. 131B: 241-249.
Mills G.A., Greenwood R., Allan I.J., Lopuchin E., Brümmer J., Knutsson J., Vrana B., 2010. Application of passive sampling techniques for monitoring the aquatic environment. . In *Analytical measurements in aquatic environments*, Namiesnik & Szefer, eds., CRC Press, Boca Raton, Fl;, 41-68.
Moore M.N., Allen J.I., McVeigh A., 2006. Environmental prognostics: An integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. Mar. Environ. Res. 61: 278-304.
Moreira S.M., Lima I., Ribeiro R., Guilhermino L., 2006. Effects of estuarine sediment contamination on feeding and on key physiological functions of the polychaete *Hediste diversicolor*: Laboratory and *in situ* assays. Aquat. Toxicol. 78: 186-201.
Moreira S.M., Moreira-Santos M., Guilhermino L., Ribeiro R., 2005. A short-term sublethal *in situ* toxicity assay with *Hediste diversicolor* (polychaeta) for estuarine sediments based on postexposure feeding. Environ. Toxicol. Chem. 24: 2010-18.
Mouneyrac C., Mastain O., Amiard J.C., Amiard-Triquet C., Beaunier P., Jeantet A.Y., Smith B.D., Rainbow P.S., 2003. Physico-chemical forms of storage and the tolerance of the estuarine worm *Nereis diversicolor* chronically exposed to trace metals in the environment. Mar. Biol. 143, 731-744.
Mouneyrac C., Perrein-Ettajani H., Amiard-Triquet C., 2010. Influence of anthropogenic stress on fitness and behaviour of a key-species of estuarine ecosystems, the ragworm *Nereis diversicolor*. Environ. Pollut. 158: 121-128.
NAS, 1980. *The International Mussel Watch*. Report of a workshop sponsored by the environment studies board. Washington, DC: Natural Resources Commission National Research Council National Academy of Sciences.
Neuparth, T., Correia A.D., Costa F.O., Lima G., Costa M.H., 2005. Multi-level assessment of chronic toxicity of estuarine sediments with the amphipod *Gammarus locusta*: I. Biochemical endpoints. Mar. Environ. Res. 60: 69-91.
Ng T.Y.T., Rainbow P.S., Amiard-Triquet C., Amiard J.C., Wang W.X., 2008. Decoupling of cadmium biokinetics and metallothionein turnover in a marine polychaete after metal exposure. Aquat. Toxicol. 89: 47-54.
Olive P.J.W., 1983. Oogenesis, Oviposition and Oosorption, John Wiley & Sons Ltd., pp. 357-422.
Olive P.J.W., Lewis C., Beardall V., 2000. Fitness components of seasonal reproduction: an analysis using *Nereis virens* as a life history model. Oceanol. Acta. 23: 377-389.
Olla B.L., Bedja A.J., Pearson W.H., 1983. Effects of oiled sediment on the burrowing behaviour of the hard clam, *Mercenaria mercenaria*. Mar. Environ. Res. 9: 183-193.
Olla B.L., Bedja A.J., Studholme A.L., Pearson W.H., 1984. Sublethal effects of oiled sediment on the sand worm, *Nereis (Neanthes) virens*: Induced changes in burrowing and emergence. Mar. Environ. Res. 13: 121-139.
Orvain F., 2005. A model of sediment transport under the influence of surface bioturbation: generalisation to the facultative suspension-feeder *Scrobicularia plana*. Mar. Ecol. Prog. Ser. 286: 43-56.
Pearson W.H., Woodruff D.L., Sugarman P.C., Olla B.L., 1981. Effects of oiled sediment on predation on the littleneck clam, *Protothaca staminea*, by the Dungeness crab, *Cancer magister*. Estuar. Coast. Shelf Sci. 13: 445-454.
Pérez E., Blasco J., Solé M., 2004. Biomarker responses to pollution in two invertebrate species: *Scrobicularia plana* and *Nereis diversicolor* from the Cadiz bay (SW Spain). Mar. Environ. Res. 58: 275-79.
Poirier L., Berthet B., Amiard J.C., Jeantet A.Y., Amiard-Triquet C., 2006. A suitable model for the biomonitoring of trace metals bioavailabilities in estuarine sediment: the annelid polychaete *Nereis diversicolor*. J. Mar. Biol. Ass. U. K.: 86, 71-82.
Romero-Ruiz A., Alhama J., Blasco J., Gomez-Ariza J.L., Lopez-Barea J., 2008. New metallothionein assay in *Scrobicularia plana*: Heating effect and correlation with other biomarkers. Environ. Pollut. 156: 1340-1347.
RNO, 2006. Surveillance de la qualité du milieu marin. Ministère de l'Environnement et Institut français de recherche pour l'exploitation de la mer (Ifremer), Paris & Nantes.
Ruus A., Schaanning M., Øxnevad S., Hylland K., 2005. Experimental results on bioaccumulation of metals and organic contaminants from marine sediments. Aquat. Toxicol. 72 : 273-292.
Siah A., Pellerin J., Amiard J.C., Pelletier E., Viglino L., 2003. Delayed gametogenesis and progesterone levels in soft-shell clams (*Mya arenaria*) in relation to *in situ* contamination to organotins and heavy metals in the St Lawrence river (Canada). Comp. Biochem. Physiol. 135C: 145-156.
Scaps P., Borot O., 2000. Acetylcholinesterase activity of the polychaete *Nereis diversicolor*: effects of temperature and salinity. Comp. Biochem. Physiol. 125C: 377-383.
Soares C., Sobral P., 2009. Density-dependent effects of bioturbation by the clam, *Scrobicularia plana*, on the erodability of estuarine sediments. Mar. Freshwater Res. 60: 737-744.
Solé M., Kopecka-Pilarczyk J., Blasco J., 2009. Pollution biomarkers in two estuarine invertebrates, *Nereis diversicolor* and *Scrobicularia plana*, from a Marsh ecosystem in SW Spain. Environ. Int. 35: 523-531.

Spooner G.M., Moore H.B., 1940. The ecology of the Tamar estuary. VI. An account of the macrofauna of the intertidal mudflats. J. Mar. Biol. Ass. U.K. 24: 283-330.

Stuer-Lauridsen F., 2005. Review of passive accumulation devices for monitoring organic micropollutants in the aquatic environment. Environ. Pollut. 136: 503-524.

Suthers I.M., Fraser A., Frank K.T., 1992. Comparison of lipid, otolith and morphometric condition indices of pelagic juvenile cod *Gadus morhua* from the Canadian Atlantic. Mar. Ecol. Prog. Ser. 84: 31-40.

Tannebaum L.V., 2005. A Critical assessment of the ecological risk assessment process: A review of misapplied concepts. Integr. Environ. Assess. Manag. 1: 66-72.

van der Oost, R., Porte-Visa C., van den Brink N.W. 2005. Biomarkers in environmental assessment. In *Ecotoxicological testing of marine and freshwater ecosystems : emerging techniques, trends and strategies*, den Besten, P.J. and Munawar, M., eds., CRC Press, Boca Raton, Fl., USA, 87-152.

Vasseur P., Cossu-Leguille C., Atienzar F., Rodius F., Lemière S., 2008. Marqueurs de génotoxicité et effets *in situ*, individuals et populationnnels. In *Les biomarqueurs dans l'évaluation de l'état écologique des milieux aquatiques*, Amiard & Amiard-Triquet, eds., Lavoisier TEC & Doc, Paris, 295-330.

Wallace W.G., Hoexum Broewer T.M., Brouwer M., et al. 2000. Alterations in prey capture and induction of metallothioneins in grass shrimp fed cadmium-contaminated prey. Environ. Toxicol. Chem. 19: 962-71.

Weis J.S., Smith G.M., Zhou T., et al. 2001. Effects of contaminants on behavior: biochemical mechanisms and ecological consequences. BioScience 51: 209-17

Wilding, J., Maltby, L., 2006. Relative toxicological importance of aqueous and dietary metal exposure to a freshwater crustacean: implications for risk assessment. Environ. Toxicol. Chem. 25: 1795-1801.

Wosniok W., Broeg K., Feist S.W., 2005. On the applicability of the various available 'health indices' for the interpretation of data ob-tained from biological effects monitoring activities and associated research studies using pathology and disease endpoints. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), 8–12 March 2005, La Tremblade, France. ICES CM 2005/F:02, Annex 8, 76-84.

Yeom D.H., Adams S.M., 2007. Assessing effects of stress across levels of biological organization using an aquatic ecosystem health index. Ecotoxicol. Environ. Saf. 67:286-295.

Zhang H., Davison W., 1995. Performance characteristics of diffusion gradients in thin films for the *in situ* measurement of trace metals in aqueous solution. Anal. Chem. 67: 3391-3400.

5.3 Publication 3

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Intersite variations of a battery of biomarkers at different levels of biological organisation in the estuarine endobenthic worm *Nereis diversicolor* (Polychaeta, Nereididae)

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The present paper is based on the recognized need of considering stress effects at different levels of biological organisation. This strategy has been developed in estuarine areas, crucial for the ecological and economic health of coastal regions. Biochemical, physiological, behavioural biomarkers and population effects were examined over more than one year in the ragworm *Nereis diversicolor*, a sentinel species important for the structure and functioning of mudflat ecosystems.

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Intersite variations of a battery of biomarkers at different levels of biological organisation in the estuarine endobenthic worm *Nereis diversicolor* (Polychaeta, Nereididae).

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Abstract

The ragworm *Nereis diversicolor* has been proposed as a sentinel species for the assessment of estuarine sediment quality. The aim of this study was to test the responsiveness of the worms using a battery of biomarkers in specimens from a moderately contaminated site (Loire estuary, Fr.) and a comparatively cleaner site (Bay of Bourgneuf, Fr.) as a reference site. Ragworms were collected on 7 occasions from April 2008 to October 2009 for the determination of biochemical (GST, AChE, digestive enzymes), physiological (energy reserves (glycogen, lipids and proteins), relationship between length and weight), and behavioural (feeding and burrowing) biomarkers. The biomarker responses were tentatively interpreted in terms of the concept of cascading events potentially responsible for local depletion/extinction of populations submitted to chemical stress. Impairments of AChE and amylase activities, feeding rate, energy reserve concentrations (glycogen and lipids), and the relationship between length and weight showed up differences between the reference site and the Loire estuary despite the latter being far from the most contaminated estuary in France or internationally. However, no links could be established in the Loire estuary ragworms between effects at infra-individual and individual levels, nor with worm population density, even though a small oil spill had occurred in the Loire estuary only one month before the beginning of sampling.

Keywords : Biomonitoring, estuarine sediment, sentinel species, biochemical impairments, physiological responses, behavioural tests

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1. Introduction

Paradoxically, throughout the world, estuaries are among the most productive ecosystems while being strongly impacted by anthropogenic activities. When considering the value of world ecosystem services and natural capital, estuaries are among the most productive ecosystems (MEA, 2005). More than 76% of all commercially and recreationally important fish and shellfish species are estuarine-dependent (Scott and Klaine, 2001). On the other hand, use of the specific search term "estuary" in the Marine Pollution Bulletin database, unearthed 551 titles to-date (July 2011), highlighting the importance of pollution threats to these ecosystems.

The polychaetes form a taxonomic group that is very important in the structure and functioning of marine and estuarine ecosystems, particularly in intertidal mudflats, being represented by several species exhibiting high densities and biomasses. Many polychaetes are endobenthic species, living in close contact with sediments which are the final sink for many organic and inorganic contaminants introduced into the environment as a consequence of anthropogenic pressure. Thus they can be used as biological models for the determination of biomarkers at different levels of biological organization (Dean, 2008; Amiard-Triquet et al., in press). In the ECOMAN project, an approach to defining sustainable ecosystem function, worm species were selected as sentinels (Galloway et al., 2006).

A biomarker is defined by Depledge (1994) as "a biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical pollutants (and/or radiations)". For many biomarkers of interest in the present study, the ragworm *Nereis diversicolor* has been shown to be very responsive (Moreira et al., 2006; Durou et al., 2007a; Kalman et al., 2009; Solé et al., 2009) and particularly robust even after the consideration of the influence of confounding factors (Kalman et al., 2010).

The aim of the present paper is to design a comprehensive study based upon partial approaches carried out previously to assess the health status of *N. diversicolor* in different estuarine sites, differentially exposed to anthropogenic pressures (Durou et al., 2007a, b; Kalman et al., 2009; Mouneyrac et al. 2010). Biomarkers were examined at different levels of biological organization in ragworms from the multi-polluted Loire estuary and the comparatively clean Bay of Bourgneuf (RNO, 2006; Marchand et al., 2009) between April

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2008 and October 2009. Biochemical responses included the activities of the biotransformation enzyme glutathione S-transferase (GST); of acetylcholinesterase (AChE) able to reveal neurotoxic impairment; of digestive enzymes involved in the acquisition of energy (amylase, carboxymethylcellulase CMCase). Physiological responses included the levels of energy reserves (as glycogen, lipids and proteins) and growth (relationships between weight and length). Behavioural responses included feeding rate (important for the acquisition of energy) and burrowing (important for predator avoidance). In addition, the population density and structure of the worms was examined in the Loire estuary which had been submitted to a small oil spill on 16 March 2008,. In order to distinguish between potential effects of this event and the responses of ragworms to chronic pollution, the initial study by Kalman et al. (2009) from April to May 2008 was extended to October 2009.

2. Materials and Methods

2.1. Sampling sites

Animals were collected from April 2008 to October 2009 at two sites in the Gulf of Biscay, Fr., differentially exposed to anthropogenic pressure. The Loire estuary (2° 07' 59.76" W, 47° 16' 29.66" N) is a large estuary exposed to multiple anthropogenic sources of chemicals, whereas the Bay of Bourgneuf (2° 04' 40.60" W, 46° 56' 23.08" N) devoted to oyster farming, extensive agriculture and tourism is considered to be comparatively less contaminated and suitable as a reference site. Furthermore,, the Loire estuary was submitted to a small oil spill (approximately 180 t of bunker fuel) on 16 March 2008 due to a pipe leak at the Donges Refinery (www.cedre.fr/en/spill/donges/donges.php). The choice of these sites took into account information available in the framework of the French "Mussel Watch" Programme (RNO, 2006; Marchand et al., 2009) and provided by the regulation authorities for the implementation of the European Community Water Framework Directive (ECWFD). In the WFD, water masses are classified according to their ecological status and their chemical status. For both criteria, the Bay of Bourgneuf has been classified as good whereas the Loire estuary has a good ecological status but a bad chemical status (http://envlit.ifremer.fr/var/envlit/storage/documents/atlas_DCE/scripts/site/carte.php?map=LB). For metals, previous studies have shown moderate contamination of the worms from the Loire compared to those from the Bay of Bourgneuf (Kalman et al. 2009).

Residual water remaining on the sediment surface at low tide was used to determine salinity. In the Loire estuary, salinity varied seasonally from 15 in winter and spring to 25.5

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in summer. In the Bay of Bourgneuf, salinity was less fluctuating (29.1 to 32.5). Temperature was not systematically documented since the two sampling sites are located only 50 km apart. Granulometry of sediment cores (≈ 20 cm deep, equivalent to the vertical distribution of the ragworms in the sediment) was determined according to the French standard method NF X31-107 by an official laboratory (Institut Départemental d'Analyses et de Conseil, IDAC, Nantes, Fr.) working under Good Laboratory Practices (Fig. 1).

2.2. Collection

Samples were collected in the intertidal zone at low tide. For both sites, and on each of the 7 sampling dates, the surface oxygenated layer (a few mm deep and about 300 cm^2 area) of sediments destined for analysis of organic contaminants, TOC (total organic carbon), Chl *a* (chlorophyll *a*) and Phaeo *a* (phaeopigments *a*) was scraped off at three places a few meters apart and placed in an aluminium box. At the same time, individuals of *N. diversicolor* were handpicked from the intertidal mudflats. Worms of similar weights were taken from the two sites at each period for each test (behavioural, biochemical and physiological). At each occasion, a further 50 specimens were collected at random in order to determine their condition based on the length of the first three segments named L3 (peristomium, prostomium and first chaetiger segment) and the wet weight as recommended by Durou et al. (2008). After collection, sediments and animals were transported in cold containers with wet algae or sediment from the site of origin without seawater for the ragworms. In the laboratory, sediments were frozen at -80°C until analysis. Animals were kept at ambient temperature of the time of collection. Animals destined for biometric measurements (L3, wet weight) were maintained in aerated containers filled with natural filtered seawater adjusted to the salinity of the site of origin for 24 hours to allow the elimination of their gut contents. Those destined for analysis of enzyme activities and concentrations of energy reserves were washed with filtered seawater to eliminate sediment and kept at -80°C until use.

2.3. Quantification of TOC, chlorophyll *a* and phaeopigments *a*

Total organic carbon (TOC) concentrations were determined in the superficial sediment by ashing (NF EN 13137 (X30-404) (IDAC, Nantes, Fr.). Pigment analyses were performed

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using a spectrophotometer (Uvikon XS UV/VIS, software Lab Power Junior, Bio-Tek Instruments (Wavelength range: 190-1100nm; wavelength percision: 0.3 nm). Lipid soluble pigments including Chl *a* and Phaeo *a* were extracted from sediment samples using the methods of Swain (1985) and Waters (2000) modified. Pigments were extracted from 31-55 g of wet sediment in each sample using 30 mL of acetone 100% rather than 90%. This was used to prevent excessive solvent dilution due to the high water content of the sediment that would otherwise make extraction less effective. Samples were shaken and kept in the dark at 4°C for a period of 24 hours. Supernatants containing dissolved pigments were filtered using a syringe through a Millipore hydrophobic membrane (MINISART PTFE) of 0.2 µm porosity and 28 mm diameter). Chl *a* and Phaeo *a* concentrations were determined following Lorenzen (1967) using DO measured at 665 and 750 nm, before and after acidifying the sample with hydrochloric acid (0.1 mL of HCl 0.1 N). This corrects for interference due to the presence of Phaeo *a*, absorbing at the same wavelength as Chl *a*, and does not require calibration with a pure solution of Chl *a* to retrieve the concentration of Chl *a* alone. In order to express Chl *a* and Phaeo *a* concentrations per unit weight of dry sediment, a sub-sample of each sediment was dried for 48 hours at 60°C to estimate the sediment water content, and eliminate its contribution to the wet sediment weight used for pigment extraction.

Phaeo *a* concentration is considered by several authors to be a useful marker for intertidal microphytobenthos grazed by meio- and macrofauna (Abele-Oeschger & Theede 1991, Buffan-Dubau et al. 1996, Cartaxana et al. 2003; Méléder et al. 2005). Thus, the quality of potentially available food sources is estimated in this study using the ratio Chl *a*/Phaeo *a* but also Chl *a*/TOC.

2.4. Assessment of exposure

Organic contaminant analyses in the superficial sediments were performed by the laboratory IDAC. Mineral oils were quantified by infra-red spectrometry according to the standardized method ISO11046 (X31-410), polycyclic aromatic hydrocarbons (PAHs) and polychlorobiphenyls (PCBs) by GC/MS(/MS) according to the standardized method XP X33-012.

2.5. Determination of biochemical markers (enzyme activities)

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For GST, AChE, amylase and CMcase activities, determinations were carried out in the whole soft tissues of 10 worms per site and collection occasion.

The procedure for homogenization adapted to each enzyme and the recovery of supernatant have been described in detail by Kalman et al. (2009). Briefly, enzyme activities were analysed in triplicate using a microplate reader as described by Galgani and Bocquené (1991) for AChE/ChE, Frasco and Guilhermino (2002) for GST. In the case of GST, an aliquot of the homogenate was centrifuged (9000g for 30 min at 4 °C) and the resulting supernatant was used directly in the enzyme assay. GST activity was determined spectrophotometrically at 340 nm ($e = 9.6\ mM^{-1}\ cm^{-1}$) by monitoring the formation of 1-glutathione- 2,4-dinitrobenzene, resulting from the conjugation of the substrate, 1-chloro-2,4-dinitrobenzene (CDNB), with glutathione in reduced form (GSH), as described by Habig et al. (1974). The α-amylase and cellulase activities were determined by the method of Bernfeld (1955) adapted by Kalman et al. (2009). All the enzyme activities were calibrated to protein concentrations which were measured in the soft tissue supernatant by the method of Bradford (1976) adapted for microplate use with bovine γ-globulin as standard protein.

2.6. Determination of physiological markers

2.6.1. Glycogen and lipid quantification

Glycogen and lipids were quantified in the whole soft tissues of 10 worms for each sampling month and each site. After homogenization in liquid nitrogen, the powder obtained was then homogenized with 1.5 mL citrate buffer at pH 5.0 for glycogen and lipid analysis. Total lipids were determined by a sulpho-phosphovanillin reaction according to the method of Frings et al. (1972). Glycogen concentrations were estimated in two aliquots of the homogenate, one of which was submitted to enzymatic digestion by amyloglucosidase according to Carr and Neff (1984). Olive oil and glycogen from oyster (Sigma, Type III) were used as standards for each method, respectively.

2.6.2. Biometric indices

The wet weight of depurated worms was determined and then worms were fixed with

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10% formalized seawater for storage until analyses. The length of the first three segments named L3 (peristomium, prostomium and first chaetiger) was measured under a binocular microscope (Gillet and Torresani, 2003).

2.7. Behavioural tests

All the behavioural tests were set up in a dark, temperature controlled room (temperature set to ambient temperature at the time of collection) and conducted within one week of collection.

The feeding rate was quantified according to the methodology described by Moreira et al. (2006). A food ration comprised 100 larvae of *Artemia salina*, kept frozen until use. Twenty worms from each site were placed into plastic beakers containing about 30 mL seawater adjusted to the salinity of the site of origin and left for 24 hours without disturbance. Worms were allowed to feed for 1 h. At the end of the test, the remaining larvae were counted and results were expressed as the number of larvae fed per hour.

Burrowing experiments were carried out as described by Bonnard et al. (2009). Briefly, worms were placed on the sediment in plastic containers of 100 mL filled with 5 cm of wet sediment from the site of origin. Their positions were recorded every two minutes. Twenty worms were submitted to each test. Individuals from the burrowing tests were recovered for AChE activity measurements.

2.8. Statistical treatment

Kinetic curves obtained from burrowing tests were ln-transformed to produce straight lines. Thus the slope of the curves in absolute value may be used as surrogates for burrowing speed. Analysis of covariance (ANCOVA) between regression coefficients of the least-square best-fit regression lines was used for comparisons, followed by Tukey's HSD post-hoc method.

For all the other biological parmeters, results are presented as mean ± SD. Normality was verified using the Shapiro-Wilk test and homoscedasticity using Fisher test. To define significant differences between sites for each sampling date, normally distributed data were analyzed by one-way ANOVA (multiple comparisons of means) followed by Tukey's HSD

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post hoc method. When normality was not established, the non-parametric Kruskal-Wallis and Mann-Whitney comparison tests were used. In addition, to compare the levels of biochemical markers over the whole sampling period, bilateral Mann-Whitney tests were carried out. The level of significance was established at $P < 0.05$. Statistical analyses were performed using XLSTAT 2011.

3. Results

3.1. Characterisation of superficial sediments

3.1.1. Exposure to contaminants

The concentrations of PCBs in sediments from both sites were always below the quantification limits which were chosen by the laboratory IDAC in order to be able to detect concentrations above the quality thresholds for sediments according to French regulations (25 µg kg^{-1} dw for CB 28, 52, 118, 180; 50 µg kg^{-1} dw for CB 101, 138, 158; 500 µg kg^{-1} dw for ΣPCBs).

The concentrations of PAHs in sediments from both sites have been standardized to the concentrations of TOC (Figure 2). On the average for the whole sampling period, the concentrations of individual compounds were higher in sediments from the Loire than in those from the Bay of Bourgneuf. Considering the sum of the 16 PAH priority pollutants listed by US EPA (US Environmental Protection Agency), the concentrations were higher in the Loire estuary than in the Bay of Bourgneuf with average concentrations reaching, respectively, 35.2 and 13.4 µg PAHs g^{-1} TOC (corresponding to 1031 and 413 µg.g^{-1} sediment d.w.).

3.1.2. Organic matter as chlorophyll *a*, phaeopigments *a* and total organic carbon TOC

The spatio-temporal variations of these three categories of organic matter are shown in Fig. 3. The highest concentrations of Chl *a* were obtained in May 2008 in the Loire estuary (73.5 µg.g^{-1} of dry sediment) whereas it was in October 2009 in the Bay of Bourgneuf (118.5 µg.g^{-1} of dry sediment). The lowest concentrations (7.7 and 11.8 µg.g^{-1} of dry sediment) were obtained in January 2009 and May 2008 for the Loire estuary and the Bay of Bourgneuf,

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respectively. Chl *a* and Phaeo *a* concentrations were higher in sediments from the Bay of Bourgneuf than in those from the Loire, with the exception of Chl *a* in May 2008. In contrast, TOC concentrations were usually higher in the Loire estuary than in the Bay of Bourgneuf except in June 2008.

The ratios Chl *a*/Phaeo *a* and Chl *a*/TOC are shown in Table 1. Both of these ratios are consistently higher for superficial sediments from the Bay of Bourgneuf except in May 2008 when levels of Chl *a* were very high in the Loire estuary.

3.2. Biochemical markers (enzyme activities)

For digestive enzymes, significant and consistent intersite differences were shown for *Nereis diversicolor* (Fig. 4). For amylase, one month after the small oil spill which occurred in the Loire estuary in March 2008, the activity was 65 times lower in local worms than in worms from the reference site (Bay of Bourgneuf). This ratio decreased to 13 and 20 for the two following months, and then stabilized at 3 to 4 after October 2008. The pattern for CMCase activity was typically the same, even if intersite differences were not as large as in the case of amylase (Fig. 4). The activity of AChE was always significantly higher in the worms from the Bay of Bourgneuf (Fig. 4). In the case of GST activity, no significant intersite difference was observed (not shown).

3.3. Physiological markers

3.3.1. Energy reserves (glycogen, lipids, proteins)

Generally, energy reserves were higher in the Loire estuary than in the Bay of Bourgneuf (Fig. 5). In the case of glycogen, monthly intersite differences were significant over the whole sampling period, except in March 2009. Despite a significant inverse ranking in April 2008, for both lipids and proteins, a significant difference, more important in the case of lipids, was revealed by the bilateral tests over the whole sampling period (with $p<0.001$ and $p = 0.045$ respectively).

3.3.2. Biometric measurements

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The length L3 and the wet weight of the 50 specimens collected at random from each site are depicted in Fig. 6, showing the minimum, median and maximum values determined for each sampling date. No significant intersite difference was shown when L3 was considered. On the other hand, the wet weight was significantly higher for the worms from the Bay of Bourgneuf belonging to the median ($p = 0.013$) and maximum ($p = 0.001$) categories compared to the worms from the Loire estuary. No intersite difference in wet weight was revealed for the smallest individuals.

3.4. Behavioural tests

The results of the burrowing tests carried out with worms from both sites allowed to burrow in the sediment of their site of origin are shown in Table 2. The intersite differences between the slopes of the curves in absolute values – a surrogate for burrowing speed – were small even if statistically different on two occasions (April and June 2008).

The results of the post-exposure feeding tests are depicted in Fig. 7. The number of larvae consumed per hour was generally higher for ragworms originating from the Bay of Bourgneuf, and the difference was significant except on two occasions. The mean number of larvae h^{-1} at each date and each site ($N=12$) were positively correlated to the corresponding values of AChE activity (Pearson's correlation coefficient $r = 0.3487$) but the correlation was not significant ($p = 0.27$).

4. Discussion

Sites of collection in the multi-polluted Loire estuary and the reference site Bay of Bourgneuf exhibited different salinities, varying from 15.0 to 25.5 in the former and from 29.1 to 32.5 in the latter. Among the biomarkers investigated in the present study, amylase, AChE and GST activities, glycogen and lipid concentrations, and burrowing behaviour are not influenced by differences in salinity (Kalman et al., 2010 and literature cited therein). Moreira et al. (2005) have shown a moderate effect of salinity on post-exposure feeding but in the range of salinities observed in the Loire and the Bay of Bourgneuf, feeding was not affected. Consequently, intersite differences observed in the present study for these parameters were

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not due to salinity as a confounding factor. For protein concentrations, Durou et al. (2007b) did not show significant differences in ragworms from sites with different salinities in the Authie estuary, France whereas Kalman et al. (2010) showed protein concentrations significantly lower at high salinity in the Goyen estuary, France, both of these sites being relatively uncontaminated. CMcase activity was nearly two times lower at 13‰ than at 30 ‰ (Kalman et al., 2010). Thus the intersite differences shown for proteins and CMcase activities between the ragworms from the Loire and Bay of Bourgneuf may be attributable to contrasted salinities rather than to a pollution effect.

Cascading effects of chemical stress at different levels of biological organisation have been documented, particularly in the case of behavioural impairments (Weis et al., 2001; Amiard-Triquet, 2009).

Behavioural disturbances were observed in ragworms from the multi-polluted Loire estuary when compared to controls from a reference site (Bay of Bourgneuf) in the case of feeding behaviour whereas burrowing activities were not so clearly affected. Feeding has been shown as a responsive behavioural biomarker in different species (see review by Amiard-Triquet, 2009), in particular in the ragworm *Nereis diversicolor* as demonstrated in specimens exposed to copper in the laboratory or to field contaminations in estuaries (Moreira et al., 2005; Moreira et al. 2006). Impairments of energy metabolism or neurotransmitters have been evoked as sources of behavioural disturbances. In the present study, the energy reserves glycogen, lipids and proteins were not depleted in specimens from the Loire estuary compared to those from the reference site. On the other hand, AChE activity which is a responsive biochemical marker in ragworms (Durou et al., 2007a; Solé et al., 2009), was significantly affected in the Loire estuary but the correlation with the feeding rate was not significant.

Burrowing behaviour was not strongly affected in ragworms, even after the small oil spill which occurred in the Loire estuary just a month before the beginning of the present study. This is in agreement with the findings of Moureyrac et al. (2010) in a study including our sites of interest, at a period (January-September 2006) when only chronic pollution was present. Sediment grain size which was coarser in the Bay of Bourgneuf and organic matter (as TOC) which was less abundant could act as confounding factors. However, Bonnard et al. (2009) have shown that ragworms acclimate well in artificial sand devoid of organic matter. In addition, cross tests carried out by Kalman et al. (2009) showed that ragworms from the Loire had a higher burrowing rate when they were allowed to burrow in the cleaner sediment from the Bay of Bourgneuf compared to their sediment of origin. Burrowing impairments in

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ragworms can be revealed only when a wider gradient of pollution is considered and such disturbances are attributable to avoidance of contaminated sediments, not to physiological impairments (Kalman et al., 2009; Moureyrac et al., 2010).

The cascading of the effects of chemical stress at different levels of biological organisation is also an important concept considering the acquisition and physiological allocation of energy (Fossi Tankoua et al., 2010). In the present study, we have examined food availability, digestive enzymes involved in food assimilation, contaminant defence (the biotransformation enzyme GST) which could contribute to an energy cost of tolerance, energy reserves and biometric measurements able to reveal growth patterns whereas a previous study examined the influence of anthropogenic stress on sexual maturation patterns (Mouneyrac et al., 2010).

Except in April 2008, ragworms from the Bay of Bourgneuf can have access to fresher organic matter represented by high ratio of Chl *a*/Phaeo *a* or Chl *a*/TOC whereas sediments from the Loire estuary showed more degraded organic matter content with the lowest values of these ratios. Méléder et al. (2005) reported the same higher level of Chl *a* in the Bay of Bourgneuf with a percentage of Phaeo *a* of no more than 30% of the total non-degraded pigments. Results obtained in the two mudflats suggest a weaker degradation of microphytobenthos in Bay of Bourgneuf compared to the Loire estuary. This can be due to the fineness of particles in the Loire estuary which allows larger deposits of organic matter than in the Bay of Bourgneuf. This hypothesis is supported by the work of Lucas and Holligan (1999) in the Westerschelde estuary. They have reported that, in the first 2 mm of sediment, sandy stations had similar concentrations of Chl *a* and Phaeo *a*, whereas muddy stations showed Phaeo *a* concentrations higher than Chl *a* concentrations.

Nereis diversicolor, as an opportunistic species, is considered able to fulfill its energy needs using different feeding strategies: carnivorous as well as filter- and deposit-feeding (Meziane & Retière 2002 and literature cited therein). Its diet may be composed of phytoplankton, microphytobenthos, and/or detritus of halophytic plants. Thus, to assay any assimilation impairment, digestive amylolytic and cellulolytic activities of amylase and cellulase enzymes are considered as relevant biomarkers (Kalman et al., 2009). Impairments of digestive enzyme functioning in invertebrates exposed to many classes of contaminants are well-established (Dedourge et al., 2008). The lower activity of amylase observed in ragworms from the Loire estuary is in agreement with this theory, but, as shown for CMcase, digestive enzymes can be also influenced by salinity and also respond to the nutritional status of invertebrates (Albentosa and Moyano, 2008) Amylase in particular could be used as

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indicative of nutritional condition.

Considering energy reserves expressed as concentrations of glycogen, lipids and proteins, the quality and quantity of available food (represented at least partly by chl *a*, phaeo *a* and TOC) and impaired digestive enzymes did not penalize the ragworms from the Loire estuary but the story was not the same when considering biometric measurements. Indeed, at the same length, specimens from the Loire showed reduced weight compared to ragworms from the Bay of Bourgneuf. According to the findings of Mouneyrac et al. (2010), it is likely that specimens from the Bay of Bourgneuf are growing more slowly and reproducing later (favouring weight increase instead of reproduction) whereas for worms from the Loire estuary, energy reserves are allocated preferentially to gametogenesis and length growth.

When a wider gradient of pollution iss considered, in ragworms from the multi-polluted Seine estuary energy reserves (glycogen, lipid and protein concentrations) as well as the relationship between length and weight were consistently impaired when compared to specimens from a reference site (Durou et al., 2007a; 2008), a situation which was attributed to a cost of tolerance. It is classically considered that energy is allocated to maintenance, growth and reproduction. In contaminated environments, physiological acclimation often generates an additional cost, required for the increased transcription and production (i.e. up-regulation) of detoxifying proteins and enzymes for combating toxic metals and xenobiotics (see review by Mouneyrac et al., 2011). In the present study, the biomarker of defence GST did not show any significant intersite difference despite the role of this enzyme in biotransformation of organic compounds such as PAHs, whereas increased GST activity was observed in ragworms from contaminated sites in the Sado estuary, Portugal (Moreira et al., 2006), the Seine estuary, France (Durou et al., 2007a) and a marsh ecosystem in SW Spain (Solé et al., 2009). In the case of the Seine and the Loire, the contrasted responses of GST may be linked to the degree of pollution documented by the French "Mussel Watch" program (RNO, 2006). In particular, mussels originating from the mouth of the Seine river exhibit very high levels of CB 153 (representative of PCB contamination) and fluoranthene (representative of PAH contamination) which are one order of magnitude higher than in the mouth of the Loire river.

In the case of ragworm population densities in the Loire estuary, in October when the annual recruitment has been completed, identical means (1500 individuals per m^2) were determined in 2008 and 2009 and did not differ significantly from those registered in April 2008, one month after the pipe leak on 16 March 2008 (Gillet, pers. comm.). These values are in the top part of the density range observed from 1998 to 2000 at the same site (Gillet and

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Torresani, 2003). This indicates that the bunker fuel leak had not had any significant population impact. This is not surprising since a low impact of oil spills on polychaetes is well-documented (Dauvin and Ruellet, 2007). Again in agreement with the different degrees of chronic pollution in the Loire and Seine estuaries, the ragworm population status is much more affected in the latter, with local almost extinction in a sector of the mesohaline area (Gillet et al., 2008) and a general depletion at the scale of the whole mudflat ecosystem over the past 25 years (Bessineton, 2009).

The effects of macrofaunal bioturbation on the chemical and physical properties of sediments have been well documented in the literature. Bioturbation has been shown to influence fluxes of organic matter, nutrients, and contaminants in estuarine sediments and across the sediment–water interface (see review by Bianchi, 2007). The depletion of ragworm populations from the Seine estuary was responsible for a reduction of sediment reworking and pumping rate with potential effects on biogeochemical cycles (Gillet et al., 2008). Thus the conservation of ragworm populations as well as other bioturbators (Michaud et al., 2005) is important for a normal functioning of estuarine mudflat ecosystems. Because the present study shows that several biomarkers in *N. diversicolor* are highly responsive, providing an early warning system well before populations may be affected, this work establishes the usefulness of the ragworm as a sentinel for the assessment of the health status of estuaries.

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Literature cited

Abele-Oeschger, D., Theede, H., 1991. Digestion of algal pigments by the common periwinkle *Littorina littorea* L. (Gasteropoda). J. Exp. Mar. Biol. Ecol. 147, 177–184.

Albentosa, M, Moyano, F.J., 2008. Influence of nutritional stress on digestive enzyme activities in juveniles of two marine clam species, *Ruditapes decussatus* and *Venerupis*

ACCEPTED MANUSCRIPT

pullastra. J. Sea Res. 59, 249-258.

Amiard-Triquet, C., Mouneyrac, C., Berthet, B., 2011. Polychaetes in ecotoxicology, in: Blaise, C., Férard, J.F. (eds.), Encyclopedia of Aquatic Ecotoxicology. Springer, Berlin, (in press).

Amiard-Triquet, C., 2009. Behavioral disturbances: the missing link between sub-organismal and supra-organismal responses to stress? Prospects based on aquatic research. Hum. Ecol. Risk Assess. 15, 87-110.

Bernfeld, P., 1955. Amylases, α and β, in: Colowick, S.P., Kaplan, N.O. (eds.), Methods Enzymol. Vol 1. Academic Press, New York, pp 149-158.

Bessineton, C., 2009. Historical records of the *Nereis diversicolor* population in the Seine estuary, in: Amiard-Triquet, C., Rainbow, P.S. (eds.), Environmental Assessment of Estuarine Ecosystems. A Case Study. CRC Press, Boca Raton, pp 183-197.

Bianchi, T.S., 2007. Biogeochemistry of Estuaries. Oxford University Press, Oxford, UK, 706 p.

Bonnard, M., Roméo, M., Amiard-Triquet, C., 2009. Effects of copper on the burrowing behavior of estuarine and coastal invertebrates, the polychaete *Nereis diversicolor* and the bivalve *Scrobicularia plana*. Hum. Ecol. Risk Assess. 15, 11-26.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Buffan-Dubau, E., de Wit, R., Castel, J. 1996. Feeding selectivity of harpacticoid copepod *Canuella perplexa* in benthic muddy environments demonstrated by HPLC analyses of chlorin and carotenoid pigments. Mar. Ecol. Prog. Ser. 137, 71–82.

Carr, R.S., Neff, J.M., 1984. Quantitative semi-automated enzymatic assay for tissue glycogen. Comp. Biochem. Physiol. 77B, 447-449.

Cartaxana, P., Jesus, B., Brotas, V. 2003. Phaeophorbide and phaeophytin a-like pigments as useful markers for intertidal microphytobenthos grazing by *Hydrobia ulvae*. Estuar. Coast. Shelf Sci. 58, 293–297

Dauvin, J.C., Ruellet, T., 2007. Polychaete/amphipod ratio revisited. Mar. Pollut. Bull. 55, 215-224.

Dean, H.K. 2008. The use of polychaetes (Annelida) as indicators species of marine pollution: a review. Rev. Biol. Trop. 56 (Suppl.4), 11-38.

Dedourge, O., Geffard, A., Amiard-Triquet, C., (2008). Origine des perturbations du

ACCEPTED MANUSCRIPT

métabolisme énergétique, in: Amiard, J.C., Amiard-Triquet, C., (Eds.), Les biomarqueurs dans l'évaluation de l'état écologique des milieux aquatiques. Lavoisier, Tec&Doc, Paris, pp. 241-71.

Depledge, M.H., 1994. The rational basis for the use of biomarkers as ecotoxicological tools, in Fossi, M.C., Leonzio, C. (Eds.), Nondestructive biomarkers in vertebrates. Lewis Publishers, Boca Raton, pp 261-285.

Durou, C., Mouneyrac, C., Amiard-Triquet, C., 2008. Environmental quality assessment in estuarine ecosystems: Use of biometric measurements and fecundity of the ragworm *Nereis diversicolor* (Polychaeta, Nereididae). Water Res. 42, 2157-2165.

Durou, C., Poirier, L., Amiard, J.C., Budzinski, H., Gnassia-Barelli, M., Lemenach, K., Peluhet, L., Mouneyrac, C., Romeo, M., Amiard-Triquet, C., 2007a. Biomonitoring in a clean and a multi-contaminated estuary based on biomarkers and chemical analyses in the endobenthic worm *Nereis diversicolor*. Environ. Pollut. 148, 445-458.

Durou, C., Smith, B.D., Roméo, M., Rainbow, P.S., Mouneyrac, C., Mouloud, M., Gnassia-Barelli, M., Gillet, P., Deutch, B., Amiard-Triquet, C., 2007b. From biomarkers to population responses in *Nereis diversicolor*: assessment of stress in estuarine ecosystems. Ecotoxicol. Environ. Saf. 66, 402-411.

Fossi Tankoua, O., Amiard, J.C., Amiard-Triquet, C., Berthet, B., Mouneyrac, C., Rainbow, P.S., 2010. Towards a comprehensive methodology for assessing the health status of coastal and estuarine ecosystems. ICES CM 2010/F:15, E-paper http://www.ices.dk/products/CMdocs/CM-2010/CM2010.pdf

Frasco, M.F., Guilhermino, L., 2002. Effects of dimethoate and beta-naphthoflavone on selected biomarkers of Poecilia reticulata. Fish Physiol. Biochem. 26, 149-156.

Frings, C.S., Fendley, T.W., Dunn, R.T., Queen, C.A., 1972. Improved determination of total serum lipids by the sulpho-phospho-vanilin reaction. Clin. Chem. 18, 673-674.

Galgani, F., Bocquené, G., 1991. Semi-automated colorimetric and enzymatic assays for aquatic organisms using microplate readers. Water Res. 25, 147-150.

Galloway, T.S., Brown, R.J., Browne, M.A., Dissanayake, A., Lowe, D., Depledge, M.H., Jones, M.B., 2006. The ECOMAN project : – a novel approach to defining sustainable ecosystem function. Mar. Pollut. Bull. 53, 186-194.

Gillet, P., Mouloud, M., Durou, C., Deutsch, B., 2008. Response of *Nereis diversicolor* population (Polychaeta, Nereididae) to the pollution impact – Authie and Seine estuaries (France). Estuar. Coast. Shelf Sci. 76, 201-210.

Gillet, P., Torresani, S., 2003. Structure of the population and secondary production of

ACCEPTED MANUSCRIPT

Hediste diversicolor (O.F. Müller, 1776), (Polychaeta, Nereidae) in the Loire estuary, Atlantic Coast, France. Estuar. Coast. Shelf Sci. 56, 621-628.

Habig, W., Pabst, M., Jakoby, W., 1974. Glutathione S-transferases. J. Biol. Chem. 249, 7130-7139.

Kalman, J., Buffet, P.E., Amiard, J.C., Denis, F., Mouneyrac, C., Amiard-Triquet, C., 2010. Assessment of the influence of confounding factors (weight, salinity) on the response of biomarkers in the estuarine polychaete *Nereis diversicolor*. Biomarkers 15, 462-469.

Kalman, J., Palais, F., Amiard, J.C., Mouneyrac, C., Muntz, A., Blasco, J., Riba, I., Amiard-Triquet C., 2009. Assessment of the health status of populations of the ragworm *Nereis diversicolor* using biomarkers at different levels of biological organisation. Mar. Ecol Prog. Ser. 393, 55-67.

Lorenzen, C.J., 1967. Determination of chlorophyll and phaeopigments: spectrophotometric equations. Limnol. Oceanogr. 12, 343-346.

Lucas, C.H., Holligan, P.M., 1999. Nature and ecological implications of algal pigment diversity on the Molenplaat tidal flat (Westerscheldt estuary, SW Netherlands). Mar. Ecol. Prog. Ser. 180, 51–64.

Marchand, M., Amouroux, I., Bédier, E., Belin, C., Claisse, D., Durand, G., Soudant, D., 2009. Qualité du Milieu Marin Littoral, Synthèse Nationale de la Surveillance. Institut Français de Recherche pour l'exploitation de la Mer (IFREMER), Nantes.

MEA (Millenium Ecosystem Assesment), 2005. Ecosystems and human well-being: Synthesis. Island Press, Washington, DC. World Resources Institute.

Méléder, V., Barillé, L., Rincé, Y., Morançais, M., Rosa, P., Gaudin, P., 2005. Spatio-temporal changes in microphytobenthos strucutre analysed by pigement composition in a macrotidal flat (Bourgneuf Bay, France). Mar. Ecol. Prog. Ser. 297, 83-99.

Meziane, T., Retière, C., 2002. Croissance de juvéniles de *Nereis diversicolor* nourris avec des de´ tritus d'halophytes. Oceanol. Acta 25, 119–124.

Michaud, E., Desrosiers, G., Mermillod-Blondin, F., Sundby, B., Stora G., 2005. The functional group approach to bioturbation: The effects of biodiffusers and gallery-diffusers of the *Macoma balthica* community on sediment oxygen uptake. J. Exp. Mar. Biol. Ecol. 326, 77-88.

Moreira, S., Lima, I., Ribeiro, R., Guilhermino, L., 2006. Effects of estuarine sediment contamination on feeding and on key physiological functions of the polychaete *Hediste diversicolor*: Laboratory and *in situ* assays. Aquat. Toxicol. 78, 186-201.

Moreira, S., Moreira-Santos, M., Guilhermino, L., Ribeiro, R., 2005. A short-term sublethal

ACCEPTED MANUSCRIPT

in situ toxicity assay with *Hediste diversicolor* (polychaeta) for estuarine sediments based on postexposure feeding. Environ. Toxicol. Chem. 24, 2010-2018.

Mouneyrac, C., Perrein-Ettajani, H., Amiard-Triquet, C., 2010. Influence of anthropogenic stress on fitness and behaviour of a key-species of estuarine ecosystems, the ragworm *Nereis diversicolor*. Environ. Pollut. 158, 121-128.

Mouneyrac, C., Leung P.T.Y., Leung, K.M.Y., 2011. Cost of tolerance. In: Amiard-Triquet, C., Rainbow, P.S., Roméo, M. (Eds.), Tolerance to environmental contaminants. CRC Press, Boca Raton, pp 265-297.

RNO, 2006. Surveillance de la qualité du milieu marin. Ministère de l'Environnement and Institut français de recherche pour l'exploitation de la mer (Ifremer), Paris & Nantes.

Scott, G., Klaine, S.J., 2001. Marine and estuarine toxicology and chemistry. Environ. Toxicol. Chem. 20, 1-2.

Solé, M., Kopecka-Pilarczyk, J., Blasco, J., 2009. Pollution biomarkers in two estuarine invertebrates, *Nereis diversicolor* and *Scrobicularia plana*, from a Marsh ecosystem in SW Spain. Environ. Int. 35, 523-531.

Swain, E.B., 1985. Measurement and interpretation of sedimentary pigments. Freshwater Biol. 15, 53-75.

Waters, M.N., 2000. The use of sedimentary algal pigments to infer historic algal communities in Lake Apopka, Florida. M.S. Thesis, University of Florida, 56 pp.

Weis, J.S., Smith, G.M., Zhou, T., Santiago-Bass, C., Weis, P., 2001. Effects of contaminants on behavior: biochemical mechanisms and ecological. BioScience 51, 209-217.

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Table 1. Quality of potentially available food sources estimated by using the ratios Chl *a*/Phaeo *a* and Chl *a*/TOC in superficial sediments.

Sampling month	Chl *a*/Phaeo *a*		Chl *a*/TOC	
	Bourgneuf	Loire	Bourgneuf	Loire
April 2008	0.30	0.13	2.95	0.50
May 2008	0.04	0.33	0.57	2.83
June 2008	0.42	-	4.45	-
October 2008	0.96	0.21	3.68	0.30
January 2009	0.56	0.32	1.45	0.27
March 2009	0.45	0.34	2.27	0.53
October 2009	2.56	1.25	5.78	2.00

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Table 2. Slopes and regression coefficients of the least-square regression lines for behavioural tests carried out with worms *Nereis diversicolor* from the Bay of Bourgneuf and the Loire estuary. * significant intersite difference between the slopes with p = 0.011; **p = 0.001

		April 08*	May 08	June 08**	Oct 08	Jan 09	March 09	Oct 09
	Slope	-0.217	-0.149	-0.183	-0.289	-0.181	-0.334	-0.207
Bourgneuf								
	R^2	0.94	0.9	0.84	0.92	0.82	0.95	0.91
	Slope	-0.238	-0.185	-0.220	-0.370	-0.144	-0.218	-0.200
Loire								
	R^2	0.87	0.84	0.76	0.95	0.86	0.90	0.82

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Figure 1 Distribution of different size fractions in sediment cores collected over the whole sampling period from the Bay of Bourgneuf and the Loire estuary. Mean values expressed in g kg^{-1} dry weight and SD. N = 7 for each site.

Figure 2 Concentrations of individual PAH compounds in superficial sediments from the Bay of Bourgneuf and the Loire estuary. Means and SD in calculated from samples collected from April 2008 to October 2009. N = 7 for each site. Ant: anthracene; BaA: benzo (a) anthracene; BaP: benzo (a) pyrene; BbF: benzo (b) fluoranthene; BghiP: benzo (ghi) perilene; BkF: benzo (k) fluoranthene; Chrys: chrysene; DaA: dibenz (ah) anthracene; Fluo: fluoranthene; IP: indeno (1,2,3,cd) pyrene; Napht: naphthalene; Phe: phenanthrene; Pyr: pyrene.

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Figure 5. Intersite variations of glycogen, lipid and protein concentrations in the whole soft tissues of the worm *Nereis diversicolor*. Mean values and SD calculated from 10 specimens from each site.* statistically significant differences.

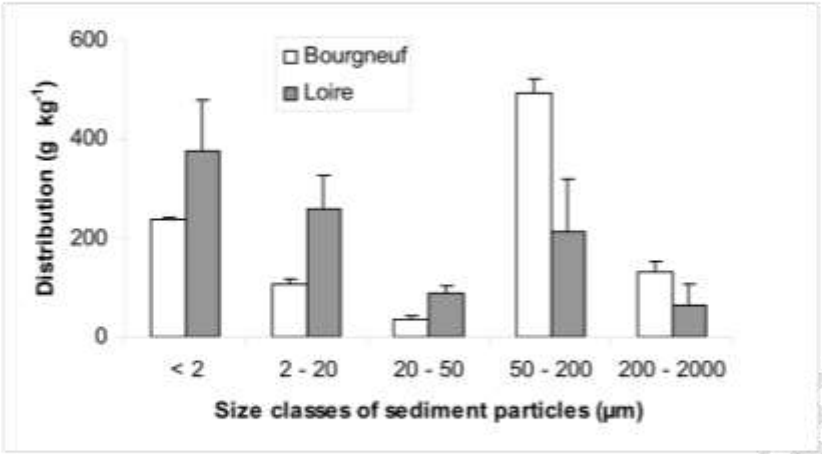
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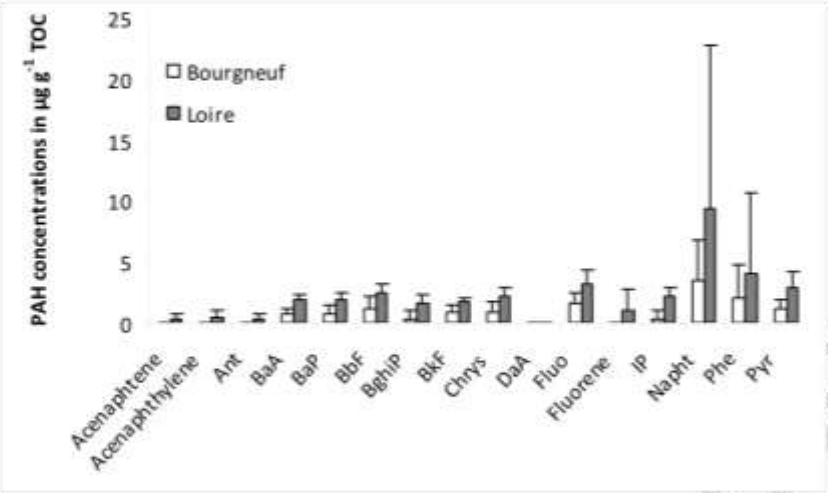
Figure 1



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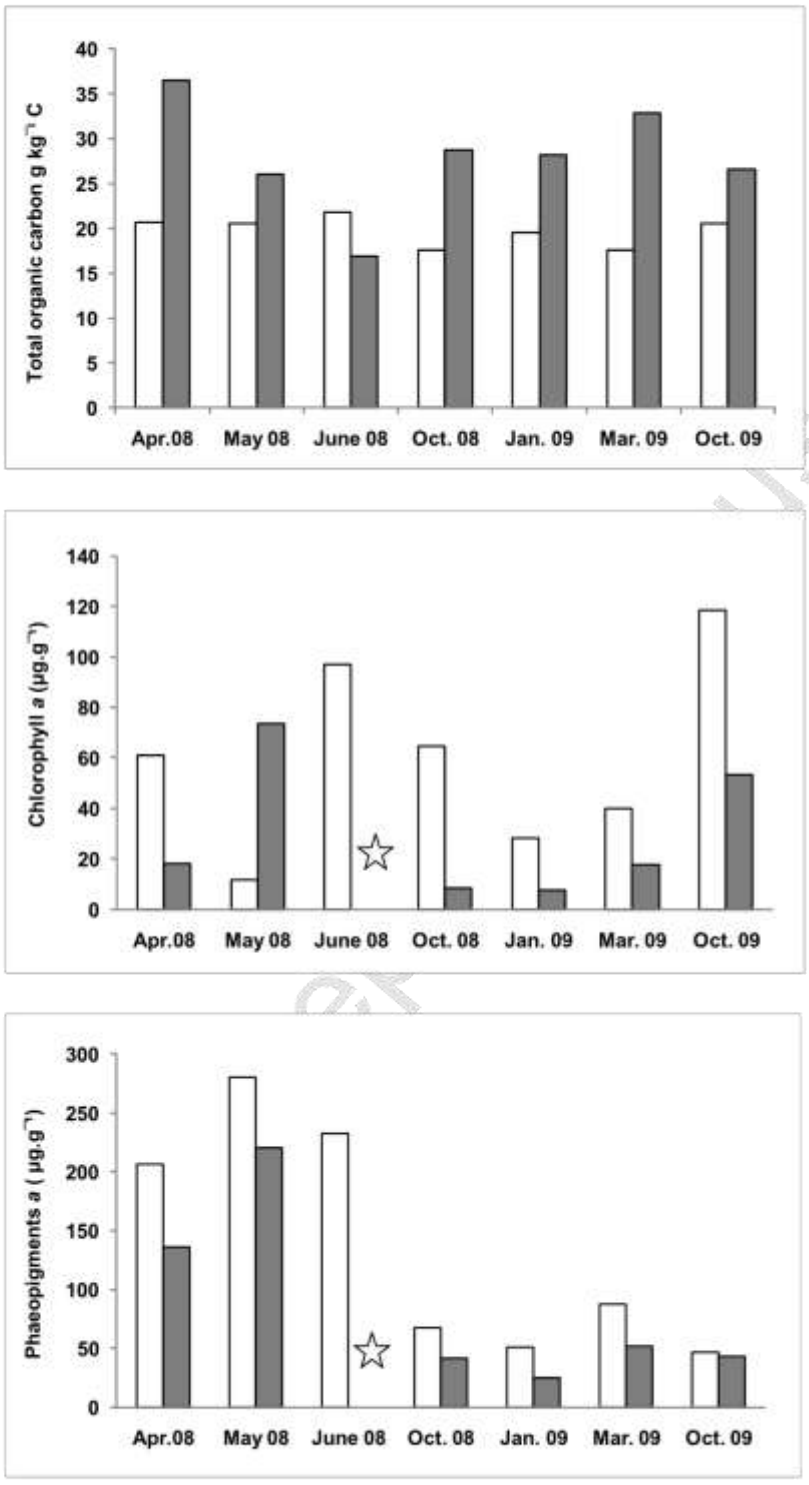
Figure 2



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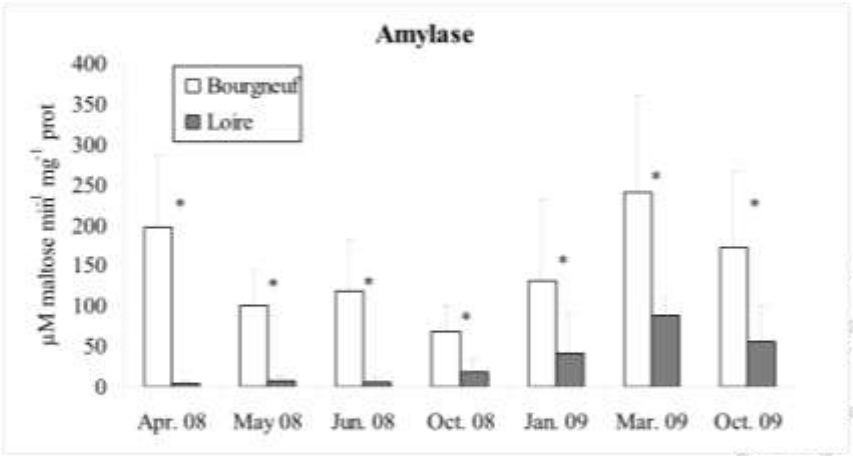
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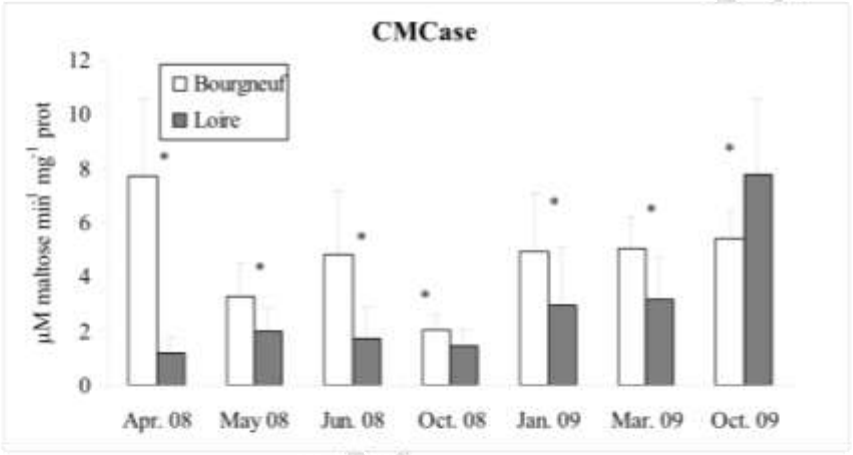
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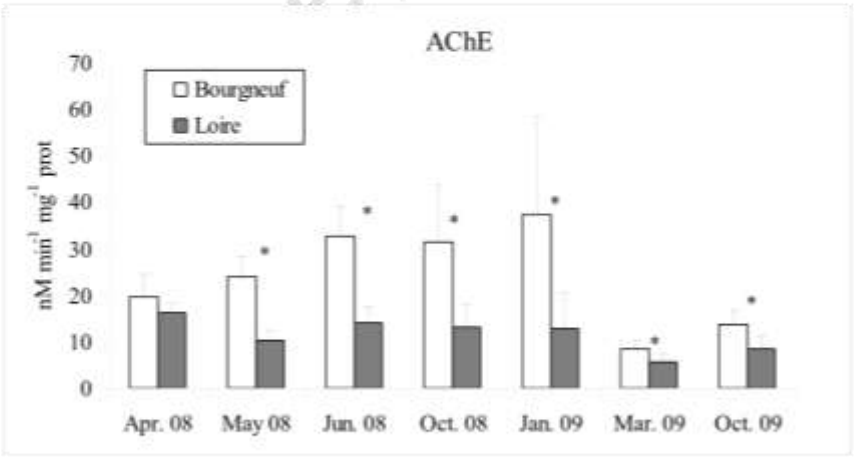


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Figure 4

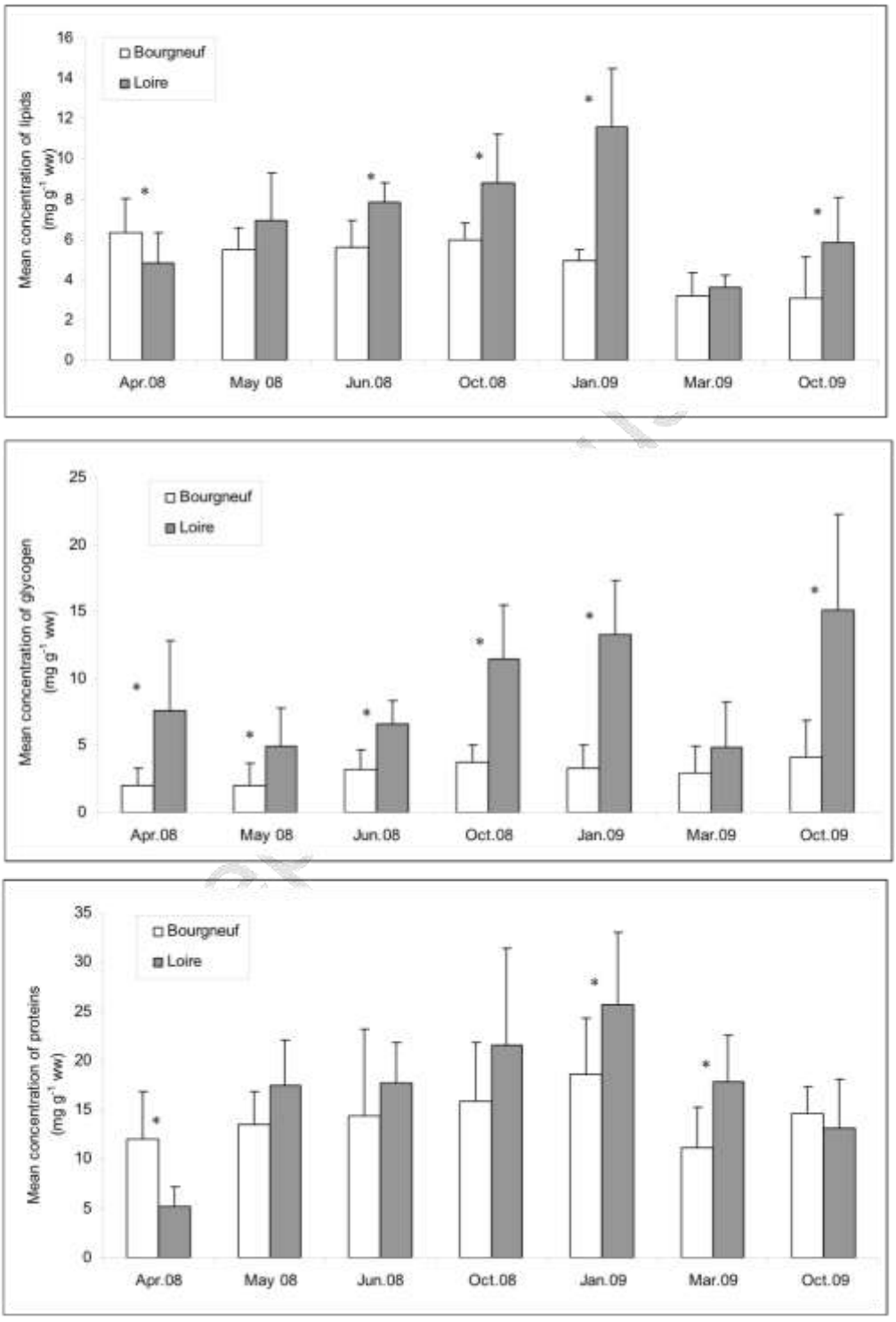






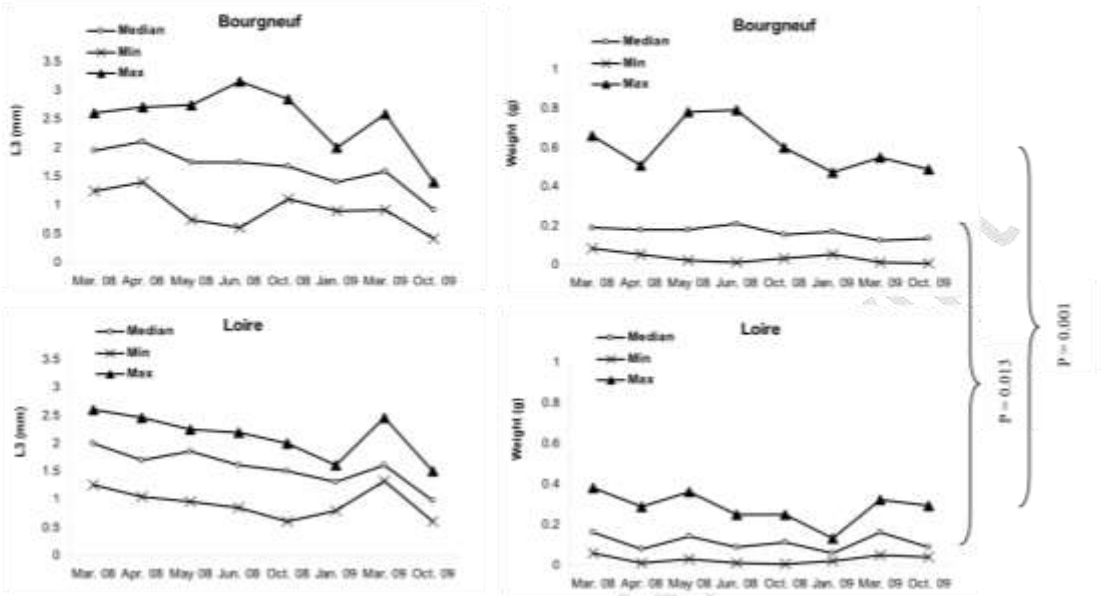
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Figure 5



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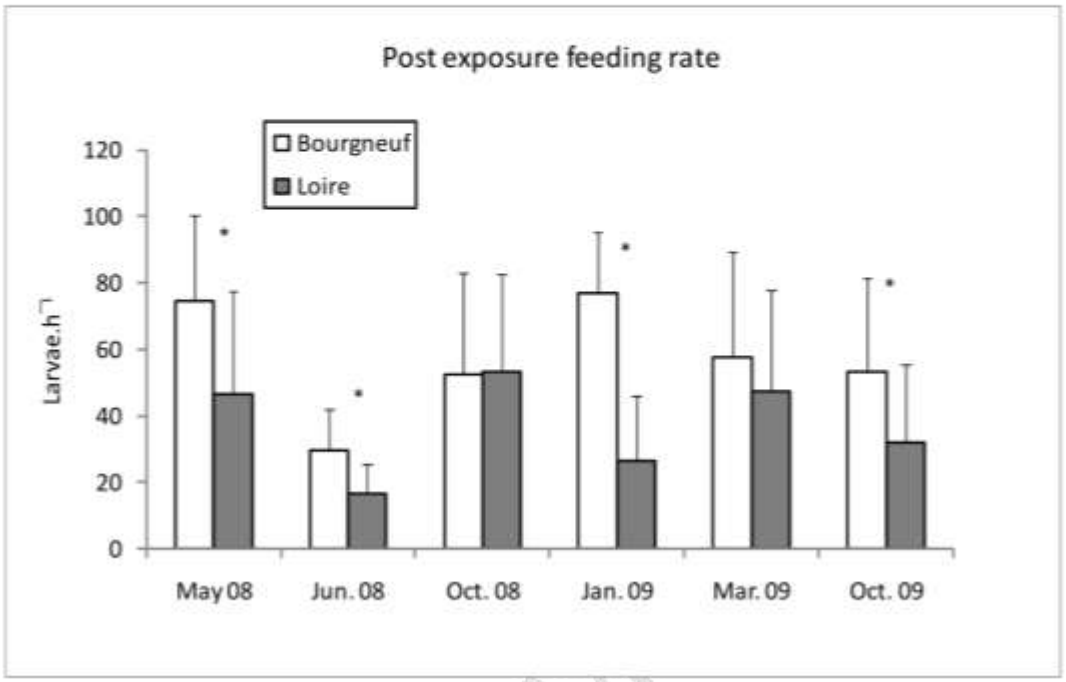
Figure 6



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Figure 7



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5.4 Publication 4 (soumise à *Aquatic Toxicology*)

Comprehensive assessment of estuarine sediment quality by using a battery of biomarkers in the bivalve *Scrobicularia plana* and the characterization of natural and anthropogenic factors

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Abstract

The bivalve *Scrobicularia plana* has been proposed as a sentinel species for the assessment of estuarine sediment quality. After the bivalve size and salinity have been estimated as potential confounding factors in the interpretation of biomarker responses, the aim of this study was to test their responsiveness in bivalves originating from a moderately contaminated site (Loire estuary, Fr.) and a comparatively cleaner site (Bay of Bourgneuf, Fr.) used as reference. Animals were collected 8 times from March 2008 to October 2009 for the determination of biochemical (MT, GST, LDH, AChE), physiological (energy reserves as glycogen and lipids, condition, hepato-somatic and gonado-somatic indices), and behavioural biomarkers (burrowing). Characteristics of both sites (temperature, salinity, grain size, total organic carbon, chlorophyll a and phaeopigments, PAHs, PCBs in sediments were also considered. Metals in clams were examined in the aim of explaining the responses of MTs. Population structure and dynamics were examined in the Loire estuary only, to reveal any impact of a small oil spill which occurred one month before the beginning of the sampling collection. Several biochemical markers (MTs, LDH) must be used in a precautionary fashion and the comparison with a reference site remains indispensable as well as a sound appreciation of salinity effects. The clearer responses were obtained with GST activity which contributes to defence against organic compounds. However, defence is not sufficient to ensure a total protection since a number of impairments were observed at the individual level (burrowing behaviour, condition index, gonado-somatic index). The interpretation of responses involved in the cascade of energetic events (from available food, digestive enzyme activities, energy reserves to condition and reproductive status) is complex but able to reveal disturbances in the Loire estuary which is far from being the most contaminated estuary in France and over the world. However, no links can be established between individual and supra-individual effects of living in the Loire estuary, even in the case of the small oil spill experienced experience at the beginning of this study.

Keywords : Biomonitoring, estuarine sediment, sentinel species, biochemical impairments, physiological responses, burrowing behaviour

1. Introduction

According to the Millenium Ecosystem Assessment (MEA 2005), when considering the value of world's ecosystem service and natural capital, estuaries are among the most productive systems. On the other hand, estuarine ecosystems are highly impacted by the human activities like industries, domestic discharges and agriculture. Sediment is the final repository of these multi-originated contaminants in which they are accumulated. Endogenous species are then more exposed to these contaminants than species living in the water column. Thus, it is relevant to assess the estuarine health status using endogenous species like the clam *Scrobicularia plana*.

From a comprehensive study carried out in the Seine estuary, considered as the most polluted estuary in France, strategies on how to design a programme of research to assess the environmental health status of an estuary suspected to have been impacted by anthropogenic contamination have been recommended (Amiard-Triquet and Rainbow, 2009). These authors have proposed a scheme of cascading effects of chemical stress at different levels of biological organization able to lead from impairments at the biochemical level to local extinction of species. This scenario has been nearly observed in estuarine species, the polychaete annelid *Nereis diversicolor* and the fish *Fundulus heteroclitus*, the populations in impacted sites being deeply depleted but not totally eradicated (Weis et al., 2001; Mouneyrac and Amiard-Triquet, 2011 and literature cited therein).

By focusing on key species, it is assumed that impairments of their responses used as biomarkers, will reveal a risk of cascading deleterious effects right through to the ecosystem level. Disturbances at the supra-individual levels affecting these organisms may result in ecological disorders. From this point of view, *S. plana* has a key role in the structure and functioning of ecosystems. High density and biomass are well documented in estuarine and coastal mudflats. *S. plana* plays a central role in estuarine food chains as a vector between primary producers (both phytoplankton and microphytobenthos as an alternative filter- and deposit-feeder; see Hughes 1969) and secondary consumers such as for bottom-feeding fish and wading birds for which the clams are an important prey-species. *S. plana* can interfere with the biogeochemical cycle of both nutrient and contaminants through its bioturbation activity. In addition, it is a useful species for the determination of a number of biomarkers either biochemical, physiological or behavioural (e.g. Byrne and O'Halloran, 2001; Mazorra et al., 2002; Romero-Ruiz et al., 2008; Solé et al., 2009; Boldina-Cosqueric et al., 2010; Buffet et al., 2011).

Although sub-individual and/or individual biomarkers are important as sensitive tools in the evaluation of the health status of organisms (Allan et al., 2006; Hagger et al., 2008), their lack of ecological relevance is frequently underlined and their use in ecological assessment questioned (Forbes et al., 2006). One key issue for the use of biomarkers in environmental assessment may be achieved by biomarkers at lower levels of biological organization that are linked to population, community or ecosystem consequences. Ideally these biomarkers must be able to provide early warning tools well before impairments occur at higher biological organization levels (Mouneyrac and Amiard-Triquet, 2011). Thus in the present study, biomarkers were selected considering the cascading effects at different levels of biological organization described by Amiard-Triquet and Rainbow (2009).

To date, given our state of knowledge it is unacceptable to study the ecotoxicological status of an estuary without comparison against a reference site. Such a reference site needs to be chosen to be as close as possible to the first estuary, in terms of salinity regime, temperature, granulometry and organic content of sediment, etc in order to mitigate the importance of confounding factors (Amiard-Triquet and Rainbow, 2009). In practice, it is often impossible to reach perfect agreement between reference and impacted sites. Those which are submitted to strong anthropogenic pressure are generally large estuaries whereas comparatively clean areas are more often available in small estuaries or adjacent coastal areas, thus it necessary to make a careful assessment of these differences. Thus preliminary to the present study, the potential influence of confounding factors (size, salinity) on biomarker tools in the clam *S. plana* has been assessed (Fossi Tankoua et al., 2011).

The biomarkers studied in the multi-polluted Loire estuary and the comparatively clean Bay of Bourgneuf have been chosen for their potential ecological value and their representativeness of different levels of biological organization: biochemical (metallothionein MT concentration and activities of glutathione S-transferase GST, lactate dehydrogenase LDH, acetylcholinesterase AChE, digestive enzymes (cellulase and carboxymethylcellulase (CMCase – 3.2.1.4.)), physiological (energy reserves, condition, reproduction) and behavioural (burrowing) responses. Because energy metabolism and associated enzymes may be influenced by both chemical stress and food availability and the fact that *S. plana* preferentially used benthic diatoms (64.5%) (Riera et al., 1999), superficial sediments were examined for the levels of organic matter as chlorophyll *a* (Chl *a*), phaeopigments *a* (Phaeo *a*) and total organic carbon (TOC). Because impairments of these responses might be expected in the multi-polluted Loire estuary from the literature dealing with estuarine

ecosystem health, population studies (density and structure) were designed in parallel in order to examine the relative importance of effects at different levels of biological organization.

2. Materials and Methods

2.1. Sampling sites

Animals were collected 8 times from March 2008 to October 2009 at 2 sites in the Gulf of Biscay, Fr., differentially exposed to anthropogenic pressure (Locations: 2° 07' 59.76" W, 47° 16' 29.66" N in the Loire estuary; 2° 04' 40.60" W, 46° 56' 23.08" N in the Bay of Bourgneuf). The Loire estuary is exposed to toxic anthropogenic effluents transported by rivers from remote and nearby conurbations, and industrial and agricultural areas. On the other hand, the Bay of Bourgneuf is mainly devoted to oyster farming, extensive agriculture and tourism. The reference site (Bay of Bourgneuf) was chosen based on the informations given by (i) the anthropogenic pressure both locally and at the scale of the river basin; (ii) the data available for contaminants in the framework of the French ''Mussel Watch'' Programme (RNO, 2006) and (iii) the data provided by the regulation authorities (AELB, 2004) for the implementation of the European Community Water Framework Directive (ECWFD).

Residual water remaining on the sediment surface at low tide was used to determined salinity. Air temperature was calculated on the basis of the maxima and minima daily temperatures of the month preceding sample collection (www.meteociel.fr) (Table 1). Granulometry of sediment cores (≈ 20 cm deep, related to the distribution of clams) was determined according to the French standard method NF X31-107 by an official laboratory (Institut Départemental d'Analyses et de Conseil, IDAC, Nantes, Fr.) working under Good Laboratory Practices (Fig. 1).

2.2. Collection

Samples were collected in the intertidal zone at low tide. For both sites, and at the 8 sampling dates, the surface oxygenated layer (a few mm) of sediments destined to the analysis of organic contaminants, Chl *a* and Phaeo *a* were scraped off at 3 places distant by a few meters (about 300 cm^2 each) and placed in aluminium boxes. At the same time,

individuals of *Scrobicularia plana* were handpicked from intertidal mudflats. To avoid the influence of size, clams of similar shell length (3.1 ± 0.3 cm for the whole specimens of both sites) were taken for behavioral, biochemical and physiological tests. At each occasion, 50 specimens were collected at random in order to determine their condition index and water content. Sediment samples and animals were transported to the laboratory in isothermal containers, with wet algae or sediment from the site of origin without seawater for the clams. In the laboratory, sediments were frozen at -80°C until their use, whereas animals were kept at the ambient temperature measured during collection. Animals destined to metal analyses were maintained in aerated containers filled with natural filtered seawater adjusted to the salinity of the site of origin for 24 hours to allow the elimination of their gut content. Those destined for enzyme activities and concentrations of energy reserves were immediately dissected. The soft tissues were washed with filtered seawater to eliminate sediment and kept at -80°C till analyses. Specimens for population studies were obtained by the sifting (mesh size of 1 µm) of three replicated sediment samples (25x25x25 cm) collected at a distance of 10m from each other at each sampling date.

2.3. Quantification of chlorophyll *a* and phaeopigments *a*

Pigment analyses were performed using a spectrophotometer (Uvikon XS UV/VIS, software Lab Power Junior, Bio-Tek Instruments (Wavelength range: 190-1100nm; wavelength percision: 0.3 nm). Liposoluble pigments including Chl *a* and Phaeo *a* were extracted from sediment samples using the methods of Swain (1985) and Waters (2000) modified. Pigments were extracted from 31-55 g of wet sediment in each sample using 30 mL of acetone 100% rather than 90%. This was used to prevent excessive solvent dilution due to high water content that would make extraction less effective. Samples were shaken and kept in the dark at 4°C for a period of 24 hours. Supernatants containing dissolved pigments were filtrated with syringe through a Millipore hydrophobic membrane (MINISART PTFE) of 0.2 µm porosity and 28 mm diameter). Chl *a* and Phaeo *a* concentrations were determined following Lorenzen (1967) using DO measured at 665 and 750 nm, before and after acidifying the sample with hydrochloric acid (0.1 mL of HCl 0.1 N). This corrects for interference due to the presence of Phaeo *a*, absorbing at the same wavelength as Chl *a*, and does not require calibration with a pure solution of Chl *a* to retrieve the concentration of Chl

a alone. In order to express Chl *a* and Phaeo *a* concentrations per unit weight of dry sediment, a sub-sample of each sediment was dried for 48 hours at 60°C to estimate the sediment water content, and eliminate its contribution to the wet sediment weight used for pigment extraction.

Phaeo *a* are considered by several authors to be useful markers for intertidal microphytobenthos grazing by meio- and macrofauna (Abele-Oeschger & Theede 1991, Buffan-Dubau et al. 1996, Cartaxana et al. 2003; Méléder et al. 2005). Thus, the quality of potentially available food sources is estimated in this study using the ratio Chl *a*/Phaeo *a* but also Chl *a*/TOC.

2.4. Assessment of exposure

Organic contaminant analyses in superficial sediments were performed by the laboratory IDAC. Mineral oils were quantified by infra-red spectrometry according to the standardized method ISO11046 (X31-410), polycyclic aromatic hydrocarbons (PAHs) and polychlorobiphenyls (PCBs) by GC/MS(/MS) according to the standardized method XP X33-012.

2.5. Quantification of metal concentrations in the soft tissues of clams

Because MT concentrations were determined in the same specimens as metals, a fractionation of soft tissues between cytosolic and insoluble phases was needed. All labware was soaked in 10 % hydrochloric acid, rinsed three times with deionised water and dried in desiccators sheltered from atmospheric dust. Nalgene bottles were used to store all reagents.

Soft tissues from each bivalve (n=8-10 per site) were homogenized at 4°C in 20 mM TRIS, 150 mM NaCl solution adjusted to pH = 8.6 (4mL g^{-1} soft tissue). β-mercaptoethanol (10^{-5} mM) was added in order to limit oxidation, as well as phenylmethanesulfonyl fluoride (PMSF) as an anti-protease (0.1 mM). The cytosol (C1) was recovered by centrifugation (25000 g for 55 minutes at 4°C).

The pellets (P1) and the remaining supernatants (C1) were heated with suprapure concentrated nitric acid (Carlo Erba). After digestion, metal levels in these different acid solutions were determined after dilution with deionised water by flame (FAAS) (Cu, Zn) or electrothermal atomic absorption spectrophotometry (EAAS) (Ag, Cd and V) with the Zeeman effect (Varian SpectrAA55, Varian SpectrAA800 spectrophotometers). Standard addition analyses were performed in an iso-medium and added concentrations of each element were + 125, 250, 500, 1000 ng Cu or Zn mL^{-1} for FAAS and + 0.5, 1, 2 ng Cd mL^{-1}, + 3.13, 6.25, 12.5 ng Ag mL^{-1}, + 12.5, 25, 50 ng V mL^{-1} for EAAS. The total concentration was recalculated from the concentrations in the supernatant (C1) and the pellet (P1).

The analytical methods have been validated through internal quality controls by using a standard reference material provided by NRC Canada (TORT-2, lobster hepatopancreas).

2.6. Determination of biological effects

2.6.1. Determination of biochemical markers

2.6.1.1. Metallothioneins

An aliquot of C1 prepared for metal quantification was heated at 75°C for 15 minutes. Then MTs were isolated by centrifugation (15000 g for 10 minutes at 4°C). The amount of MTs was determined by differential pulse polarographic analysis (Thompson and Cosson 1984). A MDE 150 Polarographic Stand (Radiometer Copenhagen) TracelabTM 50, controlled by the computer software Tracemaster 5 through a Polarographic analyser POL 150 was used. The temperature of the cell was maintained at 4°C. The standard addition method was used for calibration with rabbit liver MT (Sigma Chemical Co., St Louis, MO) due to the lack of a marine invertebrate MT standard.

2.6.1.2. Enzyme activities

For LDH, GST and AChE, determinations were carried out in the whole soft tissues of 10 clams per site and collection month. Amylase and cellulase activities were determined on four pools of digestive glands or crystalline styles of twelve clams (three per pool).

The procedure for homogenization adapted to each enzyme and the recovery of supernatant have been described in detail by Boldina-Cosqueric et al. (2010). Briefly, enzyme activities were analysed in triplicate by using a microplate reader as described by Galgani and Bocquené (1991) for AChE/ChE, Diamantino et al. (2001) for LDH, Frasco and Guilhermino (2002) for GST. The α-amylase and cellulase activities were determined by the method of Bernfeld (1955) adapted by Boldina-Cosqueric et al. (2010). All the enzyme activities were calibrated to protein concentrations which were measured in the soft tissues' supernatant by the method of Bradford (1976) adapted for microplate use with bovine γ-globulin as standard protein.

2.6.2. Determination of physiological markers

2.6.2.1. Glycogen and lipid quantification

Glycogen and lipids were quantified specifically in each organ (digestive gland and gonad when its development was sufficient during the reproductive period) of each bivalve from each sampling month and each site (n=10). After homogenization in liquid nitrogen, the powder obtained was then homogenized with 1.5 mL citrate buffer at pH 5.0 for glycogen and lipid analysis. Total lipids were determined by a sulpho-phosphovanillin reaction according to the method of Frings et al. (1972). Glycogen concentrations were estimated in two aliquots of the homogenate, one of which was submitted to enzymatic digestion by amyloglucosidase according to Carr and Neff (1984). Olive oil and glycogen from oyster (Sigma, Type III) were used as standards for each method, respectively.

2.6.2.2. Biometric indices

The condition index (CI) of clams was determined according to the recommendation of the French Regulatory Agency (AFNOR1985):

$$CI = (\text{wet weight of soft tissues/total weight}) * 100 \qquad \text{Eq. 1}$$

The gonado-somatic index (GSI) and the hepato-somatic index (Fu-hsiung et al.) were calculated using the following equation:

GSI or HSI = (gonad or digestive gland weight/ wet weight of soft tissues)*100 Eq. 2

Then the soft tissues were oven-dried at 100°C till constant weight. The water content was calculated using the following equation:

Water content = (Wet weight - dry weight/wet weight)*100 Eq. 3

2.6.3. Behavioural tests

Burrowing tests were set up in the dark the day following the collection. Specimens from each site (N = 20 for each site) were tested in the presence of their sediment of origin. Experiments were carried out using clams of approximately same shell length according to the procedure described in details by Bonnard et al. (2009). The results of burrowing tests were expressed as kinetic curves (% of unburrowed clams determined every 5 min in the 1st half an hour; every 10 min in the 2nd half) in order to take into account the burrowing speed during the first hour of the test. At the end of the experiment (7 hours), the number of unburrowed specimens was registered.

2.6.4. Determination of population structure and dynamics

Length-frequency distributions were calculated per 1 mm size-classes over the study period for the population as a whole. The modal length of the cohorts were identified applying the Bhattacharya method (1967) using the FISAT II software package (Gayanilo and Pauly, 2005).

2.7. Statistical treatment

Kinetic curves obtained from burrowing tests were ln-transformed in order to be linearized. Thus the slope of the curves in absolute value may be used as surrogates for burrowing speed. The analysis of covariance (ANCOVA) between regression coefficients of

the least-square best-fit regression lines was used to compare them. Otherwise, results are presented as mean ± SD. To define significant differences, normally distributed data were analyzed by t test (between two groups), one-way ANOVA (multiple comparisons of means) followed by Tukey's HSD post hoc method. In other cases, the non-parametric Kruskal-Wallis and Mann-Whitney comparison tests were used. To compare the levels of metals and biochemical markers over the whole sampling period bilateral Mann-Whitney tests were carried out. Level of significance was established at $P < 0.05$. Statistical analyses were performed using XLSTAT 2010.

3. Results

3.1. Characterisation of superficial sediments

3.1.1. Exposure to contaminants

The concentrations of polychlorobiphenyls (PCBs) in sediments from both sites were always below the quantification limits which were chosen by the laboratory IDAC in order to be able to detect concentrations above the quality thresholds for sediments in the French regulation (25 µg kg^{-1} dw for CB 28, 52, 118, 180; 50 µg kg^{-1} dw for CB 101, 138, 158; 500 µg kg^{-1} dw for ΣPCBs).

The concentrations of polycyclic aromatic hydrocarbons (PAHs) in sediments from both sites are shown in Figure 2. On an average for the whole sampling period, the concentrations of individual compounds were higher in sediments from the Loire than in those from the Bay of Bourgneuf. Considering the sum of the 16 PAH priority pollutant listed by US EPA (US Environmental protection Agency), the concentrations were more important in the Loire estuary than in the Bay of Bourgneuf with a maximum concentration of total PAHs of 1926 µg g^{-1} versus 642 µg.g^{-1} and average concentrations reaching respectively 1031 and 413 µg.g^{-1}.

3.1.2. Organic matter as chlorophyll *a*, phaeopigments *a* and total organic carbon TOC

The spatio-temporal variations of these three categories of organic matter are shown in Fig. 3. The highest concentrations of Chl *a* were obtained in May 2008 in the Loire estuary (73.5 $\mu g.g^{-1}$ of dry sediment) whereas it was in October 2009 in the Bay of Bourgneuf (118.5 $\mu g.g^{-1}$ of dry sediment). The lowest concentrations (7.7 and 11.8 $\mu g.g^{-1}$ of dry sediment) were obtained in January 2009 and May 2008 for the Loire estuary and the Bay of Bourgneuf respectively. Chl *a* and Phaeo *a* concentrations were higher in sediments from the Bay of Bourgneuf than in those from the Loire, with the exception of Chl *a* in May 2008. On the contrary, TOC concentrations were usually higher in the Loire estuary than in the Bay of Bourgneuf except in June 2008.

Ratios Chl *a*/Phaeo *a* and Chl *a*/TOC are shown in Table 2. Both of these ratios are consistently higher for superficial sediments from the Bay of Bourgneuf except in May 2008 when levels of Chl *a* were very high in the Loire estuary.

3.2. Biological effects

3.2.1. Metal bioaccumulation

All five of the metals studied (Ag, Cd, Cu, V, Zn) bind to MT and might therefore contribute in association to MT induction (Amiard et al. 2006; Amiard et al. 2008). Thus, the total metal content was calculated as the sum of concentrations of Ag, Cd, Cu, V, and Zn expressed in nmol g^{-1}. Total metal concentrations in the whole soft tissues were always higher in clams from the Loire estuary (Fig. 4) even if this difference was significant only from October 2008 to October 2009. No temporal variations were observed in both of the studied sites.

In order to examine the fate of additional metals accumulated in the contaminated site versus control, the regression curves were established for each metal between total concentrations and concentrations in each fraction, cytosolic C1 and insoluble P1. The slopes are depicted in Table 3. Cadmium and Zn were nearly equally distributed between these two fractions whereas Ag, Cu and V were mainly accumulated in the insoluble fraction.

3.2.2. Biochemical markers

3.2.2.1. Metallothioneins

MT concentrations were generally higher in clams from the Loire estuary compared to those from the Bay of Bourgneuf with the exception of October 2008 when an inverse ranking was observed (for significant differences, see Fig. 4). Important temporal variations were also observed for this biomarker in both sites. In the Bay of Bourgneuf, the highest concentration was observed in October 2008 and the lowest one on March 2008. In the Loire estuary, the lowest value was determined on the same month whereas high values were observed in May, June 2008 and January 2009. Due this temporal pattern, it is not surprising that no correlation was shown between MT and total metal concentrations in the soft tissues of clams collected over the whole sampling period from the Bay of Bourgneuf and the Loire estuary.

Because MT is a cytosolic protein, the relationship with metals was also examined taking into account cytosolic metals but no more significant correlations were observed (not shown).

3.2.2.2. Enzymatic activities

The activity of GST was generally higher in clams from the Loire than in specimens from the Bay of Bourgneuf (Fig. 4). Considering individually sampling months only one significant difference was depicted (April 2008) but bilateral tests carried out for the whole sampling period showed a significant difference ($p = 0.018$). In the Bay of Bourgneuf, no significant temporal differences were shown. In the Loire estuary, except a low value in March 2008, no significant temporal fluctuations were shown.

The activity of LDH was significantly higher in the specimens from the Bay of Bourgneuf on three sampling occasions (Fig. 4) but no significant difference was revealed by bilateral tests over the whole sampling period. On both sites, the activity of LDH was significantly lower in March and April 2008 than all over the remainder of the study.

The activity of digestive enzymes (amylase, CMcase) in the digestive gland of clams was significantly higher in the Loire estuary in March and April 2008 (Fig. 4) and for both

enzymes, a significant difference was revealed by bilateral tests over the whole sampling period (respectively $p<0.0001$ and $p<0.003$). In both studied sites, amylase activity was significantly lower in March 2009 compared to all the other months except October 2008 in the Bay of Bourgneuf and October 2009 in the Loire estuary. In both studied sites, CMCase activity was significantly lower in March and October 2009 compared to all the other months. In addition, in the Loire estuary, CMCase activity was significantly higher in March and April 2008 than for all the other months.

The activity of AChE was generally higher in the specimens from the Bay of Bourgneuf but the difference was significant only in April 2008 and October 2009 (not shown). In addition, no significant intersite difference was revealed by bilateral tests over the whole sampling period. In both studied sites, no significant temporal differences were shown.

3.2.3. Physiological markers

3.2.3.1. Energy reserves as glycogen and total lipids

Spatio-temporal variations of glycogen and total lipids in the gonad and the digestive gland of the clam *Scrobicularia plana* are depicted in Fig. 5. Glycogen concentrations were always higher in gonads and the digestive gland of clams from the Bay of Bourgneuf even if these differences were not always significant (Fig. 5), but bilateral tests carried out for the whole sampling period showed a significant intersite difference for the digestive gland ($p = 0.005$).

In specimens from the Bay of Bourgneuf, glycogen concentrations fluctuated concomitantly in both organs with significantly lower values in June compared to April and May. The lowest glycogen concentrations in the digestive gland were encountered in June 2008 (statistically different from all the other months except January 2008). The highest glycogen concentrations in the digestive gland were observed in October 2009 (statistically different from April and October 2008 as well as January 2009). On the other hand no significant temporal variations were shown for glycogen concentrations in the gonads of clams from the Loire estuary and only few temporal differences were significant in the case of digestive glands (lowest values determined in March 2009 significantly different from those observed in April and May 2008).

For total lipid concentrations, in both organs, bilateral tests carried out for the whole sampling period did not show a significant intersite difference even if occasionally significantly higher concentrations were observed in the Bay of Bourgneuf (Fig. 5). For temporal variations, gonads of clams from the Bay of Bourgneuf showed significantly higher concentrations in May 2008 compared to April and June 2008. In the digestive gland of the specimens from the Bay of Bourgneuf, total lipid concentrations determined in April 2008 were significantly higher than for all of the other months (except May 2008). On the other hand, values determined in March 2009 were the lowest ever registered over the whole sampling period (significant differences with concentrations determined from April to October 2008). In contrast, in the Loire no significant temporal variation was shown in both studied organs.

3.2.3.2. Biometric measurements

The water content of clams (50 specimens collected at random) was generally higher in the Loire estuary than in the Bay of Bourgneuf (Table 4) and a significant difference was revealed by the bilateral tests over the whole sampling period ($p<0.0001$). For both sites, temporal variations were negligible (Table 4).

Whatever the way used to calculate the condition index (specimens collected at random or of selected size), this biometric variable was higher in clams from the Bay of Bourgneuf with only one exception in March 2009 (Table 4). For both modes of measurement, a significant difference was revealed by the bilateral tests over the whole sampling period (with $p<0.0001$ in each case). The lowest values were generally encountered in winter and early spring (March and April 2008, January and March 2009).

On the contrary, the hepatosomatic index was higher in clams from the Loire estuary than in those from the Bay of Bourgneuf with one exception in June 2008 (Table 4). A significant difference was revealed by the bilateral tests over the whole sampling period ($p<0.0001$). The temporal variations were negligible in the Bay of Bourgneuf (16 to 21) and comparatively more important in the Loire estuary (14-25). In both sites, the lowest values were registered in June 2008.

In order to obtain a good appreciation of the spatio-temporal variations related to reproduction processes, the gonadosomatic index of clams was determined every two weeks

during the period of reproduction in 2009 (Fig. 6). At the peak of development of the gonads, the intersite difference was significant with higher GSIs in the clams from the Bay of Bourgneuf.

3.2.4. Behavioural tests

Slopes and regression coefficient of the least-square regression lines obtained during the first hour of behavioural tests carried out with clams *S. plana* from the Bay of Bourgneuf and the Loire estuary are shown in Table 5. The burrowing speed was equivalent in both sites except in April and June 2008 when the slope of the curves in absolute value – a surrogate for burrowing speed – was consistently higher in specimens from the Bay of Bourgneuf. A clear temporal trend was shown with comparatively low burrowing speeds in winter and early summer (March and April 2008, January and March 2009) and higher speeds from May to October 2008 and in October 2009.

On a longer term, disturbances of behaviour may be assessed considering the number of clams (among the 20 specimens tested) which remained unburrowed at the end of the test (7 h). This number is always higher in the Loire estuary than in the Bay of Bourgneuf with the exception of October 2009 (Fig. 7). In the reference site, the percentage of unburrowed clams varied from 0 (March 2008) to 15% at the maximum (May and October 2009). In the Loire estuary, more marked temporal variations are depicted in Fig. 7. At the beginning of the study, the percentage of unburrowed clams was 10 whereas a value as high as 40% was reached in April 2008. This percentage decreased consistently from May to October 2008 with a second less important peak (30%) in January 2009 then again a consequent decrease.

3.2.5. Populational effects

Densities are shown in table 6. They varied from about 400 to 1,000.

Shell length frequency histograms of the population of clams from the Loire estuary are shown in Fig. 8. The presence of 3 (April and May 2008) or 4 cohorts (June and October 2008, October 2009) including the individuals born at the same period (within a few days) may be recognized. For each cohort identified in Fig. 9, the mean size of the shell is shown in

table 6). Among the three cohorts present in April 2008, the biggest one (L1) had disappeared whereas cohorts L2 and L3 are growing progressively till October 2008 for L2 and October 2009 for L3. Additional cohorts L4 and L5, settled in May and June 2008, have grown till October 2009 and at this date, an additional cohort L6 is present, showing the same size as L5 at the same period of the year in 2008.

4. Discussion

In this paper devoted to biomarkers, metals were examined in the aim of explaining the responses of metallothioneins. This is the reason why the metal concentrations were not shown in detail. The sum of metals able to induce MTs were higher in clams from the Loire estuary than in those from the Bay of Bourgneuf. We compared our results for individual metals with those obtained by Solé et al. (2009). In agreement with these authors, at such moderate levels of contamination no clear relationships may be established between MT and metal concentrations depending on different sites. In areas impacted by an industrial accident, significant correlations between MT and metal concentrations were observed for Zn, Pb, Ni, Mn and Fe (Romero-Ruiz et al., 2008) among which only Zn is known as a primary inducer of MT (Roesijadi, 1996). In the present study, among metal recognized as MT inducers, Ag, Cu, and V were mainly present in the insoluble fraction of tissues whereas Cd and Zn were nearly equally distributed between insoluble and cytosolic fractions. Thus an important fraction of bioaccumulated metals were not linked to MTs. For this biomarker, temporal variations were more important than intersite differences. In the nearly allied species *Macoma balthica*, Leiniö and Lehtonen (2005) have shown a highly significant seasonal variation. In addition, our previous works have shown that, independently of any contamination pressure, MT concentrations were higher in brackish sites than in marine sites (Fossi Tankoua et al., 2011). Simulating a heavy metal spill under estuarine conditions, García-Luque et al. (2004) did not reveal significant correlations between Cd, Cu, Pb and Zn concentrations in water on the one hand and MT concentrations in the clam *Scrobicularia plana* on the other hand (recalculated from their table 1). In 2007, the same authors suggest that interactions between the influence of salinity and contamination respectively are at the origin of the observed results. Consequently, this biomarker in *S. plana* must be used in a precautionary fashion, and the comparison with a reference site (at the same salinity) remains

indispensable. In the absence of such a site, the use of a correction factor for salinity effect must be envisaged.

The concentrations of PCBs in sediments from both sites were always below the quality thresholds for sediments in the French regulation. PAH levels were comparable with or lower than those of moderately contaminated estuaries reported in several other studies (Woodhead et al., 1999; Kowalewsha et al., 2003; Fu et al., 2003; Grabe and Baron, 2004; Amiard et al., 2009). Concerning the ecotoxicological significance of the micropollutant levels recorded in the sediments from the Bay of Bourgneuf and the Loire estuary, comparison can be made between data recorded in this study and different proposed guideline values for adverse biological effects to living resources in marine and estuarine sediments, such as the Effects Range Low/Median (ERL/ERM) (Long et al., 1995). All the sediments never reached the ERL guideline values, showing a moderate risk when considering this class of contaminants.

Despite this low level of organic contaminants in the Loire estuary, GST which in a biotransformation enzyme involved in the degradation of this type of molecules, was generally higher by comparison with the reference site. Temporal variations were very limited whereas in the study by Solé et al. (2009), GST experienced temporal fluctuations which were always larger than within stations for the same period. It has been also shown that GST activity was higher in marine areas compared to brackish areas (Fossi Tankoua et al., 2011). In the present study, despite lower salinity, highest activities were shown in the Loire estuary and were thus most probably due to contamination, showing the high responsiveness of this biomarker.

Among less specific biomarkers, LDH activity was generally higher in specimens from the marine Bay of Bourgneuf than in those from the brackish Loire estuary. However, the differences were not so marked as in a relatively clean site at different salinities where LDH activity was 133% higher at 30.1 than at 11.5 (Fossi Tankoua et al., 2011). This suggests an effect of hypoxia in the Loire which is classically described in macrotidal estuaries (Marchand et al., 1993).

Because a significant influence of salinity was shown on the response of digestive enzymes in the crystalline style (Fossi Tankoua et al., 2011), in the present study, analyses were carried out in the digestive gland without the style in order to avoid this confounding factor. Impairments of digestive enzyme functioning in bivalves exposed to many classes of contaminants are well-established (Dedourge et al., 2008). Thus the fact that for both

enzymes, activities were significantly higher in specimens from the Loire over the whole sampling period was not expected. However, digestive activities also respond to the nutritional status of bivalves as shown for instance in juveniles of two marine clam species, *Ruditapes decussatus* and *Venerupis pullastra* (Albentosa and Moyano, 2008) Amylase in particular could be used as indicative of nutritional condition. This can be linked to significantly higher hepatosomatic index in clams from the Loire. A possible explanation is the quality of potential sources of food in the Bay of Bourgneuf and the Loire estuary. Except in April 2008, clams from the Bay of Bourgneuf can have access to fresher organic matter represented by high ratio value of Chl *a*/Phaeo *a* or Chl *a*/TOC whereas sediments from the Loire estuary showed more degraded organic matter content with the lowest ratio values. Méléder et al. (2005) reported the same higher level of Chl *a* in the Bay of Bourgneuf with a percentage of Phaeo *a* no more than 30% of the total non-degraded pigments. Results obtained in the two mudflats suggest a weaker degradation of microphytobenthos in Bay of Bourgneuf compared to the Loire estuary. This can be due to the fineness of particles in the Loire estuary which allows larger deposits of organic matter than in the Bay of Bourgneuf. This hypothesis is supported by the work of Lucas and Holligan (1999) in the Westerschelde estuary. They have reported that, in the first 2 mm of sediment, sandy stations had similar concentrations of Chl *a* and Phaeo *a*, whereas muddy stations showed Phaeo *a* concentrations higher than Chl *a* concentrations. Thus it is necessary for clams from the Loire to improve the use of degraded organic matter in order to acquire energy needed to fulfil all their biological functions (maintenance, growth and reproduction). Consequently, total lipid concentrations in both organs did not show a significant intersite difference even if occasionally significantly higher concentrations were observed in the Bay of Bourgneuf. On the other hand, glycogen which is the more easily remobilized source of energy was always higher in both organs of the clams of the reference site. This is in agreement with the highest condition index generally observed for specimens from the reference site. In the gonads, an increase of reserves as glycogen and lipids was observed between April and May 2008 in parallel with an increasing of the GSI (from 3.5 to 9.8 in the Bay of Bourgneuf; from 4.1 to 6.6 in the Loire estuary). Surprisingly, in June despite levels of GSI increasing again (12 and 11.3 respectively in the Bay of Bourgneuf and the Loire estuary), levels of reserves were decreasing. When the gonadosomatic index of clams was described with more details from April to August 2009, at the peak of development of the gonads, the intersite difference was significant with higher GSIs in the clams from the Bay of Bourgneuf. As underlined previously by Mouneyrac et al. (2008), the interpretation of results is complex due to

interferences between natural and anthropogenic factors. Among the natural factors, temperature and food availability and quality as it may be partly reflected by the presence of Chl *a* in sediment, are important in governing the reproductive cycle (Rodríguez-Rúa et al., 2003 and literature cited therein). Marked differences in the maximum of gonadal development are exemplified by comparing these two studies carried out medium and southern Europe. In addition, inter-annual differences can occur as shown by the comparison of the results of the present study and those obtained by Mouneyrac et al. (2008).

For behaviour, intersite differences were not marked when the burrowing speed was taken into account whereas they were clearly shown considering the number of unburrowed specimens at the end of the test (7 hours). It has been shown previously that salinity differences were able to reduced burrowing speed of clams originating from brackish areas such as those from the Loire estuary. On the contrary, the number of specimens was not affected by different salinities (Fossi Tankoua et al., 2011). Similarly, Byrne and O'Halloran (2001) have shown intersite differences by using the number of specimens remaining unburrowed after 24 hours. As mentioned by Fossi Tankoua et al. (2011) the quality of sediments may act as a confounding factor in burrowing tests. However, the optimum range of sediment grain size for burrowing activity of *S. plana* is much larger (medium sand to silt and mud) than the difference observed between the Bay of Bourgneuf and the Loire estuary (Fig. 1), and more generally among mudflats harboring *S. plana* (Alexander et al., 1993). Bonnard et al. (2009) have shown that burrowing of *S. plana* was favored in a natural mud with more organic matter and finer grain size compared to an artificial sediment (mean grain size 0.25 mm). Thus in the Loire estuary, the sediment which was finer and had higher concentrations of total organic carbon (Fig. 3) should be preferred to the sediment of the Bay of Bourgneuf. Since the reverse was observed, it may be concluded that contamination was the more important driving parameter affecting burrowing. In other studies, such behavioural impairments were attributed to a reaction of avoidance but a previous study with *S. plana* based on the methodology of cross-tests (Wilding and Maltby, 2006) have shown that hypoactivity of the clams in contaminated estuaries, including the Loire, was due to physiological damage rather than to avoidance (Boldina-Cosqueric et al., 2010). Links are clearly established between behavioural disruption and metabolic impairments and/or neurological dysfunction which can be revealed by decreased AChE activity (for a review, see Amiard-Triquet, 2009). In the Loire estuary, impairments of AChE were limited and behavioural disturbances are more likely due to energy metabolism dysfunction.

In both studied sites, AChE activity did not show any significant temporal difference. In a prolonged study covering three years with monthly sampling, AChE activity in mussels followed the temperature changes and the annual physiological variations of these bivalves (Burgeot et al., 2010). This study illustrates how establishing a sound background model for a given biomarker presents great resource implications. Thus another strategy to study the ecological status of an estuary may be based on the comparison against a reference site as exemplified in the present study in agreement with the recommendations of Amiard-Triquet and Rainbow (2009). Such a reference site must be chosen to be as close as possible to the estuary of interest, in terms of salinity regime, temperature, sediment quality, etc. in order to mitigate the importance of confounding factors. In practice it will probably be impossible to reach perfect agreement between estuaries and it remains necessary to make a careful assessment of these differences which cannot be controlled.

S. plana is known as a tolerant species, able to survive in strongly contaminated areas such as the United Kingdom estuaries heavily contaminated by past mining activities in Cornwall (Bryan et al., 1980). In the present study, which has expanded over one year and a half the previous work by Boldina-Cosqueric et al. (2010), it has been confirmed that *S. plana* is able to activate defence mechanisms against organic compounds, in particular (GST). However, defence is not sufficient to ensure a total protection since a number of impairments were observed at the individual level (burrowing behaviour, condition index, gonado-somatic index).

Potential population effects were examined in the Loire estuary only, to reveal any impact of a small oil spill which occurred one month before the beginning of the sampling collection (http://www.cedre.fr/uk/spill/donges/donges.htm). The study of population density, the distribution of size classes and the number of cohorts did not reveal anomalies in clams from the Loire estuary. As in other European populations, an annual period of recruitment was recognized followed by a slight decrease of density attributed to post-reproduction mortality (Verdelhos et al., 2005 and literature cited therein). Densities registered in the Loire estuary one month after the spillage of fuel (408 ± 124 ind m^{-2}) and at the maximum (1083 ± 237) were of the same order of magnitude as those published for different sites in the UK and France (Tamar estuary, Spooner and Moore, 1940 ; Gwendraeth estuary, South Wales, Green, 1957 ; Charentes area, Bouchet et al., 2005), and even higher than in southerner regions (Tagus estuary, Spain, Cabral and Murta, 2004 ; Ichkeul Lake, Tunisia, Casagranda and Boudouresque, 2005).

Contrary to previous achievements with the endobenthic annelid *Nereis diversicolor* (Amiard-Triquet and Rainbow, 2009), no links can be established between individual and supra-individual effects of living in the contaminated Loire estuary, even in the case of a small chemical accident. However, considering chronic pollution, even if interpretation of results is complex due to interferences between natural and anthropogenic factors, *S. plana* appears as a robust sentinel in estuarine ecosystems, able to reveal disturbances in the Loire estuary which is not one of the most contaminated estuary in France and over the world.

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Literature cited

Abele-Oeschger, D., Theede, H. 1991. Digestion of algal pigments by the common periwinkle *Littorina littorea* L. (Gasteropoda). J. Exp. Mar. Biol. Ecol. 147, 177–184.

AELB, (Agence de l'Eau Loire Bretagne), 2004. Mise en place d'un diagnostic de surveillance du milieu littoral conforme a la directive cadre.

AFNOR, 1985. Huîtres creuses – Dénominations et classification, NF V45-056, Septembre 1985.

Albentosa, M, Moyano, F.J., 2008. Influence of nutritional stress on digestive enzyme activities in juveniles of two marine clam species, *Ruditapes decussatus* and *Venerupis pullastra*. J Sea Res 59, 249-258.

Alexander, R.R, Stanton, R.J. Jr, Dodd, J.R., 1993. Influence of sediment grain size on the burrowing of bivalves: correlation with distribution and stratigraphic persistence of selected neogene clams. Palaios 8, 289-303.

Allan, I.J., Vrana, B., Greenwood, R., Mills, G.A., Roig, B., Gonzalez, C., 2006 A toolbox for biological and chemical monitoring requirements for the European Union's Water framework Directive. Talanta 69, 302–322.

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76, 160-202.

Amiard, J.C., Bodineau, L., Bragigand, V., Minier, C., Budzinski, H., 2009. Quantification of Contaminants. In: Amiard-Triquet, C., Rainbow, P.S. (eds.), Environmental Assessment of Estuarine Ecosystems: A Case Study. CRC Press, Boca Raton, FL, 2009, pp. 31-57.

Amiard, J.C., Journel, R., Bacheley, H., 2008. Influence of field and experimental exposure of mussels (*Mytilus sp.*) to nickel and vanadium on metallothionein concentration. Comp. Biochem. Physiol. 147C, 378-385.

Amiard-Triquet, C., 2009. Behavioral disturbances: the missing link between sub-organismal and supra-organismal responses to stress? Prospects based on aquatic research. Hum. Ecol. Risk Assess. 15, 87-110.

Amiard-Triquet, C., Rainbow, P.S. (eds.), 2009. Environmental Assessment of Estuarine Ecosystems. A Case Study. CRC Press, Boca Raton, 355 p.

Bhattacharya, C.G., 1967. A simple method of resolution of a distribution into gaussian components. Biometrics 23, 115–135.

Bernfeld; P., 1955. Amylases, α and β. In: Colowick, S.P., Kaplan, N.O. (eds.), Methods Enzymol. Vol 1. Academic Press, New York, pp 149-158.

Boldina-Cosqueric, I., Amiard, J.C., Amiard-Triquet, C., Dedourge-Geffard, O., Métais, I., Mouneyrac, C., Moutel, B., Berthet, B., 2010. Biochemical, physiological and behavioural markers in the endobenthic bivalve *Scrobicularia plana* as tools for the assessment of estuarine sediment quality. Ecotox. Environ. Saf. 73, 1733-1741.

Bonnard, M., Roméo, M., Amiard-Triquet, C., 2009. Effects of copper on the burrowing behavior of estuarine and coastal invertebrates, the polychaete *Nereis diversicolor* and the bivalve *Scrobicularia plana*. Hum. Ecol. Risk Assess. 15, 11-26.

Bouchet,, V., Degre, D., Sauriau, P.G., 2005. Population dynamic factors of *Scrobicularia plana* (Da Costa 1778) of Aiguillon Cove (Charente-Maritime, Vendee) under influence of predation by wintering limicoles. In: Richard, G. (Ed.), Proceedings, Third International Congress of European Societies of Malacology: Univ. La Rochelle, Laboratoire de Biologie et Environnement Marins (LBEM), pp. 221-225.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Bryan, G.W., Langston, W.J., Hummerstone, L.G., 1980. The use of biological indicators of heavy metal contamination in estuaries. Occasional Publication. Mar. Biol. Ass. U.K., 73 pp.

Buffan-Dubau E., de Wit, R., Castel, J. 1996. Feeding selectivity of harpacticoid copepod *Canuella perplexa* in benthic muddy environments demonstrated by HPLC analyses of chlorin and carotenoid pigments. Mar. Ecol. Prog. Ser. 137, 71–82.

Buffet, P.E., Fossi Tankoua, O., Pan, J.F., Berhanu, D., Herrenknecht, C., Poirier, L., Amiard-Triquet, C., Amiard, J.C., Bérard, J.B., Risso, C., Guibbolini, M., Roméo, M., Reip, P., Valsami-Jones, E., Mouneyrac, C., 2011. Behavioural and biochemical responses of two marine invertebrates Scrobicularia plana and Hediste diversicolor to copper oxide nanoparticles. Chemosphere (doi:10.1016/j.chemosphere.2011.02.003)

Burgeot, T., Gagné, F., Forget-Leray, J., Bocquené, G., 2010. Acetylcholinesterase : Methodology development of a biomarker and challenges of its application for

biomonitoring. ICES CM 2010/F:25, e-paper http://www.ices.dk/products/CMdocs/CM-2010/CM2010.pdf

Byrne, P.A., O'Halloran, J., 2001. The role of bivalve molluscs as tools in estuarine sediment toxicity testing: a review. Hydrobiologia 465, 209-217.

Cabral, H.N., Murta, A.G., 2004. Effect of sampling design on abundance estimates of benthic invertebrates in environmental monitoring studies. Mar. Ecol. Prog. Ser. 276, 19-24.

Cariou-Le Gall, V., Blanchard, G. 1995. Monthly measurements of pigment concentration from an intertidal muddy sediment of Marennes-Oleron, France. Mar Ecol Prog Ser. 121,171–179.

Carr, R.S., Neff, J.M., 1984. Quantitative semi-automated enzymatic assay for tissue glycogen. Comp. Biochem. Physiol. 77B, 447-449.

Cartaxana, P., Jesus, B., Brotas, V. 2003. Phaeophorbide and phaeophytin a-like pigments as useful markers for intertidal microphytobenthos grazing by *Hydrobia ulvae*. Estuar Coast. Shelf. Sci. 58, 293–297

Casagranda, C., Boudouresque, C.F., 2005. Abundance, Population Structure and Production of *Scrobicularia plana* and *Abra tenuis* (Bivalvia: Scrobicularidae) in a Mediterranean Brackish Lagoon, Lake Ichkeul, Tunisia. Internat. Rev. Hydrobiol. 90, 376-391.

Dedourge, O., Geffard, A., Amiard-Triquet, C., 2008. Origine des perturbations du métabolisme énergétique. In : Amiard, J.C., Amiard-Triquet, C. (eds.), Les biomarqueurs dans l'évaluation de l'état écologique des milieux aquatiques. Lavoisier, Tec&Doc, Paris, pp 241-271.

Diamantino, T.C., Almeida, E., Soares, A.M.V.M., Guilhermino, L., 2001. Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* Straus. Chemosphere 45, 553-560.

Forbes, V.E., Palmqvist, A., Bach, L., 2006 The use and misuse of biomarkers in ectoxicology. Environ. Toxico.l Chem. 25, 272-280.

Fossi Tankoua, O., Buffet, P.E., Amiard, J.C., Amiard-Triquet, C., Mouneyrac, C., Berthet, B., 2011. Potential influence of confounding factors (size, salinity) on biomarker tools in the sentinel species Scrobicularia plana used in monitoring programmes of estuarine quality. Environ. Sci. Pollut. Res. (accepted)

Frasco, M.F., Guilhermino, L., 2002. Effects of dimethoate and beta-naphthoflavone on selected biomarkers of Poecilia reticulata. Fish Physiol. Biochem. 26, 149-156.

Frings, C.S., Fendley, T.W., Dunn, R.T., Queen, C.A., 1972. Improved determination of total serum lipids by the sulpho-phospho-vanilin reaction. Clin. Chem. 18, 673-674.

Fu, J., Mai, B., Sheng, G., Zhang, G., Wang, X., Peng, P., Xiao, X., Ran, R., Cheng , F., Peng, X., Wang, Z., Tang, U.W., 2003. Persistent organic pollutants in environment of the Pearl River Delta, China: an overview. Chemosphere 52, 1411-1422.

Galgani, F., Bocquené, G., 1991. Semi-automated colorimetric and enzymatic assays for aquatic organisms using microplate readers. Water Res. 25, 147-150.

García-Luque, E., DelValls, T.A., Casado-Martínez, C., Forja, J.M., Gómez-Parra, A., 2004. Simulating a heavy metal spill under estuarine conditions: Effects on the clam *Scrobicularia plana*. Mar. Environ. Res. 58, 671-674.

García-Luque, E., DelValls, T.A., Forja, J.M., Gómez-Parra, A., 2007. Biological adverse effects on bivalves associated with trace metals under estuarine environments. Environ. Monit. Assess. 131, 27-35.

Gayanilo, F.C.J., Pauly, D., 2005. The FAO-ICLARM Stock Assessment Tools (FISAT II) Reference Manual, 8th edition .FAO (Food and Agricultural organisation) Computerized Information Series (Fisheries), Rome.

Grabe, S.A., Barron, J., 2004. Sediment contamination, by habitat, in the Tampa Bay estuarine system (1993–1999): PAHs, pesticides and PCBs. Environ. Monit. Assess. 91, 105-144.

Green, J., 1957. The growth of *Scrobicularia plana* (Da Costa) in the Gwendraeth estuary. J. Mar. Biol. Ass. U.K. 36, 41-47.

Hagger, J.A., Jones, M.B., Lowe, D., Leonard, P., Owen, R., Galloway, T.S., 2008 Application of biomarkers for improving risk assessments of chemicals under the Water Framework Directive: A case study. Mar. Poll. Bull. 56, 1111-1118.

Hughes, R.N., 1969. A study of feeding in *Scrobicularia plana*. J. Mar. Biol. Ass. U.K. 49, 805-823.

Kowalewska, G., Konat-Stepowicz, J., Wawrzyniak-Wydrowska, B., Szymczak-Zyła, M., 2003. Transfer of organic contaminants to the Baltic in the Odra Estuary. Mar. Pollut. Bull. 46, 703-718.

Leiniö, S., Lehtonen, K.K., 2005. Seasonal variability in biomarkers in the bivalves *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea. Comp. Biochem. Physiol. 140C, 408 – 421

Long, E.R., MacDonald, D.D., Smith, S.L., Calder, F.D., 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. Environ. Management 19, 81-97.

Lorenzen, C.J., 1967. Determination of chlorophyll and phaeopigments: spectrophotometric equations. Limnol. Oceanogr. 12, 343-346.

Lucas C.H., Holligan P.M. 1999. Nature and ecological implications of algal pigment diversity on the Molenplaat tidal flat (Westerscheldt estuary, SW Netherlands). Mar. Ecol. Prog. Ser. 180, 51–64

Marchand, J., 1993. The influence of seasonal salinity and turbidity maximum variations on the nursery function of the Loire estuary (France). Neth. J. Aquat. Ecol. 27, 427-436.

Mazorra, M.T., Rubio, J.A., Blasco, J., 2002. Acid and alkaline phosphatase activities in the clam Scrobicularia plana: kinetic characteristics and effects of heavy metals. Comp. Biochem. Physiol. 131B, 241-249.

MEA (Millenium Ecosystem Assesment) 2005. Ecosystems and human well-being: Synthesis. Island Press, Washington, DC. World Resources Institute.

Méléder, V., Barillé, L., Rincé, Y., Morançais, M., Rosa, P., Gaudin, P., 2005. Spatio-temporal changes in microphytobenthos strucutre analysed by pigement composition in a macrotidal flat (Bourgneuf Bay, France). Mar. Ecol. Prog. Ser. 297, 83-99.

Mouneyrac, C., Amiard-Triquet, C., 2011 Biomarkers of ecological relevance. In: Blaise, C., Férard, J.F. (eds.), Encyclopedia of Aquatic Ecotoxicology. Springer, Berlin (in press).

Mouneyrac, C., Linot, S., Amiard, J.C., Amiard-Triquet, C., Métais, I., Durou, C., Minier, C., Pellerin, J., 2008. Biological indices, energy reserves, steroid hormones and sexual maturity in the infaunal bivalve *Scrobicularia plana* from three sites differing by their level of contamination. Gen. Comp. Endocrinol. 157, 133-141.

Riera, P., Stal, L.J., Nieuwenhuize, J., Richard, P., Blanchard, G., Gentil, F. 1999. Determination of food sources for benthic invertebrates in a salt marsh (Aiguillon Bay, France) by carbon and nitrogen stable isotopes: importance of locally produced sources. Mar. Ecol. Prog. Ser. 187, 301–307.

RNO, 2006. Surveillance de la qualité du milieu marin. Ministère de l'Environnement and Institut français de recherche pour l'exploitation de la mer (Ifremer), Paris & Nantes.

Rodríguez-Rúa, A., Prado, M.A., Romero, Z., Bruzón, M., 2003. The gametogenic cycle of *Scrobicularia plana* (da Costa, 1778) (Mollusc: Bivalve) in Guadalquivir estuary (Cádiz, SW Spain). Aquaculture 217, 157-166.

Roesijadi, G., 1996 Metallothionein and its role in toxic metal regulation. Comp. Biochem. Physiol. 113C, 117–123.

Romero-Ruiz, A., Alhama, J., Blasco, J., Gomez-Ariza, J.L., Lopez-Barea, J., 2008. New metallothionein assay in Scrobicularia plana: Heating effect and correlation with other biomarkers. Environ. Pollut. 156, 1340-1347.

Solé, M., Kopecka-Pilarczyk, J., Blasco, J., 2009. Pollution biomarkers in two estuarine invertebrates, *Nereis diversicolor* and *Scrobicularia plana*, from a Marsh ecosystem in SW Spain. Environ. Int. 35, 523-531.

Spooner, G.M., Moore, H.B., 1940. The ecology of the Tamar estuary. VI. An account of the macrofauna of the intertidal mudflats. J. Mar. Biol. Ass. U.K. 24, 283-330.

Swain, E.B. 1985. Measurement and interpretation of sedimentary pigments. Freshwater Biology 15: 53-75.

Thompson, J.A.J., Cosson, R.P., 1984. An improved electrochemical method for the quantification of metallothioneins in marine organisms. Mar. Environ. Res. 11, 137-152.

Verdlhos, T., Neto, J.M., Marques, J.C., Pardal, M.A., 2005. The effect of eutrophication abatement on the bivalve *Scrobicularia plana*. Estuar. Coast. Shelf Sci. 63, 261-268.

Waters, M.N. 2000. The use of sedimentary algal pigments to infer historic algal communities in Lake Apopka, Florida. M.S. Thesis, University of Florida, 56 pp.

Weis, J.S., Smith, G.M., Zhou, T., Santiago-Bass, C., Weis, P., 2001. Effects of contaminants on behavior: biochemical mechanisms and ecological. BioScience 51: 209-217..

Wilding, J., Maltby, L., 2006. Relative toxicological importance of aqueous and dietary metal exposure to a freshwater crustacean: implications for risk assessment. Environ. Toxicol. Chem. 25, 1795-1801.

Woodhead, R.J., Law, R.J., Matthiessen, P., 1999. Polycyclic aromatic hydrocarbons in surface sediments around England and Wales, and their possible biological significance. Mar. Pollut. Bull. 38, 773-790.

Table 1. Temporal fluctuations of ecological parameters (salinity and temperature)

Sampling month	Temperature		Salinity	
	Minimum	Maximum	Loire	Bourgneuf
March 2008	5.2 (4.0)	12.3 (2.1)	15	30.5
April 2008	5.4 (3.1)	21.0 (205)	15.0	32
May 2008	10.6 (2.5)	19.4 (4.1)	15.9	32.5
June 2008	11.0 (2.4)	20.6 (2.5)	25.5	29.1
October 2008	8.3 (4.3)	17.5 (2.4)	21.4	31.4
January 2009	-0.3 (4.4)	6.4 (4.0)	17.8	31.1
March 2009	3.2 (3.2)	11.9 (2.4)	17	30.4
October 2009	12.1 (2.8)	21.5 (2.3)	22.2	31.0

*Mean and SD of air temperature registered daily during the month before sampling at Saint Nazaire (3 km from the sampling site in the Loire estuary; 49 km from the sampling place in the Bay of Bourgneuf).

Table 2. Quality of potentially available food sources estimated by using the ratio Chl *a*/Phaeo *a* and Chl *a*/TOC in superficial sediments.

Sampling month	Chl *a*/Phaeo *a*		Chl *a*/TOC	
	Bourgneuf	Loire	Bourgneuf	Loire
April 2008	0.30	0.13	2.95	0.50
May 2008	0.04	0.33	0.57	2.83
June 2008	0.42	-	4.45	-
October 2008	0.96	0.21	3.68	0.30
January 2009	0.56	0.32	1.45	0.27
March 2009	0.45	0.34	2.27	0.53
October 2009	2.56	1.25	5.78	2.00

Table 3. Slope of the regression curves (and correlation coefficient R^2) between total metal concentrations and concentrations either in the cytosol C1 and the insoluble fraction of soft tissues of clams originating from both studied sites.

		Ag	Cd	Cu	V	Zn
Insoluble vs total metals	Slope	0.9571	0.5888	0.9873	0.8896	0.5139
	R^2	0.9771	0.7762	0.9762	0.9529	0.5132
Cytosolic vs total metals	Slope	0.0429	0.4612	0.0127	0.1104	0.4861
	R^2	0.0791	0.7176	0.0067	0.2374	0.4853

Table 4. Mean values of condition index (CI) of clams collected over the whole sampling period from the Bay of Bourgneuf and the Loire estuary. Standard deviations in brackets.

	March 08	April 08	May 08	June 08	Oct. 08	Jan. 09	March 09	Oct. 09
	Water content[a]							
Bourgneuf		75 (13)			81 (1)	79 (7)	82 (3)	79 (Baumard et al.)
Loire estuary		79 (5)			83 (Baumard et al.)	80 (8)	84 (3)	82 (Baumard et al.)
	Condition index[a]							
Bourgneuf		21 (6)			23 (5)	24 (4)	19 (4)	27 (5)
Loire estuary		17 (3)			19 (3)	17 (3)	24 (4)	21 (4)
	Condition index[b]							
Bourgneuf	21 (4)	18 (3)	23 (5)	23 (5)	22 (3)	19 (3)	17 (3)	25 (4)
Loire estuary	19 (4)	17 (3)	21 (4)	19 (3)	21 (3)	17 (3)	16 (3)	20 (3)
	Hepatosomatic index[c]							
Bourgneuf	21 (4)	17 (3)	17 (5)	16 (5)	21 (3)	21 (5)	19 (4)	20 (3)
Loire estuary	22 (6)	21 (3)	23 (4)	14 (4)	21 (4)	25 (4)	24 (3)	25 (3)

[a]N = 50 (clams collected at random)

[b]Calculated from clams devoted to biochemical measurements (N = 20 in March 2008, N = 50 in April 2008; N= 60 for all of the other months)

[c]Calculated from clams devoted to energy reserves and digestive enzyme activity measurements (N = 10 in March 2008; N= 22 for all of the other months)

Table 5. Slopes and regression coefficient of the least-square regression lines for behavioral tests carried out with clams *S. plana* from the Bay of Bourgneuf and the Loire estuary. * significant intersite difference between the slopes with p = 0.011; **p = 0.001

		March 08	April 08*	May 08	June 08**	Oct 08	Jan 09	March 09	Oct 09
Bourgneuf	Slope	-0.0123	-0.0121	-0.0298	-0.0527	-0.0232	-0.0198	-0.0083	-0.0222
	R^2	0.8892	0.7979	0.9175	0.1227	0.9175	0.894	0.9052	0.8353
Loire	Slope	-0.0127	-0.0027	-0.0224	-0.0296	-0.0319	-0.0125	-0.0097	-0.0264
	R^2	0.937	0.321	0.9085	0.7894	0.9279	0.9128	0.0018	0.9266

Table 6. Density (mean and SD calculated from 3 replicated sediment samples) and shell growth of each cohort constituting the population of *Scrobicularia plana* in the Loire estuary from April 2008 to October 2009 shown by using the mean length of the shell determined by modelling.

	April 08	May 08	June 08	Oct. 08	Oct. 09
Density (number of individuals m^{-2})					
	408 (124)	1029 (24)	1083 (237)	779 (182)	699 (104)
Shell length for each cohort (mm)					
Cohort L1	30				
L2	22	28	27	29	
L3	13	18	19	22	29
L4		8	12	15	24
L5			7	10	18
L6					9

List of figures

Figure 1 Distribution of different size fractions in sediment cores collected over the whole sampling period from the Bay of Bourgneuf and the Loire estuary. Mean values expressed in g kg^{-1} dry weight and SD. N = 7 for each site.

Figure 2 Concentrations of individual PAH compounds in superficial sediments from the Bay of Bourgneuf and the Loire estuary. Means and SD calculated from samples collected from April 2008 to October 2009. N = 7 for each site. Ant: anthracene; BaA: benzo (a) anthracene; BaP: benzo (a) pyrene; BbF: benzo (b) fluoranthene; BghiP: benzo (ghi) perilene; BkF: benzo (k) fluoranthene; Chrys: chrysene; DaA: dibenz (ah) anthracene; Fluo: fluoranthene; IP: indeno (1,2,3,cd) pyrene; Napht: naphthalene; Phe: phenanthrene; Pyr: pyrene.

Figure 3. Concentrations of total organic carbon (TOC), chlorophyll a and phaeopigments a in superficial sediments from the Bay of Bourgneuf (white bars)and the Loire estuary (black bars).

Figure 4. Spatio-temporal variations of Σ (Ag, Cd, Cu, V, Zn) metal concentrations, MT concentrations and GST and LDH activities in the whole soft tissues of the clam *Scrobicularia plana*; digestive enzyme activities (amylase, CMcase) in the digestive gland. Mean values and SD calculated from 10 specimens from each site. * significant intersite differences.☆ Lost samples.

Figure 5. Spatio-temporal variations of glycogen and total lipid concentrations in the digestive gland and the gonad (when sufficiently developed) of the clam *Scrobicularia plana*. Mean values and SD calculated from 10 specimens from each site.* significant intersite differences.

Figure 6. Spatio-temporal variations of the gonadosomatic index of the clam *Scrobicularia plana* during the period of reproduction in 2009. Mean and standard deviation, N = 30 for each site. * significant intersite differences.

Fig. 7. Burrowing behavior: number of clams *Scrobicularia plana* which remained unburrowed at the end the test (7 hours). 20 specimens included in the test for each site.☆ All specimens burrowed.

Fig. 8. Shell length frequency histograms of *Scrobicularia plana* from the Loire estuary (A : April 2008, B : May, C : June, D : October, E : Oct 2009)

Figure 1

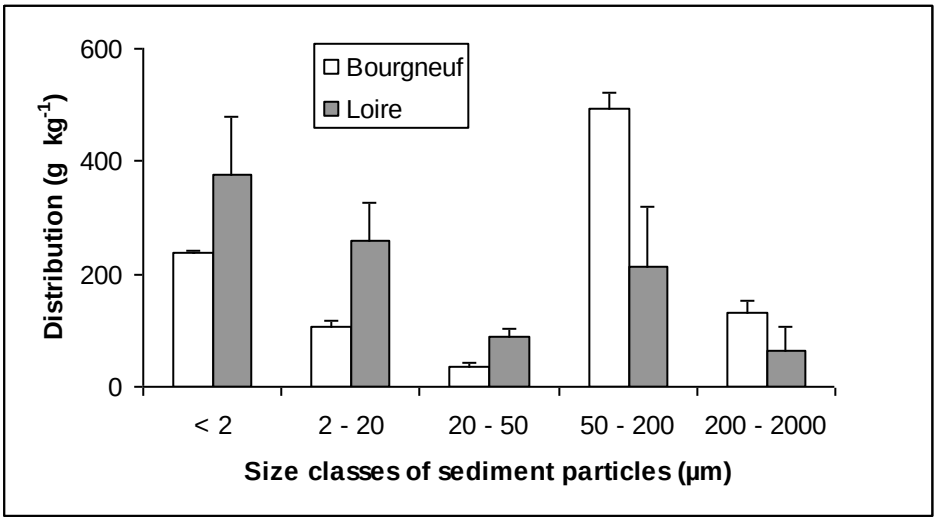

600
400
200
0
Distribution (g kg-1)
Bourgneuf
Loire
< 2
2 - 20
20 - 50
50 - 200
200 - 2000
Size classes of sediment particles (µm)

Figure 2

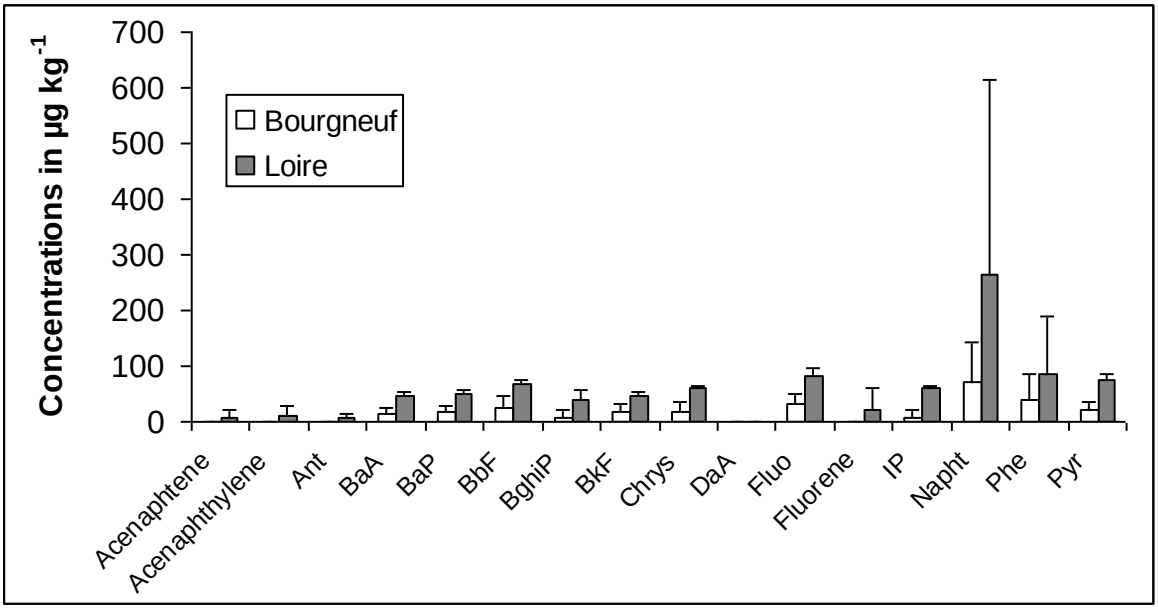
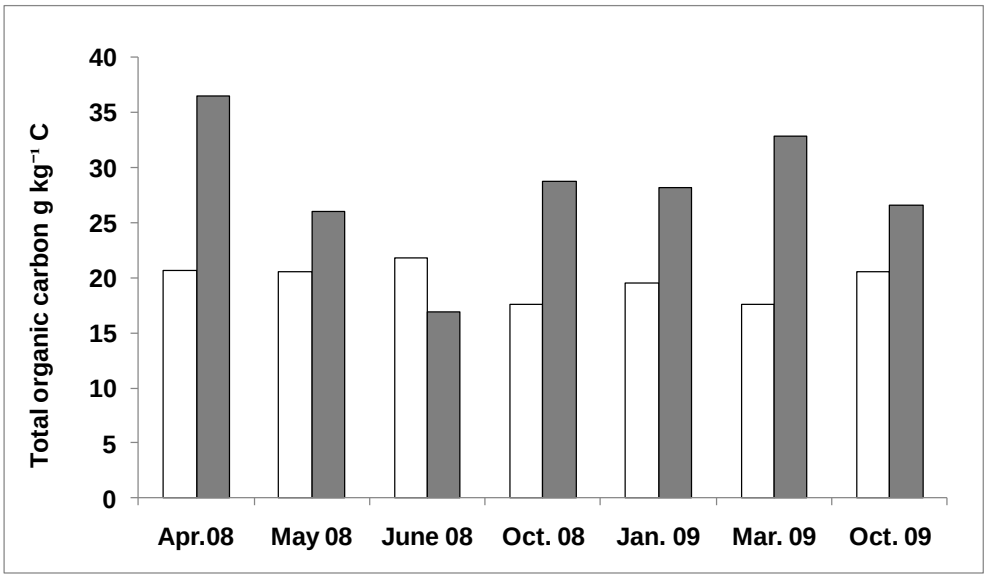
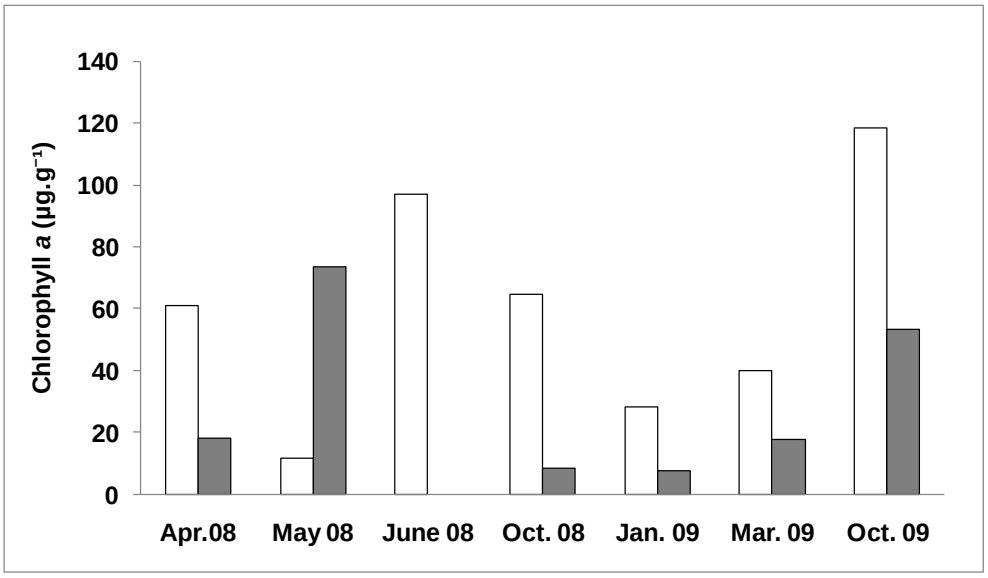

Concentrations in µg kg-1
700
600
500
400
300
200
100
0
Bourgneuf
Loire
Acenaphtene
Acenaphthylene
Ant
BaA
BaP
BbF
BghiP
BkF
Chrys
DaA
Fluo
Fluorene
IP
Napht
Phe
Pyr

Figure 3.


Total organic carbon g kg⁻¹ C
40
35
30
25
20
15
10
5
0
Apr.08
May 08
June 08
Oct. 08
Jan. 09
Mar. 09
Oct. 09


Chlorophyll a (µg.g⁻¹)
140
120
100
80
60
40
20
0
Apr.08
May 08
June 08
Oct. 08
Jan. 09
Mar. 09
Oct. 09

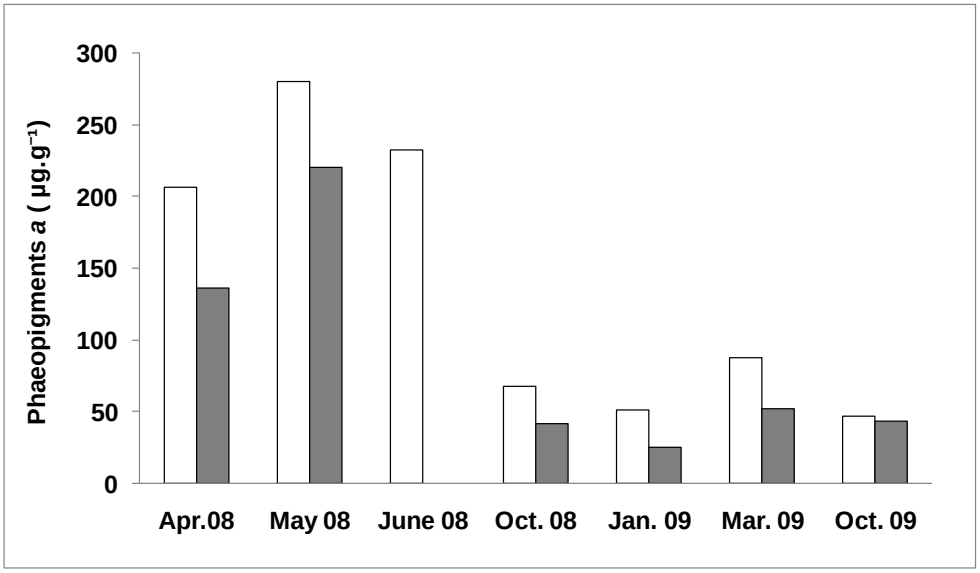

Phaeopigments a (µg.g⁻¹)
300
250
200
150
100
50
0
Apr.08
May 08
June 08
Oct. 08
Jan. 09
Mar. 09
Oct. 09

Figure 4.

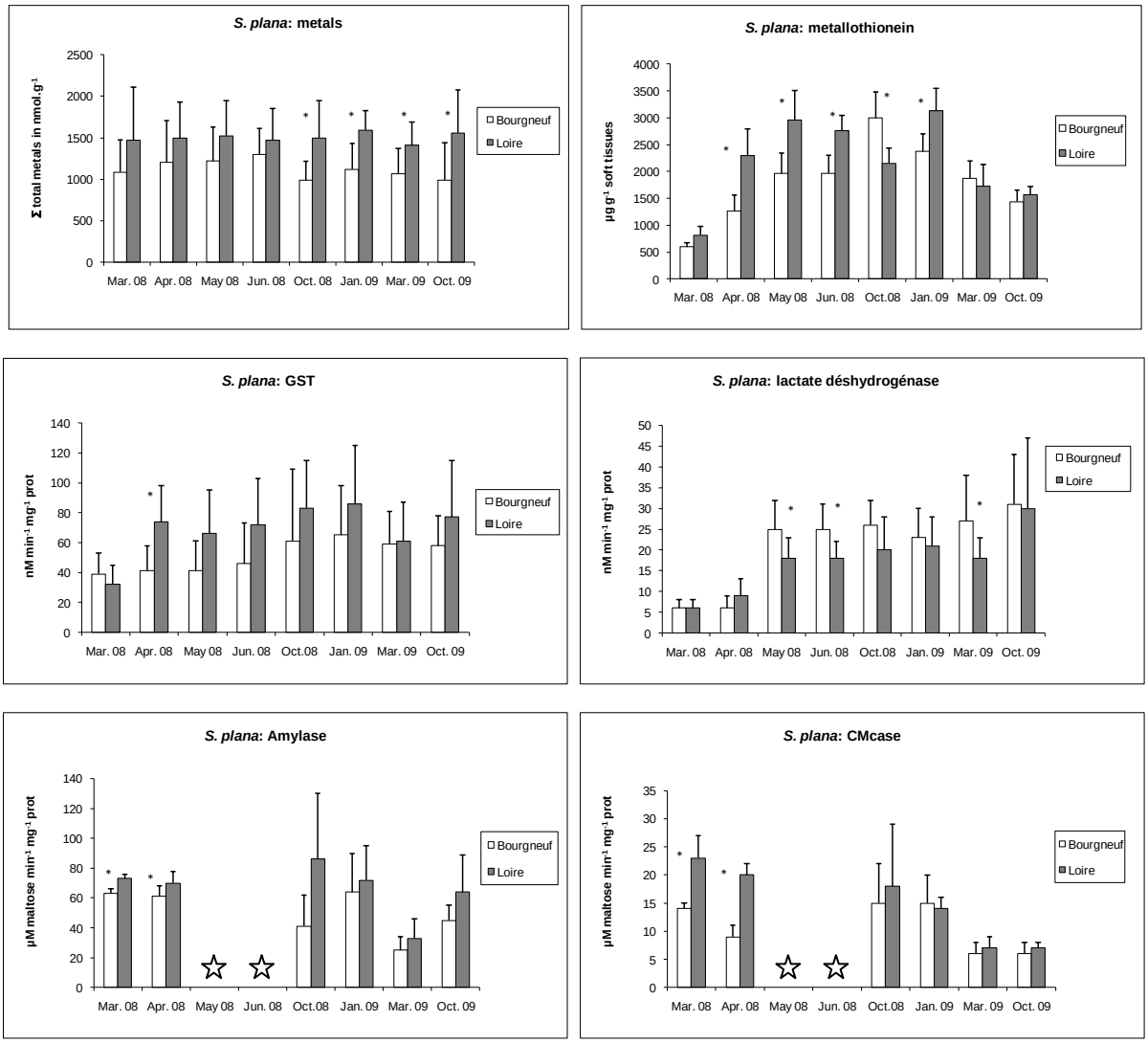
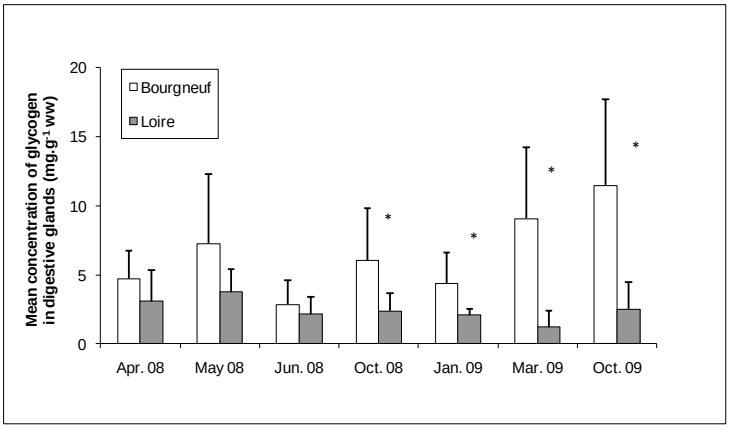
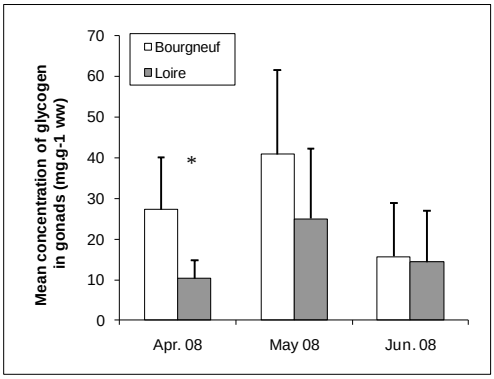
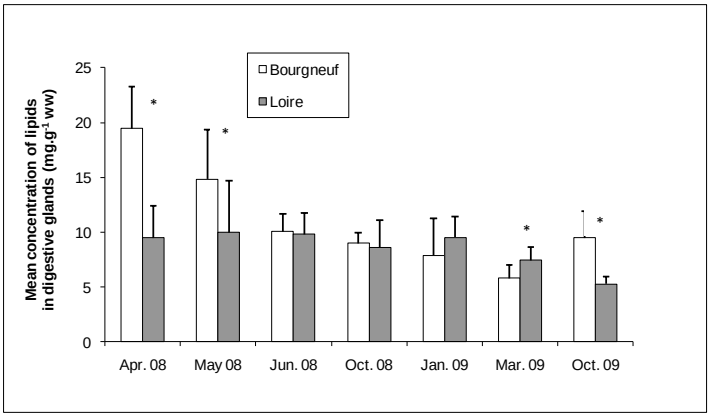

S. plana: metals
Σ total metals in nmol.g-1
Bourgneuf
Loire
Mar. 08
Apr. 08
May 08
Jun. 08
Oct. 08
Jan. 09
Mar. 09
Oct. 09
S. plana: metallothionein
µg g-1 soft tissues
Oct.08
S. plana: GST
nM min-1 mg-1 prot
S. plana: lactate déshydrogénase
S. plana: Amylase
µM maltose min-1 mg-1 prot
S. plana: CMcase

Figure 5.


Bourgneuf
Loire
Mean concentration of glycogen in digestive glands (mg.g-1 ww)
0
5
10
15
20
Apr. 08
May 08
Jun. 08
Oct. 08
Jan. 09
Mar. 09
Oct. 09


Bourgneuf
Loire
Mean concentration of glycogen in gonads (mg.g-1 ww)
0
10
20
30
40
50
60
70
Apr. 08
May 08
Jun. 08


Bourgneuf
Loire
Mean concentration of lipids in digestive glands (mg.g-1 ww)
0
5
10
15
20
25
Apr. 08
May 08
Jun. 08
Oct. 08
Jan. 09
Mar. 09
Oct. 09

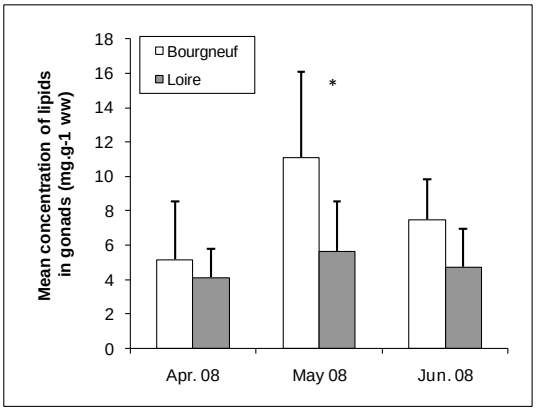

Bourgneuf
Loire
Mean concentration of lipids in gonads (mg.g-1 ww)
0
2
4
6
8
10
12
14
16
18
Apr. 08
May 08
Jun. 08

Figure 6.

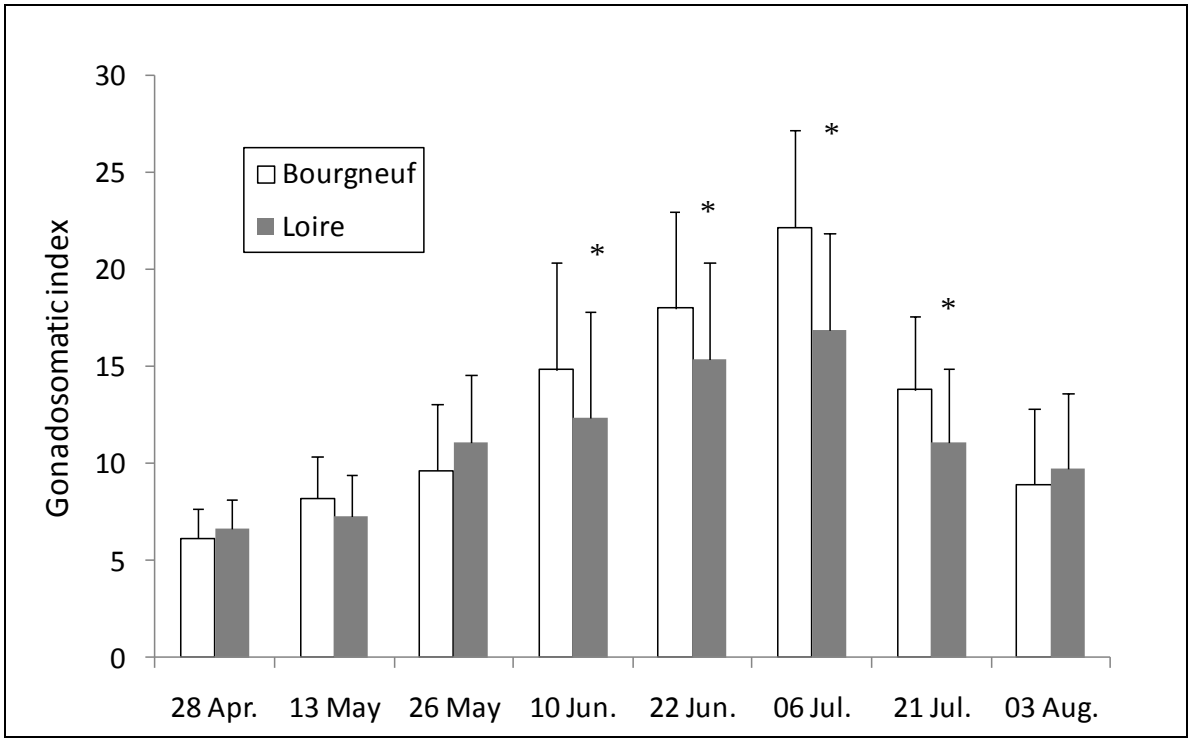

Gonadosomatic index
30
25
20
15
10
5
0
Bourgneuf
Loire
*
*
*
*
28 Apr.
13 May
26 May
10 Jun.
22 Jun.
06 Jul.
21 Jul.
03 Aug.

Fig. 7.

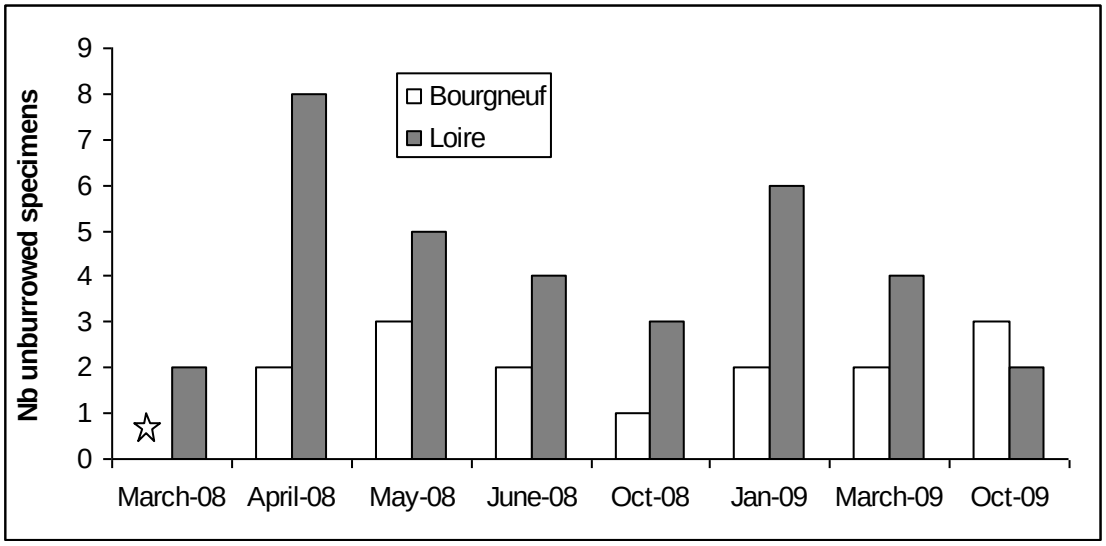
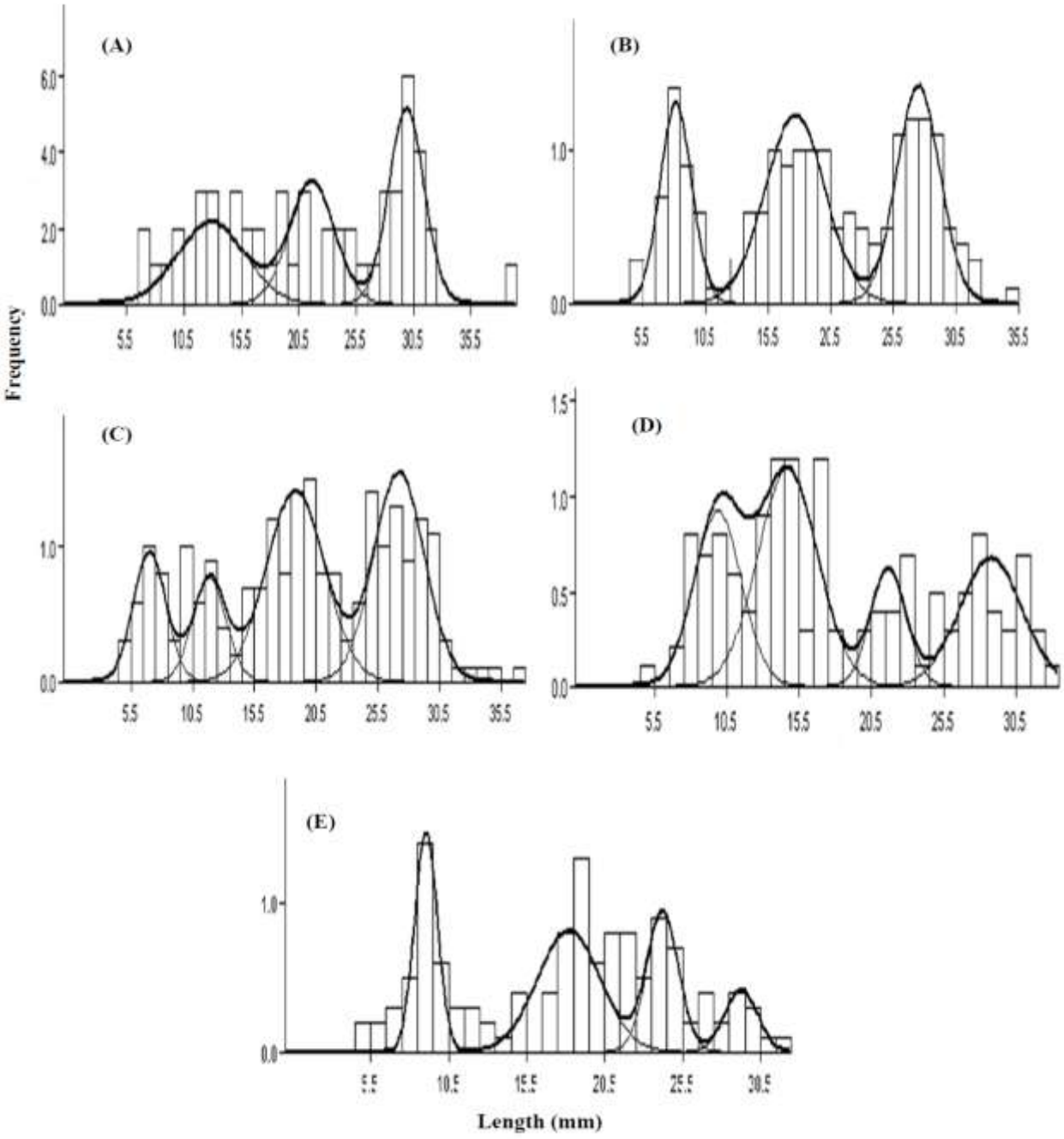

Nb unburrowed specimens
9
8
7
6
5
4
3
2
1
0
Bourgneuf
Loire
March-08
April-08
May-08
June-08
Oct-08
Jan-09
March-09
Oct-09

Fig. 8.



5.5 Conclusions

Les principaux résultats acquis sur nos deux espèces modèles ont été rassemblés dans le tableau 1 de la publication 2. Pour élargir la synthèse, ce tableau est basé non seulement sur nos propres résultats comparant les individus de Loire et de Bourgneuf mais intègre également les travaux antérieurs de l'équipe incluant d'autres sites, pollué (estuaire de la Seine) ou de référence (estuaires de l'Authie et du Goyen). Ce tableau permet également une comparaison de la réactivité des biomarqueurs selon le gradient de pollution, les deux grands estuaires de la Seine et de la Loire étant soumis à des pollutions multiples où toutes les grandes classes de contaminants sont représentées mais en terme de niveaux de contamination, la Seine est cependant beaucoup plus impactée que la Loire (sous-section 3.1).

Pour les deux espèces, les marqueurs biochimiques montrent des tendances similaires mais avec trois différences notables. L'activité de la glutathion S-transférase (biomarqueur de défense) est activée en Seine pour les deux espèces tandis qu'en Loire, ce biomarqueur de montre aucune différence avec le site de référence chez. Les activités des deux enzymes digestives examinées (amylase et CMcase) sont affectées chez *H. diversicolor* sur les deux sites tandis que chez le bivalve les différences ne sont pas significatives en Loire. L'activité de l'acétylcholinestérase qui avait déjà été présentée comme un biomarqueur extrêmement réactif par Solé et al. (2009) répond chez les deux espèces à l'exposition en estuaire de Loire comme en estuaire de Seine. La métallothionéine est induite par les contaminations estuariennes aussi bien chez les bivalves de Loire que de Seine mais dans ce cas, la comparaison interspécifique n'est pas possible puisque ce biomarqueur n'est pas fonctionnel chez l'annélide polychète (Cf. sous-section 3.3.4).

L'enfouissement est un biomarqueur qui a été assez peu utilisé par le passé mais récemment de nombreuses études ont mis en évidence ses avantages : sa simplicité d'utilisation, sa très grande sensibilité, et sa capacité à faire le lien entre des effets au niveau infra- et supra-individuel (Weis et al., Amiard et Amiard-Triquet, 2008, Amiard-Triquet, 2009, Weis et al.). Chez nos deux espèces modèles, l'enfouissement est affecté en Seine tandis qu'en Loire, seul l'enfouissement du bivalve révèle la présence de stress. De plus, en applicant la méthodologie des tests croisés (organismes du site pollué mis en présence de sédiment de référence et organismes du site de référence mis en présence de sédiment pollué) décrite par Wilding et

Maltby (2006) pour un crustacé d'eau douce, il a été montré que l'origine des perturbations de comportement était vraisemblablement un phénomène d'évitement dans le cas de *H. diversicolor* (Mouneyrac et al., 2010) et la conséquence de dommage physiologique chez *S. plana* (Boldina-Cosqueric et al., 2010). La capture des proies, testée uniquement avec les annélides polychètes de Loire s'est aussi révélée un biomarqueur comportemental sensible.

L'interprétation des données physiologiques sur les réserves énergétiques et l'indice de condition s'est révélée plus délicate. Les résultats obtenus sur dans l'estuaire de Seine (Durou et al., 2007b) étaient en parfaite adéquation avec le concept de coût de tolérance (Mouneyrac et al., 2011). Dans un estuaire moins pollué comme celui de la Loire, le patron n'est pas aussi simple en raison de l'interférence entre les différentes composantes du métabolisme énergétique (qualité de la ressource alimentaire, besoins liés au cycle sexuel, réponse au stress polluant). Dans ces conditions, la stratégie d'évaluer les réserves énergétiques sous la forme de glycogène, lipides totaux et protéines est trop basique. En ce qui concerne les lipides, il paraît pertinent de s'intéresser aux classes de lipides plutôt qu'aux lipides totaux. Ainsi, chez les larves et les jeunes stades de poissons, le stockage d'énergie se fait essentiellement sous la forme de triacylglycérols (TAG), et des études ont montré que les rapports de ceux-ci à des lipides de structure comme les phospholipides (TAG/PL) et surtout les stérols (TAG/ST) constituaient de bons indices de l'état physiologique des animaux. En estuaires, le ratio TAG/ST déterminé dans les nourriceries de soles est respectivement de 2.21, 1.55, 1.70, 0.33 et 0.27 en Seine, Vilaine, Loire, Pertuis Breton et Bourgneuf (Robert GALLOIS, Univ. La Rochelle, Comm. Pers.). Au contraire des poissons, les mollusques stockent l'essentiel de leur énergie sous forme de glycogène. Néanmoins, ils contiennent des quantités non-négligeables de triacylglycérols. Les variations du rapport TAG/ST pourrait être testé chez les invertébrés exposés à des niveaux de pollution très contrastés dans leur milieu et si cet index s'avère pertinent, il pourrait être appliqué aux populations naturelles dans des estuaires pollués et de référence, en tenant bien entendu compte du stade de maturité sexuelle des organismes (Cf Chapitre 8).

Pour les deux espèces modèles de cette thèse, une conclusion commune se dégage quand à l'évaluation de l'état de santé de l'estuaire de la Loire : manifestement un nombre important de réponses biologiques sont perturbées au niveau infra-individuel et individuel. Par contre, contrairement aux résultats de Durou et al (2007b) en Seine, aucun lien ne peut être établi avec des effets populationnels, même à la suite du petit accident pétrolier qui s'est produit au

commencement des campagnes d'échantillonnage supportant cette thèse. Il convient donc d'être extrêmement prudent dans la surveillance et la gestion de cet estuaire afin que la situation ne s'aggrave pas, contribuant cette fois à une dépletion des populations d'espèces clés pour sa structure et son fonctionnement.

En ce qui concerne le bivalve *S. plana* des travaux menés dans plusieurs estuaires britanniques et portuguais ont révélé la présence d'intersexe chez cette espèce gonochorique (Langston et al., 2007, Gomes et al., 2009). De ce point de vue, aucune donnée n'est actuellement disponible en Loire et dans les autres estuaires français et la question de l'intersexe, de ses causes et de ses conéquences fera l'objet du chapitre suivant.

Par ailleurs, parmi les biomarqueurs capables de révéler des dysfonctionnements au sein des écosystèmes, nombreux sont ceux qui sont d'un emploi relativement simple et peu coûteux, en premier lieu les biomarqueurs comportementaux dont nous avons souligné la grande sensibilité et même de nombreux marqueurs biochimiques déterminés par méthode spectrophotométrique. De ce fait, il s'agit d'un outil de choix pour la biosurveillance des écosystèmes estuariens et côtiers dans les pays en voie de développement qui ne sont actuellement pas en mesure de financer des programmes excessivement coûteux. C'est pourquoi j'ai donné une présentation à la SETAC-Afrique (Université de Buea, Cameroun, 31 mai-3 juin 2011) dont le résumé figure dans l'encadré ci-dessous.

The use of ecologically relevant and cost-effective biomarkers to assess the health status of estuarine and coastal waters

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Estuarine and coastal areas are crucial for their high productivity and thus their economical role but they are also at risk because human activities are responsible for many disturbances including chemical pollution. The methodology of biomarkers has been developed since the early nineties and may be very useful in biomonitoring programmes. Those linked directly or indirectly to reproduction are particularly interesting since they allow forecasting effects on populations well before tragic population depletion may be observed. Among these ecologically relevant biomarkers, several are of interest because they are cost-effective: biochemical markers such as acetylcholinesterase (revealing neurotoxicity), digestive enzymes (involved in energy acquisition); behavioural markers (feeding, burrowing), physiological markers based on biometric measurements (condition index, gonado-somatic index…). In addition, more specific biomarkers, used in battery, may be useful to link observed deleterious effects to causal factors (e.g. detoxificatory enzymes such as metallothionein, biotranformation enzymes such as glutathione *S*-transferase, biomarkers revealing oxidative stress such as catalase, malonedialdehyde). These biomarkers have been determined in two sentinel species, the worm *Nereis diversicolor* and the bivalve *Scrobicularia plana*, which are important in estuarine and coastal food chains. They were able to reveal intersite differences along a gradient of pollution in estuaries and bays in Northwest France. This methodology may be applied to other species recognized as sentinels for other marine ecosystems. However, it is recommended to evaluate carefully the potential effects of confounding factors such as the size or sexual status of specimens, food availability, salinity, temperature…

CHAPITRE 6. INCIDENCE DE LA CONTAMINATION DU SEDIMENT PAR DES PERTURBATEURS ENDOCRINIENS SUR LES PARAMETRES LIES A LA REPRODUCTION DE *SCROBICULARIA PLANA*

6 CHAPITRE 6. INCIDENCE DE LA CONTAMINATION DU SEDIMENT PAR DES PERTURBATEURS ENDOCRINIENS SUR LES PARAMETRES LIES A LA REPRODUCTION DE *SCROBICULARIA PLANA*

6.1 Contexte de l'étude

Nos travaux sur l'estuaire de la Loire n'ont pas mis en évidence de déplétion des populations du bivalve *Scrobicularia plana* en dépit de l'altération d'un certain nombre de réponses biologiques. Il nous a cependant paru important d'envisager un risque potentiel qui est actuellement peu documenté chez les bivalves, celui des effets des perturbateurs endocriniens. Leurs effets sur la reproduction sont subtils, se produisant à des doses très faibles dans l'environnement, souvent en l'absence de tout autre effet toxique. A la suite des travaux de Langston et al. (2007) et Gomes et al. (2009), il est établi que des perturbations de la reproduction (présence d'intersexe chez cette espèce reconnue comme gonochrorique, modifications occasionnelles du sexe ratio) affectent *S. plana* et que de telles observations peuvent s'expliquer par les effets de perturbateurs endocriniens : 17β-œstradiol (E2), 17α-ethinyloestradiol (EE2), octylphenol (OP) and nonylphenol (NP).

Dans l'état actuel des connaissances, il existe peu de données permettant de faire le lien entre de telles altérations et des effets au niveau populationnel. Chez des poissons *Pimephales promelas*, une expérience en nature dans des lacs canadiens a montré que la contamination par de l'EE2 à des doses comparables à celles effectivement trouvées dans des milieux aquatiques anthropisés faisait chuter la densité de population et aboutissait même à l'absence complète de recrutement (Kidd et al., 2007). Chez la même espèce, Miller et al. (2007) montrent qu'en laboratoire des composés tels que la 17α-trembolone et la 17β-trembolone à des concentrations de quelques ng L-1 entraînent une diminution de la concentration plasmatique de la vitellogénine (Vtg). Par modélisation, ces auteurs prévoient qu'une diminution de 50% du taux de Vtg provoquerait une extinction de la population en une vingtaine d'années. Chez de nombreuses espèces de gastéropodes, il a été montré que le tributylétain (TBT) qui fut employé dans des peintures anti-salissures pour les bateaux et certaines infrastructures marines, provoquait le développement d'un pénis non fonctionnel chez les femelles. Chez le

néogastéropode *Nucella lapillus*, ceci provoque l'obstruction du conduit génital des femelles qui deviennent alors stériles. Cette stérilité conduit à des effets populationnels drastiques, l'espèce pouvant disparaître des sites les plus impactés (Bryan et al., 1987, Gubbins et al., 2010).

A l'échelle mondiale, il est reconnu que les effluents de stations d'épuration sont une des sources majeures des molécules qui possèdent des propriétés de PE, particulièrement les œstrogènes stéroidiens et les nonylphénols (Jugan et al., 2009, Bertin et al., 2011, Gong et al., 2011, Tetreault et al., 2011). Dans les estuaires qui sont le siège d'importantes populations humaines, il paraissait donc important :

- de documenter la présence de perturbations de la reproduction, entre autres, en terme d'intersexe, de sex-ratio, d'indices gonado-somatiques ;
- de tenter de mettre en relation ces observations avec la présence de molécules à potentiel de perturbateurs endocriniens ;
- de documenter des effets populationnels éventuels dans les sites les plus impactés en terme d'altérations biologiques chez les bivalves.

Dans le cadre du Programme Interdisciplinaire Chimie Pour le Développement Durable (CPDD) du CNRS, nous avons bénéficié du soutien du groupe de recherche ERICHE «Evaluer et Réduire l'Impact de la CHimie sur l'Environnement : une vision globale allant de la chimie analytique à l'étude des cycles de vie ». Ceci nous a permis de mener une recherche en écotoxicologie associant des compétence en biologie de la reproduction disponibles au sein de MMS (Mouneyrac et al., 2008) ; des méthodes bioanalytiques adaptées à la caractérisation de différentes catégories de perturbateurs endocriniens dans les sédiments développées à l'INERIS (Kinani et al., 2010) ; des méthodes de chimie analytique performantes pour les faibles doses des principaux pollutants organiques persistants disponibles au Laboratoire de Physico et Toxico chimie de l'environnement (UMR EPOC, Université de Bordeaux 1).

Sur l'estuaire de la Loire qui a été notre principal site d'étude, nous avons voulu avoir une vision généraliste de l'importance des intrants. Pour ce faire, des sédiments ont été collectés sur l'ensemble de l'estuaire soumis à la marée (haline et/ou dynamique, Figure 14) à proximité des points de rejets de certaines usines de retraitement des eaux usées, choisies pour être représentatives de différents modes de traitement, appliqués à des volumes très variables

selon le nombre d'habitants raccordés au réseau de collecte (Thèse Letrouvé-Blanchet).



Figure 14. Localisation des sédiments collectés dans l'estuaire de la Loire (1 : St-Nazaire, 2 : Paimboeuf aval, 3 : Paimboeuf amont, 4 : Montoir de Bretagne, 5 : Coueron, 6 : Reze, 7 : Nantes CHU, 8 : Bellevue, 9 : Mauves/Loire, 10 Oudon, 11 : Ancenis, Témoin positif (T) : Aneth. Source : Thèse Isabelle Letrouvé-Blanchet)

Tous les sites présentent une activité œstrogénique (hormis St Nazaire) variant de 1.97 $ng.g^{-1}$ de carbone organique total (COT) à 28.0 $ng.g^{-1}$ de COT contenu dans le sédiment (Ancenis). Ainsi, les sites de Oudon et Ancenis (sites rurbains en partie amont de l'estuaire), présentent les plus fortes activités œstrogéniques et le site de Rezé présente une activité plus conséquente que Mauves sur Loire.

Dans le cas de l'activité anti-androgénique, elle a été observée sur huit sites parmi les douze échantillonnés (le douzième site Aneth étant le témoin positif car le sédiment avait été prélevé directement dans le ruisseau recepteur de l'effluent de la station d'épuration).

Dans le cas des activités HAP-like, des réponses allant de 1000 $\mu g.g^{-1}$ de COT à 6000 $\mu g.g^{-1}$ COT ont été obtenues sur six sites parmi les douze, la plus élevée se situant à Paimboeuf amont (point 2).Quant aux activités dioxine-like, elles sont présentes sur tous les sites (de 60 à

130 ng.g-1 COT) à l'exception de celui de Saint-Nazaire.

Cette étude montre bien l'omniprésence de perturbateurs endocriniens dans les sédiments d'un estuaire qui n'est pourtant pas le plus impacté par les activités anthropiques à l'échelle nationale et plus encore internationale.

Etant donnée la fréquence inattendue des cas d'intersexe rapportée pour les estuaires du Royaume-Uni et du Portugal (Langston et al., 2007, Gomes et al., 2009), et après qu'un essai préliminaire ait montré la présence de ce type d'anomalie non seulement en Loire mais aussi dans notre site de référence, la Baie de Bourgneuf, nous avons choisi d'étendre nos investigations à dix autres estuaires de Bretagne, présentant un état chimique et écologique variable selon les critères de la DCE (Tableau 1), ainsi qu'à l'estuaire de la Seine considéré comme témoin positif. Nous avons cherché à mettre en relation le taux d'intersexe et la présence de perturbateurs endocrininens, non seulement œstrogéniques, mais aussi androgéniques, anti-androgéniques, HAP-like et dioxine-like, à l'aide de différents tests sur lignées cellulaires après extraction des sédiments de chaque site d'étude (Cf. sous-section 3.3.3).

En estuaire de Loire, nous disposions d'une étude populationnelle relativement détaillée (Publication 4) qui ne montrait d'ailleurs pas d'anomalie notable en dépit de la présence d'intersexe (Publication 5 ci-près). Dans l'ensemble des autres estuaires, aucune analyse fine des populations n'a été menée mais à chaque prélèvement, il a été dument noté la présence ou l'absence d'individus dont la taille révèle que des recrutements ont eu lieu.

6.2 Publication 5 (Acceptée sous réserve de modifications mineures à *Environmental Pollution*)

Physiological status and intersex in the endobenthic bivalve *Scrobicularia plana* from thirteen estuaries in Northwest France

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Abstract

The bivalve *Scrobicularia plana*, an important species for the structure and functioning of estuarine and coastal mudflats, was studied in thirteen sites from NW France differing by their degree of contamination to document the presence of reproduction impairments (intersex, sex-ratio, gonado-somatic indices) in relation to the condition revealed by using hepatosomatic and condition indices. In agreement with recent studies in other European estuaries, intersex was revealed in all the studied estuaries, including sites the chemical and ecological status of which is considered "good" according to the criteria of the European Water Framework Directive. These disturbances are most probably attributable to the presence of EDCs. Our results re-inforce the concern linked to the subtle effects of EDCs, which are active at very low doses, often in the absence of any major sign of toxicity. However at this stage, no clear link may be established between intersex and populational effects.

1. Introduction

In the whole world, estuaries are witness to the same paradox: they are among the most productive ecosystems while being strongly impacted by anthropogenic activities. Intertidal mudflats are valuable ecological entities, supporting large populations of birds and acting as nurseries and feeding areas for fish and crustaceans (MEA, 2005). Bivalves, including *Scrobicularia plana*, are an essential component of these intertidal soft-sediment communities (Santos et al., 2011a). On the other hand, in the aquatic environment, sediment is the main reservoir for most of the contaminants entering ecosystems. Consequently, endobenthic invertebrates such as *S. plana* are good models for environmental assessment of estuarine ecosystems and ecotoxicological studies (e.g. Byrne et O'Halloran, 2001, Mazorra et al., 2002, Coelho et al., 2008, Romero-Ruiz et al., 2008, Solé et al., 2009, Boldina-Cosqueric et al., 2010, Berthet et al., 2011).

In ecosystems exposed to anthropogenic pressures, linking infra-individual and individual impairments to supra-individual disturbances is a challenge for ecotoxicology (Weis et al.,

2001; De Coen and Janssen 2003; Amiard-Triquet, 2009; Mouneyrac et al., 2009; Weis et al., 2011). Biological responses which contribute to the cascading effects leading from contaminant exposure to the local depletion/extinction of a given species in an estuary provide biomarkers to forecast effects at population levels, well before environmental degradation has already occurred, thus leading to expensive and technically difficult remediation processes (Amiard-Triquet and Rainbow, 2009). In this framework, responses at different levels of biological organization were observed in *S. plana* from several estuaries in France, differentially exposed to anthropogenic pressure (Boldina-Cosqueric et al., 2010; Fossi Tankoua et al., 2010). The activation of defence mechanisms towards metals (metallothionein) and other classes of contaminants (the biotransformation enzyme glutathione-S-transferase) in the most contaminated estuaries do not ensure total protection since a number of impairments were observed at the infra-individual (AChE and digestive enzyme activities) and individual (burrowing behaviour, condition index, gonado-somatic index) levels. The interpretation of responses involved in the cascade of energy events (from available food, digestive enzyme activities, and energy reserves to condition and reproductive status) is complex but reveals disturbances in the Loire estuary which is far from being the most contaminated estuary in France and over the world. However, no links can be established between individual and supra-individual effects of living in the Loire estuary, even in the case of a small oil spill. Even in the most contaminated estuary (Seine), historical records did not show a consistent decrease of *S. plana* populations (Pers. Comm. Bessineton, Réserve Naturelle Nationale de l'estuaire de la Seine).

Endocrine-related reproductive effects in molluscs are well-documented (Ketata et al., 2008) and represent a new potential threat to populations. Sex alteration (feminisation or masculinisation) were observed for instance in mussels *Mytilus edulis* (Hellou et al., 2003), *M. galloprovincialis* (Ortiz-Zarragoitia and Cajaraville 2010); soft-shell clams *Mya arenaria* (Gagné et al., 2003), freshwater mussels *Elliptio complanata* (Gagné et al., 2011), equilateral venus *Gomphina veneriformis* (Ju et al., 2009). In the clam *S. plana*, biased sex ratio towards females was observed in only 2 sites among 22 sites examined by Langston et al. (2007) in Southwest UK. On the other hand, in this gonochoric species, intersex was present in more than two-thirds of the populations screened from all these estuaries with varying severity (0 to 60% of males affected). Intersex with several degrees of intensity was detected in the Guadiana river (Portugal) with varying percentages of male clams affected (5.9, 58.3 and 71.4%) according to sexual maturation (Gomes et al., 2009).

Another impairment of reproduction could consist in delayed gametogenesis (Tlili et al., 2011 and literature quoted therein) which may result from unfavourable nutritive conditions and/or the presence of endocrine disrupting chemicals (EDCs) (Gauthier-Clerc et al. 2002). In *M. arenaria* exposed to increased temperature and herbicide mixture for 28 days, a gradual sexual maturation was observed in both sexes through experimental time, but females seemed to have a higher sensitivity to both factors compared to males (Greco et al., 2011). Thus it is not surprising that asynchronous spawning of males and females occur in contaminated environments as shown by (Smaoui-Damak et al., 2006)) in the clam *Ruditapes decussatus*, from a contaminated site in Tunisia.

Because of its role in the structure and functioning of estuarine and coastal mudflats, the reproduction success of *S. plana* is a topic of interest for environmental conservation. In thirteen sites from NW France differing by their degree of contamination, *S. plana* were collected at the beginning of gametogenesis (May) and at the peak of sexual maturity (July). The presence and degree of intersex and different parameters linked to reproduction (sex ratio, gonadosomatic index) were examined. In order to detect any delayed sexual maturation and asynchronism between males and females, the sexual maturity stages were determined in non-intersex specimens. Because energy metabolism and physiological conditions are important to insure the reproductive success, the general health of bivalves was assessed by using the determination of hepatosomatic indices and condition indices.

2. Material and Methods

2.1. Site description

Thirteen sites in the North-West of France - differentially impacted by anthropogenic pressure - were investigated (Table 1). The choice of these sites took into account information available for contaminants in the framework of the French "Mussel Watch" Programme (Marchand et al., 2009) and provided by the regulation authorities (Table 1) for the implementation of the European Community Water Framework Directive (ECWFD). In the WFD, water masses are classified according to their ecological status and their chemical status (Table 1). The Seine estuary is taken as a positive control in this study. Indeed, the

Seine basin (78,650 km^2) concentrates 40% of the economic activity of France and 50% of the river traffic; 60% of its catchments consist of arable lands. Thus pollutant inputs are very important in this estuary and their effects on biota are well-documented (Amiard-Triquet and Rainbow, 2009). The Loire estuary is exposed to toxic anthropogenic effluents transported by rivers from remote and nearby conurbations, and industrial and agricultural areas but at a lower extent than the Seine estuary. On the other hand, the Bay of Bourgneuf, mainly devoted to oyster farming, extensive agriculture and tourism is well-documented as a reference site (Fossi Tankoua et al., 2010; Geffard et al., 2001; 2005; Kalman et al., 2009).

Temperature is an ecological factor known as able to influence deeply physiological responses such as those studied in the present paper. Temperature fluctuations did not show a clear trend according to latitude. Indeed, in May, the mean daily air temperature over the month before sampling, obtained from www.meteociel.fr, showed minima between 6.4 in estuaries of South Brittany (47° N) and 9.6 in the Seine estuary (49°N) and maxima between 13.7 in the Goyen estuary (48°N) and 17.4 in the Seine (49°N). In July, the mean daily air temperature showed minima between 11.3 in the Hopital Camfrout River estuary (48° N) and 13.7 in the Goyen estuary (48°N) and maxima between 19.1 in the Goyen estuary (48°N) and 24.6 in the Vilaine (47°N). Most of the sites had salinity between 16 and 25 but two sites (Bays of Bourgneuf and St Brieuc) are more firmly marine sites (salinity > 30).

2.2. Animals and sediment collection

Two sampling trips were conducted in May and July 2010. Standard sample size of 30 clams was considered adequate for gender determination and the study of intersex (Langston et al., 2007). At each date and for each site, 30 clams were handpicked in the intertidal zone at low tide. Clams of similar shell length (3.5 ± 0.4 cm in May and 3.4 ± 0.5 in July for the whole specimens) were taken for histological analysis. Animals were transported to the laboratory in isothermal containers. In the laboratory, they were kept at ambient temperature measured during collection.

2.3. Histological observations

Animals were allowed to depurate for 2 hours before dissection. The condition index (CI), gonadosomatic index (GSI) and hepatosomatic index (HSI) were recorded. For animals

collected in May, gonads were isolated and fixed with a formaldehyde solution (10%) at the salinity of the site of origin. Fixed gonads were then dehydrated and paraffin-embedded and cut (5-7µm) with a microtome. Thin sections were colored with hemalin-eosin before observation with a light microscope coupled to an image analyzer to determine gender, reproductive development stage and to look for intersex. To categorize the different degrees of intersex in gonads of clams, the ranking system implemented by Chesman and Langston (2006) was adopted (from a predominantly male gonad: single oocyte present in a field of view to a predominantly female gonad with follicles containing bundles of sperm). For those collected in July, isolated gonads were excised, smears of the content observed under a light microscope to determine gender and to look for intersex.

Five reproductive stages (undifferentiated, development, ripe, spawning, post-emission) were characterized using morphological criteria according to Mouneyrac et al. (2008). In May, undifferentiated specimens were nearly absent. Each other stage was categorized by a maturity factor (MF = 1 for development, 2 for ripe, 3 for spawning and 4 for post-emission) and used to develop a sexual maturity index as recommended by Siah et al. (2003):

$$SMI = \Sigma \text{ (Proportion of each stage * MF)}.$$

2.4. Statistical analysis

Statistical analyses were performed using XLSTAT® 2011. Normality of data and homocedacity of variance were checked using Shapiro and Wilk's test. When these requirements were met, comparison of means was carried out using a two-way variance analysis (ANOVA) and then a Tukey's HSD post hoc method ($P < 0.05$). In other cases, the non-parametric Kruskal-Wallis and Mann-Whitney comparison tests were used. To compare the biometric measures over the two sampling period bilateral Mann-Whitney tests were carried out. Level of significance was established at $P < 0.05$. The Pearson correlation test ($P<0.05$) was used to establish relationships between biometric indices. The Z test was used to determine if the sex ratio observed at each site differed significantly from unity.

3. Results

3.1. Biometric measurements

For each biometric measurement (condition CI, hepatosomatic HSI and gonadosomatic GSI indices), we have verified that no differences existed between sexes. Thus the spatio-temporal determinations are shown for both males and females in Table 2 and Figure 1.

In May, important fluctuations of CI between sites were observed with a range of mean values between 21 and 37 (Table 2). The highest CIs were observed in specimens from the Seine, Laïta and Noyalo. In July, the CI was significantly higher than in May for specimens from Bourgneuf, Loire, Vilaine, Blavet, Bélon, and St Brieuc whereas an inverse relationship was observed in the Laïta. Again, in July, important spatial fluctuations of CI were observed with a range of mean values between 26 and 42 and the highest ones observed in the Seine and Bélon (Table 2).

In May, the highest mean values of HSI (29) were observed in the Blavet and Goyen estuaries and the Bay of St Brieuc whereas the lowest ones were shown in the Vilaine and Noyalo River (21) and Seine (17) estuaries. Temporal trends were not important with only two significant increases of HSI between May and July (Laïta and Seine). In July, the spatial differences were not so marked with the lowest value (23) recorded in the Noyalo River estuary and the maximum (32) in the Odet estuary (Table 2).

The spatio-temporal determinations of gonadosomatic (GSI) indices are shown in Fig. 1. In May, high mean values were already observed in the Seine and Noyalo River estuaries. In July, as sexual maturity gets on (Mouneyrac et al., 2008), GSIs were higher than in May in specimens from the all sites, with significant temporal differences in most of them. The highest mean values ($\geq$ 23) were encountered in the Noyalo River, Vilaine, Goyen and Seine estuaries.

Correlations between these different indices were examined by using Pearson correlation coefficients. In each sampling period, significant~~ly~~ positive correlations were observed between CI and GSI ($p<0.0001$) whereas an inverse relationship was observed between CI and HSI ($p<0.0001$). In May as well as in July, significant inverse correlations were observed between GSI and HSI ($p<0.0001$).

3.2. Sex ratio and intersex

Various degrees of intersex from focal to advanced are depicted in Fig. 2. The percentages of normal females, non affected and intersex males are shown in Fig. 3 in May and July. In May, intersex males were present in all sites, representing 13.3 % of the males in the Bélon estuary and up to 27.8 % of the males examined in the Seine. Assuming that intersex individuals are 'affected' males as recommended by Chesman and Langston (2006), sex ratios do not differ significantly from unity (Z test) in most cases (Fig. 3). In May, sex ratios were biased towards females at one (St Brieuc, $p = 0.005$) of the thirteen sites surveyed whereas a sex-ratio biased towards males was observed at the bay of Bourgneuf ($p = 0.011$). In July, a number of sites looked intersex-free and the percentages were lower than in May. In July, sex ratios were generally shifted towards females compared to May, particularly in the Noyalo River estuary ($p = 0.028$) and the Seine estuary ($p = 0.005$) where the sex ratio differed significantly from unity (Z test).

The degree of intersex is depicted in Fig. 4 for specimens collected in May. The highest degrees of intersex (advanced and enclosed) were mainly observed in the Vilaine (71%), the Bay of Bourgneuf (67%), the Blavet (43%), and the Seine estuary (40%). On the other hand, the lowest degrees of intersex (focal and diffuse) were mainly observed in the Bay of St Brieuc (75%), Fremur (71%), Loire (67%) and Bélon (50%) estuaries. At the sites where many cases of intersex were depicted (Fig. 4), the degree of intersex is not systematically higher than at the sites with a lower number of cases.

In Fig. 5, the stages of maturity are depicted for individuals collected in May (intersex males excluded). The first evidence is that females in post emission are more numerous than males in most of the studied sites. Combining different stages by using sexual maturity index (SMI), this difference between sexes is confirmed in the Vilaine, Bélon, and Goyen estuaries. On the other hand, in the Frémur estuary, sexual maturity was more precocious in males than in females.

4. Discussion

The bivalve *Scrobicularia plana* is basically considered as gonochoric. However, hermaphrodism was documented in 1996-1997 in Mweeloon Bay, Galway, West coast of Ireland (Raleigh and Keegan, 2006) but with only one case among 742 specimens. Some 50 years ago (Paes-da-Franca 1956, quoted in Langston et al., 2007) showed the presence of a small percentage (mean 2.8%) of 'hermaphrodites' in a population of *S. plana* from the highly urbanised and industrialised Tagus Estuary. In the Bou Regreb estuary, Kourradi (2007) has not observed any case of intersex in specimens collected monthly from January to December 1999. On the contrary, as observed in British (bivalves collected in 2004-2005) and Portuguese (bivalves collected in 2007) estuaries (Chesman and Langston, 2006; Gomes et al., 2009; Langston et al., 2007) intersex was frequently observed in the bivalve *Scrobicularia plana* from estuaries and bays in northwest France. Experimental exposures to sediment spiked with mixtures of 17β-oestradiol (E2), 17α-ethinyloestradiol (EE2), octylphenol (OP) and nonylphenol (NP) carried out by Langston et al. (2007) indicated that (xeno)estrogens could be a contributory factor in the induction of intersex. Thus it is expected that intersex may be present in areas exposed to urban, industrial and agricultural influences. Surprisingly, intersex was observed in areas where the health status, both chemical and ecological, was rated as good according to the assessments carried out under the Water Framework Directive (Table 1). Because the WFD recommends direct measurements of contaminants in water, where concentrations of many contaminants are extremely low, combined with too high quantification limits, thousands of analytical results are non significant (Knoery and Claisse, 2010). Approximately 550 substances are suspected to have an action on the endocrine system (CCE, 2001) and all of them are not analysed at each site. Consequently, areas currently quoted as comparatively clean may be in fact contaminated with such substances which may be efficient at low doses.

For the ecological health status, there is no clear link to-date between the presence of EDCs, their effects on individuals and consequences on invertebrate populations with the exception of imposex due to exposure of neogastropods to tributyltin (Bryan et al., 1987; Gubbins et al., 2010). In fish, experiments in the field (Kidd et al., 2007) and modelling (Miller et al., 2007) have shown that EDCs (EE2, α and β-trembolone) impair the normal levels of vitellogenin with population effects which can nearly lead to local extinction. On the other hand, as far as

we know, no causal relationship was demonstrated between intersex, either in fish or invertebrates, and populational effects. In the course of the collection of *S. plana* in the estuaries of northwest France, we have examined if small specimens which reveal the existence of recent recruitement (Verdelhos et al., 2005) were present. The presence of specimens with a shell length < 2cm was also noted since sexual maturity occurs at 2–3 years after settlement corresponding to a shell length greater than 20 mm (Hughes, 1971; Rodríguez-Rúa et al., 2003; Ruiz, 1993). Among the 13 visited sites, no recruitment occurred over the previous 2-3 years in the Noyalo River estuary, the Laïta and the Goyen estuaries which are not those exhibiting the highest percentages of intersex (respectively 21, 19 and 17%).

However, it must be taken into account that *S. plana* is generally distributed according to a patchy pattern, implying that settlement areas may not be homogeneous (Santos et al., 2011a). According to these authors, if annual spat settlement is a random process and successful every year, patches composed of single age classes would be expected due to dispersal of larvae and random settlement. However, for *S. plana*, such age composition is generally not observed, with several age classes being found in the patches (Santos et al., 2011a and literature cited therein) and this is also the situation observed in most of the sites explored in the present study (with the exception of the Noyalo River estuary, the Laïta and the Goyen estuaries). Thus Santos et al. (2011a) suggest that successful settlement is a spatially non-random process for this species and that it would be determined by specific environmental or biological factors. A combination of factors such as temperature, salinity, sediment type, hydrographic conditions and predation have been proposed to explain the species distribution pattern. In addition to these natural factors, it must be considered that avoidance towards contaminated sediments may be at work since it has been demonstrated that re-burrowing is profoundly affected in the presence of sediments originating from contaminated sites (Boldina-Cosqueric et al., 2010; Byrne and O'Halloran, 2001).

In most populations of *S. plana* equal proportions of males and females were recorded in the Guadalquivir estuary, Spain (Rodriguez-Rúa et al., 2003), Guadiana Estuary, Portugal (Gomes et al. 2009), Mira and Tagus estuary, Portugal (Guerreiro, 1998), Bidassoa estuary, Spain (Sola, 1997), Mweeloon Bay, Galway, West coast of Ireland (Raleigh and Keegan, 2006), Menai Bridge, North Wales (Hughes, 1971). In contrast, sex ratios were biased towards females at two (Cargreen and St. Germans) of the six sites surveyed by Langston et al. (2007) in Devon and Cornwall estuaries as also observed in the present study for bivalves

originating from the Bay of St Brieuc in May. The same trend was shown in the Seine in July but it has been suspected that deeply affected males may have been counted as females, particularly those with advanced intersex stages, because the small number of bundles of sperm might be concealed amongst the great number of large oocytes. Surprisingly, there was a preponderance of males among the bivalves from the Bay of Bourgneuf in May, a situation which has never been reported in the literature. Recently, in the framework of the European programme INTERREG, Minier et al. (2011) have reported that a bias towards males was seen at Saltash in the Tamar Estuary (UK). In some sites and at some sampling dates, non significant trends have been shown, with 60% of males observed (Chesman and Langston, 2006; Langston et al., 2007).

In addition an asynchronous period of sexual maturity has been frequently observed in the estuaries of northern France with different patterns depending on different sites. Such asynchronous in spawning between sexes have been observed in other bivalve species living in contaminated sites and it has been argued that it could affect recruitment (Smaoui-Damak et al., 2006 and literature cited therein). Differences between sexes have been shown in clams *Mya arenaria* exposed to contrasted temperatures and a herbicide mixture (Greco et al., 2011). A gradual sexual maturation was observed in both sexes through experimental time, but females had a higher sensitivity to both stressors compared to males. The impairment of gonad maturation may be due to direct effects of contaminants with properties of EDCs (Cooper and Wintermyer, 2009; Gagné et al., 2003; Gauthier-Clerc et al., 2002; Siah et al., 2003) or to indirect effects on energy metabolism (Gauthier-Clerc et al., 2002). The development of gonads is controlled at least partly by the availability of energy reserves (particularly glycogen and triacylglycerols) as illustrated by the inverse relationship between hepato- and gonado-somatic indices: reserves initially stored in the hepatopancreas are transferred to gonads and used for the maturation of sexual products. In populations of *S. plana* from Portugal, the Netherlands and Norway, differences in annual cycles were attributed to different temperatures and food availabilities (Santos et al. 2011b). *In situ* investigations examining biological indices, energy reserves and sexual maturity have shown differences between bivalve populations from contaminated and cleaner sites (Mouneyrac et al., 2008; Tlili et al., 2011). They could be attributed to differences in the level of pollution of the site of origin as indicated by biomarkers. However, these authors underline that it is needed to take into account also the trophic status of the system since at low levels of nutrients or food availability, the ability of organisms to cope with pollutants is generally

decreased (Heugens et al., 2001). A previous study carried out over more than one year in the Loire estuary and the Bay of Bourgneuf has shown how complex was the relationship between food availability (as chlorophyll, phaeopigments and total organic carbon in sediment) and the condition and the reproductive status of *S. plana* (Fossi Tankoua, 2011). Because occasional data on food availability have a poor significance (due to high temporal variability), it would be preferable to assess directly the energy reserves which may be allocated to reproduction.

5. Conclusion

The main contribution of the present study is to document the presence of intersex in *Scrobicularia plana* from all the studied estuaries of northwest France, in agreement with recent studies in other European estuaries whereas older studies showed hermaphrodism rarely and at much lower levels. These disturbances are most probably attributable to the presence of EDCs. Our results – obtained in sites the chemical and ecological status of which is considered "good" according to the criteria of the WFD – re-inforce the concern linked to the subtle effects of EDCs, which are active at very low doses, often in the absence of any other sign of toxicity. However at this stage, no link may be established between intersex and populational effects.

The next steps to be accomplished are i) an assessment of the ratio between lipids contributing to energy storage (triacylglycerols) and structural lipids (phospholipids and sterols) providing good indices of the physiological status; ii) a characterization of the levels of chemicals with a potential of EDCs (estrogenic, androgenic and anti-androgenic) in the sediments of the studied sites in order to relate them to the incidence of intersex in *Scrobicularia plana*.

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References

Amiard-Triquet, C., 2009 Behavioral Disturbances: The Missing Link between Sub-Organismal and Supra-Organismal Responses to Stress? Prospects Based on Aquatic Research. Human and Ecological Risk Assessment 15, 87-110

Amiard-Triquet, C., Rainbow, P.S., 2009. Environmental Assessment of Estuarine Ecosystems. A Case Study. CRC Press, Boca Raton.

Berthet, B., Leung, K., Amiard-Triquet, C., 2011. Inter- and Intraspecific Variability of Tolerance, Tolerance to Environmental Contaminants. in: Amiard-Triquet, C., Rainbow, P.S., Roméo, M. (Eds), Tolerance to Environmental Contaminants. CRC Press, Boca Raton, pp. 49-84.

Boldina-Cosqueric, I., Amiard, J.-C., Amiard-Triquet, C., Dedourge-Geffard, O., Métais, I., Mouneyrac, C., Moutel, B., Berthet, B., 2010. Biochemical, physiological and behavioural markers in the endobenthic bivalve Scrobicularia plana as tools for the assessment of estuarine sediment quality. Ecotoxicology and Environmental Safety 73, 1733-1741.

Bryan, G.W., Gibbs, P.E., Hummerstone, L.G., Burt, G.R. 1987. Copper, zinc, and organotin as long-term factors governing the distribution of organisms in the Fal estuary in Southwest England. Estuaries 10, 208-219.

Buffan-Dubau, E., de Wit, R., Castel, J., 1996. Feeding selectivity of the harpacticoid copepod Canuella perplexa in benthic muddy environments demonstrated by HPLC analyses of chlorin and carotenoid pigments. Marine Ecology Progress Series 137, 71-82.

Byrne, P.A., O'Halloran, J., 2001. The role of bivalve molluscs as tools in estuarine sediment toxicity testing: a review. Hydrobiologia 465, 209-217.

Cartaxana, P., Jesus, B., Brotas, V., 2003. Pheophorbide and pheophytin a-like pigments as useful markers for intertidal microphytobenthos grazing by Hydrobia ulvae. Estuarine, Coastal and Shelf Science 58, 293-297.

CCE, 2001. Communication de la Commission au Conseil et au Parlement européen sur la mise en œuvre de la stratégie communautaire concernant les perturbateurs endocriniens. COM(2001) 262 final.

Chesman, B.S., Langston, W.J., 2006. Intersex in the clam *Scrobicularia plana*: a sign of endocrine disruption in estuaries? Biology letters 2, 420-422.

Coelho, J.P., Nunes, M., Dolbeth, M., Pereira, M.E., Duarte, A.C., Pardal, M.A., 2008. The role of two sediment-dwelling invertebrates on the mercury transfer from sediments to the estuarine trophic web. Estuarine, Coastal and Shelf Science 78, 505-512.

Cooper, K.R., Wintermyer, M. 2009. A critical review: 2,3,7,8 -Tetrachlorodibenzo-p-dioxin (2,3,7,8-tcdd) effects on gonad development in bivalve mollusks. Journal of Environmental Science and Health - Part C Environmental Carcinogenesis and Ecotoxicology Reviews 27, 226-245

Dauvin, J.-C., Ruellet, T., Desroy, N., Janson, A.-L. 2007. The ecological quality status of the Bay of Seine and the Seine estuary: Use of biotic indices. Marine Pollution Bulletin 55, 241-257.

De Coen, W.M., Janssen, C.R., 2003. The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environmental Toxicology and Chemistry 22, 1632-1641.

Fossi Tankoua, O., Amiard, J.-C., Amiard-Triquet, C., Betthet, B., Mouneyrac, C., Rainbow, P.S., 2010. Towards a comprehensive methodology for assessing the health status of coastal and estuarine ecosystems. ICES CM 2010/F:15. In: ICES Annual Science Conference, 20-24 september 2010, Nantes, France. http://www.ices.dk/products/CMdocs/CM-2010/F/F1510.pdf

Fossi Tankoua, O., Buffet, P.E., Amiard, J.C., Berthet, B., Méléder, V., Mouneyrac, C., Amiard-Triquet. C., 2011. Comprehensive assessment of estuarine sediment quality by using a battery of biomarkers in the bivalve *Scrobicularia plana* and the characterization of natural and anthropogenic factors (submitted).

Gagné, F., Blaise, C., Pellerin, J., Pelletier, E., Douville, M., Gauthier-Clerc, S., Viglino, L., 2003. Sex alteration in soft-shell clams (*Mya arenaria*) in an intertidal zone of the Saint Lawrence River (Quebec, Canada). Comparative Biochemistry and Physiology Part C 134, 189-198.

Gagné, F., Bouchard, B., André, C., Farcy, E., Fournier M., 2011. Evidence of feminization in wild *Elliptio complanata* mussels in the receiving waters downstream of a municipal effluent outfall. Comparative Biochemistry and Physiology, Part C 153, 99-106.

Gauthier-Clerc, S., Pellerin, J., Blaise, C., Gagné, F., 2002. Delayed gametogenesis of *Mya arenaria* in the Saguenay fjord (Canada): a consequence of endocrine disruptors? Comparative Biochemistry and Physiology, Part C 131, 457–467.

Geffard, A., Amiard-Triquet, C., Amiard, J.-C., 2005. Do seasonal changes interfere with metallothionein induction by metals in mussels *Mytilus edulis*? Ecotoxicology and Environmental Safety 61, 209-220.

Geffard, A., Amiard-Triquet, C., Amiard, J.-C., Mouneyrac, C., 2001. Temporal variations of metallothionein and metal concentrations in the digestive gland of oysters *Crassostrea gigas* from a clean and a metal-rich sites. Biomarkers 6, 91-107.

Gomes, T., Gonzalez-Rey, M., Bebianno, M.J., 2009. Incidence of intersex in male clams *Scrobicularia plana* in the Guadiana Estuary (Portugal). Ecotoxicology (London, England) 18, 1104-1109.

Greco, L., Pellerin, J., Capri, E., Garnerot, F., Louis, S., Fournier, M., Sacchi, A., Fusi, M., Lapointe, D., Couture, P., 2011. Physiological effects of temperature and a herbicide mixture on the soft-shell clam *Mya arenaria* (Mollusca, Bivalvia). Environmental

Toxicology and Chemistry 30, 132-141.

Gubbins, M.J., Moore, J., Fryer, R., Davies, I.M., 2010 Long time series data showing recent recovery of gastropod populations from effects of tri-butyl tin at the shetland oil terminal. ICES CM 2010/F:09. In: ICES Annual Science Conference, 20-24 september 2010, Nantes, France. http://www.ices.dk/products/CMdocs/CM-2010/F/F0910.pdf

Guerreiro, J., 1998. Growth and production of the bivalve *Scrobicularia plana* in two southern European estuaries. Vie et Milieu 48, 121–131.

Hellou, J., Yeats, P., Steller, S., Gagné, F., 2003. Chemical contaminants and biological indicators of mussel health during gametogenesis. Environmental Toxicology and Chemistry 22, 2080-2087.

Heugens, E.H.W., Jan Hendricks, A., Dekker, T., Van Straalen, N.M., Admiraal W., 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Critical Reviews in Toxicology, 31, 247-284.

Hughes, R.N., 1971 Reproduction of *Scrobicularia plana* Da Costa (Pelecypoda: Semelidae) in North Wales. Veliger 14, 77–81.

Ju, S.M., Park, J.J., Lee, J.S., 2009. Induction of intersex and masculinization of the equilateral venus, *Gomphina veneriformis* (Bivalvia: Veneridae) by zinc. Animal Cells and Systems 13, 339-344.

Kalman, J., Palais, F., Amiard, J.-C., Mouneyrac, C., Muntz, A., Blasco, J., Riba, I., Amiard-Triquet, C., 2009. Assessment of the health status of populations of the ragworm *Nereis diversicolor* using biomarkers at different levels of biological organisation. Marine Ecology Progress Series 393, 55-67.

Ketata, I., Denier, X., Hamza-Chaffai, A., Minier, C., 2008. Endocrine-related reproductive effects in molluscs. Comparative Biochemistry and Physiology, Part C 147, 261–270.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences 104, 8897-8901.

Knoery, J., Claisse, D., 2010. Insights from 30 years of experience in running the French marine chemical monitoring network. ICES-CM 2010 / F08. In: ICES Annual Science Conference, 20-24 september 2010, Nantes, France. http://www.ices.dk/products/CMdocs/CM-2010/F/F0810.pdf

Kourradi, R., 2007. Evaluation du degré de la pollution anthropique de l'estuaire de Bou Regreb et impact sur la biologie et dynamique de *Scrobicularia plana* et *Solen marginatus*. PhD Thesis, University Mohammed V-Agdal, Rabat.

Langston, W.J., Burt, G.R., Chesman, B.S., 2007. Feminisation of male clams *Scrobicularia plana* from estuaries in Southwest UK and its induction by endocrine-disrupting chemicals. Marine Ecology Progress Series 333, 173-184.

Lorenzen, C.J., 1967. Determination of chlorophyll and pheo-pigments:spectrophotometric equations. Limnology and Oceanography 12, 343-346.

Marchand, M., Amouroux, I., Bédier, E., Belin, C., Claisse, D., Durand, G., Soudant, D., 2009. Qualité du Milieu Marin Littoral, Synthèse Nationale de la Surveillance. Institut Français de Recherche pour l'exploitation de la Mer (IFREMER), Nantes.

Mazorra, M.T., Rubio, J.A., Blasco, J., 2002. Acid and alkaline phosphatase activities in the

clam *Scrobicularia plana*: kinetic characteristics and effects of heavy metals. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 131, 241-249.

MEA (Millenium Ecosystem Assesment) 2005. Ecosystems and human well-being: Synthesis. Island Press, Washington, DC. World Resources Institute.

Miller, D.H., Jensen, K.M., Villeneuve, D.L., Kahl, M.D., Makynen, E.A., Durhan, E.J., Ankley, G.T., 2007. Linkage of biochemical responses to population-level effects: a case study with vitellogenine in the fathead minnow (*Pimephales promelas*). Environmental Toxicology and Chemistry 26, 521-527.

Minier, C et al. Determination of pertinent indicators: a strategy for Europe (DIESE). Progress Report, University of Le Havre, October 2011, 15pp.

Mouneyrac, C., Durou, C., Gillet, P., Hummel, H., Amiard-Triquet, C., 2009. Linking energy metabolism, reproduction, abundance and structure of *Nereis diversicolor* populations. in: Amiard-Triquet, C., Rainbow, P.S. (Eds), Environmental Assessment of Estuarine Ecosystems. A Case Study. CRC Press, Boca Raton, pp. 159-181.

Mouneyrac, C., Linot, S., Amiard, J.-C., Amiard-Triquet, C., Métais, I., Durou, C., Minier, C., Pellerin, J., 2008. Biological indices, energy reserves, steroid hormones and sexual maturity in the infaunal bivalve *Scrobicularia plana* from three sites differing by their level of contamination. General and Comparative Endocrinology 157, 133-141.

Ortiz-Zarragoitia, Cajaraville, M.P., 2010.Intersex and oocyte atresia in a mussel population from the Biosphere's Reserve of Urdaibai (Bay of Biscay). Ecotoxicology and Environmental Safety 73, 693-701.

Raleigh, J., Keegan, B. F., 2006. The gametogenic cycle of *Scrobicularia plana* (Mollusca: Bivalvia) in Mweeloon Bay (Galway, west coast of Ireland). Journal of the Marine Biological Association of the United Kingdom 86, 1157-1162.

RNO, 2006. Surveillance du Milieu Marin, Travaux du Réseau National d'Observation de la qualité du milieu marin. Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), Paris, Nantes, p. 52.

Rodríguez-Rúa, A., Prado, M.A., Romero, Z., Bruzon, M., 2003. The gametogenic cycle of *Scrobicularia plana* (da Costa, 1778) (Mollusc: Bivalve) in Guadalquivir estuary (Cadiz, SW Spain). Aquaculture 217, 157–166.

Romero-Ruiz, A., Alhama, J., Blasco, J., Gómez-Ariza, J.L., López-Barea, J., 2008. New metallothionein assay in *Scrobicularia plana*: Heating effect and correlation with other biomarkers. Environmental Pollution 156, 1340-1347.

Ruiz, J.M., 1993. Metallic pollution in estuaries, with special reference to the effects of tributyltin (TBT) and copper on the early life stages of *Scrobicularia plana* (Mollusca: Bivalvia). PhD, University of Plymouth.

Santos, S., Luttikhuizen, P.C., Campos, J., Heip, C.H.R., van der Veer, H.W., 2011a. Spatial distribution patterns of the peppery furrow shell *Scrobicularia plana* (da Costa, 1778) along the European coast: a review. Journal of Sea Research 66, 238-247.

Santos, S., Cardoso, J.F.M.F., Cardoso, C. Luttikhuizen, P.C., van der Veer, H.W., 2011b. Seasonal variability in somatic and reproductive investment of the bivalve *Scrobicularia plana* (da Costa, 1778) along a latitudinal gradient. Estuarine, Coastal and Shelf Science 92, 19-26.

Siah, A., Pellerion, J., Amiard, J.-C., Pelletier, E., Viglino, L., 2003. Delayed gametogenesis and progesterone levels in soft-shell clams (*Mya arenaria)* in relation to *in situ* contamination to organotins and heavy metals in the St Lawrence river (Canada). Comparative Biochemistry and Physioogy, Part C: Toxicology & Pharmacology 135, 145-156.

Smaoui-Damak, W., Rebai, T., Berthet, B., Hamza-Chaffai, A., 2006. Does cadmium pollution affect reproduction in the clam *Ruditapes decussatus*? A one-year case study. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 143, 252-261.

Sola, J.C. 1997. Reproduction, population dynamics, growth and production of *Scrobicularia plana* da Costa (pelecypoda) in the Bidasoa estuary, Spain. Aquatic Ecology 30, 283-296.

Solé, M., Kopecka-Pilarczyk, J., Blasco, J., 2009. Pollution biomarkers in two estuarine invertebrates, *Nereis diversicolor* and *Scrobicularia plana*, from a Marsh ecosystem in SW Spain. Environment International 35, 523-531.

Tlili, S., Métais, I., Ayache, N., Boussetta, H., Mouneyrac, C., 2011. Is the reproduction of *Donax trunculus* affected by their sites of origin contrasted by their level of contamination? Chemosphere 84, 1362-1370.

Verdelhos, T., Neto, J.M., Marques, J.C., Pardal, M.A., 2005. The effect of eutrophication abatement on the bivalve *Scrobicularia plana*. Estuarine, Coastal and Shelf Science 63, 261-268.

Weis, J.S., Smith, G., Zhou, T., Santiago-Bass, C., Weis, P., 2001. Effects of contaminants on behaviour: biochemical mechanisms and ecological consequences. BioScience 51**,** 209-217.

Weis, J.S., Bergey, L., Reichmuth, J., Candelmo, A., 2011, Living in a Contaminated Estuary: Behavioral Changes and Ecological Consequences for Five Species. BioScience 61, 375-385.

Table 1. Characteristics of sampling sites

Sites	GPS coordinates	Ecological health status	Chemical health status
Bay of Bourgneuf	46° 56' 23.08" N, 2° 04' 40.60" W	Good	Good
Loire estuary	47° 16' 29.66" N, 2° 07' 59.76" W	Good	Bad
Vilaine estuary	47° 29' 54.15" N, 2° 26' 22.22" W	Good	Good
River of Noyalo	47° 37' 02.18" N, 2° 41' 07.9" W	Good	Good
Blavet estuary	47° 46' 23.22" N, 3° 18' 1.67" W	Moderate	Good
Laïta estuary	47° 48' 13" N, 3° 31' 37" W	Good	Good
Bélon estuary	47° 49' 17.89" N, 3° 39' 04.47" W	Good	Good
Odet estuary	47° 58' 11" N, 4° 05' 48" W	Moderate	Good
Goyen estuary	48° 02' 17"N, 4° 29' 16" W	Good	Bad
River of L'Hôpital Canfrout	48° 19' 15.36" N, 4° 15' 59.55" W	Good	Good
Saint-Brieuc bay	48° 31' 35.3" N, 2° 37' 50,6" W	Poor	Good
Frémur estuary	48° 36' 18.1" N, 2° 07' 25" W	Poor	Good
Seine estuary	49° 26' 36.19" N, 0° 16' 05.36" E	Moderate	Bad*

Sources : (http://envlit.ifremer.fr/var/envlit/storage/documents/atlas_DCE/scripts/site/carte.php?map=LB; * : Dauvin et al., 2007).

Table 2. Biometric measures: Condition index (CI), and hepatosomatic index (HSI)

	Bourgneuf	Loire estuary	Vilaine	River of Noyalo	Blavet	Laïta	Bélon	Odet	Goyen	River of L'Hôpital-Camfrout	Saint Brieuc	Frémur	Seine
CI													
MAY	23 (5) ^{abc}	23 (5) ^{ab}	23 (5) ^{ab}	33 (7) ^{fg}	26 (5) ^{bcd}	37 (4) ^{g}	31 (4)^{ef}	31 (5) ^{fg}	28 (4) ^{cde}	27 (5) ^{bcde}	21 (3)^{a}	28 (4) ^{de}	37 (4) ^{g}
JULY	33 (5) ^{ef}	29 (6) ^{abcde}	30 (6) ^{bcde}	30 (7) ^{cde}	32 (5) ^{ef}	29 (7) ^{abcde}	38 (3)^{fg}	28 (3) ^{abc}	31 (5) ^{de}	26 (4) ^{ab}	26 (5)^{a}	28 (3) ^{abcd}	42 (6) ^{g}
HSI													
MAY	-	-	21 (9) ^{bc}	21 (3) ^{b}	29 (5) ^{ef}	25 (4) ^{cd}	25 (6) ^{cd}	27 (6) ^{def}	29 (4) ^{ef}	26 (4) ^{de}	29 (6) ^{f}	27 (6) ^{def}	17 (4) ^{a}
JULY	25 (5) ^{ab}	30 (7) ^{bc}	27 (10) ^{abc}	23 (6) ^{a}	28 (7) ^{abc}	31 (7) ^{bc}	28 (7) ^{abc}	32 (7) ^{c}	30 (6) ^{bc}	29 (9) ^{bc}	29 (12) ^{bc}	29 (6) ^{abc}	26 (10) ^{abc}

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FIGURE 1. Spatio-temporal variations of the gonadosomatic index of the clam *Scrobicularia plana*. Mean and standard deviation, N = 30 for each site. * significant temporal differences.

FIGURE 2. Light micrographs of the portion of the gonads of *S. plana*. a) female, ovarian tissue; b) non affected male, testicular tissue; c to f male testicular tissue showing the presence of oocytes, with different degrees of intersex from focal to advanced (O: oocytes; S: spermatozoa with the lumen of follicle filled with tails; FO: follicle containing oocytes; FS: follicle containing spermatozoa)

FIGURE 3. Proportion of female, male and intersex clams found in May and July. * indicates significant departure from unity in ratio of males (non affected + intersex) to females ($p<0.05$, Z test)

FIGURE 4. Different degrees of intersex in the gonads of *S. plana* collected in May termed according to Chesman and Langston (2006). The number above each bar indicates the percentage of intersex males observed at each site among a sample of 30 clams.

FIGURE 5. Sexual maturation with partitioning expressed in percent between each observed development stages of gonads and sexual maturity index (SMI) from *S. plana* collected in the 13 sites (with the exception of Hôpital-Camfrout River estuary) in may. SMI =Σ (% of each sexual state X maturity factor). Each stage was categorized by a maturity factor (0, spent; 1, indifferent; 2, development; 3, mature; 4, spawning), according to Mouneyrac et al. (2008).

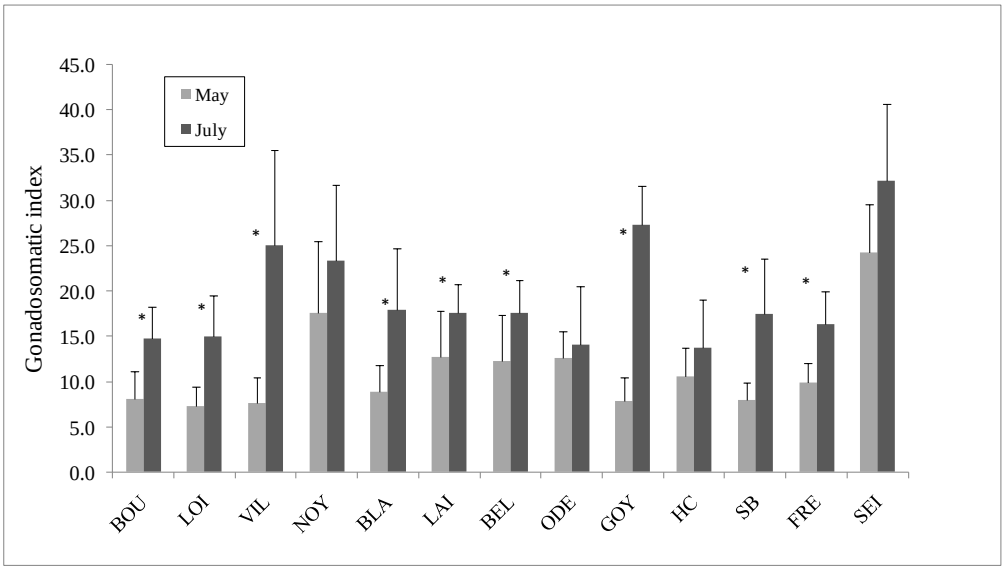

Gonadosomatic index
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
May
July
*
BOU
LOI
VIL
NOY
BLA
LAI
BEL
ODE
GOY
HC
SB
FRE
SEI

FIGURE 1.

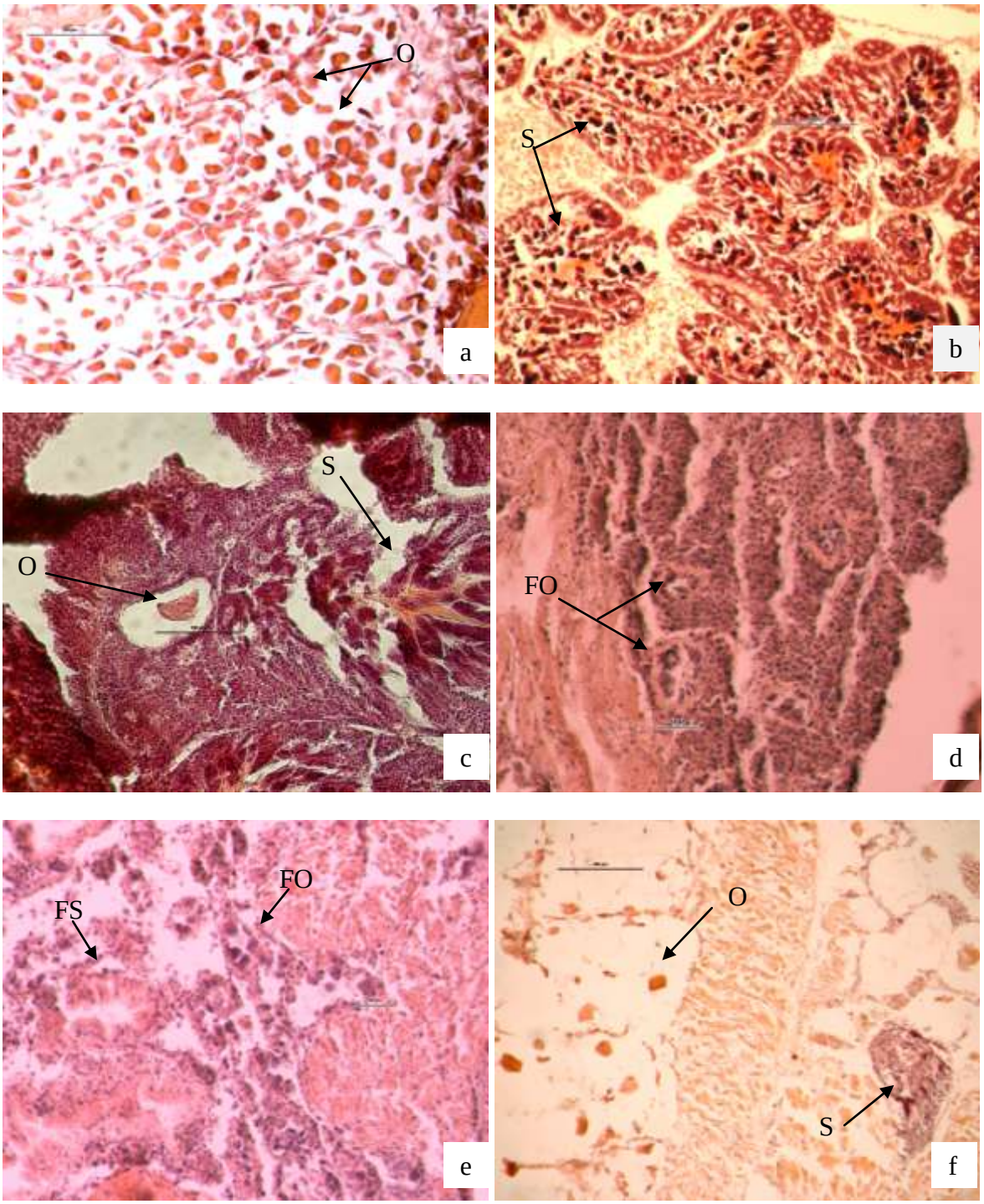
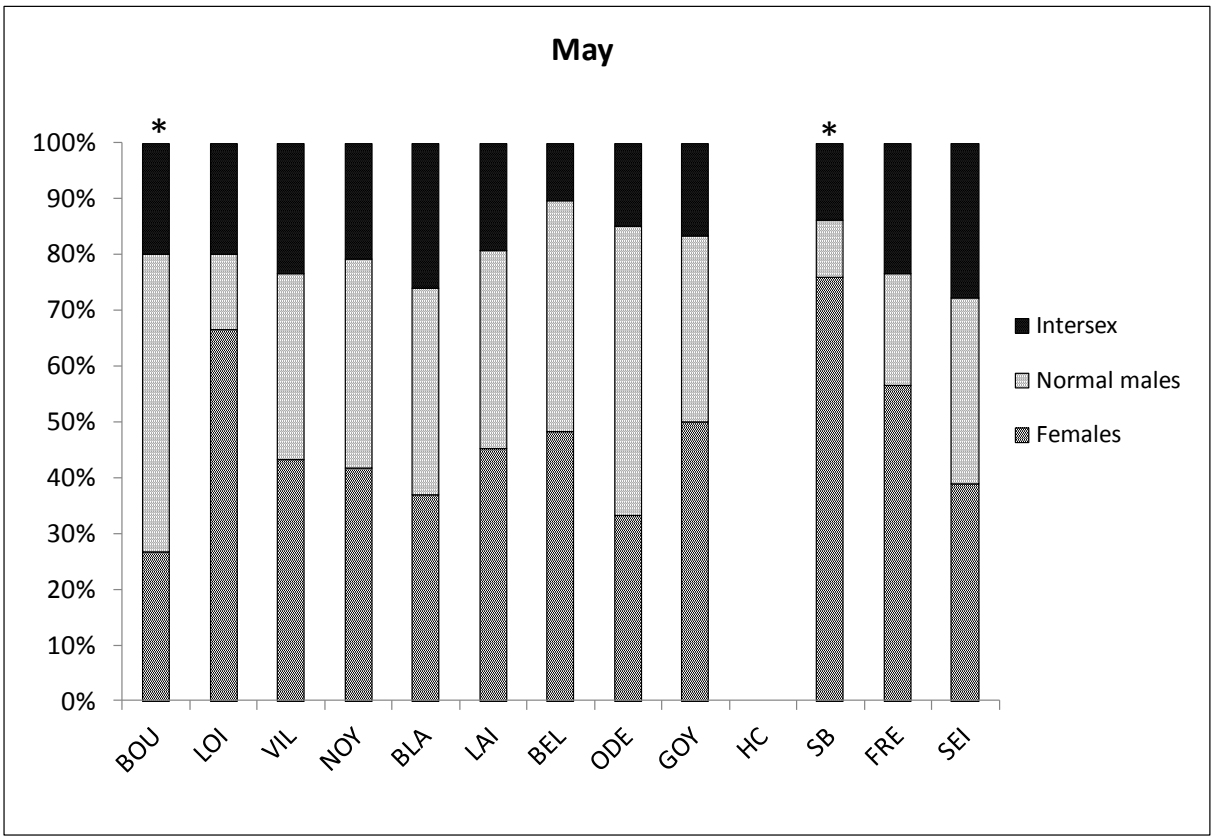


FIGURE 2.


May
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
*
*
BOU
LOI
VIL
NOY
BLA
LAI
BEL
ODE
GOY
HC
SB
FRE
SEI
Intersex
Normal males
Females

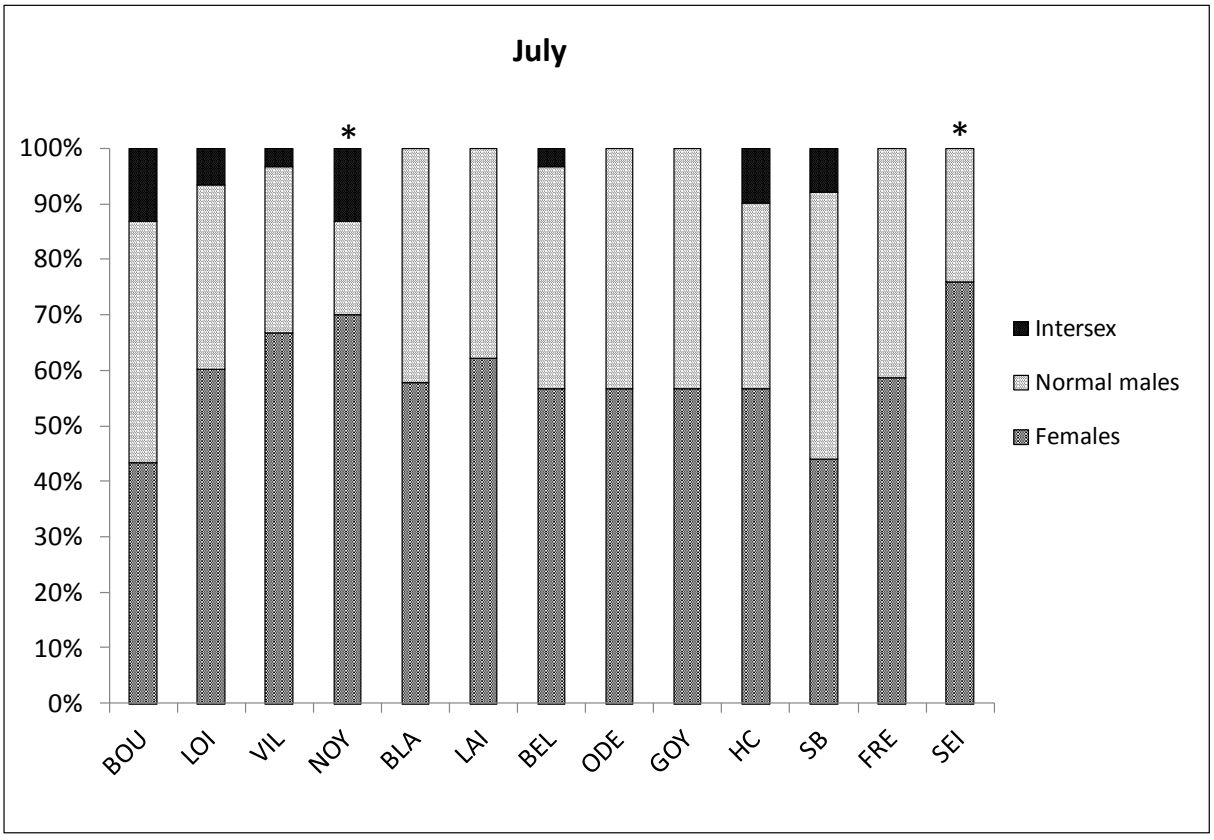

July
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
*
*
BOU
LOI
VIL
NOY
BLA
LAI
BEL
ODE
GOY
HC
SB
FRE
SEI
Intersex
Normal males
Females

FIGURE 3.

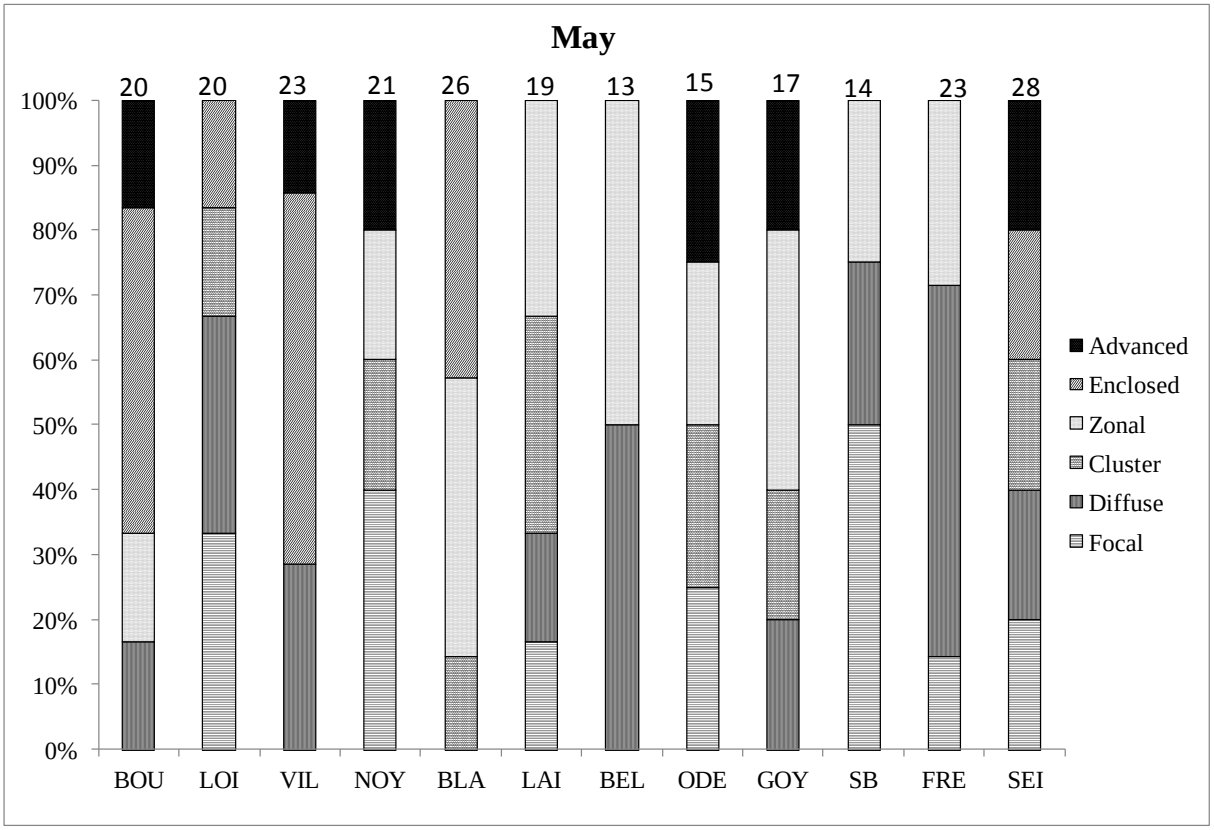

May
20
20
23
21
26
19
13
15
17
14
23
28
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
BOU
LOI
VIL
NOY
BLA
LAI
BEL
ODE
GOY
SB
FRE
SEI
Advanced
Enclosed
Zonal
Cluster
Diffuse
Focal

FIGURE 4.

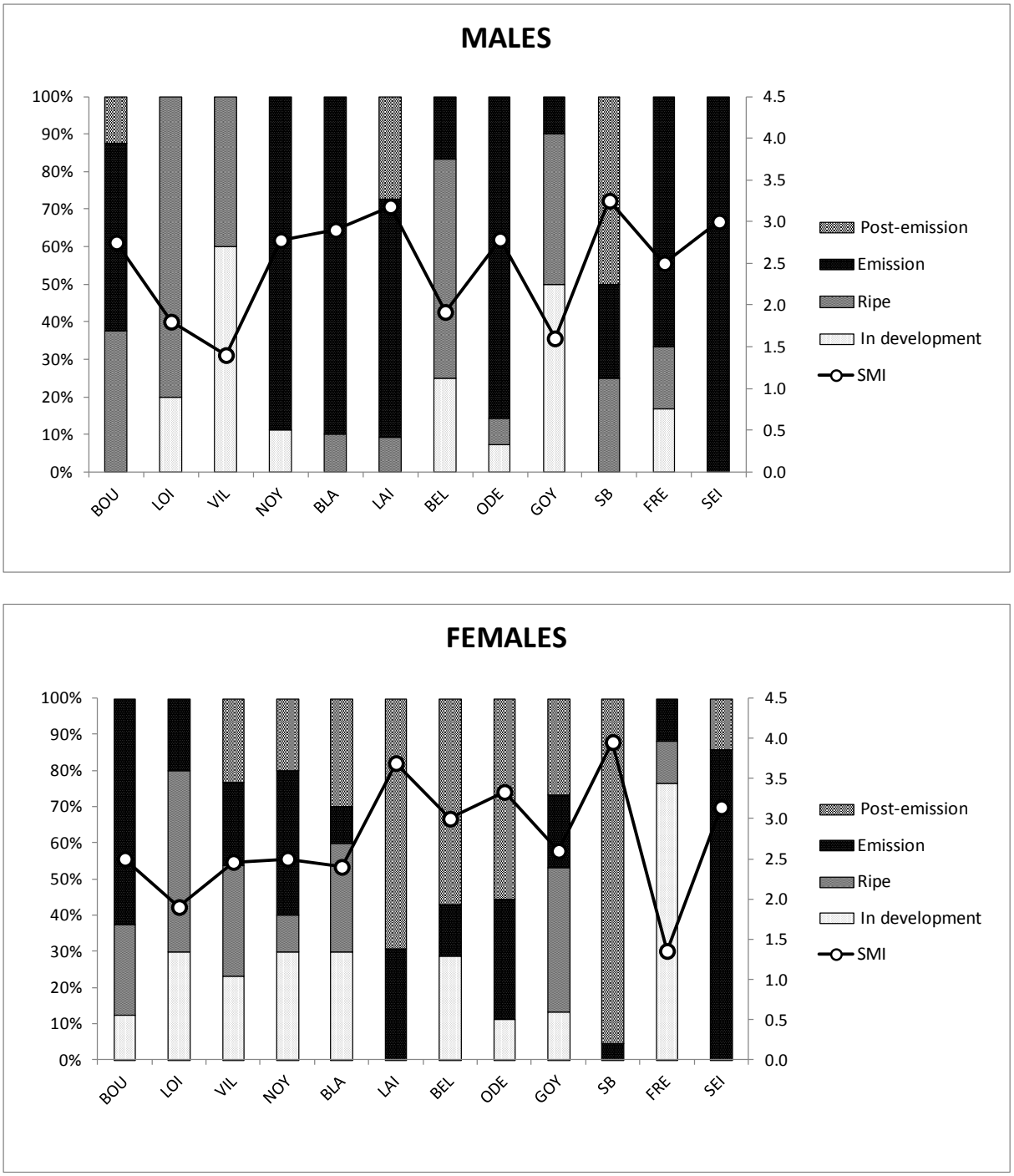

MALES
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
BOU
LOI
VIL
NOY
BLA
LAI
BEL
ODE
GOY
SB
FRE
SEI
Post-emission
Emission
Ripe
In development
SMI
FEMALES
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
BOU
LOI
VIL
NOY
BLA
LAI
BEL
ODE
GOY
SB
FRE
SEI
Post-emission
Emission
Ripe
In development
SMI

FIGURE 5.

6.3 Publication 6 (en préparation)

Presence of endocrine disrupting compounds along a contamination gradient in twelve estuaries in France: Is it a relation with occurence of intersex in the clam *Scrobicularia plana*?

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Abstract

The aim of this study was to investigate for the prevalence of intersex in *Scrobicularia plana* and a possible link with the presence of Endocrine Disrupting Compounds (EDCs) in superficial sediments. In twelve estuaries from NW France differing by their degree of contamination (as documented by the data of "Réseau National d'Observation" (Marchand. et al., 2009) intersex and sex-ratio were examined in *S. plana* collected during the maturation of sexual products. The presence of EDCs in superficial sediments collected at the same sites was investigated by using a battery of *in vitro* bioassays allowing the quantification of receptor-mediated activities, namely estrogen (ER), androgen (AR) and dioxin (AhR) receptors. Chemical analysis were carried out on superficial sediment after *in vitro* bioassays, in order to determine the concentrations of organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) which were normalized to the total organic carbon (TOC) content. Intersex cases were recorded in clams from all the studied sites, at a percentage varying from 22.2 to 60.0% of affected males. Significant estrogenic, anti-androgenic, PAH-like and dioxin-like activities were also recorded in all the sampling sites but at different intensities. On the other hand, significant androgenic activities were reported only in 6 over the 12 sites. The sum of PCBs was in the range of 67 to 6052 $ng.g^{-1}$ TOC (i.e. 0.4 to 184 $ng.g^{-1}$ d.w.). The sum of organochlorine pesticides varied from 0 to 564 $ng.g^{-1}$ TOC (corresponding to 0 to 17 $ng.g^{-1}$ d.w). 2,4-Dichlorodiphenyldichloroethylene and 4,4-Dichlorodiphenyldichloroethane occurred in the highest concentrations. The sum of PAHs was in the range of 7510 to 561331 $ng.g^{-1}$ TOC (i.e. 48 to 17064 $ng.g^{-1}$ d.w.). Fluoranthene was found in the highest concentration and acenaphthylene in the lowest. No direct correlation was found between percentage of intersex and proportion of cellular responses in bioassays or organic compound concentrations detected by chemical analyses.

1. Introduction

Estuaries are the most productive ecosystems in the world, but they are also among the most threatened by human activities. Estuarine sediment is the final repository of many classes of contaminants issuing from human activities. Among these pollutants, the so-called "endocrine disrupting compounds" (EDCs) have emerged these last three decades. EDCs have the potential to interact with the endocrine system by interfering with the synthesis, secretion,

transport, binding, action or elimination of natural hormones (Kavlock. et al., 1996). In december 1999, European community has established a priority list of 553 man-made substances and 9 hormones suspect to act as endocrine disrupters (http://ec.europa.eu/environment/endocrine/documents/sec_2007_1635_en.pdf). Among a number of 600 chemical substances screened for their possible endocrine action, 320 showed an evidence or potential evidence for ED effects, while 109 were not retained and 147 have been completely excluded from the evaluation. EDCs differ by their origin, structure, nature and mode of action. Numerous studies dealing with the effects EDCs on animals as well as humans have been conducted (Scott and Sloman, 2004; Cevasco. et al., 2008). It is thus well-known that some organic pollutants can disrupt the endocrine system of humans and biota by binding to the estrogen receptor, ER, the androgen receptor, AR or the Aryl hydrocarbon receptor, AhR (Ohtake. et al., 2003). Exposure to EDCs may lead to adverse health effects, such as increased rates of specific cancers and reproductive system abnormalities. In the aquatic species, these studies concern principally fish (Park. et al., 2009), gastropods or bivalve species of economic interest (Matthiessen and Sumpter, 1998; Puy-Azurmendi. et al., 2010; Zou, 2010). Few studies concern the effects of EDCs on non commercial species like *Scrobicularia plana* (Chesman and Langston, 2006; Langston. et al., 2007).

The clam *S. plana* is an endobenthic organism, important for the structure and functioning of estuarine and coastal mudflats, and thus the reproduction success of this species is a topic of interest for environmental conservation. Intersex cases have been described in this specie in UK (Chesman and Langston, 2006; Langston, et al., 2007) and Portugal (Gomes. et al., 2009) estuaries. Langston et al. (2007) have also demonstrated that xenoestrogens were able to induce a feminization of male clams. The aim of the present study was to investigate for the presence of intersexe in *S. plana* and to examine if they may be linked to the presence of EDCs in 12 estuaries and bays from the NW of France. To detect respectively estrogenic, (anti)androgenic and dioxin-like and activities, different receptor-mediated activities were used (ER, AR and AhR). Based on the responses obtained with *in vitro* cellular bioassays, chemical characterization of sediment extract was then carried out to quantified selected classes of EDCs, which may be responsible for the activity observed.

2. Material and Methods

2.1. Site description

Twelve sites in the North-West of France - differentially impacted by anthropogenic pressure - were investigated (Table 1). The choice of these sites took into account information available for contaminants in the framework of the French "Mussel Watch" Programme (Marchand, et al., 2009) and provided by the regulation authorities (Table 1) for the implementation of the European Community Water Framework Directive (ECWFD). In the WFD, water masses are classified according to their ecological status and their chemical status (Table 1). The Seine estuary (49° 26' 36.19" N, 0° 16' 05.36" E) is taken as a positive control in this study. Indeed, the Seine basin (78,650 km2) concentrates 40% of the economic activity of France and 50% of the river traffic; 60% of its catchments consist of arable lands. Thus pollutant inputs including EDCs are very important in this estuary and their effects on biota, especially some species of fish and molluscs are well-documented (Tronczynski. et al., 1999; Poirier. et al., 2006; RNO, 2006; Cailleaud. et al., 2007b; Amiard-Triquet and Rainbow, 2009; Amiard. et al., 2009; Huet. et al., 2009; Marchand, et al., 2009; Amiard-Triquet. et al., 2011; Poisson. et al., 2011). The Loire estuary is exposed to toxic anthropogenic effluents transported by rivers from remote and nearby conurbations, and industrial and agricultural areas but at a lower extent than the Seine estuary. On the other hand, the Bay of Bourgneuf, mainly devoted to oyster farming, extensive agriculture and tourism is well-documented as a reference site (Geffard, 2001; Geffard. et al., 2005; Kalman. et al., 2009; Fossi Tankoua. et al., 2010). Some of these estuaries run thought large cities (Seine, Loire), others near medium-sized cities (Odet, Blavet, Noyalo, Vilaine and Saint-Brieuc) or small towns (Bourgneuf, Goyen, Frémur, Bélon and Laïta). Some of the studied sites are surrounded by watersheds dominated by a more or less intensive agriculture which constitute an important source of pesticides known to act as possible endocrine disruptors.

2.2. Animals and sediment collection

Sampling campaigns were conducted in 2010 in 12 sites of the NW France (Table 1). At each site, 30 clams of similar shell length (3.5 ± 0.4 cm) were handpicked in the intertidal zone at low tide for histological analysis. The surface oxygenated layer (a few mm) of

sediments destined to *in vitro* bioassays, total organic carbon (TOC) concentration and grain size determination, and analysis of organic contaminants, were scraped off at three places distant by a few meters (about 300 cm^2 each) and placed in three aluminum boxes. Sediment samples and animals were transported to the laboratory in isothermal containers, with wet algae or sediment from the site of origin without seawater for the clams. In the laboratory, sediments were frozen at -80°C until their use, whereas animals were dissected after 2 hours of depuration.

2.3. Histological observations

Gonads were isolated and fixed with a formaldehyde solution (10%) at the salinity of the site of origin. Fixed gonads were then dehydrated and paraffin-embedded and cut (5-7µm) with a microtom. Thick sections were colored with hemalun-eosin before observation with a light microscope coupled to an image analyzer to determine gender, and search for intersex.

2.4. Sediment characteristics

Sediment characteristics were determined in sediment samples. Dry matter and total organic carbon (TOC) were determined by an external laboratory (UMR 5805 EPOC, Bordeaux ; M.Cremer). Grain size was determined by gravimeter by a laser gravimeter. These parameters are indispensable to standardize the concentrations of persistent organic pollutants (POPs). Indeed, TOC is a factor known to influence compound partitioning/retention in soils, especially for POPs. Because of their hydrophobic nature, most of them have a strong affinity with TOCs (Nam. et al., 2008).

2.5 Chemicals and reagents

Flutamide (Flut), 3-(4,5-dimethylthiazol-2-ol)-2,5-diphenyltetrasodium bromide tetrazolium (MTT), 17β-estradiol (E2), 5α-dihydrotestosterone (DHT), Luciferin, methanol (MeOH) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St Quentin Fallavier, France). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was obtained from

Promochem (Molsheim, France). All standards were of 98.1– 99.8% purity. For bioassay experiments, stock solutions (10 mM) were prepared in either dimethylsulfoxide (DMSO) or methanol and stored at −20 °C. Polycyclic aromatic hydrocarbons (PAHs), Polychlorobiphenyls (PCBs) and pesticides (OCPs) used for chemical analyses and SR12813-EQ determination were purchased from Sigma-Aldrich. All the chemical reagents used and their abbreviation are listed in table 3.

2.6. Sediment extraction procedure for cell-culture in-vitro bioassays

Sediment extracts were prepared as summarized in annex1. All solvents used were HPLC-grade (Sigma-Aldrich). Briefly five grams of freeze-dried and sieved sediment (2mm) from each location were extracted with a mixture of 10 mL of acetone and dichloromethane (50: 50 v/v) with accelerated Solvent Extraction (Three extraction cycles in 22 mL stainless steel extraction cells, 75 °C, 103 bars, ASE 200; Dionex, France). Extracts were firstly concentrated by rotary evaporation at (51°C, vaccum bar: 600, time: 35 min) and then evaporated to complete dryness under a gentle nitrogen stream and reconstituted into 1 mL of the extraction solvent. The finals extracts were then divided gravimetrically in two equal parts. Those destined to bioassays were completely dried under nitrogen stream, dissolved in 500 µL DMSO each, homogenized and stored at -20°C until use. The second part was destined to chemical analysis.

2.6. Cell culture and in vitro *bioassays*

In vitro bioassays offer a rapid and sensitive response which take into account all the effects of the mixture of contaminants presents in the tested sample.

2.6.1. Estrogenic, (anti-.)androgenic and dioxin-like activities

The MELN reporter cell lines were used to measure the estrogenic activity of sediment extract, whereas for androgenic and antiandrogenic activities, MDA-Kb reporter cell lines were used. The MELN cell line was derived from MCF-7 breast cancer cells that have been

stably transfected with a plasmid containing the luciferase gene (Luc) driven by an ERE in front of the β-globulin promoter (Balaguer. et al., 1999). The MDA-kb-2 cell line (ATCC, #CRL-2713) was obtained by stable transfection of AR and a MMTV-driven luciferase reporter gene into the human mammary cancer MDA-MB-453 cell line (Wilson. et al., 2002). Dioxin-like activities were measure in the fish liver PLHC-1 cell line (ATCC, #CRL-2406) previously described (Ryan and Hightower, 1994). Routinely culturing condition for both cell lines have been previously described in detail (Louiz. et al., 2008; Kinani. et al., 2010).

For estrogenic and (Dedourge-Geffard et al.)androgenic activities cell were left to incubate in a phenol red free DMEM (supplemented with 3% of dextran charcoal coated-FCS, DCC medium) 48h before experiment beginning. An approximate density of 50,000 cells per well were then seeded in white-opaque 96-wells culture plates. One day later, serial dilution of organic extracts and reference chemical were added to cells in triplicate and left incubate for 16h. At the end of the incubation period, medium was removed and replace by 50µL/well of 0.3mM D-luciferine in DCC medium. After 5min, the luminescence signal was read in living cells every 2s per well with a microtiter plate luminometer (µBeta, PerkinElmer SAS, Courtaboeuf, France) (Creusot. et al., 2010; Kinani, et al., 2010).

For dioxin-like activity, cell (≈50,000/well) were exposed to extract and reference chemicals as described above and left to incubate for 4h and 24h respectively in order to differentiate rapidly-metabolized dioxin-like compounds from persistent ones (Louiz, et al., 2008; Creusot, et al., 2010; Kinani, et al., 2010). Then plates were processed for 7-ethoxyresorufin-O-deethylase activity on intact cells as previously described (Mosmann, 1983; Laville. et al., 2004).

DMSO concentrations in all culture media were 0.5% (for agonist test) and 0.6% (for antagonist tests).

2.6.2. Cell viability

After exposition, cellular viability in different cell lines was estimated with the methyl-thiazol-tetrazolium (MTT) assay following the Mossman modified protocol previously described (Laville, et al., 2004).

2.6.3. Data analysis and determination of bioassay-derived toxic-equivalents

As described by precedent authors (Creusot, et al., 2010; Kinani, et al., 2010), efficient concentrations (i.e. EC20 and EC50, the concentrations of sediment extracts and chemical standards that give respectively 25 and 50% of the maximum luciferase or EROD activities) and sigmoid dose-response curves and were determined with the REGTOX 7.5 Microsoft Excel macro (freely available at http://eric.vindimian.9online.fr), by using Hill equation (Vindimian. et al., 1983). For compounds with incomplete dose/response curves, i.e. which does not reached a plateau; the Hill parameter for maximal effect was fixed at 100% (maximal response given by the positive control) before modeling of the data. In all bioassays, significant responses were defined as those greater than two times the standard deviation of the response obtained with solvent control (DMSO). The estrogenic, (anti)androgenic and dioxin-like activities in samples derived from bioassays were respectively expressed as E2-, DHT-, Flu- and BaP- or TCDD- equivalents (Bio-ref-EQs). To overcome the possible influence of sediment type (granulometry and TOC content), these activities were calculated as the ratio of the EC20 of the reference chemicals expressed as ng/L or mg/L to that of the sample expressed as equivalent gram of TOC per liter (g TOC EQ/L).

2.7. Chemical analyses

PAHs were analyzed by GC/MS. PCBs and organochlorinated pesticides (OCPs) were analysed by GC/ECD. The analytical procedures were adapted from (Budzinski. et al., 2000) for PAHs; (Alder. et al., 2006) for pesticides; and (Cailleaud. et al., 2007a) for PCBs.

2.8. Statistical analysis

Statistical analyses were performed using XLSTAT® 2011. Normality of data was checked using Shapiro and Wilk's test. To assess comparisons of means, a one-way variance analysis (ANOVA) and then a Dunnett's unilateral test were performed ($P < 0.05$). A principal component analysis (PCA) was carried out to discriminate the intersite variations of the studied parameters (intersex in clams, concentration of luciferase induction in treated cells and organic compound concentration revealed by chemical analysis), and to put in evidence if

any relationship existed between them. In cell bioassays, each sample was tested at different concentrations in at least two independent experiments, with a negative (solvent) control and complete dose/response for reference chemicals (E2, Fluta, DHT, BaP, TCDD) at each time.

3. Results

3.1. Characterization of superficial sediments

Grain size and TOC of sediments from the twelve sites are presented in table 2. The TOC content in superficial sediments varied from 4.3 to 65.8 mg of C per gram of sediment dry weight with the highest content in the Laïta and Odet estuaries (65.8 and 56.4 mg $C.g^{-1}$ sediment d.w.) and the lowest in the Saint-Brieuc and Bourgneuf bays (4.3 and 6.3 mg $C.g^{-1}$ sediment d.w.). The TOC content was correlated to the grain size, the sites with the higher percentage of big size particles (200-630) where those with less TOC content.

3.2. Intersex

The percentages of normal females, non affected and intersex males are shown in Fig. 1. As recommended by (Chesman and Langston, 2006)), it was assumed that intersex individuals were 'affected' males. Intersex males were present in all sites, representing 22.2% at the minimum in the Odet estuary to 60.0% of the total number of males at the maximum in the Loire estuary. When intersex is expressed taking into account the total population, this percentage varied from 13.3 % at the minimum in the Bélon estuary to 27.8 % at the maximum in the Seine estuary. Sex ratios did not differ significantly from unity (Z test) in most cases (Fig. 1). Sex ratios were biased towards females at one (St Brieuc, $p < 0.05$) of the twelve sites surveyed whereas a sex-ratio biased towards males was observed in the bay of Bourgneuf ($p = .0.011$).

The degree of intersex is depicted in Figure 1. The highest degrees of intersex (advanced and enclosed) were mainly observed in the Vilaine (71%), the Bay of Bourgneuf (67%), the Blavet (43%), and the Seine estuary (40%). On the other hand, the lowest degrees of intersex (focal and diffuse) were mainly observed in the Bay of St Brieuc (75%), Fremur (71%), Loire (67%) and Bélon (50%) estuaries. At the sites where many cases of intersex

were depicted (Fig. 1), the degree of intersex is not systematically higher than at the sites with a lower number of cases.

3.3. Cellular activities of organic sediment extracts

Because the slopes of dose-response curves for different sediment extracts were not parallel to that of references (β -E2, Flutamid, TCDD and BaP) the use of EC_{25} was chosen to derive Bio- Ref-EQs. All the cellular activities obtained are summarized in figures 2 and 3.

3.3.1. Estrogenic activity

Significant dose dependent-induction of luciferase activity was obtained for all tested sediment extracts. The response magnitudes varied between 33% and 78% of the maximal response elicited by β-E2. The highest values of estrogenic activities (Figure 2) were obtain in Seine, Frémur and Blavet estuaries (39 to 41 $ng.g^{-1}$ COT), followed by Bélon, Goyen and Saint-Brieuc (respectively 27, 21 and 18 $ng.g^{-1}$ COT). The lowest values were observed in Bourgneuf (7 $ng.g^{-1}$ COT) and Odet (9 $ng.g^{-1}$ COT).

3.3.2. (Dedourge-Geffard et al.) androgenic activity

All the sediment extracts showed an anti-androgenic activity (Figure 2). The highest activity was obtained in the Seine estuary with 1 628 578 Flu-EQs ($ng.g^{-1}$ COT). This value was 3.5 to 4.8 times higher than in other sites. Odet and Vilaine have shown the lowest values, respectively (165 641 and 168 912 Flu-EQs ($ng.g^{-1}$ COT). When expressed in Flu-EQs, $ng.g^{-1}$sediment d.w. (Annex 1), the highest activity is maintained in the Seine estuary (16188 Flu-EQs $ng.g^{-1}$sdt), and the lowest values in the bays of Bourgneuf and Saint-Brieuc.

A lower androgenic activity varying from 31 to 57 DHT-EQ ($ng.g^{-1}$ COT) was induced by sediment extract from 6 sites over the 12 studied (Noyalo, Frémur, St-Brieuc, Loire and Seine estuary).

3.3.3. Dioxin-like activity

As observed in the previous bioassays, all the sediment extracts have shown significant HAP-like and dioxin-like activities (Figure 3). The profile was approximately similar for both activities. The Seine estuary showed the highest BaP and TCDD-EQs values with respectively 1787 BaP EQ (µg.g^{-1} TOC) and 1053 TCDD-EQ (ng.g^{-1} TOC). In the Loire, Bélon, Blavet and Goyen estuaries values were comprised between 569 and 619 BaP-EQ (µg.g^{-1} TOC). The remaining sites showed low values comprised between 81 and 263 BaP-EQ. µg g^{-1}TOC) with the lowest in the Noyalo river. In the case of dioxin-like activities, lowest values (74 to 190 TCDD-EQ in ng.g^{-1} TOC) occured in the same sites as for BaP-EQ activity, with the exception of the Bélon estuary where TCDD-EQ activity was lower.

3.6. Chemical analyses

3.3.1. Polyaromatic hydrocarbons

All the PAHs analysed were present in the studied sediment extracts (table 4).

When taking in account the seventeen PAHs analysed (Figure 4), the Goyen and Seine estuaries have shown the highest values, respectively 136 and 114 µg.g^{-1} COT. In the decreasing order the Blavet, Loire, Frémur, Bélon and Vilaine estuaries have shown medium values whereas the lowest were obtained in the Noyalo river estuary and the Bourgneuf bay. The total PAH concentrations showed a profile closed to that obtained when PAH-like activities were tested on PLHC1 cells. When looking at the results expressed in ng.g^{-1} of sediment (Annexe 2), the profile was different. The Goyen estuary still had the highest value, but the PAH concentration was 3.1 times higher than in the Blavet estuary, which occupied the second position just before the Bélon and Seine estuaries. But the sites with the lowest PAH concentration remained the same (Bourgneuf, Saint-Brieuc and Noyalo).

3.3.1. Polychlorobiphenyls

When taking in account the seven PCBi analysed, concentrations (Figure 4) varied between sites with the highest values in the Seine estuary (2736 ng.g^{-1} COT), followed by

Frémur, Blavet, St Brieuc and Loire (1290, 805, 707 and 479 ng.g^{-1} COT respectively). The lowest concentration was recorded in the bay of Bourgneuf (65 ng.g^{-1} COT). As for PAH concentrations, the profile was different when the results are expressed per gram of sediment, with the highest values recorded in the Blavet, Odet and Seine estuaries. The lowest value was again recorded in the bay of Bourgneuf as when concentrations are expressed per gram of TOC (Figure 5).

3.3.2. Organochlorine pesticides

OCPs were not detected in Bourgneuf and Saint-Brieuc bays as depicted in Figure 4. In the ascending order, concentrations of 24.2 to 108.5 ng.g^{-1} COT were observed respectively in the Laïta, Vilaine, Odet, Blavet, Bélon, Noyalo river, Loire and Goyen estuaries. The highest concentration (300.5 ng.g^{-1} COT) occured in the Seine estuary. The total PCB concentrations showed a profile closed to that obtained when dioxin-like activities were tested on PLHC1 cells, with the exception of the Fremur estuary. As in the case of PAH and PCB concentrations, the profile was different when the results are expressed per gram of sediment (Figure 5). In particular the Goyen estuary showed the highest OCP concentration (4.7 ng.g^{-1} sdt) when expressed in ng.g^{-1} of sediment, whereas the Frémur estuary remain the lowest (0.1 ng.g^{-1} sdt, with the Bays of Bourgneuf and St-Brieuc where OCPs were not detected.

3.3.4. Contribution of different classes of organic compounds to the activities revealed by in vitro *bioassays*

3.4. Principal components analyses

Data taken into consideration for the first Principal Components Analyses (PCA) were: the percentage of intersex (number of affected males to the total number of males) and the results of *.in vitro* bioassay tests (oestrogenic, anti-androgenic, PAH and dioxin-like activities, Figure 5). For the second PCA, data taking into consideration were: the percentage of intersex as in the first PCA and the sum of different organic compound classes detected in the sediment extracts by chemical analysis (PAHs, PCBs, OCPs, Figure 6). For two types of ACP, variables were very good represented with at least 85% of the variance represented in the first

two axes F1 and F2. The plots of scores in the co-ordinates of principal components F1 and F2 for each PCA done are presented in figures 5 and 6. In the two cases, the plots of scores in the co-ordinates of principal components F1 and F3 on the one hand, and F2 and F3 on the other hand, were not shown since these representations were mainly redundant with those of F1 vs. F2 (F1 vs F3) or retained less information (< 36% for F2 vs. F3). The correlation circles are also displayed the corresponding figures. F1 is positively correlated with *in vitro* bioassay activities (Figure 5) or organic compound concentrations (Figure6), where as F2 axe was correlated with the percentage of intersex (circle of correlations). This orthogonal disposition provides us information about the independence of these two parameters. The rate of intersex males seems independent of the EDC activities and concentrations reveal by bioassay or by chemical analysis.

In the figures 5 and 6 there was a clear separation between the results of the Seine from those of the other sites. The centroid of the cloud of Seine scores is located at the extremity of the positive part of F1 (positively correlated with high bioassays activities and organic compound concentrations), whereas the other sites are located in the medium or negative part of F1 (correlated with moderate to lower bioassays activities or organic compound concentration). The centroid of the clouds of St-Brieuc, Loire and Frémur scores are located at the upper of the positive part of F2, positively correlated with high percentage of intersex.

4. Discussion

5. Conclusion

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References

AELB, SCE, CREOCEAN, 2003. Etude de délimitation et de Caractérisation des masses d'eau du bassin Loire Bretagne: Atlas cartographique des masses d'eaux côtières. Mise en place d'un diagnostic de surveillance du milieu littoral conforme à la Directive Cadre. Agence de l'eau Loire Bretagne, pp. 1-106.

Alder, L., Greulich, K., Kempe, G., Vieth, B., 2006. Residue analysis of 500 high priority pesticides: Better by GC–MS or LC–MS/MS? Mass Spectrometry Reviews 25, 838-865.

Amiard-Triquet, C., Rainbow, P.S., 2009. Environmental Assessment of Estuarine Ecosystems: A Case Study Taylor & Francis, Inc. CRC Press.

Amiard-Triquet, C., Rainbow, P.S., Romeo, M., 2011. Tolerance to Environmental Contaminants. CRC Press, Boca Raton.

Amiard, J.-C., Bodineau, L., Bragigand, V., Minier, C., Budzinski, H., 2009. Quantification of Contaminants, in: Amiard-Triquet, C., Rainbow, P.S. (Eds.), Environmental Assessment of Estuarine Ecosystems. CRC Press, Boca Raton, pp. 31-57.

Balaguer, P., François, F., Comunale, F., Fenet, H., Boussioux, A.-M., Pons, M., Nicolas, J.-C., Casellas, C., 1999. Reporter cell lines to study the estrogenic effects of xenoestrogens. The Science of The Total Environment 233, 47-56.

Budzinski, H., Letellier, M., Thompson, S., LeMenach, K., Garrigues, P., 2000. Combined protocol for the analysis of polycyclic aromatic hydrocarbons (PAHs) and polychlorobiphenyls (PCBs) from sediments using focussed microwave assisted (FMW) extraction at atmospheric pressure. Fresenius' Journal of Analytical Chemistry 367, 165-171.

Cailleaud, K., Forget-Leray, J., Souissi, S., Hilde, D., LeMenach, K., Budzinski, H., 2007a.

Seasonal variations of hydrophobic organic contaminant concentrations in the water-column of the Seine Estuary and their transfer to a planktonic species *Eurytemora affinis* (Calanoïda, copepoda). Part 1: PCBs and PAHs. Chemosphere 70, 270-280.

Cailleaud, K., Forget-Leray, J., Souissi, S., Lardy, S., Augagneur, S., Budzinski, H., 2007b. Seasonal variation of hydrophobic organic contaminant concentrations in the water-column of the Seine Estuary and their transfer to a planktonic species *Eurytemora affinis* (Calanoïd, copepod). Part 2: Alkylphenol-polyethoxylates. Chemosphere 70, 281-287.

Cevasco, A., Urbatzka, R., Bottero, S., Massari, A., Pedemonte, F., Kloas, W., Mandich, A., 2008. Endocrine disrupting chemicals (EDC) with (anti)estrogenic and (anti)androgenic modes of action affecting reproductive biology of *Xenopus laevis*: II. Effects on gonad histomorphology. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 147, 241-251.

Chesman, B.S., Langston, W.J., 2006. Intersex in the clam *Scrobicularia plana*: a sign of endocrine disruption in estuaries? Biology letters 2, 420-422.

Creusot, N., Kinani, S., Balaguer, P., Tapie, N., LeMenach, K., Maillot-Maréchal, E., Porcher, J.-M., Budzinski, H., Aït-Aïssa, S., 2010. Evaluation of an hPXR reporter gene assay for the detection of aquatic emerging pollutants: screening of chemicals and application to water samples. Analytical and Bioanalytical Chemistry 396, 569-583.

Fossi Tankoua, O., Amiard, J.C., Amiard- Triquet, C., Berthet, B., Mouneyrac, C., Rainbow, P.S., 2010. Towards a comprehensive methodology for assessing the health status of coastal and estuarine ecosystems International Council for the Exploration of the Sea, Nantes, pp. 1-20.

Geffard, A., 2001. Réponse du biota à la contamination polymétallique d'un milieu estuarien, La Gironde, FR: Exposition, Imprégnation, Induction d'une protéine de détoxication, la métallothionéine, impact au niveau individuel et populationnel, Faculté de Pharmacie. Université de Nantes, Nantes, p. 251.

Geffard, A., Amiard-Triquet, C., Amiard, J.-C., 2005. Do seasonal changes affect metallothionein induction by metals in mussels, *Mytilus edulis*? Ecotoxicology and Environmental Safety 61, 209-220.

Gomes, T., Gonzalez-Rey, M., Bebianno, M.J., 2009. Incidence of intersex in male clams

Scrobicularia plana in the Guadiana Estuary (Portugal). Ecotoxicology (London, England) 18, 1104-1109.

Huet, M., Le Goïc, N., Koken, M., 2009. Intensité de l'imposex chez *Nucella lapillus* le long des côtes de la manche et de l'atlantique en 2009. Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), Paris, Nantes.

Kalman, J., Palais, F., Amiard, J.C., Mouneyrac, C., Muntz, A., Blasco, J., Riba, I., Amiard-Triquet, C., 2009. Assessment of the health status of populations of the ragworm *Nereis diversicolor* using biomarkers at different levels of biological organisation. Marine Ecology Progress Series 393, 55-67.

Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster, M., Mac, M.J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D.M., Sinks, T., Tilson, H.A., 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ Health Perspect 104, 715-740.

Kinani, S., Bouchonnet, S., Creusot, N., Bourcier, S., Balaguer, P., Porcher, J.-M., Aït-Aïssa, S., 2010. Bioanalytical characterisation of multiple endocrine- and dioxin-like activities in sediments from reference and impacted small rivers. Environmental Pollution 158, 74-83.

Langston, W.J., Burt, G.R., Chesman, B.S., 2007. Feminisation of male clams *Scrobicularia plana* from estuaries in Southwest UK and its induction by endocrine-disrupting chemicals. Marine Ecology Progress Series 333, 173-184.

Laville, N., Aït-Aïssa, S., Gomez, E., Casellas, C., Porcher, J.M., 2004. Effects of human pharmaceuticals on cytotoxicity, EROD activity and ROS production in fish hepatocytes. Toxicology 196, 41-55.

Louiz, I., Kinani, S., Gouze, M.E., Ben-Attia, M., Menif, D., Bouchonnet, S., Porcher, J.M., Ben-Hassine, O.K., Aït-Aïssa, S., 2008. Monitoring of dioxin-like, estrogenic and anti-androgenic activities in sediments of the Bizerta lagoon (Tunisia) by means of in vitro cell-based bioassays: Contribution of low concentrations of polynuclear aromatic hydrocarbons (PAHs). Science of The Total Environment 402, 318-329.

Marchand, M., Amouroux, I., Bédier, E., Belin, C., Claisse, D., Durand, G., Soudant, D., 2009. Qualité du Milieu Marin Littoral, Synthèse Nationale de la Surveillance. Institut

Français de Recherche pour l'exploitation de la Mer (IFREMER), Nantes.

Matthiessen, P., Sumpter, P., 1998. Effect of Estrogenic Substances in the Aquatic Environment, in: Braunbeck, T., Hinton, D.E., Streit, B. (Eds.), Fish Ecotoxicology. Birkhäuser, Verlag Basel, pp. 319-336.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55-63.

Nam, J.J., Gustafsson, O., Kurt-Karakus, P., Breivik, K., Steinnes, E., Jones, K.C., 2008. Relationships between organic matter, black carbon and persistent organic pollutants in European background soils: Implications for sources and environmental fate. Environmental Pollution 156, 809-817.

Ohtake, F., Takeyama, K.-i., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., Tohyama, C., Krust, A., Mimura, J., Chambon, P., Yanagisawa, J., Fujii-Kuriyama, Y., Kato, S., 2003. Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. Nature 423, 545-550.

Park, J.-W., Tompsett, A.R., Zhang, X., Newsted, J.L., Jones, P.D., Au, D.W.T., Kong, R., Wu, R.S.S., Giesy, J.P., Hecker, M., 2009. Advanced fluorescence in situ hybridization to localize and quantify gene expression in Japanese medaka (*Oryzias latipes*) exposed to endocrine-disrupting compounds. Environmental Toxicology and Chemistry 28, 1951-1962.

Poirier, L., Berthet, B., Amiard, J.-C., Jeantet, A.-Y., Amiard-Triquet, C., 2006. A suitable model for the biomonitoring of trace metal bioavailabilities in estuarine sediments: the annelid polychaete *Nereis diversicolor*. Journal of the Marine Biological Association of the United Kingdom 86, 71-82.

Poisson, E., Fisson, C., Amiard-Triquet, C., Burgeot, T., Couteau, J., Dur, G., Durand, F., Forget-Leray, J., Letendre, J., Souissi, S., Xuereb, B., 2011. Effet de la contamination chimique: des organismes en danger?, in: Seine-Aval, G. (Ed.). Seine-Aval, Programme scientifique.

Puy-Azurmendi, E., Ortiz-Zarragoitia, M., Kuster, M., Martínez, E., Guillamón, M., Domínguez, C., Serrano, T., Barbero, M.C., Alda, M.L.d., Bayona, J.M., Barceló, D., Cajaraville, M.P., 2010. An integrated study of endocrine disruptors in sediments and reproduction-related parameters in bivalve molluscs from the Biosphere's Reserve of

Urdaibai (Bay of Biscay). Marine Environmental Research 69, Supplement 1, S63-S66.

RNO, 2006. Surveillance du Milieu Marin, Travaux du Réseau National d'Observation de la qualité du milieu marin. Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER)

Ministère de l'Ecologie et du Développement Durable, Paris, Nantes.

Ryan, J.A., Hightower, L.E., 1994. Evaluation of heavy-metal ion toxicity in fish cells using a combined stress protein and cytotoxicity assay. Environmental Toxicology and Chemistry 13, 1231-1240.

Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquatic Toxicology 68, 369-392.

Tronczynski, J., Munschy, C., Moisan, K., 1999. Les contaminants organiques qui laissent des traces: source, transport et devenir.

Vindimian, E., Robaut, C., Fillion, G., 1983. A method for cooperative and non comparative binding studies using non regression analysis on a microcomputer. J. Appl. Biochem. 5, 261-268.

Wilson, V.S., Bobseine, K., Lambright, C.R., Gray, L.E., 2002. A Novel Cell Line, MDA-kb2, That Stably Expresses an Androgen- and Glucocorticoid-Responsive Reporter for the Detection of Hormone Receptor Agonists and Antagonists. Toxicological Sciences 66, 69-81.

Zou, E., 2010. Aquatic Invertebrate Endocrine Disruption, in: Breed, M.D., Moore, J. (Eds.), Encyclopedia of Animal Behavior. Academic Press, Oxford, pp. 112-123.

Table1. Characterization of sampling sites (BOU: Bourgneuf Bay; LOI: Loire estuary; VIL: Vilaine estuary; NOY: River of Noyalo; BLA: Blavet estuary; LAI: Laïta estuary; BEL: Belon Estuary; ODE: Odet estuary; GOY: Goyen estuary; SB: Saint Brieuc Bay; FRE: Frémur estuary; SEI: Seine estuary)

Sites	GPS Coordinates	Ecological health status	Chemical health status	Potential impact of bordering rivers on the coastal waters	Potential impact of wastewater treatment plants	Potential impact of population density of the surrounding townships	Importance of harbour activities
Bay of Bourgneuf	2° 04' 40.60" W, 46° 56' 23.08" N	Good	Good	Moderate	Moderate	Low	Moderate
Loire estuary	2° 07' 59.76" W, 47° 16' 29.66" N	Good	Bad	High	High	High	High
Vilaine estuary	47° 29' 54.15" N, 2° 26' 22.22" W	Good	Good	Moderate	Low	Low	Moderate
River of Noyalo	47° 37' 02.18" N, 2° 41' 07.9" W	Good	Good	High	Moderate	Moderate	Low
Blavet estuary	47° 46' 23.22" N, 3° 18’ 1.67" W	Moderate	Good	High	High	Moderate	High
Laïta estuary	47° 48' 13" N, 3° 31' 37" W	Good	Good	Moderate	Low	Moderate	Low
Bélon estuary	47° 49' 17.89" N, 3° 39' 04.47" W	Good	Good	Moderate	Low	Low	Low
Odet estuary	47° 58' 11" N, 4° 05' 48" W	Moderate	Good	Moderate	Moderate	High	Moderate
Goyen estuary	48° 02' 17"N, 4° 29' 16" W	Good	Bad	Low	Moderate	Low	Low
Saint-Brieuc bay	48° 31’ 35.3" N, 2° 37’ 50,6" W	Poor	Good	Moderate	High	High	High
Frémur estuary	48° 36’ 18.1" N, 2° 07’ 25" W	Poor	Good	Moderate	High	Moderate	Low

Sources : http://envlit.ifremer.fr/var/envlit/storage/documents/atlas_DCE/scripts/site/carte.php?map=LB (AELB. et al., 2003)

http://www.marais-breton-baie-bourgneuf.com/sage/iso_album/document_1-diagnostic_du_territoire_v4_2008_10_03.pdf

Table 2. Characterization of superficial sediments

	Bourgneuf	Loire	Vilaine	Séné	Blavet	Laïta	Bélon	Odet	Goyen	Brest	Saint Brieuc	Frémur	Seine
Granulometry (µm)													
< 2	1.90	4.86	4.50	5.25	3.83	2.28	3.16	3.07	2.39	3.33	1.35	3.96	3.27
2-20	15.95	49.67	49.04	56.69	43.40	28.93	39.54	38.05	26.77	38.7	8.27	35.57	26.53
21-50	6.47	33.08	32.37	43.40	34.74	29.67	36.50	37.40	35.98	27.95	9.93	33.94	30.70
51-200	26.10	11.18	13.14	8.74	16.43	32.58	19.06	19.33	30.82	24.38	44.2	24.61	37.86
200-630	49.61	1.21	0.92	0.62	1.56	6.55	1.74	2.15	4.03	5.67	36.24	1.89	1.64
TOC (mg $C.g^{-1}$ sdt)	6.3	26.1	23.3	34.3	35.9	65.8	50.0	56.4	42.7	30.4	4.3	14.5	9.9

Table 3. Overview of investigated chemicals: chemicals families, analytical standards, sources and methods used for their quantification in samples

Chemical classes	Chemicals	Analytical methods (Reference)
PAHs (Origin)	Naphthalene (Owen et al.), acenaphthylene (Scholz et al.), acenaphthene (**Ace**), fluorene (**Flu**), phenanthrene (**Phe**), anthracene (**Ant**), fluoranthene (**Flt**), pyrene (Papaspyrou et al.), benz[a]anthracene (**B[a]A**), chrysene (**Chr**), benzo[b]fluoranthene (**B[b]F**), benzo[k]fluoranthene (**B[k]F**), benzo[a]pyrene (**B[a]P**), indeno[1,2,3-c,d]pyrene (Ind), dibenz[a,h]anthracene (**DBA)**, benzo[g,h,i]perylene (**B[ghi]P**)	
Organochlorine pesticides (Origin)	HCB, 2,4'DDE , Cis Chlordane, Trans Nonachlor, 4,4'DDE+Dieldrin, 4,4'DDD, 2,4'DDT, 4,4'DDT, Lindane	
Polychlorobiphenyls (Origin)	CB 50+28, CB 52, CB 101, CB 115, CB 153, CB 138, CB 180	

Table 4. Concentrations of the 17 PAHs and their relative contribution to the total dioxin-like activity in sediment organic extract from the twelve studied sites

Chemicals	LOQ (ng.g^{-1})	IEF		Concentrations in sampling sites (ng.g^{-1} COT)											
				BGF	LOI	VIL	NOY	BLA	LAI	BEL	ODE	GOY	SB	FRE	SEI
		BaP 4h	TCDD 24h												
Nap	0.11	n.i.	n.i.	164	490	186	87	324	106	105	96	416	53	221	882
Acpy	0.17	5.56^{E-03}	n.i.	Nd.	82	43	24	93	47	75	49	239	46	94	333
Acp	0.08	n.i.	n.i.	72	173	179	36	232	47	108	63	251	26	114	410
Flu	0.09	1.44^{E-02}	n.i.	90	231	204	62	328	62	151	81	388	64	185	729
Phe	0.06	n.i.	n.i.	568	2372	1389	460	3221	775	1720	897	6302	957	2214	6833
Ant	0.11	n.i.	n.i.	135	456	263	83	627	316	261	154	1216	227	328	3752
Flt	0.05	n.i.	n.i.	889	5187	3676	1318	6848	2090	4746	2314	19368	3191	5403	13720
Pyr	0.17	3.58^{E-03}	n.i.	694	4186	2759	977	5552	1665	3795	2003	16269	2512	4407	11201
BaA	0.06	2.58^{E-01}	3.58^{E-05}	368	2596	1695	531	3388	1160	2207	1154	10137	1750	2585	7217
Triph+Chrys	0.06	2.92^{E-01}	3.58^{E-05}	613	3186	1961	757	3888	1220	2532	1302	9955	1814	2813	8930
BbF+BkF+BjF				1170	6138	4059	1787	8803	1923	5276	3185	23575	3511	6201	20455
BeP				521	2428	1507	610	3311	1077	1922	1244	8932	1231	2306	7938
BaP	0.20	1	4.23^{E-03}	518	3038	1848	668	3892	1415	2439	1462	11597	1739	2719	8430
Per				542	5249	1873	487	2176	708	1381	811	3248	491	758	3191
IP	0.07	8.43^{E-01}	2.79^{E-02}	564	3206	2211	1026	4586	1619	2930	2036	12672	1927	3081	10325
DaA+DaC				102	611	399	174	885	265	494	363	2333	332	552	2085
BP	0.16	n.i.	n.i.	501	2395	1515	697	3325	1148	2061	1584	9195	1293	2188	7340
Σ HAP (ng.g^{-1} COT)				7510	42025	25766	9784	51478	15644	32202	18797	136092	21164	36171	1137772
Chem-BaP-EQ (ng.g^{-1} COT)															
Bio-BaP-EQ (ng.g^{-1} COT)				100	619	263	81	596	262	611	125	569	109	259	1781
Chem-BaP-EQ/Bio-BaP-EQ (%)															
Chem-TCDD-EQ															
Bio-TCDD-EQ				77	404	190	74	306	107	163	81	512	136	277	1053
Chem-TCDD-EQ/Bio-TCDD-EQ (%)															

Table 5. Concentrations of organochlorine pesticides and polychlorobiphenyls in the sediment organic extract from the twelve studied sites

Chemical classes	Chemicals	SREF	Concentrations in sampling sites											
			BGF	LOI	VIL	NOY	BLA	LAI	BEL	ODE	GOY	SB	FRE	SEI
Organochlorine pesticides	HCB		< 2.1	8.0	3.8	4.0	3.6	1.6	**25.0**	8.8	2.1	n.d.	< 2.1	9.9
	2,4'DDE		n.d.	8.7	< 2.3	18.4	10.2	2.3	3.3	4.9	9.2	n.d.	n.d.	**64.1**
	Cis Chlordane		n.d.	17.0	n.d.	31.1	1.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**108.4**
	Trans Nonachlor		n.d.	3.7	4.9	4.5	4.3	2.5	16.0	4.7	2.9	n.d.	n.d.	**19.1**
	4,4'DDE+Dieldrin		< 2.1	12.3	6.8	8.2	11.2	12.8	6.1	9.2	44.4	< 2.1	5.0	**28.8**
	4,4'DDD		< 2.1	11.6	2.5	1.1	8.9	3.2	2.9	8.3	42.8	n.d.	4.04	**48.2**
	2,4'DDT	1.8^{E-02}	n.d.	3.4	6.3	< 3.4	< 2.1	< 2.1	< 2.1	< 2.1	n.d.	n.d.	n.d.	**8.8**
	4,4'DDT		n.d.	< 2.1	n.d.	n.d.	< 2.1	n.d.	2.2	2.5	**3.5**	n.d.	n.d.	n.d.
	Lindane	2.2^{E-02}	< 2.1	3.4	< 2.1	4.7	4.7	2.4	5.1	3.8	3.6	< 2.1	< 2.1	**13.3**
	Σ OCP ($ng.g^{-1}$ COT)		0.0	65.7	24.2	68.2	40.0	22.4	55.4	38.5	104.8	0.0	9.0	**287**
Polychloro biphenyls	CB 50+28		n.d.	14.9	6.7	3.3	85.0	21.3	3.44	5.5	24.4	< 3.3	7.5	**346.7**
	CB 52		< 3.3	23.0	5.4	6.9	40.9	14.6	12.4	14.6	23.9	< 3.3	7.5	**238.4**
	CB 101	3.4^{E-03}	< 3.3	68.4	18.4	26.3	70.7	43.8	30.8	53.6	58.6	641.1	**1174.7**	394.2
	CB 118		8.1	50.0	14.1	15.6	96.5	42.4	38.3	35.2	73.0	30.2	31.6	**335.5**
	CB 153	6.0^{E-03}	33.6	140.0	43.4	44.2	204.9	55.6	45.3	139.1	95.8	18.4	31.1	**608.5**
	CB 138	5.3^{E-03}	23.0	124.8	35.6	40.7	192.2	59.8	47.8	127.6	82.2	16.9	28.4	**539.8**
	CB 180	6.1^{E-03}	< 3.3	59.0	18.6	24.0	115.3	21.9	19.8	93.9	33.7	< 3.3	9.2	**272.7**
	Σ PCB ($ng.g^{-1}$ COT)		64	480	142	161	805	259	198	469	392	707	1290	**2736**
	Chem-OCP-EQ ($ng.g^{-1}$ COT)													
	Bio-OCP-EQ ($ng.g^{-1}$ COT)													
	Chem-OCP-EQ/Bio-OCP-EQ (%)													
	Chem-PCB-EQ													
	Bio-PCB-EQ													
	Chem-PCB-EQ/Bio-PCB-EQ (%)													

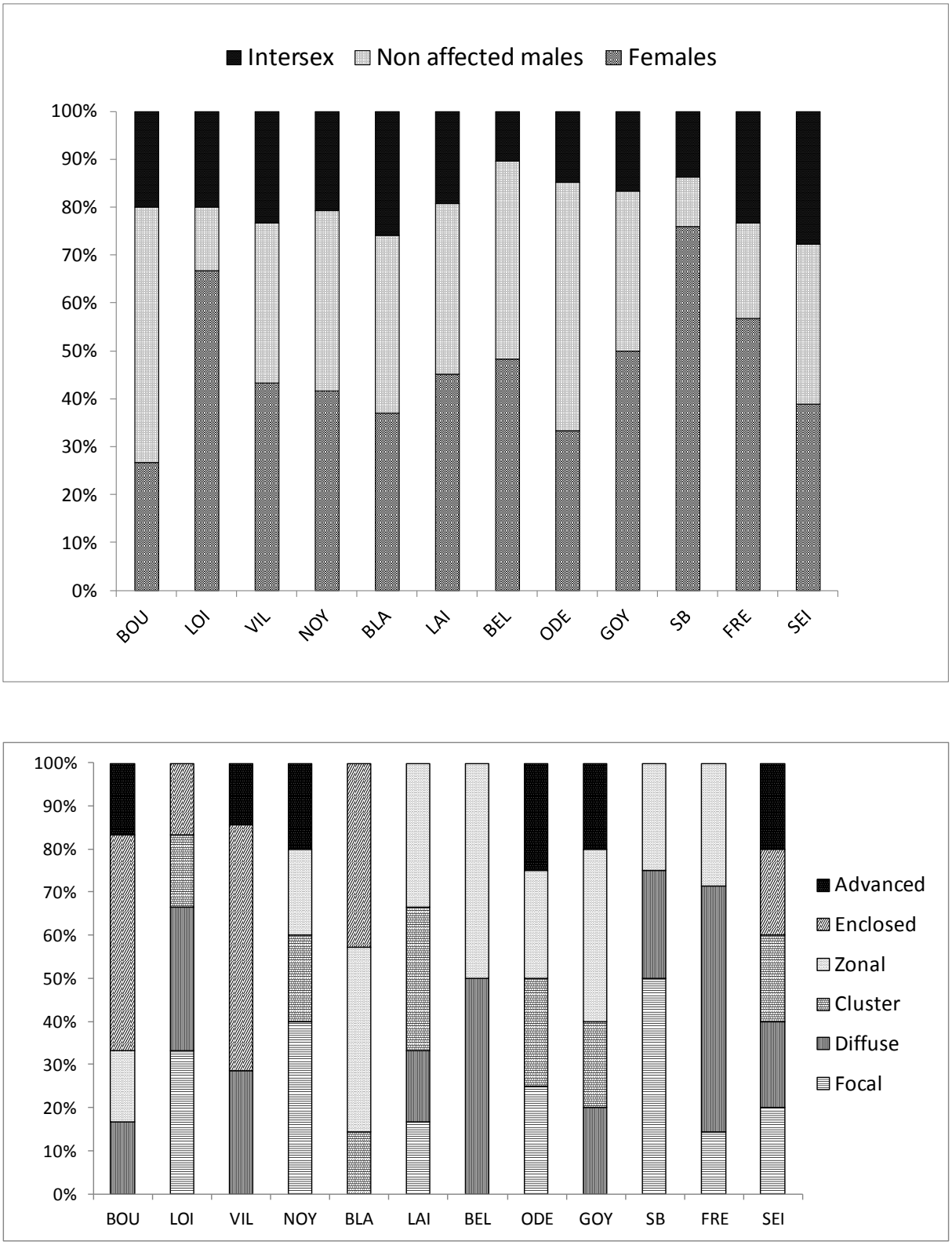


Figure 1. Sex ratio, occurrence and degree of intersex in clams from twelve sites in the NW France

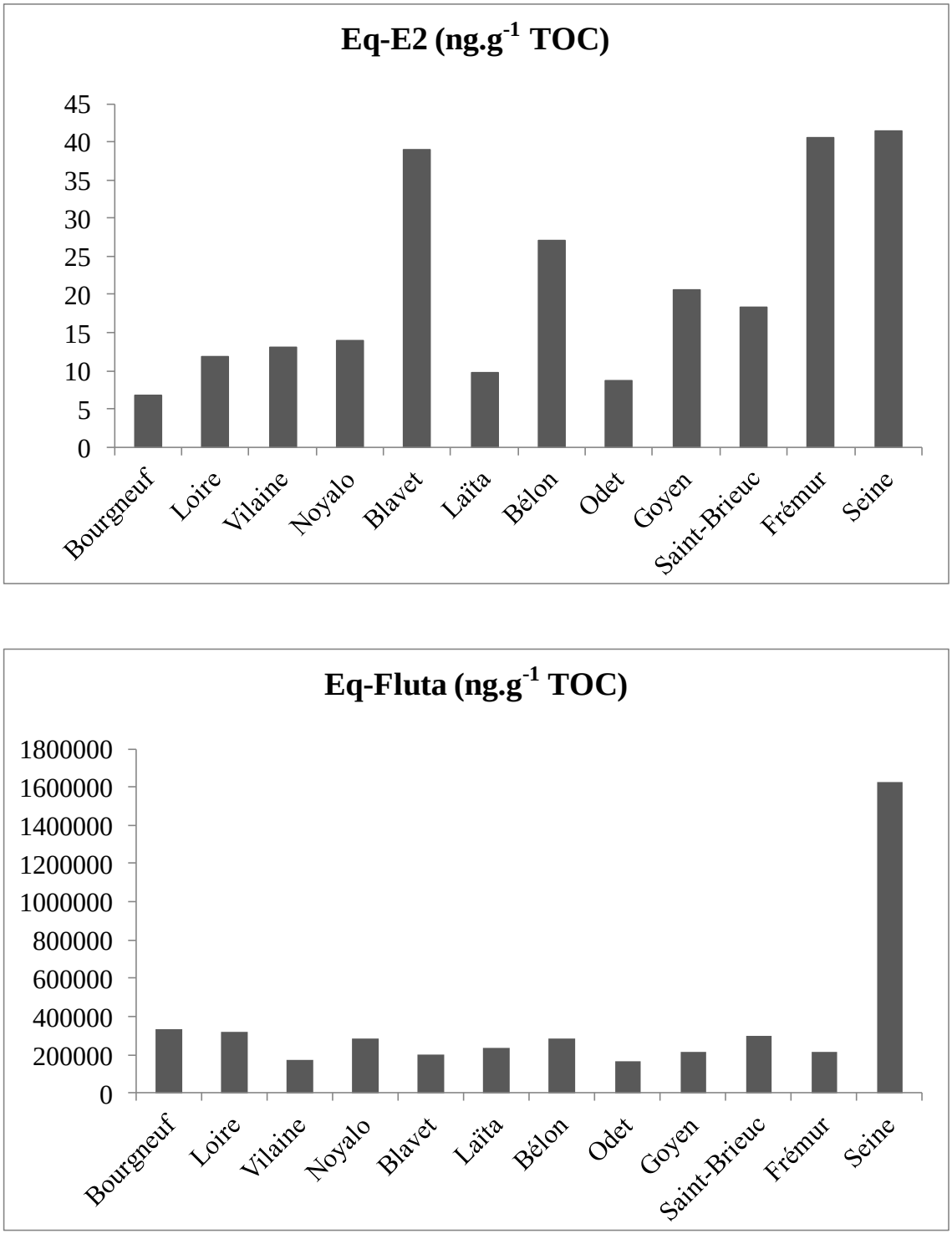


Figure 2. Estrogenic and anti-androgenic activities of the sediment extracts revealed respectively by using MELN and MDA-Kb2 reporter cell lines

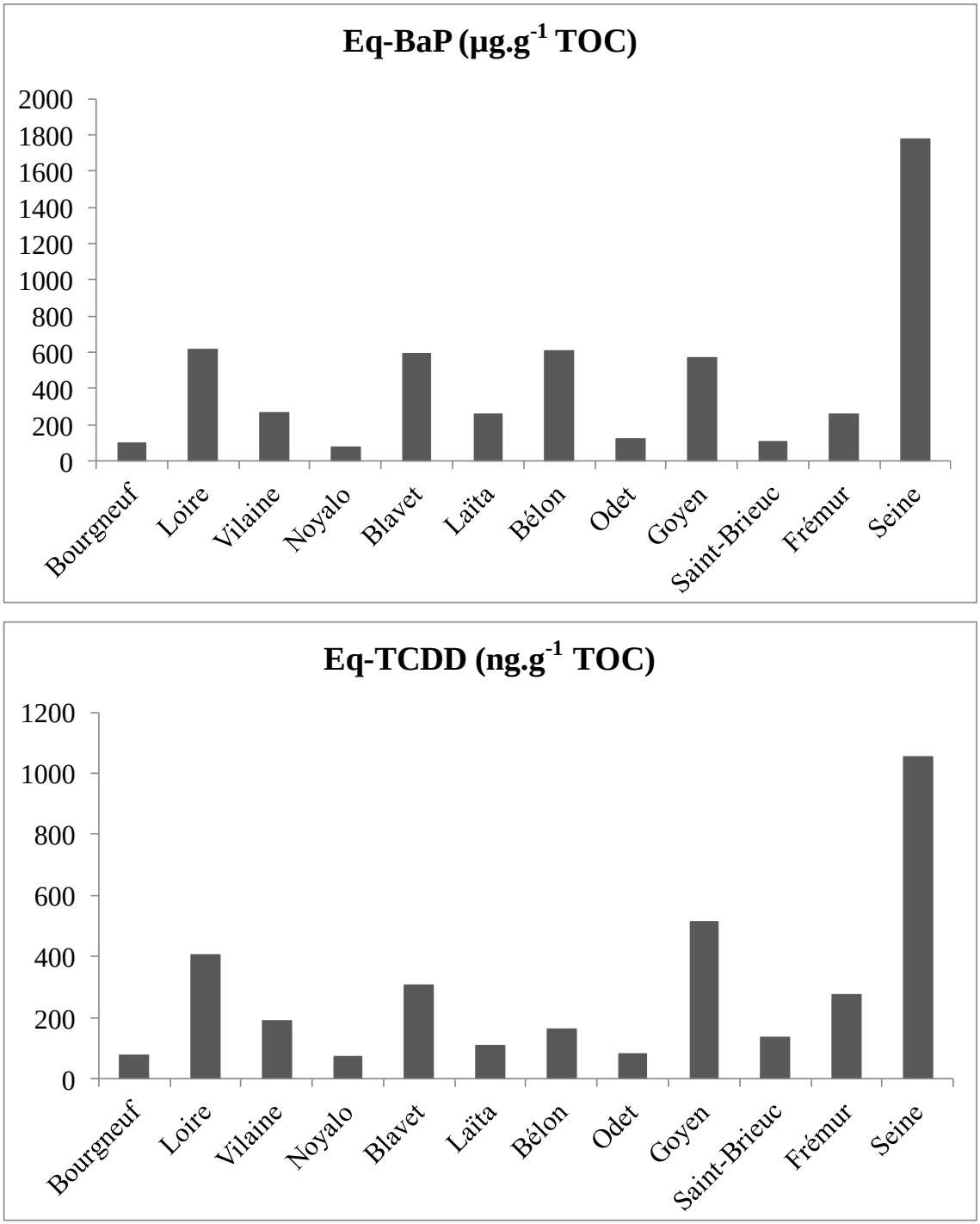


Figure 3. PAH and dioxin- like activities revealed by using PLHC1 reporter cell lines

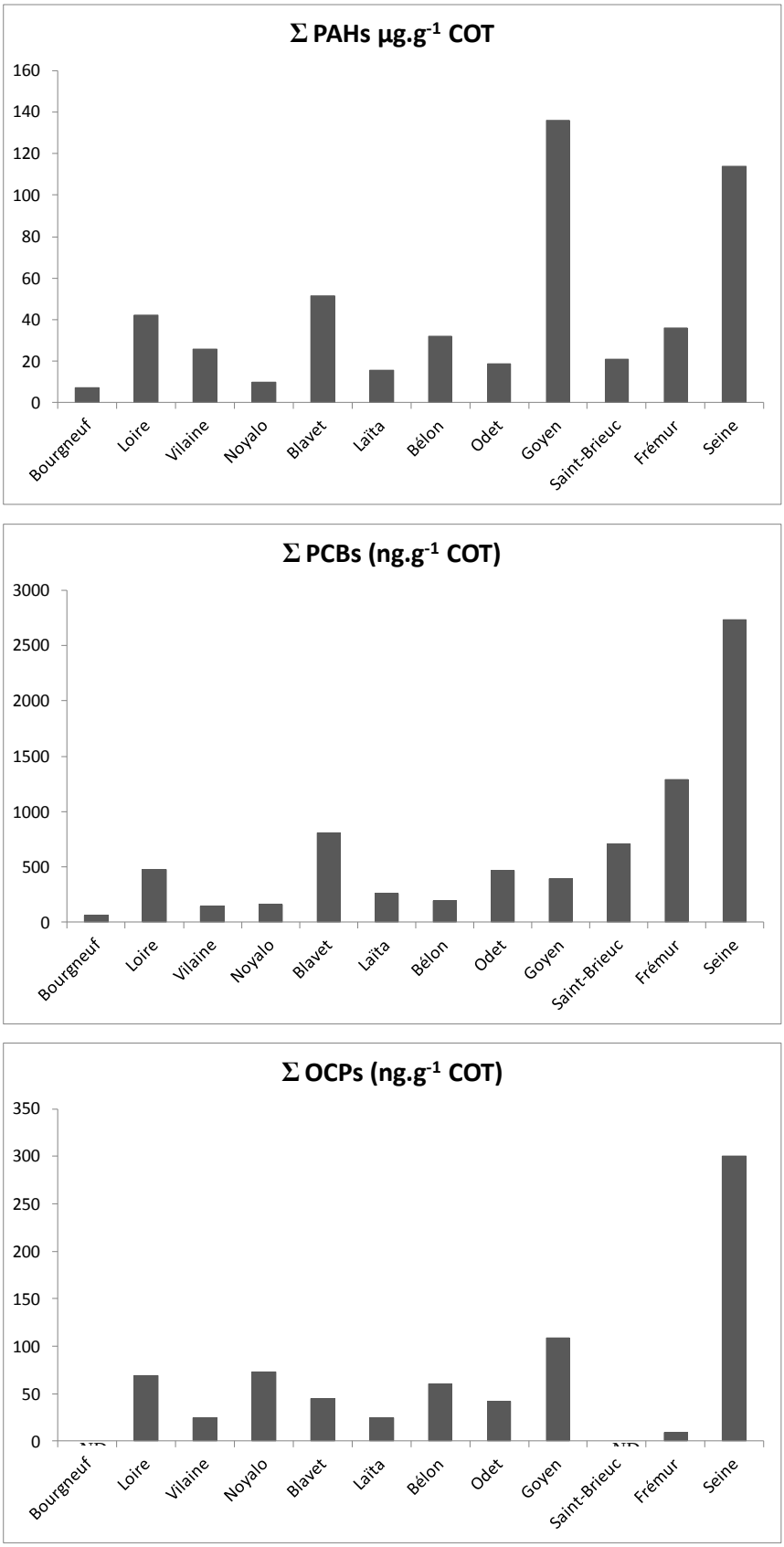


Figure 4. Sum of PAHs , PCBs and OCPs in ng.g-1 TOC determined in sediment organic extracts of the twelve sites by using chemicals analysis (ND: non detected)

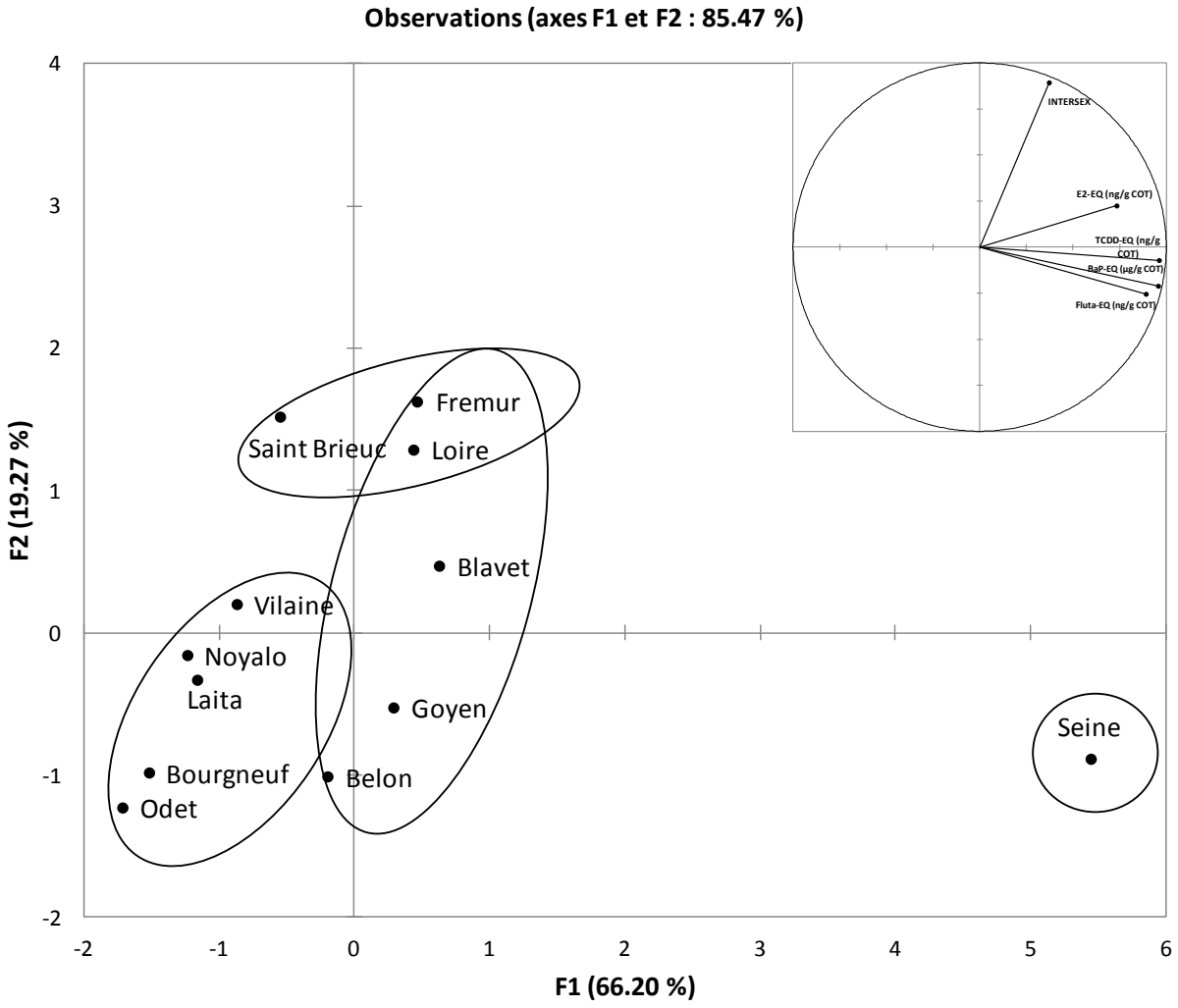


Figure 5. Principal component analysis (principal plan 1&2) including in vitro bioassays and intersex data of the twelve sites. The correlation circle is shown in the vignette. Bioassays data: significant correlation with F1; Intersex: significant correlation with F2

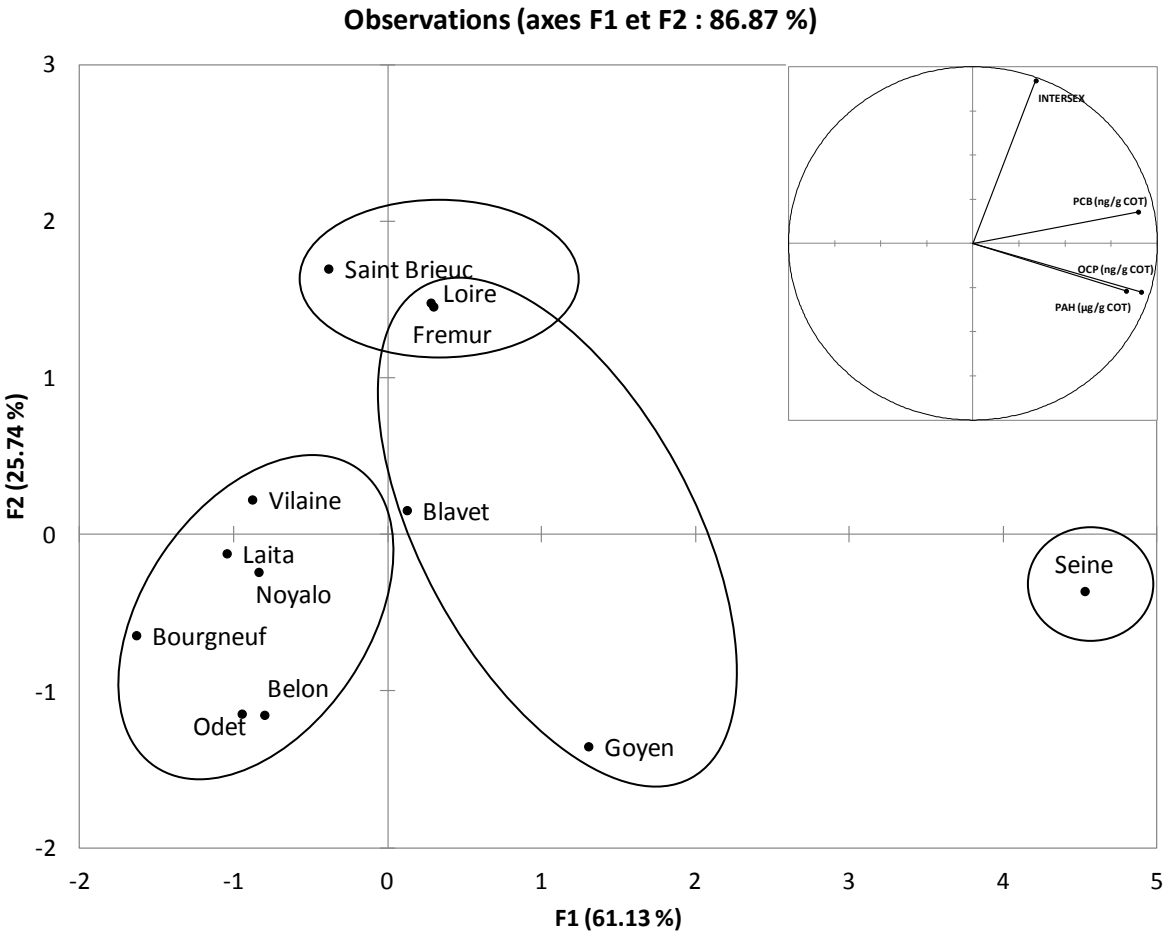
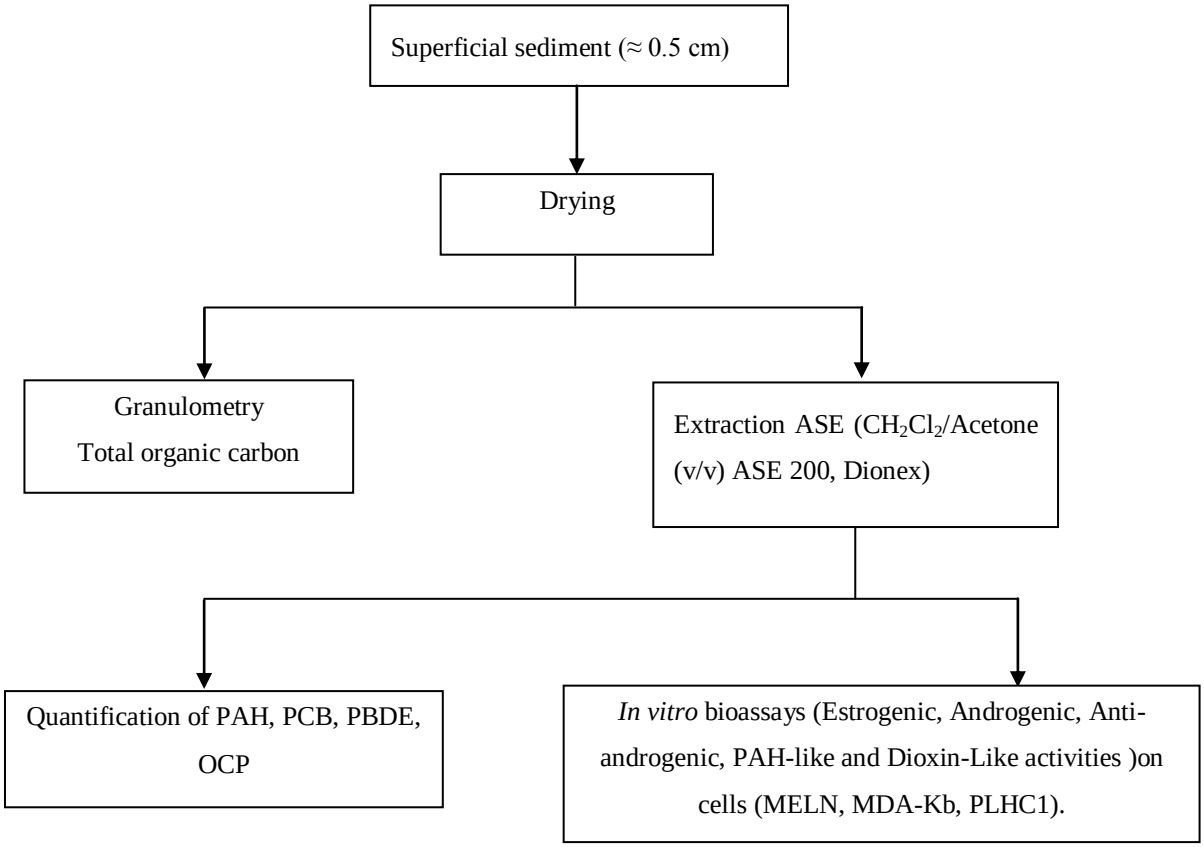


Figure 6. Principal component analysis (principal plan 1&2) including sum of each class of organic compound and intersex data of the twelve sites. The correlation circle is shown in the vignette. PAH, PBC, OCP concentrations: significant correlation with F1; Intersex: significant correlation with F2

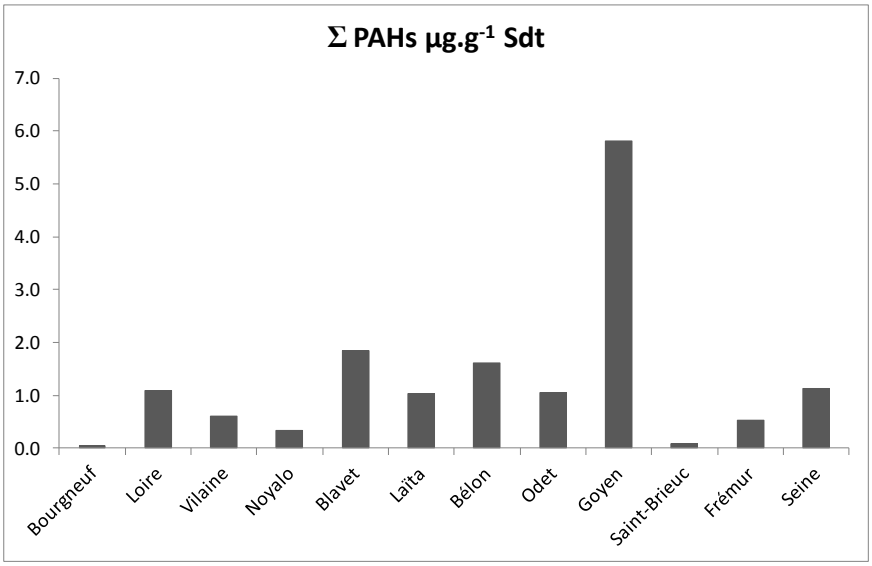
Annex 1. Sediment extraction procedure

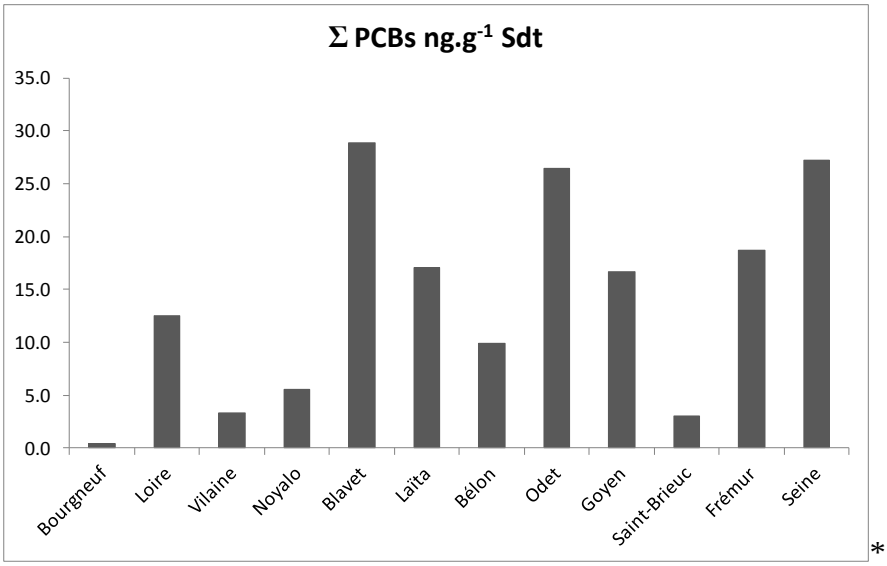


Annex 2. *In vitro* assays activities expressed in ng or µg per gram of sediment

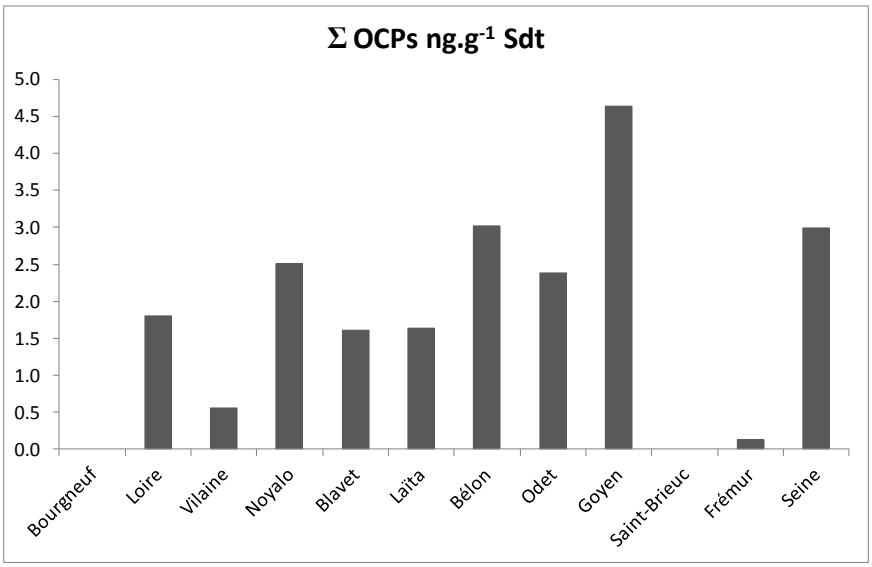
	Measured activities				
	Estrogenic	Androgenic	Anti-androgenic	PAH-like	Dioxin-like
	E2-EQ ($ng.g^{-1}$ sdt)	DHT-EQ ($ng.g^{-1}$ sdt)	FLU-EQ ($ng.g^{-1}$ sdt)	BaP-EQ ($\mu g.g^{-1}$ sdt)	TCDD-EQ ($ng.g^{-1}$ sdt)
Bourgneuf	0.04		2130	0.6	0.5
Loire	0.31	0.8	8310	16.2	10.5
Vilaine	0.31	1.2	3936	6.1	4.4
Noyalo	0.48	1.9	9653	2.8	2.5
Blavet	1.40		7101	21.4	11.0
Laïta	0.64		15410	17.2	7.1
Bélon	1.35		14094	30.5	8.1
Odet	0.49		9342	7.1	4.6
Goyen	0.88		9259	24.3	21.9
Saint-Brieuc	0.08	0.2	1278	0.5	0.6
Frémur	0.59	0.8	3089	3.8	4.0
Seine	0.41	0.4	16188	17.7	10.5
D.L.					

Annex 3. Sum of PAHs, PCBs and OCPs in $ng.g^{-1}$ sediment determined in sediment organic extracts of the twelve sites by using chemicals analysis





*



6.4 Conclusions

Les critères d'évaluation de la reproduction établis sur les populations de *Scrobicularia plana* issues de treize sites du nord ouest de la France ont montré un certain nombre d'altérations telles que le développement asynchrone des produits génitaux des mâles et des femelles (la maturité étant globalement plus précoce pour ces dernières), des biais dans le sex-ratio qui est normalement équilibré entre les deux sexes et surtout, dans tous les sites, la présence d'individus intersexués qui ont été interprétés comme des mâles féminisés d'après Langston et al. (2007). En début de maturité sexuelle (mai), l'observation de l'intersexe a été menée sur des coupes histologiques représentatives des différentes zones de la gonade. A proximité du pic de maturité sexuelle (juillet), l'examen de l'intersexe a été réalisé à l'aide de frottis selon la procédure utilisée par Langston et al. (2007). Chacune de ces techniques présente des avantages et des inconvénients. Les coupes sont consommatrices de temps et de produits mais en contre-partie, elles permettent non seulement de distinguer le sexe et la présence d'intersexe, mais aussi de caractériser les stades de maturité sexuelle. Par contre, lorsque le bivalve est prêt à pondre, il est impossible de réaliser des inclusions correctes et dans ce cas, les frottis apparaissent comme la technique à privilégier. Cette détermination beaucoup plus rapide présente aussi quelques inconvénients. Ainsi, nous suspectons que, lorsque l'intersexe est très avancé, les ovocytes nombreux et de grande taille soient susceptibles de masquer les quelques groupes de spermatozoïdes restant, amenant à conclure qu'il s'agit d'une femelle au lieu d'un mâle intersexué (ce qui expliquerait l'absence d'intersexe en juillet dans la population de la Seine, choisie comme site témoin positif, alors que l'intersexe était le plus fort de tous les sites en mai, à 28% de la population totale). En début de maturité sexuelle, nous avons observé sur les coupes que les ovocytes étaient bien apparents quand les spermatozoïdes n'étaient pas encore clairement différenciés. L'observation sur frottis dans ce cas paraît plus délicate à interpréter ainsi qu'en fin d'émission des produits génitaux quand il reste très peu de gamètes dans la gonade.

D'après les travaux publiés par Langston et al. (2007), nous nous attendions à ce que les cas d'intersexe observés chez *S. plana* des sites du nord-ouest de la France soient explicables par la présence et le niveau de contamination des sédiments dans lesquels elles vivent par des (xéno)œstrogènes. Toutefois aucune corrélation n'a pu être établie entre les niveaux d'activité en termes d'équivalents estradiol (E2) déterminés par les tests cellulaires *in vitro* et les pourcentages d'intersex. En second lieu, la littérature indique que les composés de type

dioxine peuvent avoir un effet soit œstrogénique, soit anti-œstrogénique selon que des oestrogènes soient absents ou présents dans le milieu, au travers des effets croisés entre le récepteur Ah et le récepteur aux œstrogènes ER. Dans une revue récente, (Cooper et Wintermyer, 2009) considèrent qu'il est nécessaire d'élucider les relations entre les dioxines et les effets d'autres composés apparentés aux dioxines sur le contrôle hormonal impliqué dans le développement des gonades. Il a été proposé dans la littérature que les HAP et leurs métabolites aient aussi des activités œstrogéniques et anti-œstrogéniques (Kummer et al., 2008) et littérature citée par ces auteurs), variables selon la structure moléculaire (Hayakawa et al., 2011). Plus généralement, une évaluation compréhensive des perturbateurs endocriniens (PE) doit prendre en compte la capacité des contaminants chimiques à interférer avec les différentes cibles du système hormonal. Aucune corrélation n'a pu être mise en évidence entre les données de reproduction de *S. plana* et les résultats des différents tests cellulaires visant à mettre en évidence les activités antiandrogéniques, dioxine-like et HAP-like.

Il faut toutefois noter que les données issues des tests *in vitro* ne permettent pas toujours de préjuger des effets qui seront observés *in vivo* (e. g. Kummer et al., 2008) A un autre niveau, lorsque l'on passe de l'expérimentation où les PE potentiels sont testés individuellement à la situation dans l'environnement estuarien, l'interprétation se complexifie en raison de la présence concomitante de molécules pouvant agir en synergie ou avoir des effets antagonistes.

Les tests cellulaires ayant démontré la présence de réponses pour la totalité des sédiments issus des estuaires du nord ouest de la France, les analyses chimiques ont ciblé trois grandes catégories de contaminants, PCB, HAP et pesticides organochlorés. Deux sites ressortent comme particulièrement contaminés : l'estuaire de la Seine pour les trois classes de contaminants et le Goyen pour les pesticides et les HAP. Pour les PCB et les HAP, ces résultats sont en parfait accord avec les tests cellulaires. Pour la Seine et le Goyen qui sont classés comme ayant un « mauvais » état chimique au regard des critères de la Directive Cadre sur l'Eau, ces résultats n'ont rien de surprenant tandis que pour les estuaires du Blavet et de Frémur, classés comme ayant un « bon » état chimique, il est frappant que les taux des contaminants analysés y soient relativement élevés.

De même, il existe une certaine distorsion entre l'évaluation de l'état écologique de nos sites selon les critères de la DCE et la présence de perturbations de la reproduction observées chez une espèce clé pour le fonctionnement de l'écosystème. Nous avons mentionné dans la publication 5 certains aspects de l'approche DCE qui sont à l'origine de ces distorsions.

Comme cela apparaît clairement dans la figure 7, les réponses des communautés aux stress environnementaux – sur lesquelles se base la caractérisation du bon état écologique –ne deviennent significatives que sur le long terme, bien après que des effets à de plus faibles niveaux d'organisation biologique se soient déjà produits, lesquels pourraient être mis à profit comme signaux d'alerte, en particulier ceux qui sont suspectés de pouvoir affecter le succès de la reproduction. Ainsi Lewis et Watson (2011) recommandent d'inclure dans la « boîte à outils écotoxicologique » des critères d'évaluation de la reproduction chez les polychètes mais leur propos pourraient être étendus aux bivalves, d'autant plus que ce taxon comporte de nombreuses espèces importantes pour la pêche et l'aquaculture dont la biologie de la reproduction est très étudiée. C'est le cas en particulier des espèces de moules chez lesquelles des altérations sexuelles (féminisation ou masculinisation) ont aussi été observées (Hellou et al., 2003, Ortiz-Zarragoitia et Cajaraville, 2010) ainsi que chez la mye *Mya arenaria* (Gagné et al., 2003).

Toutefois, dans l'état actuel des connaissances, il n'a pas été mis en évidence un lien direct entre les perturbations recensées chez *S. plana*, en particulier l'intersexe, et des effets sur les populations des estuaires étudiés. En effet, l'absence de recrutement au cours des 2 ou 3 dernières années a été observée dans trois sites qui n'étaient pas les plus affectés par l'intersexe.

CHAPITRE 7. MISE EN ŒUVRE DES BIOMARQUEURS POUR L'EVALUATION DE L'ECOTOXICITE DE NANOPARTICULES METALLIQUES

7 CHAPITRE 7. MISE EN ŒUVRE DES BIOMARQUEURS POUR L'EVALUATION DE L'ECOTOXICITE DE NANOPARTICULES METALLIQUES

7.1 Contexte de l'étude

La publication présentée dans ce chapitre s'inscrit dans une démarche de **transfert de compétence** nécessitée par le développement au sein de notre équipe de recherches consacrées à l'écotoxicologie de contaminants émergents et dans ce cas précis, de nanoparticules métalliques (NP). Ces recherches sont menées dans le cadre d'un programme européen "THE REACTIVITY AND TOXICITY OF ENGINEERED NANOPARTICLES: RISKS TO THE ENVIRONMENT AND HUMAN HEALTH" (NanoReTox, FP7). Notre équipe, au travers de C. Mouneyrac, est leader du WP consacré à l'écotoxicologie.

Jusqu'à très récemment, les tests réalisés pour évaluer le risque associé au relargage de NP dans l'environnement étaient menés selon les pratiques de la toxicologie plutôt que de l'écotoxicologie, en particulier l'exposition des organismes à des doses massives de contaminants. Dans le cadre de NanoReTox, les écotoxicologues se sont fixé comme ligne directrice de mener des expériences prenant au maximum en compte la réalité du terrain, en tout premier lieu l'utilisation de modèles biologiques issus de l'environnement naturel et des doses faibles, susceptibles d'être à terme rencontrées dans l'environnement.

La nécessité de travailler sur les faibles doses découle de l'existence d'un phénomène de saturation des mécanismes de défense permettant aux organismes de se maintenir dans des milieux pollués (Amiard-Triquet et Roméo in Amiard-Triquet et al., 2011). Ceci concerne la métallothionéine (détoxication des métaux), l'EROD et la GST (biotransformation), la catalase et la SOD (défenses anti-oxidantes), la résistance multixénobiotique et les protéines chaperones (HSP). Les tests toxicologiques utilisant des doses aiguës ne permettent pas à ces mécanismes de fonctionner, provoquant leur saturation immédiate et, en conséquence, des effets toxiques qui ne sont ni de la même nature, ni de la même intensité que ceux qui se produisent à faible dose.

Comme la plupart des contaminants introduits dans l'environnement, il est très vraisemblable

que les nanoparticules aient pour destination finale les sédiments comme nous pouvons le voir sur la figure 14 (Klaine et al., 2008). Les modèles biologiques utilisés tout au long de cette thèse (l'annélide polychète *Hediste diversicolor* et le bivalve *Scrobicularia plana*) apparaissaient donc comme pertinents pour l'évaluation de l'écotoxicité des nanoparticules métalliques. En ce qui concerne les doses à tester, dans l'état actuel des connaissances, il n'existe pas de méthodes qui permettent la mesure des concentrations environnementales de nanoparticules. Pour certaines, la modélisation fournie des concentrations susceptibles d'être rencontrées dans l'environnement (Tiede et al., 2009) mais ce type de données n'était pas disponible pour le cuivre. Il y a actuellement un débat quant aux rôles respectifs des caractéristiques physiques des nanoparticules et de la toxicité des métaux qu'elles sont susceptibles de libérer dans le milieu (Auffan et al., 2009). Nous avons adopté l'hypothèse du pire cas et nous avons établi les doses à tester comme si tout le cuivre des NP pouvait être libéré dans le milieu. Nous avons réalisé en parallèle des expositions avec des NP de CuO ou du cuivre soluble avec une concentration identique de cuivre qui était de 10 µg L^{-1}, c'est-à-dire du niveau de celles qui peuvent être mesurées dans des milieux contaminés.

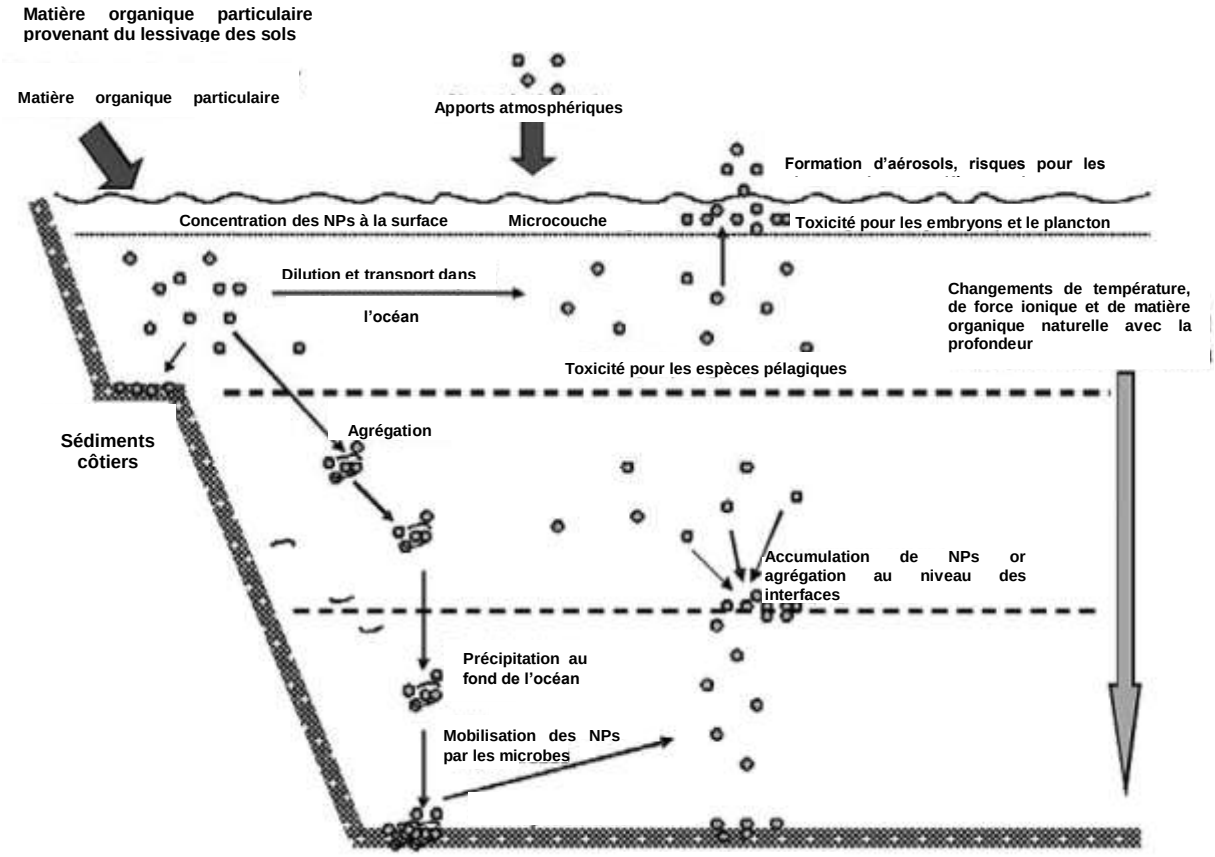


Figure 15. Schéma illustrant le devenir possible des nanoparticules (NPs) dans l'environnement marin,

ainsi que le risque qu'ils représentent pour les organismes d'après Klaine et al. (2008).

Dans ce contexte de réalisme environnemental, il nous a été demandé de contribuer à l'évaluation de l'écotoxicité des NP d'oxyde de cuivre en mettant en oeuvre les biomarqueurs comportementaux dont les données de la littérature et nos propres résultats avec *H. diversicolor* et *S. plana* ont établi qu'ils étaient réactifs à de faibles contaminations et qu'ils avaient une pertinence écologique permettant, lorsqu'ils sont perturbés, de suspecter des risques au niveau de la population, voire de la communauté.

7.2 Publication 7

Behavioural and biochemical responses of two marine invertebrates Scrobicularia plana and *Nereis diversicolor* to copper oxide nanoparticles

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abstract

Engineered nano-sized Cu oxide particles are extensively used in diverse applications. Because aquatic environments are the ultimate "sink" for all contaminants, it is expected that nanoparticles (NP) will follow the same fate. In this study, two marine invertebrates *Scrobicularia plana* and *Hediste diversicolor* were chosen as ecotoxicological models. The aim was to evaluate behavioural (burrowing kinetics, feeding rate) and biochemical (biomarkers) responses of *S. plana* and *H. diversicolor* exposed in the laboratory to Cu (10 l g L^{-1}) added in natural seawater either in the form of engineered nanoparticles (NPs) of CuO or as dissolved Cu in 2% HNO_3. Exposure was characterized by considering (i) the physico-chemical fate of NP (ii) the fraction of labile Cu in experimental media and (iii) Cu bioaccumulation. Results showed high aggregation of CuO NPs in seawater and no additional bioavailable Cu concentrations. Behavioural impairments were observed in *S. plana* exposed to CuO NPs or soluble Cu whereas in *H. diversicolor*, only the exposure to soluble Cu led to a burrowing decrease. No obvious neurotoxicity effects were revealed since in both species, no changes in cholinesterasic activity occurred in response to both forms of Cu exposure. Biomarkers of oxidative-stress catalase and glutathione-S-transferase were enhanced in both species whereas superoxide dismutase was increased only in *S. plana* exposed to CuO NPs. Metallothionein-like protein was increased in bivalves exposed to both forms of Cu. Since, no detectable release of soluble Cu from CuO NPs occurred during the time of experiment, ecotoxicity effects seem to be related to CuO NPs themselves.

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1. Introduction

Because the physicochemical properties of nanoparticles are distinct from their bulk counterparts, the fast growth of nanotechnologies has brought new industrial and business opportunities. For instance, engineered nano-sized copper oxide particles (CuO NP) are commonly used as bacteriocides and have the potential to replace noble metal catalysts for carbon monoxide oxidation (Zhou et al., 2006). CuO NP suspensions (nanofluids) have excellent thermal conductivity and are used as a heat transfer fluid in machine tools (Chang et al., 2005). Health and environmental concerns on free engineered nanoparticles (ENPs) have been highlighted in several reports (Royal Commission on Environmental Pollution, 2008; European Commission, 2009). Because aquatic environments are the ultimate "sink" for all contaminants, it is expected that ENPs will follow the same fate (Kaegi et al., 2008). Environmental monitoring data of engineered NPs, arising from use in consumer products, are currently lacking, which could be due to the difficulty to detect and quantify ENPs in complex matrices, such as water, sediments, and soils, as well as in organisms and their susceptible tissues (Wiesner et al., 2006; Nowack and Bucheli, 2007). Most of the ecotoxicological studies published are conducted with high NP concentrations, probably unrealistic from

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an environmental point of view as indicated by Predicted Environmental Concentrations of ENPs arising from use in consumer products (Tiede et al., 2009).

In order to investigate putative ecological impairments caused by ENPs, it is necessary to select species which have a key role in the structure and functioning of ecosystems and are recognized as good models for biomonitoring purposes. In this way, marine invertebrates such as the ragworm *Hediste diversicolor* and the bivalve mollusc *Scrobicularia plana* (Byrne and Halloran, 2001; Solé et al., 2009) represent good candidates. Soluble forms of copper are highly toxic to aquatic organisms (Eisler, 2007; Luoma and Rainbow, 2008). In the case of Cu-containing NPs, the release of Cu and its speciation may be a key factor in their ecotoxicity, besides that related to the nanoparticle itself. Biomarkers of defence metallothionein: MT, catalase: CAT, glutathione-S-transferase: GST, superoxide dismutase: SOD and damage thibarbyturic acid reactive substances: TBARS, cholinesterase: ChE are commonly used in environmental assessment (Lagadic et al., 2000; Amiard and Amiard-Triquet, 2008). Lactate dehydrogenase (LDH) activity, as the terminal enzyme of anaerobic glycolysis, plays an important function in anaerobic metabolism (Gagnon and Holdway, 1999; Diamantino et al., 2001). Clams (*S. plana*) are able to maintain a working anaerobic metabolism under anoxia and hydrogen sulphide exposure (Oeschger and Pedersen, 1994). Induction in LDH activity due to chemical stress has been reported in different species of bivalves such as *S. plana* (Boldina-Cosqueric et al., 2010), Perna viridis (Nicholson and Lam, 2005), Donax trunculus (Tlili et al., 2010) and polychetes such as *H. diversicolor* (Moreira et al., 2006). In addition, behavioural biomarkers are sensitive tools to assess the impact of the contaminants at concentrations far below the lethal effect (Amiard-Triquet, 2009).

Nanoparticles of CuO were identified has being important in ecotoxicological assays due to their relatively low dissolution rate but their potentially high toxicity towards organisms (Stone et al., 2010). Thus, the aim of the present study was to evaluate biochemical and behavioural responses of two marine endobenthic invertebrates, *S. plana* and *H. diversicolor*. We selected an aqueous medium because it was difficult to distribute evenly and reliably NPs in a natural sediment medium and both intra-sedimentary species studied were able to freely contact with pollutants in the aqueous medium. Indeed, *H. diversicolor* is a hemisessile polychaete living in galleries aerated by water fluxes generated by body movement. It creates a burrowing environment that may be relatively independent of the surrounding sediment since the burrow is coated by the secretion of a mucus layer. In the case of *S. plana*, the cavity oxygenate water is flushed through the burrow from time to time and within the animal the soft tissues are bathed in pallial fluid with is primarily seawater. Both species were exposed in the laboratory to environmentally relevant Cu levels (10 l g L^{-1}) added in natural filtered seawater either in the form of engineered CuO NP or as soluble Cu. This Cu level (10 l g L^{-1}) was reported in marine environments highly contaminated by this metal (Bryan and Langston, 1992). Exposure was characterized by considering the fate of NP (size distribution and fraction of labile Cu in experimental media) and Cu bioaccumulation.

2. Material and methods

2.1. Chemicals used and particle characterization

CuO nanopowder was obtained from a commercial source (Intrinsiq Materials Limited) with the particle size specified by the manufacturer as 10–100 nm. A stock suspension of CuO nanoparticles (25 mg L^{A1}) was prepared in deionized water (DIW), stored at 20 °C and used for testing within 2 weeks. Before use, this stock suspension was sonicated for 5 min (Ultrasonicator BRANSON B-1200 E2 HF 100 W). A stock solution of dissolved Cu as $CuNO_3$ (1 g Cu L^{-1}, in 2% HNO_3 w/w) was purchased from Fluka Analytical. All the other chemicals were purchased from Sigma Aldrich.

The stability of CuO NP in both DIW (stock solution used within 2 weeks after preparation) and in the experimental medium (natural seawater) in the absence (t = 0) and presence (t = 2 d) of organisms was monitored by dynamic light scattering (DLS). A suspension of CuO nanoparticles (25 mg L^{-1}) in DIW was ultrasonicated for 10 s and the hydrodynamic diameter of the particles was measured using DLS (Malvern, Zetasizer Nano ZS). Then, 5 l L of this stock solution were spiked into natural seawater (10 l g L^{-1} Cu final concentration), and subsequently the size distribution and zeta potential of CuO nanoparticles were measured.

2.2. Animal collection and acclimatation

Worms (H. diversicolor) and bivalves (S. plana) were collected by hand from an intertidal mudflat (upper 20 cm depth) in the Bay of Bourgneuf (2°04¼40.60″W, 46°56′23.08″N), located on the West Atlantic coast (France), in July 2009. This site is monitored by the French "Mussel Watch" Programme (Réseau National d'Observation, 2006) and is documented as relatively clean (Kalman et al., 2009). Only bivalves with shell length ranging from 15 to 20 mm were selected to avoid any potential influence of sexual maturity. Worms from the same size were selected to avoid a potential influence of weight (0.40 ± 0.13 g mean wet weight) and they were collected in June which did not correspond to the period of sexual maturity (Mouneyrac et al., 2010). Then, H. diversicolor and *S. plana* were transported to the laboratory in cool boxes with sediment from the collection site. In the laboratory, animals were allowed to eliminate their gut contents and acclimatize for 48 h in aerated natural seawater, UV treated and filtered through 0.45 l m. This acclimatization time period was chosen after Poirier et al. (2006) and Burlinson and Lawrence (2007).

2.3. Exposure protocol

Bivalves (*S. plana*) were placed into 2.2 L polypropylene aquaria (12 individuals/tank) filled with 2 L seawater and worms (H. diversicolor) were introduced individually in plastic beakers of 100 mL filled with 50 mL seawater. Three treatments: (i) natural seawater only; (ii) CuO NPs: 10 l g Cu L^{-1}; and (iii) dissolved Cu: 10 l g Cu L^{-1}, were carried out in a triplicate design (three tanks per treatment for each species) during 16 d for *S. plana* and 7 d for *H. diversicolor*, using a semi-static exposure regime, in the dark at the temperature they experienced in their sediment of origin at this period of the year (21 °C). The experimental media (water and contaminant) were renewed every other day. In order to avoid interferences between food and the fate of NPs, invertebrates remained unfed during the whole experiment. Previous studies carried out in our laboratory have shown that fast did not induce any decrease of the condition index of clams or histopathological changes for both species.

2.4. Metal quantification in experimental medium

The estimation of dissolved fraction of Cu was determined during S. plana exposure in laboratory to seawater only (Controls), soluble Cu or CuO NP, by using DGT tools (diffusive gradients in thin films) (Davison and Zhang, 1994).

This technique, based on mass transport control of the chemical species of interest from water or sediment pores water, uses two hydrogel layers. A polyacrylamide gel is used as the diffusive layer, and is backed up with a second thin gel layer containing a Chelex

cation-exchange resin selective for trace metals. The diffusive layer of known thickness is placed in the DGT probe on top of the binding phase and covered with a filter used to avoid biofouling. Ions diffuse through the filter and diffusive layer to reach the Chelex resin. The mass of the diffused ion, M, can be obtained by direct measurement of the ion concentration (C_r) in the resin layer with total volume of resin V_r:

$M = C_r V_r$:

The DGT disc units (2.5 cm diameter corresponding to a 3.14 cm^2 diffusive area) were purchased from DGT Research Ltd. A Chelex-100 resin beads and a diffusive gel with a pore size of about 5 nm were used (open pore diffusive gel) (Zhang and Davison, 1999). The thickness of gel was 0.82 mm. A filter of 0.14 mm thickness and 0.45 l m pores size covered the gel.

For each condition, 8 DGT units were deployed in the tanks containing S. plana individuals and placed in temperature controlled room (22 °C). In order to avoid a bias due to the presence of DGT units in the medium, these individuals were not employed for biological tests.

Measurement of metal accumulated by the DGT units was realised on day 0, 3, 7 and 16 (2 DGT per sampling day). After sampling, DGT units were washed with ultra-pure water (Milli-Q) and placed in 1.5 mL vials. One millilitre of 1 M HNO_3 was added and left for a day to elute copper from the Chelex resin. Copper determination was performed by graphite Furnace Atomic Absorption Spectrometry (Varian SpectrAA 800) in either neat or diluted samples of the 1 M HNO_3 eluent. Mass of metal accumulated (M) by the DGT unit was calculated as follows:

$$M = C_r V_r = C_e (V_g + v_e) f_e$$

where C_e is the Cu concentration in HNO_3 (µg L^{-1}), V_g and V_e, the volumes of the gel ($0.15.10^{-3}$ L) and the eluent (1.10^{-3} L) respectively and f_e, the elution factor (0.8).

2.5. Biochemical markers

For each biomarker, 10 bivalves were collected from the three different tanks (n = 3 or n = 4 taken randomly from each tank) corresponding to each experimental condition. After exposure, the length and the total weight of bivalves were recorded. After removal of the shells, the soft tissues were carefully wiped with absorbent paper and stored at - 80 °C until biochemical analysis. For worms, following exposure, they were carefully wiped with absorbent paper, weighed individually and stored at À80 °C until biochemical analysis. For MTLP and LDH, measurements were carried out individually (n = 10 for each biomarker). For GST, CAT, SOD, TBARS and ChE, determinations were carried out on 5 pools of 2 individuals per species (*H. diversicolor*, *S. plana*) per treatment.

Because previous studies revealed that metallothionein-like protein (MTLP) determination was not a relevant biomarker of metal exposure in *H. diversicolor* (Poirier et al., 2006), MTLP analyses were performed only in bivalves (S. plana). The whole soft tissues of S. plana were homogenised at 4 °C in 20 mM TRIS, 10^{-5} mM b-mercaptoethanol, 0.1 mM Phenylmethanesulfonyl Fluoride (PMSF), 150 mM NaCl adjusted to pH = 8.6 (4 mL g^{-1} soft tissue). The soluble (S1) and insoluble (P1) fractions were separated by centrifugation at 30 000g for 30 min at 4 °C. An aliquot of the soluble fraction (S1) was heated at 75 °C for 15 min. Then MTLPs (i.e. heat-stable thiolic compounds) were isolated by centrifugation (15 000g for 10 min at 4 °C) and determined by Differential Pulse Polarography (DPP) analysis (Mouneyrac et al., 2002). The standard addition method was used for calibration with rabbit liver MT (Sigma Chemical Co., St. Louis, MO) in the absence of a marine bivalve MT standard.

For GST, CAT, SOD, TBARS and ChE, pooled soft tissues were homogenised at 4 °C to prevent enzyme or tissue degradation in TRIS buffer (TRIS 50 mM, NaCl 150 mM, DTT 1 mM, antiprotease mixture (Sigma P8340, diluted in 1/1000) adjusted to pH 7.4 in a 1:3 ratio (w:v) using a motor-driven glass-Teflon homogenizer at 500 rpm. The homogenates were then centrifuged for 25 min at 9000g. Supernatants were immediately frozen at - 80 °C as 50 µL aliquots until biomarker analysis. An aliquot of the homogenate was centrifuged (9000g for 30 min at 4 °C) and the resulting supernatant was used directly in the enzyme assay. GST activity was determined spectrophotometrically at 340 nm (ε = 9.6 mM^{-1} cm^{-1}) by monitoring the formation of 1-glutathion-2,4-dinitrobenzene, resulting from the conjugation of the substrate, 1-chloro-2,4-dinitrobenzene (CDNB), with glutathione reduced form (GSH), as described by Habig et al. (1974). Results were expressed as nmoles of glutathione conjugate produced per min and per mg protein (nmoles min^{-1} mg^{-1} protein). CAT activity was estimated spectrophotometrically as the decrease in absorbance at 240 nm (ε = 0.04 mM^{-1} cm^{-1}) due to dismutation of hydrogen peroxide (H_2O_2) according to Claiborne (1985). Results were expressed as µmoles of H_2O_2 transformed per min and per mg protein (µmoles min^{-1} mg^{-1} protein). SOD activity was determined as the degree of inhibition of cytochrome C reduction by superoxide anion radicals generated by xanthine oxydase/xanthine reaction at 550 nm (McCord and Fridovich, 1969). Results were expressed as SOD Unit per mg protein (SOD Unit mg^{-1} protein). One unit of SOD activity was defined as the amount of sample producing 50% inhibition in 1 mL reaction system per mg protein. ChE activity was determined using the method of Ellman and Courtney (1961) adapted to a microplate reader by Galgani and Bocquene (1991). Results were expressed as nmoles of thiocholine produced per min and per mg protein (nmoles min^{-1} mg^{-1} protein). In the case of *H. diversicolor*, Scaps and Borot (2000) have characterized the presence of acetylcholinesterase whereas in S. plana it is not clear if only AChE or also pseudocholinesterases are able to hydrolise the substrate used. Thus in the all paper, we will use the abbreviation ChE for cholinesterases. Lipid peroxidation was estimated by the formation of thiobarbituric acid reactive substances (TBARS), quantified by reference to MDA absorbance (ε = 1.56 x 10^5 M^{-1} cm^{-1}) at 530 nm (Halliwell and Gutteridge, 1985). Results were expressed as nmoles of TBARS produced per mg protein (nmoles of TBARS $mg^{Á1}$ protein).

For LDH determination, an aliquot of the homogenate prepared as described above for GST (but with individual specimens) was centrifuged at 3300g for 5 min at 4 °C. Measurement of LDH activity was realized with a microplate reader as described in Diamantino et al. (2001). Proteins were quantified in the supernatants according to Bradford (1976).

2.6. Metal quantification in animals

For bivalves, Cu was determined in the pellets and supernatants, the preparation of which has been described above for MTLP determination. Cu bioaccumulation was estimated in the whole soft tissues of worms (H. diversicolor, n = 9 per condition). Animal tissues were digested by heating (90 °C) with Aqua Regia (HCl/HNO_3:75/25) after preliminary tests showing that pure HNO_3 was not sufficient to insure a complete dissolution of Cu from CuO NP. Then, the quantification of Cu concentrations was performed by using Flame Atomic Absorption Spectrophotometry (FAAS, Varian SpectrAA800 spectrophotometer) with deuterium lamp background correction. Standard addition analyses were performed in an isomedium and added concentrations of Cu were 125 ng mL^{-1}, 250 ng mL^{-1}, 500 ng mL^{-1}, 1000 ng mL^{-1}. All labware were cleaned in 10% hydrochloric-acid bath for 24 h and rinsed three times with DIW before being used. The accuracy of

the analyses was checked by digesting certified material (Mussel tissue, National Institute of Standards and Technology). In the case of bivalves, the total concentration was recalculated by adding concentrations of Cu in pellet and supernatant.

2.7. Behavioural experiments

For burrowing tests, 20 bivalves were collected from the three different tanks (n = 6 or n = 7 taken randomly from each tank) corresponding to each experimental condition. For feeding tests, all the bivalves were involved (n = 36 per condition distributed in three different tanks). Both burrowing and feeding tests were carried out with 20 ragworms per condition. At the end of the behavioural tests, animals were returned in their experimental medium of origin. This procedure is acceptable since Burlinson and Lawrence (2007) have shown that burrowing organisms were not affected by consecutive behavioural assays.

2.7.1. Burrowing tests

Bivalves (*S. plana*) and worms (*H. diversicolor*) were submitted to burrowing tests as described by Bonnard et al. (2009), after being previously exposed in the laboratory for 4 d to seawater only (controls), CuO NPs or dissolved Cu as described in exposure protocol sub-section (see above). For bivalves (*S. plana*), burrowing experiments were carried out in plastic containers filled with 3 cm of natural sediment (collected from sampling site) and topped up with 2 L of seawater. Natural sediment was homogenised by hand 1 d before experimentation. Burrowing behaviour was studied by placing individuals on the surface of the sediment and observing the number which had burrowed at frequent intervals; every 5 min in the 1st hour, every 10 min in the 2nd hour, every 20 min in the 3rd and 4th hour, then every hour until 6 or 7 h of test. Twenty individuals of *H. diversicolor* were tested for each studied condition. Briefly, we used plastic clean containers of 100 mL filled with 5 cm of wet sediment from the site of origin. Twenty worms were placed individually on the sediment and their positions were recorded every two min during 30 min.

2.7.2. Feeding rate

The feeding rate was estimated for bivalves (S. plana) and worms (*H. diversicolor*) previously exposed for 11 or 7 d respectively, to seawater only (Controls), CuO NPs or soluble Cu. The feeding rate of S. plana was quantified according to the methodology described by Worrall and Widdows (1983) and adapted to our conditions. Algae *Tetraselmis suecica* supplied by IFREMER were used as food at a concentration of 10 000 cell mL^{-1} in each tank. The concentration of algae not ingested by bivalves was measured after 1 h. The feeding rate of *H. diversicolor* was quantified according to the methodology described by Moreira et al. (2005). Twenty worms were fed 100 Artemia salina larvae into their individual plastic beakers containing 50 mL of seawater. They were left undisturbed for 1 h, then the remaining larvae were collected and

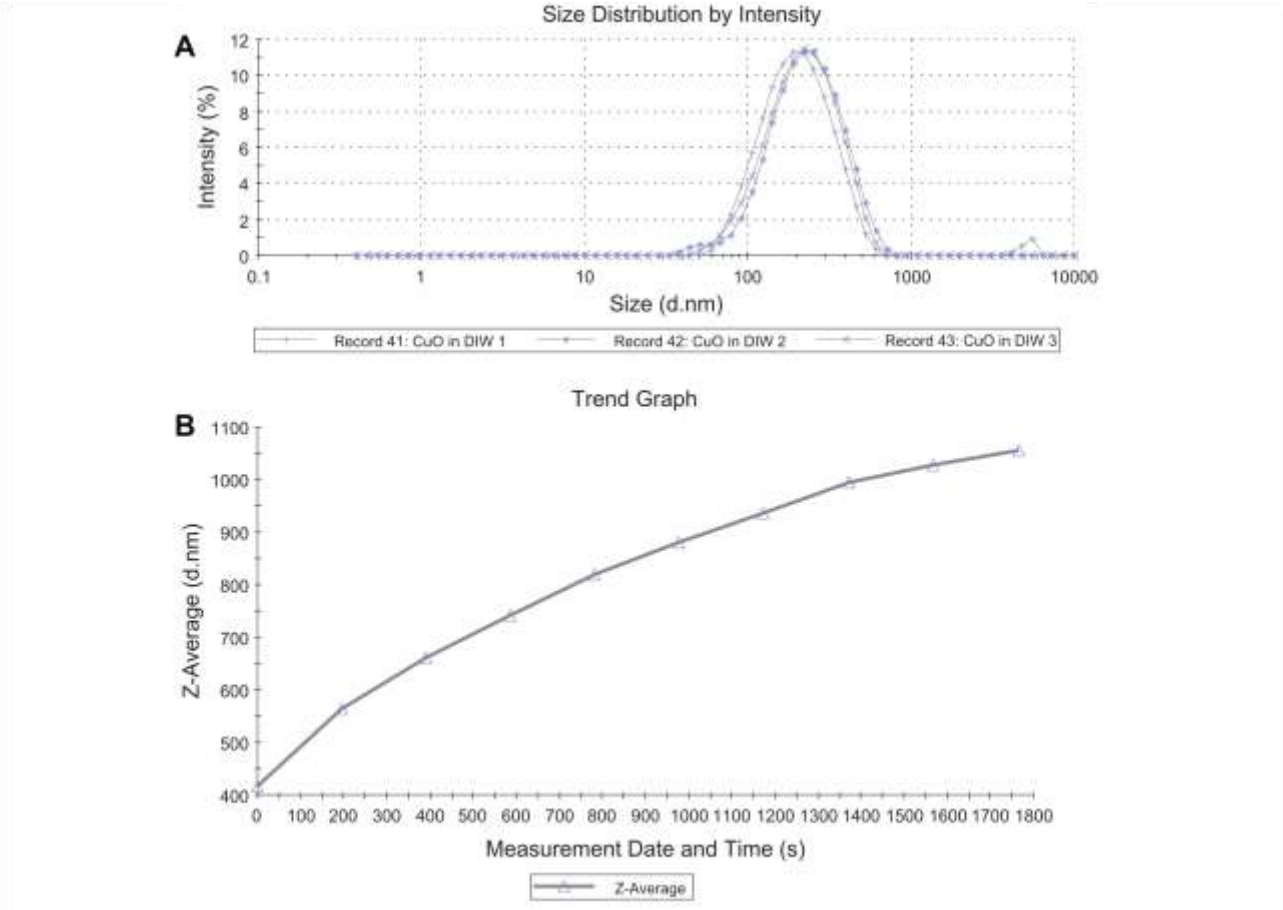


Fig. 1. Size distribution (A) of CuO nanoparticles in DIW and evolution of the size of CuO nanoparticles when a drop of sea water (t = 2 d) is incorporated in the DIW suspension (B).

counted and results were expressed as the number of larvae ingested per hour per individual.

2.8. Statistical analysis

The burrowing kinetic curves were ln-transformed in order to linearize these data and then, they were compared by using analysis of covariance (ANCOVA) between regression coefficients of the least-square best-fit regression lines. Otherwise, results are presented as mean ± SD. Significant differences were established by using one-way analysis of variance (ANOVA) or non-parametric Mann and Whitney comparison tests when variances of groups were different. Level of significance was established at p 6 0.05. Statistical analyses were performed by using XlStat pro 7.5.

3. Results

3.1. Fate of CuO nanoparticles in the experimental medium

Size distribution of CuO NP in DIW ranged from 40 nm to 500 nm with an average of 197 nm (Fig. 1A) and the prepared suspension was stable for approximately 1 month. When diluted in sea water (at both t = 0 and t = 2 d where animal stayed during 2 d), the NPs aggregate/agglomerate and the sample is not suitable for DLS measurements. Fig. 1B shows how rapidly the size of the particles increases when 5 µL of sea water is added in the DIW suspension. Zeta potential measurements show that the particles are relatively highly positively charged (26.3 mV) in DIW, which corroborates the stability of the suspension, while the NPs appear slightly negatively charged in seawater collected at t = 0 and t = 2 (- 8.69 and - 7.72 mV respectively), an indication of poor stability.

3.2. Cu in the exposure medium

Fig. 2 shows the mass of copper accumulated by DGT units during the 16 d of laboratory exposure to seawater only (Controls), dissolved Cu or CuO NPs. The results obtained for DGT exposed to CuO nanoparticles, at a nominal concentration of 10 µg of Cu L^{-1}, demonstrated that the masses of copper accumulated were similar to those accumulated by DGT units deployed in control tanks and significantly lower than those obtained with DGT units exposed to soluble copper (respectively on day 16: 0.338 ± 0.012 µg; 0.383 ± 0.055 µg, 1.017 ± 0.033 µg). This result indicates that no detectable liberation of labile copper from NPs occurred in the medium during the time of experiment.

3.3. Cu bioaccumulation

Cu concentrations in the whole soft tissues of *S. plana*, exposed in the laboratory during 16 d and H. diversicolor exposed during 7 d to seawater only (Controls), CuO NPs (10 Ì g Cu L^{-1}) and soluble Cu (10 Ì g Cu L^{-1} in 1 M of HNO_3) are illustrated in Table 1. Bivalves (S. plana) and worms (H. diversicolor) exposed to both forms of copper (CuO NPs and soluble Cu) showed significantly higher concentrations compared to controls. In addition for both species, no significant differences were observed depending on the form of Cu in the exposure medium.

3.4. Biomarkers

Measurements of biomarkers of defences and damages in bivalves (S. plana) and worms (*H. diversicolor*) are illustrated in Table 2. In both species exposed to CuO NPs, catalase and GST activities increased significantly compared to controls. In H. diversicolor, catalase activity also increased significantly after soluble Cu exposure treatments. In bivalves, SOD activity was significantly higher in animals exposed to CuO NP compared to controls or animals exposed to soluble Cu. MTLP concentrations increased in bivalves exposed to both forms of copper (soluble Cu, CuO NPs), but significantly only for soluble Cu. For both species, no damages were observed according to the results of TBARS, LDH and ChE.

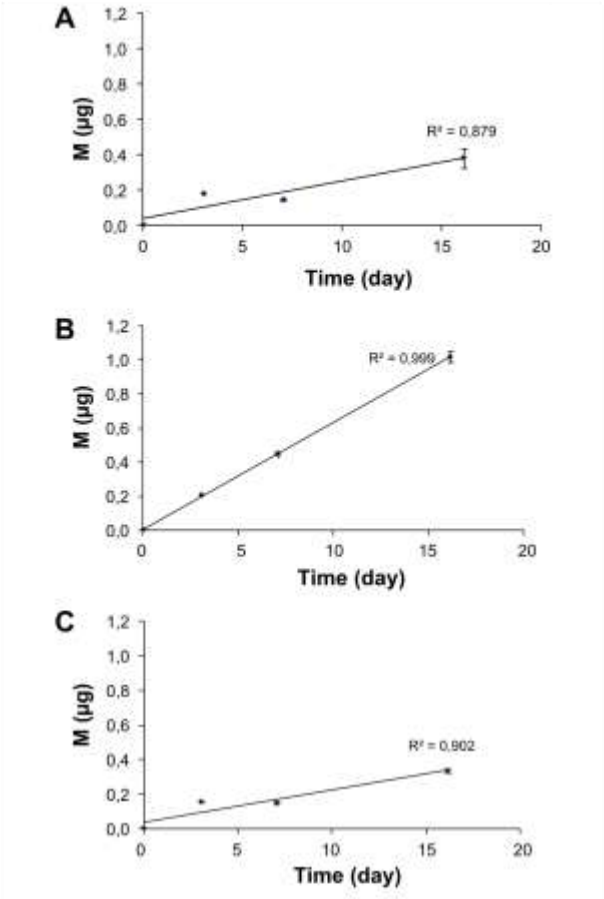


Fig. 2. Mean mass (and standard deviation) of copper accumulated (Ì g) by DGT units (n = 8) during the time of exposure. (A) Control; (B) Soluble Cu; (C) CuO NP.

Table 1
Mean (ng g^{-1} wet weight) copper concentrations (and SD between brackets) in *Scrobicularia plana* and in *Nereis diversicolor* (whole soft tissues) exposed in the laboratory to Cu (10 Ì g L^{-1}) either in the form of CuO NP (CuO NP), soluble Cu and seawater only (Controls). Concentrations with different superscripts differed significantly at the 95% level in each species.

	S. plana	*N. diversicolor*
Controls	3634 (1915)^{a}	1006 (132)^{c}
Soluble Cu	5998 (3023)^{b}	1431 (583)^{d}
CuO NP	7495 (2379)^{b}	1570 (302)^{d}

3.5. Burrowing behaviour

The burrowing behaviour of *S. plana* and H. diversicolor (previously exposed for 4 d to soluble Cu, CuO NPs as well as controls) in sediment collected from their site of origin is shown in Fig. 3A and B. Slopes and regression coefficients of the best-fit regression

Table 2
Means (and SD between brackets) of biomarkers quantified in *Nereis diversicolor* after 7 d and *Scrobicularia plana* after 16 d of exposure treatments (Controls, soluble Cu, CuO NP). For both species, biomarker levels with different superscripts differed significantly at the 95%

Biomarkers		*Scrobicularia plana*			*Nereis diversicolor*		
		Controls (n = 5)	Soluble Cu (10 l g L^{-1}) (n = 5)	CuO NP (10 l g L^{-1}) (n = 5)	Controls (n = 5)	Soluble Cu (10 l g L^{-1}) (n = 5)	CuO NP (10 l g L^{-1}) (n = 5)
Defences	CAT (µmol min^{-1} mg^{-1} protein)	66.50 (16.59)^{a}	80.98 (3.53)^{a}	119.35 (17.70)^{b}	53.27 (2.46)^{a}	78.05 (9.35)^{b}	96.51 (13.45)^{c}
	GST (nmol min^{-1} mg^{-1} protein)	260.87 (43.68)^{a}	311.40 (31.15)^{a}	423.18 (36.26)^{b}	81.19 (27.54)^{a}	94.60 (31.84)^{ab}	130.99 (11.81)^{b}
	SOD (U SOD mg^{-1} protein)	6.40 (1.25)^{a}	6.47 (0.82)^{a}	10.23 (2.67)^{b}	75.45 (9.5)	73.76 (28.00)	72.45 (3.8)
	MTLP (µg g^{A-1} tissues)	876.12 (182.23)^{a}	1060.02 (211.85)^{b}	1016.82 (130.17)^{ab}			
	LDH (nmol min^{-1} mg^{-1} protein)	20.57 (5.49)	18.97 (7.14)	23.06 (10.72)	552.03 (89.91)	514.02 (96.43)	569.50 (208.90)
Damages	TBARS (nmol MDA mg^{-1} protein)	0.52 (0.11)	0.50 (0.05)	0.53 (0.23)	0.76 (0.20)	0.77 (0.12)	0.90 (0.014)
	AChE (nmol min^{-1} mg^{-1} protein)	22.36 (6.24)	26.37 (9.28)	28.49 (10.71)	71.33 (14.23)	70.26 (17.69)	70.14 (8.61)

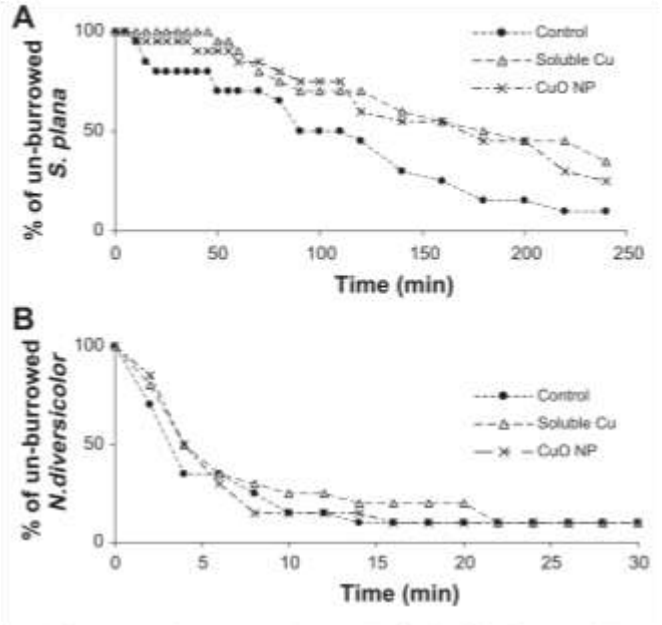


Fig. 3. Percentage of unburrowed clams (A) *Scrobicularia plana* (n = 20) and ragworms (B) *Hediste diversicolor* (n = 20) at different times (min). Animals were previously exposed to the different experimental conditions: sea water only (control o), soluble copper Δ, CuO nanoparticles x ; and allowed to burrow in their sediment of origin (Bay of Bourgneuf).

lines obtained after ln transformation of the raw data are shown in Table 3. In *S. plana,* the burrowing behaviour was significantly impaired in bivalves exposed to CuO NP or to soluble form of Cu compared to controls. In the case of H. diversicolor, only the exposure to soluble Cu led to a significant decrease of burrowing kinetics compared to controls and CuO NP exposure.

3.6. Feeding rate

Feeding rates of *S. plana* and *H. diversicolor* were exposed (controls, soluble Cu, CuO NP) for 11 d or 7 d respectively. During the first hour of feeding, bivalves (*S. plana*) in control medium showed the highest feeding rates (6051 ± 1003 cells h^{-1}) while feeding rates were significantly impaired only for specimens from CuO NP exposure medium (3680 ± 1294 cells h^{-1}).

Concerning *H. diversicolor* feeding rates were not affected by experimental conditions (not shown).

4. Discussion

4.1. Exposure

The environmental fate and ecotoxicity of engineered NPs may be influenced by a number of properties, including particle size and

Table 3
Slopes and determination coefficients of the best-fit regression lines obtained after ln transformation of the raw data shown in Fig. 3. Slopes with different superscripts differed significantly at the 95%level.

Experimental conditions	Animals	Slope	Coefficient R^2
Control	*S. plana*	- 0.0096^{a}	0.9604
Soluble Cu	*S. plana*	- 0.0043^{b}	0.9664
CuO Nanoparticles	*S. plana*	- 0.0051^{b}	0.9318
Control	*N. diversicolor*	- 0.1433^{a}	0.9478
Soluble Cu	*N. diversicolor*	- 0.0734^{b}	0.8545
CuO Nanoparticles	*N. diversicolor*	- 0.132^{a}	0.8299

size distribution, state of aggregation, charge and solubility. Following release to the environmental systems, most engineered NPs are able to aggregate to some degree. The degree and kinetics of aggregation and the size range of the aggregates is dependent on the characteristics of the particles, the characteristics of the environmental system, and the concentration of the nanoparticles. As aggregation is likely to affect toxicity, characterization of NP in experimental media is essential for nanoecotoxicology (Tiede et al., 2009). In the present study, the particle size of CuO NP specified by the manufacturer ranged from 10 to 100 nm. However, actual characterization of CuO NP in DIW showed that particle size ranged from 40 nm to 500 nm with an average of 197 nm. In seawater, the CuO NP highly aggregated/agglomerated and the hydrodynamic size (or Z-average) increased rapidly to values of around 1000 nm within the duration of the test. According to Fabrega et al. (2011) aggregation enhances the rate at which NPs sink to the bottom, where their ingestion by animals that feed on sediments is possible. This mechanism is likely to occur in the case of *S. plana* which is "primarily a deposit-feeder [which] obtains only some of its food by filtering suspended matter from the sea" Hughes (1969). *H. diversicolor*, as an opportunistic species, is considered able to fulfil its energy needs using different kinds of diet (Olivier et al., 1995). However, the aggregation/agglomeration of most NPs detected by DLS may not record the existence of some free NPs in the media, as previously expressed by Griffitt et al. (2008).

In the case of metal-containing NPs such as CuO NPs, the release of metal ions and their speciation may also be a key factor in their (eco)toxicity. The presence of organisms can affect particle dissolution in exposure medium (Griffitt et al., 2008). Thus, in this work, we studied the putative dissolution of Cu from CuO NP in real exposure (in the presence of animals) to estimate the contribution of dissolved Cu ions to overall cytotoxic effects. Solubility of copper oxide in water has been extensively studied under various pH and temperature conditions (Palmer et al., 2004). Models of bioavailability and of toxicity of copper ions have been proposed (Luoma and Rainbow, 2008). Since Zhang and Davison (1999) have described the DGT method, this has been widely used in environmental studies dealing with metal speciation in water (Pesavento et al., 2009). In the conditions of our experiment, DGT results indicated that no measurable release of labile Cu from CuO NPs occurred during the time of experiment (16 d). Griffitt et al. (2008) showed similar results, with less than 1%by mass of the original dose present in dissolved form after 48 h for copper in a freshwater medium. In contrast, Blinova et al. (2010) found, also in freshwater, higher copper dissolution (12%of copper) from CuO NP using Escherichia coli Cu-sensor. This present work demonstrates the interest of using DGT techniques as a proxy to estimate the bioavailability of metals released from NP under soluble form in the aquatic environment.

4.2. Cu bioaccumulation

As documented earlier, both bivalves and worms exposed to soluble Cu in the experimental medium have incorporated Cu. Due to the limited period of exposure, the concentrations reached at the end of the experiment were orders of magnitude lower than those uncountered in the most polluted estuaries (Bryan et al., 1980). Concentrations as high as 752 l g g^{A1} dw (about 150 l g g^{A1} ww) were determined in *S. plana* from the Erme estuary, UK and 1430 l g g^{A1} dw (about 286 l g g^{A1} ww) in *Nereis* (*Hediste*) *diversicolor* from Restronguet Creek, UK. More interestingly, the concentrations reached in each species exposed to Cu as nanoparticles were identical to those due to soluble Cu, despite DGT results did not indicate any measurable release of labile Cu from CuO NPs occurring during the time of experiment. However, NP can also be taken into the gut providing additional route for metal bioavailability. In a recent study, Galloway et al. (2010) using transmission electronic microscopy (TEM), observed that TiO_2 NPs were localized in the gut lumen in the marine polychaete *Arenicola marina*. Because we have seen that CuO NP was remarkably stable since pure HNO_3 was insufficient to solubilized them (see Section 2.6) it is less probable that they may be partially solubilized in the gut due to the moderate pH value in the gut of bivalves (pH: 4–5.2) and worms (pH: 5.5–6).

4.3. Ecotoxicity effects

The fate and ecotoxicity of particles of nanometric size in endobenthic organisms such as the bivalve mollusc *S. plana* and the ragworm *H. diversicolor* is very poorly known. Questions appeared since recent studies showed that NP can be more toxic than their bulk form (Blinova et al., 2010; Heinlaan et al., 2008) or micrometric particles (Karlsson et al., 2009). It was previously shown that the toxicity of bulk and CuO NPs was due to the solubilized bioavailable fraction, most likely Cu^{2+} ions (Heinlaan et al., 2008). Since no additional labile Cu in seawater exposure media (compared to controls) was registered during the time of experiment (16 d), we have to investigate about the specific toxicity from nanoparticulate copper.

Induction of MT, a cystein-rich protein that binds metals and thus contributes to metal detoxification, has been reported in various aquatic species under in situ or laboratory metals exposure (Amiard et al., 2006). In addition, MTLP may be involved in the defence against oxidative stress (Viarengo et al., 1999). Several studies reported that nanoparticles contribute to oxidative stress (Unfried et al., 2007). Independently of any nanotoxicity, the Cu toxicity may be caused at least partly by the generation of reactive-oxygen species (Sun et al., 2009 and literature cited therein). In this work, an increase of MTLP was observed in bivalves exposed to Cu either as CuO NPs or soluble Cu. CAT, GST and SOD have been classified as antioxidant systems of defence in various aquatic species such as bivalves (Almeida et al., 2007). CAT and GST activities increased significantly in both species (*S. plana*, *H. diversicolor*) and SOD in *S. plana* exposed to CuO NP, suggesting an oxidative stress endured by animals. In *H. diversicolor* from a contaminated estuary, an enhancement of LDH activity (up to 1.5 fold) was interpretated as an increased rate of organisms' anaerobic metabolism, suggesting a rapid need of additional energy to ameliorate chemical stress (Moreira et al., 2006). In the present study, no changes in LDH activity were observed in both species in any cases of experimental exposure. However, during progressive or prolonged anoxia, many marine invertebrates rely on different metabolic pathways (e.g. the glucose-succinate pathway) to maintain a production of metabolic energy (for a review see Livingstone, 1991). For example, succinate is a sensitive indicator of anoxia in bivalves (Oeschger and Pedersen, 1994) or in marine polychaetes (Völkel and Grieshaber, 1992).

Concerning biomarkers of damages, no alteration of TBARS levels, and ChE activities were depicted in any cases and in both species, under the experimental conditions. It may be suggested that the antioxidant defences could play their role by preventing oxidative damage. However, defence mechanisms were probably not sufficient to prevent behavioural impairments. Burrowing kinetics were significantly impaired in *S. plana* exposed to both forms of Cu (CuO NP or soluble Cu) compared to controls whereas for feeding rate only in the case of CuO NP exposure. In the case of H. diversicolor, only the exposure to soluble Cu led to a significant decrease of the burrowing kinetic of worms and feeding rate seemed not to be affected by experimental conditions. In a similar way, Wallace et al. (2000) showed that saturation of Cd-MT in grass shrimp fed cadmium-contaminated preys was associated with decreased the prey capture.

In invertebrates, tolerance to environmental contaminants is based upon a large range of chemical handling strategies. So it is not surprising that responses differ between a bivalve and a polychaete. In the case of toxic metals but also essential metals in large excess, a first line of defence is based on MT as a detoxificatory ligand. Previous works have well-established that the way MT plays a protective role is different between these taxa. The concentration of this metalloprotein is generally increased in bivalves exposed to metal pollution, providing directly a detoxificatory ligand (Amiard et al., 2006) whereas in certain polychaete species, it is MT turnover which is substantially increased in response to metal exposure, probably allowing the transfer from cytosol to detoxificatory granules (Ng et al., 2008). In *H. diversicolor* originating from highly Cu contaminated site, Cu granules were observed as a major sink for bioaccumulated Cu (Mouneyrac et al., 2003).

Links between behavioural impairment and AChE inhibition is well-documented for aquatic biota (Amiard-Triquet, 2009). In the present study, behavioural impairments were not accompanied by an inhibition of ChE activity, but metabolical or physiological disturbances due to the "cost of tolerance" could be the cause of these impairments (Bonnard et al., 2009).

5. Conclusions

In conclusion, the Cu concentrations used in this study either in the form of CuO NPs or soluble Cu were able to induce biological effects. It must be highlighted that the chosen soluble Cu concentrations of 10 Ì g L^{-1} may be encountered in polluted environments (since there is no available measured or predicted data on CuO NP concentrations). Several biomarkers of defence are activated in the presence of CuO NPs, namely GST, CAT and SOD in S. plana and CAT and GST in Hediste diversicolor. These defences may be relatively efficient since no significant effects were shown considering individually biochemical markers of neurotoxicity (ChE) or oxidative damage (TBARS). However, behavioural impairments were observed in the bivalve (*S. plana*), considering both the burrowing and feeding behaviours. For a number of biomarkers, it must be noted that biological responses are significantly more important in the presence of CuO NPs than in the presence of soluble Cu. Thus, these results suggest a specific nanoparticle effect. Finally, the use of both marine invertebrates (*S. plana*, *H. diversicolor)* and the set of biomarkers chosen in this work seem suitable to conduct ecotoxicity studies with ENPs. With a view to risk assessment it is necessary to improve the evaluation of exposure and the subsequent bioaccumulation. Future works (TEM) are in progress to localize cellular targets of CuO NPs in both species.

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References

Almeida, E.A., Bainy, A.C.D., Loureiro, A.P.M., Martinez, G.R., Miyamoto, S., Onuki, J., Barbosa, L.F., Machado Garcia, C.C., Prado, F.M., Ronsein, G.E., Sigolo, C.A., Brochini, C.B., Martins, A.M.G., Medeiros, M.H.G., DiMascio, P., 2007. Oxidative stress in *Perna perna* and other bivalves as indicators of environmental stress in the Brazilian marine environment: antioxidants, lipid peroxidation and DNA damage. Comp. Biochem. Physiol. A 146, 588–600.

Amiard, J.C., Amiard-Triquet, C., 2008. Les Biomarqueurs dans l'Évaluation de l'État Écologique des Milieux Aquatiques, Lavoisier, Paris.

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76, 160–202.

Amiard-Triquet, C., 2009. Behavioral disturbances: the missing link between sub-organismal and supra-organismal responses to stress? Prospects based on aquatic research. Hum. Ecol. Risk. Assess. 15, 87–110.

Blinova, I., Ivask, A., Heinlaan, M., Mortimer, M., Kahru, A., 2010. Ecotoxicity of nanoparticles of CuO and ZnO in natural water. Environ. Pollut. 158, 41–47.

Boldina-Cosqueric, I., Amiard, J.C., Amiard-Triquet, C., Dedourge-Geffard, O., Métais, I., Mouneyrac, C., Moutel, B., Berthet, B., 2010. Biochemical, physiological and behavioural markers in the endobenthic bivalve *Scrobicularia plana* as tools for the assessment of estuarine sediment quality. Ecotoxicol. Environ. Saf. 73, 1733–1741.

Bonnard, M., Romeo, M., Amiard-Triquet, C., 2009. Effects of copper on the burrowing behavior of estuarine and coastal invertebrates, the polychaete *Nereis diversicolor* and the bivalve *Scrobicularia plana*. Hum. Ecol. Risk Assess. 15, 11–26.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bryan, G.W., Langston, W.J., 1992. Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. Environ. Pollut. 76, 89–131.

Bryan, G.W., Langston, W.J., Hummerstone, L.G., 1980. The use of biological indicators of heavy metal contamination in estuaries. Mar. Biol. Ass. U.K., Occ. Publ. no. 1, 73 pp.

Burlinson, F.C., Lawrence, A.J., 2007. A comparison of acute and chronic toxicity tests used to examine the temporal stability of a gradient in copper tolerance of Hediste diversicolor from the Fal estuary, Cornwall, UK. Mar. Pollut. Bull. 54, 66–71.

Byrne, P.A., Halloran, J.O., 2001. The role of bivalve molluscs as tools in estuarine sediment toxicity testing: a review. Hydriobiologia 465, 209–217.

Chang, H., Jwo, C., Lo, C., Tsung, T., Kao, M., Lin, H., 2005. Rheology of CuO nanoparticle suspension prepared by ASNSS. Rev. Adv. Mater. Sci. 10, 128–132.

Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), Handbook of Methods for Oxygen Radical Research. CRC Press, Boca Raton London New York, pp. 283–284.

Davison, W., Zhang, H., 1994. In situ speciation measurements of trace components in natural waters using thin-film gels. Nature 367, 545–548.

Diamantino, T.C., Almeida, E., Soares, A.M.V.M., Guilhermino, L., 2001. Lactate deshydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* straus. Chemosphere 45, 553–560.

Eisler, R., 2007. Eisler's Encyclopedia of Environmentally Hazardous Priority Chemicals. Elsevier Science Ltd., Oxford.

Ellman, G., Courtney, K., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–90.

European Commission, 2009. Nanosciences and Nanotechnologies: An Action Plan for Europe 2005–2009. Second Implementation Raport 2007–2009. COM(2009)607 Final, Brussels, 29.10.2009.

Fabrega, J., Luoma, S., Tyler, C., Galloway, T., Lead, J., 2011. Silver nanoparticles: behaviour and effects in the aquatic environment. Environ. Int. 37, 517–531.

Gagnon, M.M., Holdway, D.A., 1999. Metabolic enzyme activities in fish gills as biomarkers of exposure to petroleum hydrocarbons. Ecotoxicol. Environ. Saf. 44, 92–99.

Galgani, F., Bocquene, G., 1991. Semi-automated colorimetric and enzymatic assays for aquatic organisms using microplate readers. Water Res. 25, 147–150.

Galloway, T., Lewis, C., Dolciotti, I., Johnston, B.D., Moger, J., Regoli, F., 2010. Sublethal toxicity of nano-titanium dioxide and carbon nanotubes in a sediment dwelling marine polychaete. Environ. Pollut. 158, 1748–1755.

Griffitt, R.J., Luo, J., Gao, J., Bonzongo, J.C., Barber, D.S., 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. Environ. Toxicol. Chem. 27, 1972–1978.

Habig, W., Pabst, M., Jakoby, W., 1974. Glutathione S-transferases. J. Biol. Chem. 249, 7130–7139.

Halliwell, B., Gutteridge, J., 1985. Free Radicals in Biology and Medicine Printed Resource. Oxford University Press, Oxford.

Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.C., Kahru, A., 2008. Toxicity of nanosized and bulk ZnO, CuO and TiO2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Chemosphere 71, 1308–1316.

Hughes, R.N., 1969. A study of feeding in Scrobicularia plana. J. Mar. Biol. Ass. UK 49, 805–823.

Kaegi, R., Ulrich, A., Sinnet, B., Vonbank, R., Wichser, A., Zuleeg, S., Simmler, H., Brunner, S., Vonmont, H., Burkhardt, M., Boller, M., 2008. Synthetic TiO_2 nanoparticle emission from exterior facades into the aquatic environment. Environ. Pollut. 156, 233–239.

Kalman, J., Palais, F., Amiard, J.C., Mouneyrac, C., Muntz, A., Blasco, J., Riba, I., Amiard-Triquet, C., 2009. Assessment of the health status of populations of the ragworm Nereis diversicolor using biomarkers at different levels of biological organisation. Mar. Ecol. Prog. Ser. 393, 55–67.

Karlsson, H.L., Gustafsson, J., Cronholm, P., Möller, L., 2009. Size-dependent toxicity of metal oxide particles: a comparison between nano- and micrometer size. Toxicol. Lett. 188, 112–118.

Lagadic, L., Caquet, T., Amiard, J.C., Ramade, F., 2000. Use of Biomarkers for Environmental Quality Assessment, first ed. CRC Press, USA.
Livingstone, D.R., 1991. Origins and evolution of pathways of anaerobic metabolism in the animal kingdom. Am. Zool. 31, 522–534 (Mangum. C. & W. van Winkle, 1973).
Luoma, S., Rainbow, P.S., 2008. Metal Contamination in Aquatic Environments: Science and Lateral Management, Cambridge University Press, Cambridge.
McCord, J., Fridovich, I., 1969. An enzymatic function for erythrocuprein. J. Biol. Chem. 244, 6049–6055.
Moreira, S.M., Moreira-Santos, M., Guilhermino, L., Ribeiro, R., 2005. A short-term sublethal in situ toxicity assay with *Hediste diversicolor* (polychaeta) for estuarine sediments based on postexposure feeding. Environ. Toxicol. Chem. 24, 2010–2018.
Moreira, S.M., Limaa, I., Ribeiro, R., Guilhermino, L., 2006. Effects of estuarine sediment contamination on feeding and on key physiological functions of the polychaete Hediste diversicolor: laboratory and in situ assays. Aquat. Toxicol. 78, 186–201.
Mouneyrac, C., Amiard, J.C., Amiard-Triquet, C., Cottier, A., Rainbow, P.S., Smith, B.D., 2002. Partitioning of accumulated trace metals in the talitrid amphipod crustacean *Orchestia gammarellus*: a cautionary tale on the use of metallothionein-like proteins as biomarkers. Aquat. Toxicol. 57, 225–242.
Mouneyrac, C., Mastain, O., Amiard, J.C., Amiard-Triquet, C., Beaunier, P., Jeantet, A.Y., Smith, B.D., Rainbow, P.S., 2003. Trace-metal detoxification and tolerance of the estuarine worm *Hediste diversicolor* chronically exposed in their environment. Mar. Biol. 143, 73–744.
Mouneyrac, C., Perrein-Ettajani, H., Amiard-Triquet, C., 2010. Influence of anthropogenic stress on fitness and behaviour of a key-species of estuarine ecosystems, the ragworm *Nereis diversicolor*. Environ. Pollut. 158, 121–128.
Ng, T.Y.T., Rainbow, P.S., Amiard-Triquet, C., Amiard, J.C., Wang, W.-X., 2008. Decoupling of cadmium biokinetics and metallothionein turnover in a marine polychaete after metal exposure. Aquat. Toxicol. 89, 47–54.
Nicholson, S., Lam, P.K.S., 2005. Pollution monitoring in Southeast Asia using biomarkers in the mytilid mussel *Perna viridis* (Mytilidae: Bivalvia). Environ. Int. 31, 121–132.
Nowack, B., Bucheli, T.D., 2007. Occurrence, behavior and effects of nanoparticles in the environment. Environ. Pollut. 150, 5–22.
Oeschger, R., Pedersen, T.F., 1994. Influence of anoxia and hydrogen sulphide on the energy metabolism of *Scrobicularia plana* (da Costa) (Bivalvia). J. Exp. Mar. Biol. Ecol. 184, 255–268.
Olivier, M., Desrosiers, G., Caron, A., Retière, C., Caillou, A., 1995. Réponses comportementales des polychètes *Nereis diversicolor* (O.F. Müller) et *Nereis virens* (Sars) aux stimuli d'ordre alimentaire: utilisation de la matière organique particulaire (algues et halophytes). Can. J. Zool 73, 2307–2317.
Palmer, D.A., Bénézeth, P., Simonson, J.M., 2004. Solubility of copper oxides in water and steam. In: Nakahara, M., Matubayasi, N., Ueno, M., Yasuoka, K., Watanabe, K. (Eds.), Proceedings of the 14th International Conference in the Properties of Water and Steam, Kyoto, pp. 491–496.
Pesavento, M., Alberti, G., Biesuz, R., 2009. Analytical methods for determination of free metal ion concentration, labile species fraction and metal complexation capacity of environmental waters: a review. Anal. Chim. Acta 631, 129–141.
Poirier, L., Berthet, B., Amiard, J.C., Jeantet, A.Y., Amiard-Triquet, C., 2006. A suitable model for the biomonitoring of trace metal bioavailabilities in estuarine sediments: the annelid polychaete *Nereis diversicolor*. J. Mar. Biol. Assoc. UK 86, 71–82.
RNO. French National Mussel Watch Programme, Ifremer, Bulletin 2006. <http://www.ifremer.fr/envlit/documents/bulletins/rno>.
Royal Commission on Environmental Pollution, 2008. Novel Materials in the Environment: The Case of Nanotechnology. Twenty Ninth Report.
Scaps, P., Borot, O., 2000. Acetylcholinesterase activity of the polychaete *Nereis diversicolor*: effects of temperature and salinity. Comp. Biochem. Physiol. 125, 377–383.
Solé, M., Kopecka-Pilarczyk, J., Blasco, J., 2009. Pollution biomarkers in two estuarine invertebrates, *Nereis diversicolor* and *Scrobicularia plana*, from a Marsh ecosystem in SW Spain. Environ. Int. 35, 523–531.
Stone, V., Nowack, B., Baun, A., van den Brink, N., Kammer, F., Dusinska, M., Handy, R., Hankin, S., Hassellöv, M., Joner, E., Fernandes, T.F., 2010. Nanomaterials for environmental studies: classification, reference material issues, and strategies for physico-chemical characterisation. Sci. Total Environ. 408, 1745–1754.
Sun, F., Zhou, Q., Wang, M., An, J., 2009. Joint stress of copper and petroleum hydrocarbons on the polychaete *Perinereis aibuhitensis* at biochemical levels. Ecotox. Environ. Saf. 72, 1887–1892.
Tiede, K., Hassellöv, M., Breitbarth, E., Chaudhry, Q., Boxall, A.B.A., 2009. Considerations for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles. J. Chromatogr. A 1216, 503–509.
Tlili, S., Métais, I., Boussetta, H., Mouneyrac, C., 2010. Linking changes at sub-individual and population levels in *Donax trunculus*: assessment of marine stress. Chemosphere 81, 692–700.
Unfried, K., Albrecht, C., Klotz, L.O., Von Mikecz, A., Grether-Beck, S., Schins, R.P.F., 2007. Cellular responses to nanoparticles: target structures and mechanisms. Nanotoxicology 1, 52–71.
Viarengo, A., Burlando, B., Cavaletto, M., Marchi, B., Ponzano, E., Blasco, J., 1999. Role of metallothionein against oxidative stress in the mussel *Mytilus galloprovincialis*. Am. J. Physiol. Regul. Integr. Comp. Physiol. 277, 1612–1619.
Völkel, S., Grieshaber, M.K., 1992. Mechanisms of sulphide tolerance in the peanut worm, *Sipunculus nudus* (Sipunculidae) and in the lugworm *Arenicola marina* (Polychaeta). J. Comp. Physiol. B 162, 469–477.
Wallace, W.G., Brouwer, T.M.H., Brouwer, M., Lopez, G.R., 2000. Alterations in prey capture and induction of metallothioneins in grass shrimp fed cadmium-contaminated prey. Environ. Toxicol. Chem. 19, 962–971.
Wiesner, M.R., Lowry, G.V., Alvarez, P., Dionysiou, D., Biswas, P., 2006. Assessing the risks of manufactured nanomaterials. Environ. Sci. Technol. 40, 4336–4345.
Worrall, C.M., Widdows, J., 1983. Physiological changes following transplantation of the bivalve *Scrobicularia plana* between three populations. Mar. Ecol. Prog. Ser. 12, 281–287.
Zhang, H., Davison, W., 1999. Diffusional characteristics of hydrogels used in DGT and DET techniques. Anal. Chim. Acta 398, 329–340.
Zhou, K., Wang, R., Xu, B., Li, Y., 2006. Synthesis, characterization and catalytic properties of CuO nanocrystals with various shapes. Nanotechnology 17, 3939–3943.

7.3 Conclusions

En présence de doses environnementales de cuivre, le comportement d'enfouissement était altéré chez le bivalve *Scrobicularia plana* quelque soit le mode d'exposition au cuivre soluble ou nanoparticulaire. Pour l'annélide polychète *Hediste diversicolor,* la perturbation n'était pas aussi marquée et elle n'était significative que pour le cuivre ajouté sous forme soluble. L'utilisation de la méthodologie des DGT (diffusive gradient in thin films) ayant montré que les nanoparticules de CuO ne libéraient pas de quantités mesurables de cuivre sous forme soluble, il apparaît que les perturbations de l'enfouissement chez la scrobiculaire sont dues aux caractéristiques physiques des NP et non à une toxicité du métal.

Le comportement d'alimentation post-exposition qui s'était révélé sensible après une exposition de *H. diversicolor* en nature, en Loire (publication 3) et dans les estuaires portuguais étudiés par Moreira et al. (2005, 2006) n'a pas mis en évidence d'effet du soluble ou nanoparticulaire.

Les expériences ultérieures menées par Pierre-Emmanuel Buffet dans le cadre de NanoReTox ont confirmé l'utilité des biomarqueurs comportementaux mis au point au cours de ma thèse pour révéler l'écotoxicité des NP (tableau 3).

Tableau 3. Réponses des biomarqueurs comportementaux dans les invertébrés endogés exposés aux nanoparticules métalliques ou aux métaux solubles. ↘ perturbation du comportement. NS pas d'effet significatif (Pierre-Emmanuel Buffet, Comm. Pers.).

NP testées et conditions d'exposition	Comportement d'enfouissement		Tests d'alimentation
	Scrobicularia plana	*Hediste diversicolor*	*Hediste diversicolor*
NP d'or	↘	↘	↘
Diamètre 5 nm	↘	↘	↘
Diamètre 15 nm	↘	↘	NS
Diamètre 40 nm			
NP d'Ag			
Voie aqueuse	NS	↘	NS
Voie alimentaire	NS	↘	NS
Ag soluble			
Voie aqueuse	NS	↘	NS
Voie alimentaire	NS	↘	NS
NP de ZnO (sédiment)	↘	↘	NS
DEG	↘	↘	↘
DEG + NP			
Cu en mésocosmes			
Nanoparticulaire	↘	NS	NS
Soluble	↘	↘	NS

CHAPITRE 8. CONCLUSIONS GENERALES ET PERSPECTIVES

8 CHAPITRE 8. CONCLUSION GENERALES ET PERSPECTIVES

8.1 Perspectives de recherche

8.1.1 Indices lipidiques de condition

Depuis que le coût de tolérance a été conceptualisé par (Calow, 1991) de nombreuses études sont venues conforter cette vision (Mouneyrac et al., 2011). Le développement de la tolérance peut-être associé à une modification de l'allocation énergétique, la part de l'énergie employée pour faire face au stress chimique n'étant plus disponible pour la croissance et la reproduction. En ce qui concerne l'une de nos espèces d'intérêt, l'annélide polychète *Hediste diversicolor*, les travaux de Durou et al. (2007b) dans l'estuaire de Seine avaient montré que l'analyse des réserves énergétiques sous forme de glycogène, lipides, et protéines s'inscrivait parfaitement dans le cadre conceptuel du coût de tolérance. Nous avons vu que pour les deux invertébrés étudiés dans l'estuaire de la Loire, avec des niveaux de contaminations moins forts, le stress chimique n'entraîne pas nécessairement des réponses claires à ce niveau global (Chapitre 5), la qualité de la ressource alimentaire et les besoins liés au cycle sexuel venant masquer l'effet du stress polluant. Dans la continuité de ce travail de thèse, nous avons donc initié une recherche pourtant sur l'importance relative des lipides de structures comme les phospholipides et les stérols et des lipides de stockage de l'énergie comme les triaglycérols.

Les indices de condition lipidiques seront examinés dans les tissus du bivalve intrasédimentaire *Scrobicularia plana* exposés à des niveaux de pollution très contrastés dans leur milieu (par exemple Seine vs Baie de Bourgneuf). Si cet indice s'avère pertinent, il pourrait être appliqué aux populations naturelles du bivalve dans des estuaires pollués et de référence. Dans cette optique, des prélèvements ont déjà été effectués en parallèle avec l'étude de l'intersexe dans treize estuaires (depuis la baie de Bourgneuf, au sud, à la baie du Mont Saint Michel au Nord ainsi que la Seine comme témoin positif). Les indices de condition lipidiques pourront être déterminés sur deux campagnes de prélèvements, en début de maturation des produits sexuels et au moment de la dissémination des produits sexuels. L'identification structurale des stérols et des acides gras sera menée séparément dans la glande digestive (organe de métabolisation) et dans la gonade où les lipides sont remobilisés

pour la production des produits génitaux. Pour chaque site et chaque stade de maturité, les analytises seront menées sur des individus dont le sexe aura été déterminé ainsi que les indices gonadosomatiques (IGS qui renseignent sur le degré de développement de la gonade). Les résultats pourront être interprétés en fonction de la qualité du milieu (présence de contaminants et de nourriture dans les sédiments superficiels, Cf Chapitre 6) et de la biologie de l'espèce. En plus de la connaissance précise du degré de maturité sexuelle qui a été évalué pour chaque population à l'aide de l'IMS (indice de maturité sexuel établi comme recommandé par Siah et al., 2003, Cf. publication 5), ces bivalves ont déjà été étudiés par Françoise DENIS (MMS Le Mans et MNHM Concarneau) qui a établi leur patron génétique. Ce projet sera mené dans le cadre du master de recherche d'Emilie Perrat (2011-2012). Il s'appuiera sur les résultats acquis par deux stagiaires co-dirigés (avec Gaëtane Wielgosz-Colin et Aurélie Couzinet-Mossion, MMS) dans le cadre de cette thèse (Sarah Dupouey, Guillaume Meynard) qui ont miniaturisé le protocole d'extraction lipidique classiquement utilisé au laboratoire pour l'adapter aux quantités comparativement minimes de tissus que représente la gonade ou la glande digestive de notre modèle de bivalve, permettant d'analyser les acides gras et les stérols des gonades et des glandes digestives de *S. plana* grâce à la chromatographie en phase gazeuse couplée à la spectrométrie de masse. Les premiers résultats montrent que, en accord avec leur rôle en tant que lipides de structure, les proportions de stérols entre les organes et les sites sont relativement identiques malgré de fortes variations d'abondance, et que le cholestérol est le stérol le plus abondant (± 40 %). La prédominance de certains acides gras a été établie (16:0; 16:1 (Δ9); 17:0 iso et 18:0) ainsi que la spécificité du 4,8,12-triméthyltridécanoate dans la glande digestive. La séparation des lipides en classes grâce à la chromatographie liquide haute performance, pourrait ouvrir d'intéressantes perspectives pour la suite de l'étude.

8.1.1.1 Automatisation des tests comportementaux

Il est bien établi d'après les données de la littérature récente que les biomarqueurs comportementaux sont d'un très grand intérêt en écotoxicologie par leur simplicité d'utilisation, leur très grande sensibilité, et leur capacité à faire le lien entre des effets au niveau infra- et supra-individuel. De plus, cette méthode non-destructive permet de mener sur les mêmes individus la détermination d'autres biomarqueurs (analyses, biochimiques,

physiologiques ou génétiques) ce qui présente un avantage manifeste dans le contexte des approches multi-marqueurs. Nos travaux dans l'estuaire de la Loire ont confirmé l'interêt des tests comportementaux chez les invertébrés endogés (Publications 2 à 4) et l'utilisation de ces biomarqueurs s'est avérée très pertinente pour l'étude en laboratoire de l'écotoxicité des nanoparticules dans des conditions aussi réalistes que possible, en particulier en terme de doses testées (Publication 7).

La littérature montre des exemples assez nombreux dans lesquels les études comportementales utilisent des techniques automatisées de capture d'images ou de traitement l'enregistrement des mouvements des animaux. Si on considère uniquement le cas des invertébrés, les plus anciennes ont porté sur la détection des contaminants par l'observation de la fermeture des valves des bivalves (Musselmonitor®, Dreissena monitor®) décrites par Baldwin et Kramer (1994). Plus récemment des techniques adaptées au bivalve endobenthique *Corbicula fluminea* ont été proposées (Tran et al., 2003, Fournier et al., 2004, Tran et al., 2004, 2007). En étroite complémentarité avec les techniques plus simples utilisées dans le cadre de cette thèse, il paraît adéquat de considérer le comportement des organismes exposés (soit en nature, soit au laboratoire) une fois qu'ils se sont ré-enfouis. Pour ce faire, le « Multispecies Freshwater Biomonitor" (MFB), un système commercialisé conçu par (Gerhardt et Schmidt, 2002), semble offrir une bonne faisabilité.

8.1.2 Perturbations de la reproduction

8.1.2.1 Perturbations endocrines : causes et conséquences

Plusieurs paramètres de la reproduction de *S. plana* sont perturbés dans les estuaires du nord-ouest de la France, avec en particulier la présence d'intersexe à tous les sites examinés chez une espèce normalement considérée comme gonochorique. Les études datant de quelques décennies ne montraient généralement pas de telles anomalies et quand c'était le cas, les taux d'intersexe restaient très limités (publication 5). Les observations de taux élevés d'intersexe ont été publiées à partir de 2006 pour les estuaires britanniques et portugais et le projet DIESE (determination of pertinent indicators for environmental monitoring : a strategy for Europe) coordonné par C. Minier dans le cadre du programme européen Interreg montre aussi à ce

jour la présence d'intersexe dans des sites situés sur la côte française de la Manche. Le phénomène n'est pas limité à *S. plana* et des altérations du sexe (féminisation ou masculinisation) ont été observées par exemple chez les moules *Mytilus edulis* (Hellou et al., 2003), *M. galloprovincialis* (Ortiz-Zarragoitia et Cajaraville, 2010); la mye *Mya arenaria* (Gagné et al., 2003), la moule d'eau douce *Elliptio complanata* (Gagné et al., 2011), ou la palourde *Gomphina veneriformis* (Ju et al., 2009). Les données de la littérature font suspecter que des perturbateurs endocriniens soient à l'origine des anomalies de la reproduction des bivalves.

Différentes molécules ayant un potentiel PE sont connues pour être des contaminants de l'environnement. En particulier, les (xéno)œstrogènes 17β-œstradiol (E2), 17α-ethinyloestradiol (EE2), octylphenol (OP) and nonylphenol (NP) dont il a été montré par (Langston et al., 2007) qu'ils contribuaient à l'induction de l'intersexe, sont fréquemment présents dans les effluents de stations d'épuration. Il n'est donc pas étonnant que la présence de PE soit ubiquitaire dans les milieux estuariens comme cela a été démontré dans des sédiments prélevés dans tout l'estuaire de la Loire à proximité de stations d'épuration (Cf sous-section 6.1) mais aussi dans la zone soumise à la marée saline des estuaires du nord-ouest de la France et dans les baies de Bourgneuf et de Saint Brieuc (bien qu'à un plus faible niveau) (publication 6).

Toutefois, nos travaux et ceux des autres équipes impliquées dans cette thématique ne montrent pas relation simple entre le taux d'intersexe et la présence de perturbateurs endocrininens (œstrogéniques, anti-androgéniques et androgéniques). Globalement, on peut invoquer des synergies ou des antogonismes entre des PE ayant des modes d'action différents mais plus fondamentalement, nous manquons de connaissances sur le mode d'action des PE. L'essentiel des modèles décrivant les interactions des PE avec leurs cibles biologiques ont été établis pour des espéces mammaliennes. Chez les poissons, les effets de molécules modèles de PE ont été bien étudiés sur l'expression des gènes réactifs à ce type de substance, en particulier chez le medaka *Oryzias latipes* (Park et al., 2009). Les effets moléculaires ont pu être au moins partiellement élucidés en associant les changements de l'expression génique avec des effets histologiques. Dans le cas des invertébrés, il est délicat d'extrapoler des connaissances acquises chez les vertébrés, même chez les poissons. D'une manière générale, les mécanismes fondamentaux de la vie sont hautement conservés au cours de l'évolution mais manifestement ce raisonnement est insuffisant et étant donné les enjeux en termes de

conservation de la qualité de l'environnement, il est essentiel de développer des recherches mécanistiques chez les invertébrés comme cela a été proposé par exemple pour les effets de la dioxine sur le développement des gonades de bivalves (Cooper et Wintermyer, 2009).

Par rapport au risque environnemental, la question fondamentale est de savoir dans quelle mesure les altérations telles que l'intersexe, la modification du sex-ratio ou l'asynchronisme de la maturation des gamètes mâles et femelles vont se traduire par des effets sur les populations. Bien que l'absence de recrutement de *S. plana* au cours des 2 ou 3 dernières années ait été observée sur trois de nos sites d'études, rien ne permet d'établir un lien direct avec les altérations de la reproduction constatées. A notre connaissance, aucune évidence nouvelle n'a été apportée depuis que (Huet et al., 2008) mentionnaient que des baisses d'effectifs pouvaient être attendues dans les populations dont la reproduction était affectée mais que, les effets populationnels dans les milieux naturels restaient largement spéculatifs. Les seuls cas avérés résultent d'une expérience en nature sur la contamination de poissons par l'EE2 (Kidd et al., 2007) et de l'exposition dans des milieux contaminés par le tributylétain (TBT) de néogastéropodes (*Purpura lapillus*) aboutissant à l'extinction locale de populations (Gubbins et al., 2010). Toutefois, selon (Cooper et Wintermyer, 2009), l'altération de la capacité de reproduction des bivalves suite à l'exposition à des perturbateurs endocriniens tels que dioxines et PCB pourrait être l'une des raisons majeures du déclin des populations de bivalves observé aux Etats-Unis dans les milieux dulcicoles et estuariens.

La présence très fréquente de molécules ayant des effets œstrogéniques, anti-androgéniques et androgéniques dans les sédiments estuariens révélée par les tests cellulaires *in vitro*, dans la présente étude mais aussi dans bien d'autres milieux aquatiques (Louiz et al., 2008, Kinani et al., 2010) même si elle ne se traduit pas systématiquement par des effets *in vivo*, est préoccupante pour l'état de santé de l'environnement. En effet, certaines de ces molécules sont susceptibles d'être incorporées dans les tissues des bivalves comme cela a été montré pour diverses espèces (e.g. TBT chez la mye *Mya arenaria* étudiée par Gagné et al. 2003 ; alkylphénol polyéthoxylates chez le bivalve d'eau douce *Elliptio complanata*, (Sabik et al., 2003); 17β-estradiol et nonylphénol chez la moule d'eau douce *Dreissena polymorpha*, (Peck et al., 2007). La préoccupation liée au risque des PE est renforcée du fait qu'il suffit de faibles doses, de l'ordre de celles rencontrées dans l'environnement, pour que se produisent des effets sur la reproduction, souvent en l'absence de tout autre évidence de toxicité.

8.1.2.2 Qualité des gamètes et des larves

Nos investigations au cours de cette thèse ont largement contribué à montrer que dans les estuaires soumis à une pression anthropique relativement importante comme c'est le cas en Loire, un nombre important de réponses biologiques sont affectées chez *S. plana* et *H. diversicolor*. Tout au long de ce travail, nous nous sommes focalisés sur des processus biologiques individuels ou infra-individuels dont le dérèglement est en lien direct ou indirect avec la reproduction. Parce qu'ils contribuent au succès de la reproduction, nous pouvons donc leur accorder une certaine valeur écologique, permettant de prévoir des effets populationnels avant que la dégradation du milieu soit telle que de telles altérations apparaissent effectivement.

En complément aux résultats acquis, il nous semblait intéressant de caractériser la qualité des gamètes et/ou des larves issues de parents ayant eu leur cycle de vie dans des milieux plus ou moins contaminés, ce qui serait encore plus directement en lien avec le succès de la reproduction. Cet objectif rencontre les conclusions d'une synthèse publiée récemment par (Lewis et Watson, 2011), recommandant d'intégrer dans les recherches écotoxicologiques des critères d'évaluation liés à la reproduction des annélides polychètes, en particulier des espèces appartenant comme *H. diversicolor* à la famille des Néréidés.

Pour ce faire, dans le cadre d'un stage de formation continue à l'international (Francesca Perisi, Bac +5, Sardaigne) que j'ai co-dirigé, nous avons mené en laboratoire des expériences visant à obtenir des pontes de *H. diversicolor* selon la méthode proposée par Marty (1997) à partir de spécimens originaires de quatre sites différant par leur degré de contamination (Baie de Bourgneuf, estuaires de la Loire, du Goyen et de la Seine, Cf. Sous-section 3.1). Dans un premier temps, nous souhaitions comparer le profil moyen de chaque ponte de femelles de *H. diversicolor* selon leur site d'origine : nombre de pontes par femelle, durée inter-ponte, temps de survie de la génitrice à partir de la première émission d'ovocytes, nombre d'ovocytes par ponte et le nombre total d'ovocytes pondus/femelle. Dans un second temps, nous souhaitions comparer la viabilité des larves issues des géniteurs de *H. diversicolor* des quatre sites d'intérêt. En dépit du concours de l'inventeur de la méthode, et bien que l'aspect des individus ait témoigné d'un développement des produits génitaux, aucune ponte n'a été obtenue sur une période de deux mois. Des fiches techniques détaillées ont été rédigées afin

de permettre la ré-édition de ces essais à MMS ultérieurement.

Les larves de bivalves sont des organismes extrêmement sensibles à la qualité de l'environnement, en particulier à la présence de contaminants chimiques, ce qui a conduit à une large utilisation des tests sur larves de bivalves en lien avec les préconisations des organismes réglementaires (Quiniou et al., 2007). Par ailleurs, l'importance des activités aquacoles basées sur les bivalves ont nécessité la mise au point de méthodes et techniques très élaborées pour estimer la capacité des géniteurs à se reproduire, en particulier la détermination de la motilité (vitesse de déplacement) des gamètes mâles à l'aide d'analyseurs d'image (Marc Suquet, écloserie d'Argenton, IFREMER). Chez *S. plana*, les paramètres principaux qu'il conviendrait de mesurer pour estimer la capacité de l'espèce à se reproduire sont i) la motilité des gamètes mâles et ii) la viabilité des larves D évaluée par le pourcentage d'anomalies larvaires détectées après fécondation et incubation des œufs selon la méthode déjà largement utilisée pour les tests règlementaires. Il a été montré que la reproduction en laboratoire était possible (Frenkiel et Mouëza, 1979) mais selon Ruiz et al. (Ruiz et al.) dans toutes les fertilisations tentées après une ponte réussie, seul un petit pourcentage de larves nageuses était observé à côté d'une majorité de matériel inerte enfermé dans le chorion. Dans le cadre d'une coopération avec Françoise Quiniou (IFREMER, Brest), nous nous sommes également trouvés confrontés à cette situation ce qui ne nous a pas permis de mener d'expériences concluantes quand à une viabilité différentielle des larves selon l'origine des géniteurs (Baie de Bourgneuf, estuaires du Bélon, de la Loire et de la Seine). Comme pour les annélides, des fiches techniques détaillées ont été rédigées afin de permettre la ré-édition de ces essais à MMS ultérieurement. Il semble que pour la motilité et la viabilité, il soit indispensable de se situer au pic de maturité sexuelle. Or ce n'est pas aisé de prévoir à quel moment se situe ce pic puisque pour un même site il change d'année en année (probablement avec les conditions de température et d'éclairement conditionnant la production primaire et la nourriture disponible) et au cours d'une même année, il ne se situe pas au même moment, dans différents sites, même proches, entre lesquels il n'y a manifestement pas de gradient latitudinal (Cf. publication 5).

8.2 Perspectives opérationnelles

8.2.1 Des biomarqueurs efficaces et économiques pour la biosurveillance

Nous avons souligné dans le chapitre 5 que parmi les biomarqueurs développés au cours de cette thèse, les biomarqueurs comportementaux ainsi que les biomarqueurs biochimiques déterminés par photospectrométrie étaient à la fois pertinents pour la surveillance de la qualité de l'environnement et d'un coût raisonnable pour une application dans les pays en développement.

Nous avons également souligné que pour les biomarqueurs à vocation écologique, leur capacité à prévoir des effets au niveau supra-individuel serait d'autant plus évidente qu'il s'agirait d'espèces ayant un rôle important dans la structure et le fonctionnement des écosystèmes.

La Chine qui connaît un grand développement économique a pris conscience de la nécessité de sauvegarder la qualité environnementale. A ce jour toutefois, les moyens pour la surveillance demeurent limités. Dans le cadre d'une coopération Université Océanique de Chine à Qingdao et Université de Nantes, il est prévu de développer un projet d'évaluation de la qualité du milieu basée sur les résultats acquis à l'aide des biomarqueurs comportementaux et biochimiques testés au cours de cette thèse. Le modèle biologique sera un bivalve endogé *Ruditapes philippinarum* très abondant en baie de Qingdao et qui donne lieu à une intense activité de pêche à pied. Il sera également possible de s'appuyer sur les espèces sentinelles classiquement utilisées dans les cadres des programmes de surveillance de type « Mussel Watch », la moule *Mytilus* sp. et l'huître *Crassostrea ariakensis*.

8.2.2 Vers une méthodologie compréhensive pour l'évaluation de l'état de santé des écosystèmes estuariens

Nous ne reviendrons pas en détail sur la méthodologie compréhensive qui a été largement explicitée dans la publication 2. Brièvement, elle comporte trois étapes :

- dans la première, il est recommandé d'utiliser des biomarqueurs à vocation écologique, capable de révéler un risque populationnel bien avant que l'état de santé

du milieu se soit tellement dégradé que des effets se manifestent sur les communautés car à ce stade la remédiation est très difficile et excessivement coûteuse. Comme nous l'avons déjà souligné, l'utilisation de ces biomarqueurs sera d'autant plus pertinente qu'ils seront déterminés dans des espèces clés pour la structure et le fonctionnement de l'écosystème étudié. Il s'agit généralement de biomarqueurs en lien avec la reproduction et beaucoup se situent à un niveau d'intégration biologique relativement élevé (biomarqueurs physiologiques et comportementaux). De ce fait, ils peuvent être affectés par différents types de stress et ne sont donc pas spécifiques, c'est-à-dire capable de révéler la (les) classe(s) de polluants majoritairement responsables des effets observés.

- Dans une seconde étape, le gestionnaire a besoin – pour pouvoir prendre des mesures de protection pertinentes – de connaître aussi précisément que possible les contaminants impliqués. Il faut alors se baser sur des biomarqueurs classiques (core biomarkers), plus spécifiques, et utilisés en batterie pour proposer des indices intégratifs ou des systèmes experts qui peuvent conduire un classement s'apparentant à l'échelle de qualité utilisée dans le cadre de la Directive Cadre sur l'Eau.

- La troisième étape consistera à valider les hypothèses basées sur les résultats des biomarqueurs en quantifiant dans le milieu les contaminants potentiellement responsables de l'altération des réponses biologiques. Toutefois, il est recommandé de ne pas se limiter à une analyse globale des contaminants car cette dose globale que ce soit dans l'eau, dans les sédiments ou dans les organismes n'a pas de signification écotoxicologique. En ce qui concerne le milieu physique, le développement de la méthodologie des capteurs passifs (tels que les DGT utilisées dans la publication 7) permet, en plus de la quantification, d'avoir accès à un certain nombre de caractéristiques physico-chimiques du contaminant qui conditionnent sa biodisponibilité et sa toxicité. En ce qui concerne les matrices biologiques, il est pertinent de s'intéresser aux métabolites (métabolites de HAP dans la bile des poissons dont l'analyse est recommandée dans le cadre d'OSPAR) et aux formes physico-chimiques de stockage dans les tissus.

Tous les écotoxicologues mais aussi les écologues qui ont cherché à développer divers indicateurs et indices ont accordé une grande importance à la formulation de la référence. Selon la terminologie de la DCE pour les éléments de qualité physico-chimique et les

éléments de qualité biologique, il convient de se référer « aux conditions non perturbées". Or à ce jour, il n'existe plus de sites « vierges » et dans le meilleur des cas, les sites de référence seront des sites aussi propres que possible, qui n'existeront manifestement pas dans les grands estuaires autour desquels vit la plus grande partie de la population mondiale mais seulement dans de petits estuaires, moins exposés aux impacts humains, mais qui diffèrent des grands par de nombreux facteurs naturels. Nous ne reviendrons pas en détail sur l'importance de prendre en compte les facteurs de confusion (publication 1), de réaliser une sélection précautionneuse pour ceux que l'on peut contrôler (taille, sexe des animaux) et pour ceux qui ne peuvent pas être contrôlés (salinité, granulométrie, taux de matière organique, hydrodynamisme, disponibilité alimentaire…), de les mesurer en parallèle des déterminations écologiques et écotoxicologiques pour en minimiser l'impact sur l'interprétation des indicateurs et indices.

Nous avons recensé dans l'introduction l'utilisation qui était faite – ou non – des biomarqueurs dans les programmes de surveillance. Nous avons souligné que la DCE qui régit la surveillance des masses d'eau de transition, incluant les estuaires, ignorait totalement l'utilisation des biomarqueurs en dépit des efforts des scientifiques européens qui ont démontré la pertinence de ces outils pour mieux atteindre les objectifs de cette directive (Allan et al., 2006, Hagger et al., 2006, Sanchez et Porchez, 2009, Fossi Tankoua et al., 2010), en particulier sous l'égide de l'ICES. Dans la mesure où i) il est absolument impossible de quantifier les milliers de molécules susceptibles de présenter un risque pour l'environnement, ii) où pour celles qui sont recherchées, la méthodologie préconisée par le DCE est peu efficace (Knoery et Claisse, 2010); iii) où les indicateurs de la DCE au niveau des assemblages et des communautés ne révèlent des effets que sur le long terme, il n'est pas surprenant de découvrir des effets néfastes comme ceux dûs aux perturbateurs endocriniens dans des sites réputés selon la DCE pour leur bon état chimique et écologique (publication 5).

Si la méthodologie préconisée par la DCE est utile pour l'évaluation rétrospective du risque, elle ne procure pas au gestionnaire de l'environnement les éléments de connaissance qui lui permettrait de faire une évaluation prospective du risque et de prendre les mesures adéquates bien avant que la dégradation de l'environnement aient pris des proportions telles qu'elles se manifestent clairement dans la structure et le fonctionnement des écosystèmes. Ceci est particulièrement vrai dans les estuaires qui sont des systèmes complexes et hautement dynamiques où – à ce niveau d'organisation biologique élevé – il est spécialement difficile de

mettre en évidence des changements dûs aux stress chimiques dans un environnement naturellement très fluctuant (Dauvin, 2007, Elliott et Quintino, 2007, Borja et al., 2011). Même si nous avons beaucoup insisté sur le problème des facteurs de confusion, il est plus maîtrisable lorsque les réponses biologiques sont examinées au niveau individuel ou infra-individuel.

L'état des connaissances permettrait une amélioration considérable de l'évaluation du risque et des procédures de surveillance environnementale. On ne peut que regretter le délai mis à intégrer les avancées scientifiques dans la réglementation et dans la gestion. Cette situation qui est aussi rencontrée dans le domaine de la santé publique, a donné lieu à la création d'une nouvelle discipline, l'agnotologie, définie en 1992 par l'historien des Sciences Robert N. Proctor. Elle consiste à étudier la manière dont la société met en œuvre des puissants mécanismes d'oblitération du savoir. Ainsi les agences de régulation ont tendance à privilégier les démarches standardisées menées selon les bonnes pratiques de laboratoire plutôt que les démarches de la recherche académique, moins standardisées mais flexibles et mieux adaptées à la recherche d'effets subtils à long terme et à faible dose. Quelle est par exemple la pertinence d'établir une CL_{50} pour un perturbateur endocrinien ? Plus précisémment dans notre domaine, l'une des raisons de cet état de fait est la plus haute technicité et parfois le coût plus élevé de certains nouveaux outils. La société est-elle prête à financer une meilleure conservation de l'environnement ? Et si oui, quel type de conservation : retrouver les sites « vierges » du Jardin d'Eden ou maintenir les fonctionnalités des estuaires en termes de bien et de services pour notre propre espèce ?

CHAPITRE 9. REFERENCES BIBLIOGRAPHIQUES

9 REFERENCES BIBLIOGRAPHIQUES

1. **Aarab N., 2004. Les biomarqueurs chez les poissons et les bivalves: de l'exposition à l'effet et du laboratoire au terrain Bordeaux: Université de Bordeaux 1. 233 pages.**

2. **Abrantes A., Pinto F., Moreira M.H., 1999. Ecology of the polychaete *Nereis diversicolor* in the Canal de Mira (Ria de Aveiro, Portugal): Population dynamics, production and oogenic cycle. Acta Oecologica 20, 267-283.**

3. **Adams S.M., Shepard K.L., Greeley M.S., Jimenez B.D., Ryon M.G., Shugart L.R., McCarthy J.F., Hinton D.E., 1989. The use of bioindicators for assessing the effects of pollutant stress on fish. Elsevier, Oxford, Royaume-Uni. 563 pages.**

4. **Alizier S., Aulert C., Bessineton C., Cuvilliez A., Denis L., Dauvin J.-C., Garcia C., Janson A.-L., Jourde J., Lesourd S. *et al.*, 2010. Le Benthos de l'estuaire de la Seine, in: Dauvin, Janson (Eds.), Fascicules Seines-Aval. Groupement d'interêt public Seine-Aval, 72 pages.**

5. **Allan I.J., Vrana B., Greenwood R., Mills G.A., Roig B., Gonzalez C., 2006. A "toolbox" • for biological and chemical monitoring requirements for the European Union's Water Framework Directive. Talanta 69, 302-322.**

6. **Alongi D.M., 1998. Coastal ecosystem processes. CRC Press, Boca Raton.**

7. **Amiard-Triquet C., 2009. Behavioral Disturbances: The Missing Link between Sub-Organismal and Supra-Organismal Responses to Stress? Prospects Based on Aquatic Research. Human and Ecological Risk Assessment: An International Journal 15, 87-110.**

8. **Amiard-Triquet C., Dauvin J.-C., 2009. Introduction, in: Amiard-Triquet, Rainbow (Eds.), Environmental Assessment of Estuarine Ecosystems. CRC Press, Boca Raton, pp. 1-17.**

9. **Amiard-Triquet C., Hummel H., Mouneyrac C., Durou C., Gillet P., 2009. Linking energy metabolism, reproduction, abundance, and structure of *Nereis diversicolor* populations, Environmental Assessment of Estuarine Ecosystems. CRC Press, Boca Raton, pp. 159-181.**

10. **Amiard-Triquet C., Rainbow P.S., Romeo M., 2011. Tolerance to Environmental Contaminants. CRC Press, Boca Raton. 464 pages.**

11. **Amiard J.-C., Amiard-Triquet C., 2008. Les biomarqueurs dans l'évaluation de l'état écologique des milieux aquatiques, Tec & Doc ed. Lavoisier, Paris. 375 pages.**

12. **Amiard J.-C., Bacheley H., Barillé A.-L., Barillé L., Geffard A., Himery N., 2004. Temporal changes in nickel and vanadium concentrations and in condition index and metallothionein levels in three species of molluscs following the "Erika" oil spill. Aquatic Living Resources 17, 281-288.**

13. **Amiard J.-C., Bodineau L., Bragigand V., Minier C., Budzinski H., 2009. Quantification of Contaminants, in: Amiard-Triquet, Rainbow (Eds.), Environmental Assessment of Estuarine Ecosystems. CRC Press, Boca Raton, pp. 31-57.**

14. **Amiard J.C., Amiard-Triquet C., Barka S., Pellerin J., Rainbow P.S., 2006. Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. Aquatic Toxicology 76, 160-202.**

15. **Amiard J.C., Caquet T., Lagadic L., 1998. Les biomarqueurs parmi les méthodes d'évaluation de la qualité de l'environnement, in: Lagadic, Caquet, Amiard, Ramade (Eds.), Utilisation de biomarqueurs pour la surveillance de la qualité de l'environnement. Lavoisier, Paris, pp. XXI-XXXI.**

16. **Andral B., Stanisiere J.Y., Sauzade D., Damier E., Thebault H., Galgani F., Boissery P., 2004. Monitoring chemical contamination levels in the Mediterranean based on the use of mussel caging. Marine Pollution Bulletin 49, 704-712.**

17. **Andrew M., O'Connor W., Dunstan R., MacFarlane G., 2010. Exposure to 17α-ethynylestradiol causes dose and temporally dependent changes in intersex, females and vitellogenin production in the Sydney rock oyster. Ecotoxicology 19, 1440-1451.**

18. **Auffan M., Rose J., Wiesner M.R., Bottero J.-Y., 2009. Chemical stability of metallic nanoparticles: A parameter controlling their potential cellular toxicity *in vitro*. Environmental**

Pollution 157, 1127-1133.

19. Baird D.J., Barber I., Calow P., 1990. Clonal Variation in General Responses of *Daphnia magna* Straus to Toxic Stress. I. Chronic Life-History Effects. Functional Ecology 4, 399-407.

20. Balaguer P., François F., Comunale F., Fenet H., Boussioux A.-M., Pons M., Nicolas J.-C., Casellas C., 1999. Reporter cell lines to study the estrogenic effects of xenoestrogens. The Science of The Total Environment 233, 47-56.

21. Baldwin I.G., Kramer K.J.M., 1994. Biological early warning systems, in: Kramer (Ed.), Biomonitoring of coastal waters and estuaries. CRC Press, Boca Raton, pp. 1-23.

22. Banta G.T., Andersen O., 2003. Bioturbation and the fate of sediment pollutants: Experimental case studies of selected infauna species. 53, 16.

23. Banta G.T., Holmer M., Jensen M.H., Kristensen E., 1999. Effects of two polychaete worms, *Nereis diversicolor* and *Arenicola marina*, on aerobic and anaerobic decomposition in a sandy marine sediment. Aquatic Microbial Ecology 19, 189-204.

24. Bard S.M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquatic Toxicology 48, 357-389.

25. Batten S.D., Hawkins L.E., Hutchinson S., Desprez M., Rybarczyk H., Paterson G.L.J., Lambshead P.J.D., Ducrotoy J.P., 1994. The ecophysiology of *Hediste (Nereis) diversicolor* O.F. Muller (Polychaeta) in the Baie de Somme, France. Mémoires du Muséum national D'histoire naturelle, 162-613.

26. Baumard P., Budzinski H., Garrigues P., Raoux C., Bellocq J., Thompson S., Philippe G., Hartmut B., Colin H.W., Jean FranÃ§ois NarbonneA2 - Philippe Garrigues H.B.C.H.W. *et al.*, 2001. Chapter 6 - Comparative study of sediment and mussel aromatic compound content in European coastal environments. Relationship with specific biomarkers, Biomarkers in Marine Organisms. Elsevier Science, Amsterdam, pp. 131-177.

27. Beliaeff B., Burgeot T., 2002. Integrated biomarker response: A useful tool for ecological risk assessment. Environmental Toxicology and Chemistry 21, 1316-1322.

28. Bergayou H., Mouneyrac C., Pellerin J., Moukrim A., 2009. Oxidative stress responses in bivalves (*Scrobicularia plana, Cerastoderma edule*) from the Oued Souss estuary (Morocco). Ecotoxicology and Environmental Safety 72, 765-769.

29. Berthet B., Leung K., Amiard-Triquet C., 2011. Inter- and Intraspecific Variability of Tolerance, in: Amiard-Triquet, Rainbow, Roméo (Eds.), Tolerance to Environmental Contaminants. CRC Press, Boca Raton, pp. 49-84.

30. Berthet B., Mouneyrac C., Amiard J.C., Amiard-Triquet C., Berthelot Y., Le Hen A., Mastain O., Rainbow P.S., Smith B.D., 2003. Accumulation and Soluble Binding of Cadmium, Copper, and Zinc in the Polychaete *Hediste diversicolor* from Coastal Sites with Different Trace Metal Bioavailabilities. Archives of Environmental Contamination and Toxicology 45, 468-478.

31. Bertin A., Inostroza P.A., Quiñones R.A., 2011. Estrogen pollution in a highly productive ecosystem off central-south Chile. Marine Pollution Bulletin 62, 1530-1537.

32. Bessineton C., 2009. Historical Records of the *Nereis diversicolor* Population in the Seine Estuary, Environmental Assessment of Estuarine Ecosystems. CRC Press, pp. 188-197.

33. Bianchi T.S., 2006. Biogeochemistry of Estuaries. Oxford University Press. 706 pages.

34. Bjerregaard L.B., Korsgaard B., Bjerregaard P., 2006. Intersex in wild roach (*Rutilus rutilus*) from Danish sewage effluent-receiving streams. Ecotoxicology and Environmental Safety 64, 321-328.

35. Bocchetti R., Fattorini D., Gambi M.C., Regoli F., 2004. Trace Metal Concentrations and Susceptibility to Oxidative Stress in the Polychaete *Sabella spallanzan* (Gmelin) (Sabellidae): Potential Role of Antioxidants in Revealing Stressful Environmental Conditions in the Mediterranean. Archives of Environmental Contamination and Toxicology 46, 353-361.

36. Boldina-Cosqueric I., Amiard J.-C., Amiard-Triquet C., Dedourge-Geffard O., Métais I., Mouneyrac C., Moutel B., Berthet B., 2010. Biochemical, physiological and behavioural markers in the endobenthic bivalve *Scrobicularia plana* as tools for the assessment of estuarine sediment

quality. Ecotoxicology and Environmental Safety 73, 1733-1741.

37. Bonnard M., Roméo M., Amiard-Triquet C., 2009. Effects of Copper on the Burrowing Behavior of Estuarine and Coastal Invertebrates, the Polychaete *Nereis diversicolor* and the Bivalve *Scrobicularia plana*. Human and Ecological Risk Assessment: An International Journal 15, 11-26.

38. Borja A., Barbone E., Basset A., Borgersen G., Brkljacic M., Elliott M., Garmendia J.M., Marques J.C., Mazik K., Muxika I. *et al.*, 2011. Response of single benthic metrics and multi-metric methods to anthropogenic pressure gradients, in five distinct European coastal and transitional ecosystems. Marine Pollution Bulletin 62, 499-513.

39. Bretaud S., Saglio P., Toutant J.-P., 2001. Effets du carbofuran sur l'activité de l'acétylcholinestérase cérébrale et sur l'activité de nage chez *Carassius auratus* (Cyprinidae). Cybium, International Journal of Ichthyology 25, 8.

40. Broeg K., Westernhagen H.v., Zander S., Körting W., Koehler A., 2005. The"bioeffect assessment index" (BAI): A concept for the quantification of effects of marine pollution by an integrated biomarker approach. Marine Pollution Bulletin 50, 495-503.

41. Bryan G.W., Gibbs P.E., Hummerstone L.G., Burt G.R., 1987. Copper, zinc, and organotin as long-term factors governing the distribution of organisms in the Fal Estuary in Southwest England. Estuarine Research Federation 10, 208-219.

42. Bryan G.W., Langston W.J., Hummerstone L.G., Burt G.R., 1985. A guide to the assessment of heavy metal contamination in estuaries using biological indicators. Occasional Publication of the Marine Biological Association 4. Marine Biological Association of the United Kingdom, 1-93.

43. Burgeot T., Forget-Leray J., Akcha X.F., Bocquené G., 2010. Acethylcholinesterase: methodology development of a biomarker and challenges of its application for biomonitoring International Council for the Exploration of the Sea, pp. 1-15. http://www.ices.dk/products/CMdocs/CM-2010/F/F2510.pdf

44. Bursztyka J., 2008. Métabolisme du Bisphénol A, de la Vinchlozoline et de la Génisteine dans les systèmes biologiques utilisés pour étudier les perturbateurs endocriniens: conséquences en terme de toxicité Toulouse: Institut National Polytechnique de Toulouse. 166 pages. http://ethesis.inp-toulouse.fr/archive/00000690/01/bursztyka.pdf

45. Buss D.S., Callaghan A., 2008. Interaction of pesticides with p-glycoprotein and other ABC proteins: A survey of the possible importance to insecticide, herbicide and fungicide resistance. Pesticide Biochemistry and Physiology 90, 141-153.

46. Byrne P.A., O'Halloran J., 2001. The role of bivalve molluscs as tools in estuarine sediment toxicity testing: a review. Hydrobiologia 465, 209-217.

47. Calow P., 1991. Physiological costs of combating chemical toxicants : ecological implications. Comparative biochemistry and physiology. C. Comparative pharmacology and toxicology 100, 3-6.

48. Cardoso P.G., Lillebø A.I., Pereira E., Duarte A.C., Pardal M.A., 2009. Different mercury bioaccumulation kinetics by two macrobenthic species: The bivalve *Scrobicularia plana* and the polychaete *Hediste diversicolor*. Marine Environmental Research 68, 12-18.

49. Cepede C., 1914. L euryhalinite de *Nereis (Hediste) diversicolor* O.F.M. Soc. Zool. France Bulletin 39, 276-289.

50. Chandler G.T., Shipp M.R., Donelan T.L., 1997. Bioaccumulation, growth and larval settlement effects of sediment-associated polynuclear aromatic hydrocarbons on the estuarine polychaete, *Streblospio benedicti* (Webster). Journal of Experimental Marine Biology and Ecology 213, 95-110.

51. Cherkaoui E., 2006. Structure et organisation des peuplements macrozoobenthiques de l'estuaire du Bou Regreg après la construction du barrage Rabat: Université Mohammed V – Agdal. 305 pages. http://toubkal.imist.ma/bitstream/123456789/152/3/THESE_CHERKAOUI.pdf

52. Chesman B.S., Langston W.J., 2006. Intersex in the clam *Scrobicularia plana*: a sign of endocrine disruption in estuaries? Biology letters 2, 420-422.

53. Coelho J.P., Nunes M., Dolbeth M., Pereira M.E., Duarte A.C., Pardal M.A., 2008. The role of

two sediment-dwelling invertebrates on the mercury transfer from sediments to the estuarine trophic web. Estuarine, Coastal and Shelf Science 78, 505-512.

54. Cohen J.E., Small C., Mellinger A., Gallup J., Sachs J., 1997. Estimates of Coastal Populations. Science 278, 1209-1213.

55. Colborn T., vom Saal F.S., Soto A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101, (3) 378-384.

56. Cong Y., Banta G.T., Selck H., Berhanu D., Valsami-Jones E., Forbes V.E., 2011. Toxic effects and bioaccumulation of nano-, micron- and ionic-Ag in the polychaete, *Nereis diversicolor*. Aquatic Toxicology 105, 403-411.

57. Cooper K.R., Wintermyer M., 2009. A Critical Review: 2,3,7,8-Tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) Effects on Gonad Development in Bivalve Mollusks. Journal of Environmental Science and Health, Part C 27, 226-245.

58. Cuny P., Miralles G., Cornet-Barthaux V., Acquaviva M., Stora G., Grossi V., Gilbert F., 2007. Influence of bioturbation by the polychaete *Nereis diversicolor* on the structure of bacterial communities in oil contaminated coastal sediments. Marine Pollution Bulletin 54, 452-459.

59. Dagnino A., Allen J.I., Moore M.N., Broeg K., Canesi L., Viarengo A., 2007. Development of an expert system for the integration of biomarker responses in mussels into an animal health index. Biomarkers 12, 155-172.

60. Dales R.P., 1950. The reproduction and larval development of *Nereis diversicolor* O. F. Müller. Journal of the Marine Biological Association of the United Kingdom 29, 321-360.

61. Dales R.P., Kennedy G.Y., 1954. On the diverse colours of *Nereis diversicolor*. Journal of the Marine Biological Association of the United Kingdom 33, 699-708.

62. Dauvin J.-C., 2007. Paradox of estuarine quality: Benthic indicators and indices, consensus or debate for the future. Marine Pollution Bulletin 55, 271-281.

63. Dayton P., Curran S., Kitchingman A., Wilson M., Catenazzi A., Restrepo J., Birkeland C., Blaber S., Saifullah S., Branch G. *et al.*, 2005. Coastal Systems, in: Hassan, Scholes, Ash (Eds.), Ecosystems and Human Well-being: Current State and Trends, Volume 1. Island Press, Washington, DC, pp. 513-549.

64. De Coen W.M., Janssen C.R., Segner H., 2001. The Use of Biomarkers in *Daphnia magna* Toxicity Testing V. *In Vivo* Alterations in the Carbohydrate Metabolism of *Daphnia magna* Exposed to Sublethal Concentrations of Mercury and Lindane. Ecotoxicology and Environmental Safety 48, 223-234.

65. De Lafontaine Y., Gagné F., Blaise C., Costan G., Gagnon P., Chan H.M., 2000. Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and monitoring of water quality of the St Lawrence River (Canada). Aquatic Toxicology 50, 51-71.

66. Dean H.K., 2008. The use of polychaetes (Annelida) as indicator species of marine pollution: a review. Revista de Biología Tropical (International Journal of Tropical Biology and Conservation) 56 (Suppl. 4), 11-38.

67. Dedourge-Geffard O., Palais F., Biagianti-Risbourg S., Geffard O., Geffard A., 2009. Effects of metals on feeding rate and digestive enzymes in *Gammarus fossarum*: An in situ experiment. Chemosphere 77, 1569-1576.

68. Denison M.S., Nagy S.R., 2003. Activation of the Aryl Hydrocarbon Receptor by Structurally Diverse Exogenous and Endogenous Chemicals. Annual Review of Pharmacology and Toxicology 43, 309-334.

69. Depledge M.H., 1994. The rationale basis for the use of biomarkers as ecotoxicological tools. , in: Fossi, Leonzio (Eds.), Non destructive biomarkers in vertebrates. Lewis Publishers, pp. 271-296.

70. Desprez M., Ducrotoy J.P., Sylvand B., 1986. Fluctuations naturelles et évolution artificielle des biocénoses macrozoobenthiques intertidales de trois estuaires des côtes françaises de la Manche. Hydrobiologia 142, 249-270.

71. Devaux A., Fiat L., Gillet C., Bony S., 2011. Reproduction impairment following paternal genotoxin exposure in brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*). Aquatic

Toxicology 101, 405-411.

72. Duport E., Stora G., Tremblay P., Gilbert F., 2006. Effects of population density on the sediment mixing induced by the gallery-diffusor *Hediste (Nereis) diversicolor* O.F. Müller, 1776. Journal of Experimental Marine Biology and Ecology 336, 33-41.

73. DuRant S.E., Hopkins W.A., Talent L.G., 2007. Impaired terrestrial and arboreal locomotor performance in the western fence lizard (*Sceloporus occidentalis*) after exposure to an AChE-inhibiting pesticide. Environmental Pollution 149, 18-24.

74. Durou C., 2006. Recherche d'indicateurs de l'état physiologique de l'annélide polychète endogée *Nereis diversicolor* en relation avec la qualité du milieu Nantes: Université de Nantes. 201 pages.

75. Durou C., Mouneyrac C., 2007. Linking steroid hormone levels to sexual maturity index and energy reserves in *Nereis diversicolor* from clean and polluted estuaries. General and Comparative Endocrinology 150, 106-113.

76. Durou C., Mouneyrac C., Amiard-Triquet C., 2008. Environmental quality assessment in estuarine ecosystems: Use of biometric measurements and fecundity of the ragworm *Nereis diversicolor* (Polychaeta, Nereididae). Water Research 42, 2157-2165.

77. Durou C., Poirier L., Amiard J.-C., Budzinski H., Gnassia-Barelli M., Lemenach K., Peluhet L., Mouneyrac C., Roméo M., Amiard-Triquet C., 2007a. Biomonitoring in a clean and a multi-contaminated estuary based on biomarkers and chemical analyses in the endobenthic worm *Nereis diversicolor*. Environmental Pollution 148, 445-458.

78. Durou C., Smith B.D., Roméo M., Rainbow P.S., Mouneyrac C., Mouloud M., Gnassia-Barelli M., Gillet P., Deutsch B., Amiard-Triquet C., 2007b. From biomarkers to population responses in *Nereis diversicolor*: Assessment of stress in estuarine ecosystems. Ecotoxicology and Environmental Safety 66, 402-411.

79. Eisler R., 2007. Eisler's Encyclopedia of Environmentally Hazardous Priority Chemicals. Elsevier, Potomac, MD,.

80. Elliott M., Quintino V., 2007. The Estuarine Quality Paradox, Environmental Homeostasis and the difficulty of detecting anthropogenic stress in naturally stressed areas. Marine Pollution Bulletin 54, 640-645.

81. Engelsen A., Pihl L., 2008. Grazing effects by *Nereis diversicolor* on development and growth of green algal mats. Journal of Sea Research 59, 228-236.

82. Engenheiro E.L., Hankard P.K., Sousa J.P., Lemos M.F., Weeks J.M., Soares A.M.V.M., 2005. Influence of dimethoate on acetylcholinesterase activity and locomotor function in terrestrial isopods. Environmental toxicology and chemistry 24, 603-609.

83. Esnault G., Retière C., Lambert R., 1990. Food resource partitioning in a population of *Nereis diversicolor* (Annelida, Polychaeta) under experimental conditions, in: Barnes, Gibson (Eds.), Trophic relationships in the marine environment. Aberdeen University Press, Aberdeen, pp. 453-467.

84. Esselink P., Zwarts L., 1989. Seasonal trend inburrow depth and tidal variation in feeding activity of *Nereis diversicolor*. Marine Ecology Progress Series 56, 243-254.

85. Farrington J.W., Tripp B.W., States. U., 1995. International Mussel Watch Project : initial implementation phase, final report. U.S. Dept. of Commerce, National Oceanic and Atmospheric Administration, National Ocean Service, Office of Ocean Resources Conservation and Assessment, Silver Spring, Md. 22 pages. http://www.biodiversitylibrary.org/item/22841

86. Fauchald K., Jumars P.A., 1979. The diet of worms : a study of polychaete feeding guilds. Oceanogr. Mar. Biol. Annu. Rev. 17, 193-284.

87. Fernandes S., Sobral P., Alcântara F., 2009. *Nereis diversicolor* and Copper contamination effect on the erosion of cohesive sediments: A flume experiment. Estuarine, Coastal and Shelf Science 82, 443-451.

88. Ferreira F., Santos M.M., Castro L.F.C., Reis-Henriques M.A., Lima D., Vieira M.N., Monteiro N.M., 2009. Vitellogenin gene expression in the intertidal blenny *Lipophrys pholis*: A new sentinel species for estrogenic chemical pollution monitoring in the European Atlantic coast? Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 149, 58-64.

89. **Fidalgo E Costa P., Oliveira R.F., Cancela da Fonseca L., 2006. Feeding Ecology of *Nereis diversicolor* (O.F. Müller) (Annelida, Polychaeta) on Estuarine and Lagoon Environments in the Southwest Coast of Portugal. Pan-American Journal of Aquatic Sciences 1, 114-126.**

90. **Flammarion P., 2000. Mesure d'un biomarqueur de pollution chez les poissons d'eau douce. Cemagref, Lyon. 126 pages.**

91. **Flammarion P., Devaux A., Garric J., 2000. Marqueurs biochimiques de pollution dans les écosystèmes aquatiques continentaux. Exemples d'utilisation et perspectives pour le gestionnaire. Bulletin Français de la Pêche et de la Pisciculture, 209-226.**

92. **Fong P.P., Garthwaite R.L., 1994. Allozyme electrophoretic analysis of the *Hediste limnicola — H. diversicolor — H. japonica* species complex (Polychaeta: Nereididae) Marine Biology 118, 463-470.**

93. **Fossi Tankoua O., Amiard J.C., Amiard-Triquet C., Berthet B., Mouneyrac C., Rainbow P.S., 2010. Towards a comprehensive methodology for assessing the health status of coastal and estuarine ecosystems International Council for the Exploration of the Sea, Nantes, pp. 1-20. http://www.ices.dk/products/CMdocs/CM-2010/F/F1510.pdf**

94. **Fournier E., Tran D., Denison F., Massabuau J.-C., Garnier-Laplace J., 2004. Valve closure response to uranium exposure for a freshwater bivalve (*Corbicula fluminea*): Quantification of the influence of pH. Environmental Toxicology and Chemistry 23, 1108-1114.**

95. **François F., Poggiale J.-C., Durbec J.-P., Stora G., 1997. A New Approach for the Modelling of Sediment Reworking Induced by a Macrobenthic Community. Acta Biotheor 45, 295-319.**

96. **Frenkiel L., Mouëza M., 1979. Développement larvaire de deux Tellinacea, *Scrobicularia plana* (Semelidae) et *Donax vittatus* (Donacidae). Marine Biology 55, 187-195.**

97. **Fu-hsiung L., Anthony T., Richard F., Scott M., Izabela W., 2003. CSO Considerations, Management of Combined Sewer Overflows. CRC Press. 272 pages.**

98. **Gagné F., Blaise C., Pellerin J., Gauthier-Clerc S., 2002. Alteration of the biochemical properties of female gonads and vitellins in the clam *Mya arenaria* at contaminated sites in the Saguenay Fjord. Mar Environ Res 53, 295-310.**

99. **Gagné F., Blaise C., Pellerin J., Pelletier E., Douville M., Gauthier-Clerc S., Viglino L., 2003. Sex alteration in soft-shell clams (*Mya arenaria*) in an intertidal zone of the Saint Lawrence River (Quebec, Canada). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 134, 189-198.**

100. **Gagné F., Bouchard B., André C., Farcy E., Fournier M., 2011. Evidence of feminization in wild *Elliptio complanata* mussels in the receiving waters downstream of a municipal effluent outfall. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 153, 99-106.**

101. **Galloway T., Goven A., 2006. Invertebrate Immunotoxicology. Immunotoxicology and Immunopharmacology, Third Edition, 365-383.**

102. **García-Alonso J., Hoeger U., Rebscher N., 2006. Regulation of vitellogenesis in *Nereis virens* (Annelida: Polychaeta): Effect of estradiol-17[beta] on eleocytes. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 143, 55-61.**

103. **Geffard A., 2001. Réponse du biota à la contamination polymétallique d'un milieu estuarien, La Gironde, FR: Exposition, Imprégnation, Induction d'une protéine de détoxication, la métallothionéine, impact au niveau individuel et populationnel Nantes: Université de Nantes.251 pages.**

104. **Geracitano L.A., Bocchetti R., Monserrat J.M., Regoli F., Bianchini A., 2004. Oxidative stress responses in two populations of *Laeonereis acuta* (Polychaeta, Nereididae) after acute and chronic exposure to copper. Marine Environmental Research 58, 1-17.**

105. **Gercken J., Sordyl H., 2002. Intersex in feral marine and freshwater fish from northeastern Germany. Marine Environmental Research 54, 651-655.**

106. **Geret F., Serafim A., Bebianno M.J., 2003. Antioxidant Enzyme Activities, Metallothioneins and Lipid Peroxidation as Biomarkers in *Ruditapes decussatus*? Ecotoxicology 12, 417-426.**

107. **Gerhardt A., Schmidt S., 2002. The multispecies freshwater biomonitor a potential new tool for sediment biotests and biomonitoring. Journal of Soils and Sediments 2, 67-70.**

108. **Gilbert F., François F., Stora G., Bertrand J.-C., 1997. Inhibition de la dénitrification par les hydrocarbures dans les sédiments marins. Rôle de Nereis diversicolorInhibition of denitrification by hydrocarbons in marine sediments. Role of *Nereis diversicolor*. Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie 320, 819-824.**

109. **Gillet P., Mouloud M., Durou C., Deutsch B., 2008. Response of *Nereis diversicolor* population (Polychaeta, Nereididae) to the pollution impact - Authie and Seine estuaries (France). Estuarine, Coastal and Shelf Science 76, 201-210.**

110. **Gillet P., Torresani S., 2003. Structure of the population and secondary production of *Hediste diversicolor* (O.F. Müller, 1776), (Polychaeta, Nereidae) in the Loire estuary, Atlantic Coast, France. Estuarine, Coastal and Shelf Science 56, 621-628.**

111. **GIP Loire Estuaire G.d.I.P.L.e., 2003. Les peuplements, Les indicateurs. GIP Loire Estuaire, Nantes. http://www.loire-estuaire.org/documents/pdf/CMB_040105_L2C1.pdf**

112. **Gofas S., 2011. *Scrobicularia plana* (da Costa, 1778). World Register of Marine Species. http://www.marinespecies.org/aphia.php?p=taxdetails&id=141424**

113. **Gomes T., Gonzalez-Rey M., Bebianno M.J., 2009. Incidence of intersex in male clams *Scrobicularia plana* in the Guadiana Estuary (Portugal). Ecotoxicology (London, England) 18, 1104-1109.**

114. **Gong J., Ran Y., Chen D.-Y., Yang Y., 2011. Occurrence of endocrine-disrupting chemicals in riverine sediments from the Pearl River Delta, China. Marine Pollution Bulletin 63, 556-563.**

115. **Grant A., Hateley J.G., Jones N.V., 1989. Mapping the ecological impact of heavy metals on the estuarine polychaete *Nereis diversicolor* using inherited metal tolerance. Marine Pollution Bulletin 20, 235-238.**

116. **Green J., 1957. The growth of Scrobicularia plana (da costa) in the Gwendraeth estuary. Journal of the Marine Biological Association of the United Kingdom 36, 41-47.**

117. **Grémare A., Olive P.J.W., 1986. A preliminary study of fecundity and reproductive effort in two polychaetous annelids with contrasting reproductive strategies. Anglais 9, 1-16.**

118. **Gubbins M.J., Moore J., Fryer R., Davies I.M., 2010. Long time series data showing recent recovery of gastropod populations from effects of Tri-butyl Tin at the Shetland oil terminal, International Council for the Exploration of the Sea. , pp. 1-12.**

119. **Guelorget O., Mazoyer-Mayere C., 1983. Croissance, biomasse et production de *Scrobicularia plana* dans une lagune méditerranéenne: l'étang du Prévost à Palavas (Hérault, France). Multilingue 5, 13-22.**

120. **Guerra-García J., Maestre M., González A., García-Gómez J., 2006. Assessing a Quick Monitoring Method Using Rocky Intertidal Communities as a Bioindicator: A Multivariate Approach in Algeciras Bay. Environmental Monitoring and Assessment 116, 345-361.**

121. **Guerreiro J.A., 1991. The gametogenic cycle of *Scrobicularia plana* (da Costa, 1778) (Mollusc: Bivalve) in Guadalquivir estuary (Cádiz, SW Spain). Aquaculture 217, 155-166.**

122. **Guerreiro J.A., 1998. Growth and production of the bivalve *Scrobicularia plana* in two southern European estuaries. Vie et Milieu 48, 121-131.**

123. **Guillette Jr L.J., Gunderson M., 2001. Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. Reproduction 122, 857-864.**

124. **Hagger J.A., Jones M.B., Leonard D.P., Owen R., Galloway T.S., 2006. Biomarkers and integrated environmental risk assessment: Are there more questions than answers? Wiley Periodicals, Inc., pp. 312-329.**

125. **Hagger J.A., Jones M.B., Lowe D., Leonard D.R.P., Owen R., Galloway T.S., 2008. Application of biomarkers for improving risk assessments of chemicals under the Water Framework Directive: A case study. Marine Pollution Bulletin 56, 1111-1118.**

126. **Hamza-Chaffai A., Pellerin J., Amiard J.C., 2003. Health assessment of a marine bivalve *Ruditapes decussatus* from the Gulf of Gabès (Tunisia). Environment International 28, 609-617.**

127. **Hansen K., Kristensen E., 1998. The impact of the polychaete *Nereis diversicolor* and enrichment with macroalgal (*Chaetomorpha linum*) detritus on benthic metabolism and nutrient dynamics in organic-poor and organic-rich sediment. Journal of Experimental Marine Biology and Ecology 231, 201-223.**

128. **Hateley J.G., Grant A., Jones N.V., 1989. Heavy metal tolerance in estuarine populations of *Nereis diversicolor*, in: Ryland, Tyler (Eds.), Reproduction, genetics and distribution of marine organisms. Proc 23rd Eur. Mar. Biol. Symp., pp. 379-385.**

129. **Hayakawa K., Bekki K., Yoshita M., Tachikawa C., Kameda T., Tang N., Toriba A., Hosoi S., 2011. Estrogenic/Antiestrogenic Activities of Quinoid Polycyclic Aromatic Hydrocarbons. Journal of Health Science 57, 274-280.**

130. **Hellou J., Yeats P., Steller S., Gagné F., 2003. Chemical contaminants and biological indicators of mussel health during gametogenesis. Environmental Toxicology and Chemistry 22, 2080-2087.**

131. **Heugens E.H., Hendriks A., Dekker A.J., Van Straalen N.M., Admiraal W., 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Critical Reviews in Toxicology 31, 247-284.**

132. **Hobbie J.E., 2000. Estuarine science: the key to progress in coastal ecological research, in: Hobbie (Ed.), Estuarine Science: A Synthetic Approach to Research and Practice. Island Press, Washington DC, pp. 1-11.**

133. **Holloway G.J., Sibly R.M., Povey S.R., 1990. Evolution in Toxin-Stressed Environments. Functional Ecology 4, 289-294.**

134. **Holthe T., 1991. Identification of Annelida Polychaeta from northern European and adjacent Arctic waters. NTNU Vitenskapsmuseet, Trondheim. 30 pages. http://urn.kb.se/resolve?urn=urn:nbn:no:ntnu:diva-8025**

135. **Hopkins W.A., Staub B.P., Snodgrass J.W., Taylor B.E., DeBiase A.E., Roe J.H., Jackson B.P., Congdon J.D., 2004. Responses of benthic fish exposed to contaminants in "outdoor microcosms"examining the ecological relevance of previous laboratory toxicity tests. Aquatic Toxicology 68, 1-12.**

136. **Hopkins W.A., Winne C.T., DuRant S.E., 2005. Differential swimming performance of two natricine snakes exposed to a cholinesterase-inhibiting pesticide. Environmental Pollution 133, 531-540.**

137. **Huet M., Le Goïc N., Koken M., 2009. Intensité de l'imposex chez *Nucella lapillus* le long des côtes de la manche et de l'atlantique en 2009. Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), Paris, Nantes. http://envlit.ifremer.fr/content/download/80707/550777/version/2/file/ImposexRapportFinal_2009.pdf**

138. **Huet M., Minier C., Pellerin J., 2008. Les perturbateurs endocriniens: études de cas, in: Amiard, Amiard-Triquet (Eds.), Les biomarqueurs dans l'évaluation de l'état écologique des milieux aquatiques. Lavoisier, Paris, France, pp. 179-210.**

139. **Hughes R.N., 1969. A study of feeding in *Scrobicularia plana*. Journal of the Marine Biological Association of the United Kingdom 49, 805-823.**

140. **Hughes R.N., 1970. Population Dynamics of the Bivalve *Scrobicularia plana* (Da Costa) on an Intertidal Mud-Flat in North Wales. Journal of Animal Ecology 39, 333-356.**

141. **Hughes R.N., 1971. Reproduction of *Scrobicularia plana* Da Costa (Pelecypoda: Semelidae) in North Wales. The Veliger 14, 77- 81.**

142. **ICES, 2007. Report of the ICES/OSPAR Workshop on Integrated Monitoring of Contaminants and their Effects in Coastal and Open-sea Areas (WKIMON III). ICES Headquarters, Copenhagen, 209 pages.**

143. **ICES, 2009. Report of the Joint ICES/OSPAR Study Group on Integrated Monitoring of Contaminants and Biological Effects (SGIMC), Copenhagen, 64 pages.**

144. **Isobe T., Oshihoi T., Hamada H., Nakayama K., Yamada T.K., Tajima Y., Amano M., Tanabe S., 2011. Contamination status of POPs and BFRs and relationship with parasitic infection in**

finless porpoises (*Neophocaena phocaenoides*) from Seto Inland Sea and Omura Bay, Japan. Marine Pollution Bulletin 63, 564-571.

145. Jager T., Klok C., 2010. Extrapolating toxic effects on individuals to the population level: the role of dynamic energy budgets. Philosophical Transactions of the Royal Society B: Biological Sciences 365, 3531-3540.

146. Jawich D., 2006. Etude de la toxicité de pesticites vis-àvis de deux genres de levures: approche cinétique et moléculaire Toulouse: Institut National Polytechnique de Toulouse- Université Saint-Joseph de Beyrouth. 134 pages.

147. Jørgensen A., Giessing A.M.B., Rasmussen L.J., Andersen O., 2008. Biotransformation of polycyclic aromatic hydrocarbons in marine polychaetes. Marine Environmental Research 65, 171-186.

148. Ju S.M., Park J.J., Lee J.S., 2009. Induction of Intersex and Masculinization of the Equilateral Venus, *Gomphina veneriformis* (Bivalvia: Veneridae) by Zinc. Animal Cells and Systems 13, 339-344.

149. Jugan M.L., Oziol L., Bimbot M., Huteau V., Tamisier-Karolak S., Blondeau J.P., Lévi Y., 2009. *In vitro* assessment of thyroid and estrogenic endocrine disruptors in wastewater treatment plants, rivers and drinking water supplies in the greater Paris area (France). Science of The Total Environment 407, 3579-3587.

150. Kaiser J., 2001. Bioindicators and Biomarkers of Environmental Pollution and Risk Assessment, illustrated edition ed. Taylor and Francis Inc. 240 pages.

151. Kalman J., Buffet P.E., Amiard J.C., Denis F., Mouneyrac C., Amiard-Triquet C., 2010. Assessment of the influence of confounding factors (weight, salinity) on the response of biomarkers in the estuarine polychaete *Nereis diversicolor*. Biomarkers 15, 461-469.

152. Kalman J., Palais F., Amiard J.C., Mouneyrac C., Muntz A., Blasco J., Riba I., Amiard-Triquet C., 2009. Assessment of the health status of populations of the ragworm *Nereis diversicolor* using biomarkers at different levels of biological organisation. Marine Ecology Progress Series 393, 55-67.

153. Kavlock R.J., Daston G.P., DeRosa C., Fenner-Crisp P., Gray L.E., Kaattari S., Lucier G., Luster M., Mac M.J., Maczka C. *et al.*, 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ Health Perspect 104, 715-740.

154. Kennish M.J., 1999. Estuary Restoration and Maintenance: The National Estuary Program. Taylor & Francis, Inc. CRC Press. 376 pages.

155. Kidd K.A., Blanchfield P.J., Mills K.H., Palace V.P., Evans R.E., Lazorchak J.M., Flick R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences 104, 8897-8901.

156. Kinani S., Bouchonnet S., Creusot N., Bourcier S., Balaguer P., Porcher J.-M., Aït-Aïssa S., 2010. Bioanalytical characterisation of multiple endocrine- and dioxin-like activities in sediments from reference and impacted small rivers. Environmental Pollution 158, 74-83.

157. Klaassen C.D., Liu J., Choudhuri S., 1999. METALLOTHIONEIN: An Intracellular Protein to Protect Against Cadmium Toxicity. Annual Review of Pharmacology and Toxicology 39, 267-294.

158. Klaine S.J., Alvarez P.J.J., Batley G.E., Fernandes T.F., Handy R.D., Lyon D.Y., Mahendra S., McLaughlin M.J., Lead J.R., 2008. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. Environmental Toxicology and Chemistry 27, 1825-1851.

159. Knoery J., Claisse D., 2010. Insights from 30 years of experience in running the French marine chemical monitoring network., International Council for the Exploration of the Sea, Nantes, pp. 1-9. http://www.ices.dk/products/CMdocs/CM-2010/F/F0810.pdf

160. Koger C.S., Teh S.J., Hinton D.E., 2000. Determining the sensitive developmental stages of intersex induction in medaka (*Oryzias latipes*) exposed to 17 β -estradiol or testosterone. Marine Environmental Research 50, 201-206.

161. Kooijman S.A.L.M., Baas J., Bontje D., Broerse M., Van Gestel C.A.M., Jager T., 2009.

Ecotoxicological Applications of Dynamic Energy Budget Theory, in: Devillers (Ed.), Ecotoxicology Modeling. Springer, Berlin, pp. 237-259.

162. Kourradi R., 2007. Evaluation du degré de la pollution anthropique de l'estuaire de Bou Regreg et impact sur la biologie et dynamique de *Scrobicularia plana* (Linné, 1758) et *Solen marginatus* (Linné, 1767) Rabat: Université Mohammed V – Agdal. 300 pages.

163. Kuiper G.G.J.M., Carlsson B., Grandien K., Enmark E., Häggblad J., Nilsson S., Gustafsson J.-A., 1997. Comparison of the Ligand Binding Specificity and Transcript Tissue Distribution of Estrogen Receptors α and β. Endocrinology 138, 863-870.

164. Kumar A., Chapman J.C., 1998. Profenofos toxicity to the eastern rainbow fish (*Melanotaenia duboulayi*). Environmental Toxicology and Chemistry 17, 1799-1806.

165. Kummer V., Maškováa J., Zralý Z., Neča J., Šimečková P., Vondráček J., Machala M., 2008. Estrogenic activity of environmental polycyclic aromatic hydrocarbons in uterus of immature Wistar rats. Toxicology Letters 180, 212-221.

166. Labenia J.S., Baldwin D.H., French B.L., Davis J.W., Scholz N.L., 2007. Behavioral impairment and increased predation mortality in cutthroat trout exposed to carbaryl. Marine Ecology Progress Series 329, 1-11.

167. Lacaze E., Geffard O., Bony S., Devaux A., 2010. Genotoxicity assessment in the amphipod *Gammarus fossarum* by use of the alkaline Comet assay. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 700, 32-38.

168. Lagadic L., Caquet T., Amiard J.-C., Ramade F., 1997. Biomarqueurs en écotoxicologie - Aspects fondamentaux. Masson, Paris. 419 pages.

169. Lagauzère S., 2008. Influence de la bioturbation des macro-invertébrés benthiques sur le comportement biogéochimique de l'uranium au sein des sédiments d'eau douce *PhD.* Marseille: Université de la Méditerranée Aix-Marseille II. 285 pages.

170. Langston W.J., Burt G.R., Chesman B.S., 2007. Feminisation of male clams *Scrobicularia plana* from estuaries in Southwest UK and its induction by endocrine-disrupting chemicals. Marine Ecology Progress Series 333, 173-184.

171. Lawrence A.J., Soame J.M., 2009. The endocrine control of reproduction in Nereidae: a new multi-hormonal model with implications for their functional role in a changing environment. Philosophical Transactions of the Royal Society B: Biological Sciences 364, 3363-3376.

172. Leiniö S., Lehtonen K.K., 2005. Seasonal variability in biomarkers in the bivalves *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 140, 408-421.

173. Lemière S., Cossu-Leguille C., Bispo A., Jourdain M.-J., Lanhers M.-C., Burnel D., Vasseur P., 2005. DNA damage measured by the single-cell gel electrophoresis (Comet) assay in mammals fed with mussels contaminated by the "Erika"™ oil-spill. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 581, 11-21.

174. Lewis C., Galloway T., 2009. Reproductive consequences of paternal genotoxin exposure in marine invertebrates. Environ. Sci. Technol. 43, 928-933.

175. Lewis C., Watson G.J., 2011. Expanding the ecotoxicological toolbox: The inclusion of polychaete reproductive endpoints. Mar Environ Res. *In press*.

176. Licciano M., Stabili L., Giangrande A., Cavallo R.A., 2007. Bacterial accumulation by *Branchiomma luctuosum* (Annelida: Polychaeta): A tool for biomonitoring marine systems and restoring polluted waters. Marine Environmental Research 63, 291-302.

177. Lopes C., Péry A.R.R., Chaumot A., Charles S., 2005. Ecotoxicology and population dynamics: Using DEBtox models in a Leslie modeling approach. Ecological Modelling 188, 30-40.

178. Loquet M., Dupont J.P., 1976. Etude morphologique et microbiologique des terriers à Nereis dans la slikke en Baie de Somme (Cap Hornu) - Essais préliminaires. Bull. Fr. Piscic., 170-186.

179. Louiz I., Kinani S., Gouze M.E., Ben-Attia M., Menif D., Bouchonnet S., Porcher J.M., Ben-Hassine O.K., Aït-Aïssa S., 2008. Monitoring of dioxin-like, estrogenic and anti-androgenic activities in sediments of the Bizerta lagoon (Tunisia) by means of *in vitro* cell-based bioassays:

Contribution of low concentrations of polynuclear aromatic hydrocarbons (PAHs). Science of The Total Environment 402, 318-329.

180. Lucas F., Bertru G., 1997. Bacteriolysis in the gut of *Nereis diversicolor* (O.F. Müller) and effect of the diet. Journal of Experimental Marine Biology and Ecology 215, 235-245.

181. Luengen A.C., Friedman C.S., Raimondi P.T., Flegal A.R., 2004. Evaluation of mussel immune responses as indicators of contamination in San Francisco Bay. Marine Environmental Research 57, 197-212.

182. Luis O.J., Passos A.M., 1995. Seasonal changes in lipid content and composition of the polychaete *Nereis (Hediste) diversicolor*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 111, 579-586.

183. Lye C.M., Bentley M.G., Clare A.S., Sefton E.M., 2005. Endocrine disruption in the shore crab *Carcinus maenas*-a biomarker for benthic marine invertebrates? Marine Ecology Progress Series 288, 221-232.

184. Markert B.A., Breure A.M., Zechmeister H.G., 2003. Bioindicators & biomonitors: principles, concepts, and applications. Elsevier. 997 pages. http://books.google.com/books?id=swKWsOY9CJ4C

185. Marty R., 1997. Biologie de la reproduction et du développement de deux espèces d'annélides polychètes *Nereis diversicolor* (O.F. Müller) et *Perinereis cultrifera* Grübe Dinard: Université de Rennes 1. 208 pages.

186. Matozzo V., Gagné F., Marin M.G., Ricciardi F., Blaise C., 2008. Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review. Environment International 34, 531-545.

187. Matthiessen P., Sumpter P., 1998. Effect of Estrogenic Substances in the Aquatic Environment, in: Braunbeck, Hinton, Streit (Eds.), Fish Ecotoxicology. Birkhäuser, Verlag Basel, pp. 319-336.

188. Mazorra M.T., Rubio J.A., Blasco J., 2002. Acid and alkaline phosphatase activities in the clam *Scrobicularia plana*: kinetic characteristics and effects of heavy metals. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 131, 241-249.

189. McEwen B.S., Wingfield J.C., 2003. The concept of allostasis in biology and biomedicine. Hormones and Behavior 43, 2-15.

190. McLusky D.S., Elliott M., 2004. The estuarine ecosystem: ecology, threats and management 3rd ed. Oxford University Press, Oxford. 221 pages.

191. Mettam C., Santhanam V., Havard M.S.C., 1982. The Oogenic Cycle of *Nereis Diversicolor* Under Natural Conditions. Journal of the Marine Biological Association of the United Kingdom 62, 637-645.

192. Meziane T., Retiere C., 2002. Croissance de juvéniles de *Nereis diversicolor* nourris avec des détritus d'halophytes. Oceanologica Acta 25, 119-124.

193. Meziane T., Thorin S., 2000. Macro-invertebrates food webs, in: Feunteun, Thorin, Lefeuvre (Eds.), European salt marsh modelling, . EU programme Environment and Climate, pp. 291-313.

194. Miller D.H., Jensen K.M., Villeneuve D.L., Kahl M.D., Makynen E.A., Durhan E.J., Ankley G.T., 2007. Linkage of biochemical responses to population-level effects: A case study with vitellogenin in the fathead minnow (*Pimephales promelas*). Environmental Toxicology and Chemistry 26, 521-527.

195. Mills L.J., Gutjahr-Gobell R.E., Haebler R.A., Borsay Horowitz D.J., Jayaraman S., Pruell R.J., McKinney R.A., Gardner G.R., Zaroogian G.E., 2001. Effects of estrogenic (o,p'-DDT; octylphenol) and anti-androgenic (p,p'-DDE) chemicals on indicators of endocrine status in juvenile male summer flounder (*Paralichthys dentatus*). Aquatic Toxicology 52, 157-176.

196. Minguez L., Meyer A., Molloy D.P., Giambérini L., 2009. Interactions between parasitism and biological responses in zebra mussels (*Dreissena polymorpha*): Importance in ecotoxicological studies. Environmental Research 109, 843-850.

197. Minier C., Caltot G., Leboulanger F., Hill E., M., 2000. An investigation of the incidence of intersex fish in Seine-Maritime and Sussex region. Analusis 28, 801-806.

198. **Minier C., Moore M.N., Galgani F., Claisse D., 2006. Multixenobiotic resistance protein expression in *Mytilus edulis*, *M. galloprovincialis* and *Crassostrea gigas* from the French coasts. Marine Ecology Progress Series 322, 143-154.**

199. **Moore M.N., Icarus Allen J., McVeigh A., 2006. Environmental prognostics: An integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. Marine Environmental Research 61, 278-304.**

200. **Moreau J.-L., Baudrimont M., Carrier P., Peltier G., Bourdineaud J.-P., 2008. Metal binding and antioxidant properties of chimeric tri- and tetra-domained metallothioneins. Biochimie 90, 705-716.**

201. **Moreira S.M., Lima I., Ribeiro R., Guilhermino L., 2006. Effects of estuarine sediment contamination on feeding and on key physiological functions of the polychaete *Hediste diversicolor*: Laboratory and in situ assays. Aquatic Toxicology 78, 186-201.**

202. **Moreira S.M., Moreira-Santos M., Guilhermino L., Ribeiro R., 2005. A short-term sublethal in situ toxicity assay with hediste diversicolor (polychaeta) for estuarine sediments based on postexposure feeding. Environmental Toxicology and Chemistry 24, 2010-2018.**

203. **Mouneyrac C., Durou C., Gillet P., Hummel H., Amiard-Triquet C., 2009a. Linking Energy Metabolism, Reproduction, Abundance, and Structure of *Nereis diversicolor* Populations, in: Amiard-Triquet, Rainbow (Eds.), Environmental Assessment of Estuarine Ecosystems. CRC Press, Boca Raton, pp. 159-181.**

204. **Mouneyrac C., Leung P., Leung K., 2011. Cost of Tolerance, in: Amiard-Triquet, Rainbow, Roméo (Eds.), Tolerance to Environmental Contaminants. CRC Press, Boca Raton, pp. 265-297.**

205. **Mouneyrac C., Linot S., Amiard J.C., Amiard-Triquet C., Métais I., Durou C., Minier C., Pellerin J., 2008. Biological indices, energy reserves, steroid hormones and sexual maturity in the infaunal bivalve *Scrobicularia plana* from three sites differing by their level of contamination. General and Comparative Endocrinology 157, 133-141.**

206. **Mouneyrac C., Linot S., Amiard J.C., Amiard-Triquet C., Métais I., Durou C., Minier C., Pellerin J., 2009b. Biological indices, energy reserves, steroid hormones and sexual maturity in the infaunal bivalve *Scrobicularia plana* from three sites differing by their level of contamination. General and Comparative Endocrinology 157, 133-141.**

207. **Mouneyrac C., Mastain O., Amiard J.C., Amiard-Triquet C., Beaunier P., Jeantet A.Y., Smith B.D., Rainbow P.S., 2003. Trace-metal detoxification and tolerance of the estuarine worm *Hediste diversicolor* chronically exposed in their environment. Marine Biology 143, 731-744.**

208. **Mouneyrac C., Perrein-Ettajani H., Amiard-Triquet C., 2010. Influence of anthropogenic stress on fitness and behaviour of a key-species of estuarine ecosystems, the ragworm *Nereis diversicolor*. Environmental Pollution 158, 121-128.**

209. **Muséum National d'Histoire Naturelle M., 2003-2010. Inventaire National du Patrimoine Naturel, Paris. http://inpn.mnhn.fr.**

210. **Nacci D., Huber M., Champlin D., Jayaraman S., Cohen S., Gauger E., Fong A., Gomez-Chiarri M., 2009. Evolution of tolerance to PCBs and susceptibility to a bacterial pathogen (*Vibrio harveyi*) in Atlantic killifish (*Fundulus heteroclitus*) from New Bedford (MA, USA) harbor. Environmental Pollution 157, 857-864.**

211. **Newman M.C., Roberts M.H., Hale R.C., 2002. Coastal and estuarine risk assessment. Lewis Publishers, Boca Raton, Fl. 347 pages.**

212. **Ng T.Y.T., Rainbow P.S., Amiard-Triquet C., Amiard J.-C., Wang W.-X., 2008. Decoupling of cadmium biokinetics and metallothionein turnover in a marine polychaete after metal exposure. Aquatic Toxicology 89, 47-54.**

213. **Obolewski K., Strzelczak A., Bodo K., 2009. Quantitative structure of *Hediste diversicolor* (O.F. Müller) in estuary zones of the southern Baltic Sea (Polish central coastline). Baltic Coastal Zone 13, 147-161.**

214. **Ohtake F., Takeyama K.-i., Matsumoto T., Kitagawa H., Yamamoto Y., Nohara K., Tohyama C., Krust A., Mimura J., Chambon P. *et al.*, 2003. Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. Nature 423, 545-550.**

215. **Olive P.J.W., 1983. Annelida Polychaeta, in: Adiyodi, Adiyodi (Eds.), Reproductive Biology of Invertebrates. 1: Oogenesis, Oviposition and Oosorption. John Wiley and Sons Ltd, Chichester, pp. 357-422.**

216. **Olive P.J.W., 1995. Annual breeding cycles in marine invertebrates and environmental temperature: Probing the proximate and ultimate causes of reproductive synchrony. Journal of Thermal Biology 20, 79-90.**

217. **Olive P.J.W., Garwood P.R.G., 1981. Gametogenic cycle and population structure of *Nereis (Hediste) diversicolor* and *Nereis (Nereis) pelagica* from north-east England. Journal of the Marine Biological Association of the United Kingdom 61, 193-213.**

218. **Olivier M., Desrosiers G., Caron A., Retière C., Caillou A., 1995. Réponses comportementales des polychètes *Nereis diversicolor* (O.F. Müller) et *Nereis virens* (Sars) aux stimuli d'ordre alimentaire : utilisation de la matière organique particulaire (algues et halophytes). Canadian Journal of Zoology 73, 2307-2317.**

219. **Olivier M., Desrosiers G., Caron A., Retière C., Caillou A., 1997. Juvenile growth of *Nereis diversicolor* (O.F. Müller) feeding on a range of marine vascular and macroalgal plant sources under experimental conditions. Journal of Experimental Marine Biology and Ecology 208, 1-12.**

220. **Ortiz-Zarragoitia M., Cajaraville M.P., 2010. Intersex and oocyte atresia in a mussel population from the Biosphere's Reserve of Urdaibai (Bay of Biscay). Ecotoxicology and Environmental Safety 73, 693-701.**

221. **Orvain F., 2005. A model of sediment transport under the influence of surface bioturbation: generalisation to the facultative suspension-feeder *Scrobicularia plana*. Marine Ecology Progress Series 286, 43-56.**

222. **OSPAR C., 2006. Stratégie pour un Programme conjoint d'évaluation et de surveillance continue (JAMP). OSPAR. http://qsr2010.ospar.org/media/assessments/Basic_documents/FR_03-22f_Strategie_JAMP.pdf**

223. **Owen R., Buxton L., Sarkis S., Toaspern M., Knap A., Depledge M., 2002. An evaluation of hemolymph cholinesterase activities in the tropical scallop, *Euvola (Pecten) ziczac*, for the rapid assessment of pesticide exposure. Marine Pollution Bulletin 44, 1010-1017.**

224. **Palanisamy P., Sasikala G., Mallikaraj D., Natarajan M., 2011. Study of Behavioural Response of Air-Breathing Catfish *Mystus cavasius* Exposed to Electroplating Industrial Effluent Chromium. International Journal of Pharmaceutical Sciences and Research 2, 1790-1792.**

225. **Papaspyrou S., Thessalou-Legaki M., Kristensen E., 2010. The influence of infaunal (*Nereis diversicolor*) abundance on degradation of organic matter in sandy sediments. Journal of Experimental Marine Biology and Ecology 393, 148-157.**

226. **Park J.-W., Tompsett A.R., Zhang X., Newsted J.L., Jones P.D., Au D.W.T., Kong R., Wu R.S.S., Giesy J.P., Hecker M., 2009. Advanced fluorescence in situ hybridization to localize and quantify gene expression in Japanese medaka (*Oryzias latipes*) exposed to endocrine-disrupting compounds. Environmental Toxicology and Chemistry 28, 1951-1962.**

227. **Paul-Pont I., de Montaudouin X., Gonzalez P., Jude F., Raymond N., Paillard C., Baudrimont M., 2010a. Interactive effects of metal contamination and pathogenic organisms on the introduced marine bivalve *Ruditapes philippinarum* in European populations. Environmental Pollution 158, 3401-3410.**

228. **Paul-Pont I., Gonzalez P., Baudrimont M., Jude F., Raymond N., Bourrasseau L., Le Goïc N., Haynes F., Legeay A., Paillard C. *et al.*, 2010b. Interactive effects of metal contamination and pathogenic organisms on the marine bivalve *Cerastoderma edule*. Marine Pollution Bulletin 60, 515-525.**

229. **Pavlov D.D., Chuiko G.M., Gerassimov Y.V., Tonkopiy V.D., 1992. Feeding behavior and brain acetylcholinesterase activity in bream (*Abramis brama* L.) as affected by DDVP, an organophosphorus insecticide. Comparative biochemistry and physiology. C. Comparative pharmacology and toxicology 103, 563-568.**

230. **Peck M.R., Labadie P., Minier C., Hill E.M., 2007. Profiles of environmental and endogenous estrogens in the zebra mussel *Dreissena polymorpha*. Chemosphere 69, 1-8.**

231. **Pellerin J., Gauthier-Clerc S., Siah A., Assoi-Etchian O., 2004. Stress physiologique et perturbation endocrinienne chez les bivalves marins, in: Pelletier, Campbell, Denizeau (Eds.), Écotoxicologie moléculaire. Principes fondamentaux et perspectives de développement. Presses de l'Université du Québec, Québec, pp. 350-400.**

232. **Pérez E., Blasco J., Solé M., 2004. Biomarker responses to pollution in two invertebrate species: *Scrobicularia plana* and *Nereis diversicolor* from the Cádiz bay (SW Spain). Marine Environmental Research 58, 275-279.**

233. **Petridis P., Jha A.N., Langston W.J., 2009. Measurements of the genotoxic potential of (xeno-)oestrogens in the bivalve mollusc *Scrobicularia plana*, using the Comet assay. Aquatic Toxicology 94, 8-15.**

234. **Pillon A., Servant N., Vignon F., Balaguer P., Nicolas J.-C., 2005. *In vivo* bioluminescence imaging to evaluate estrogenic activities of endocrine disrupters. Analytical Biochemistry 340, 295-302.**

235. **PNUE, 1979. Programme coordonné de surveillance continue et de recherche en matière de pollution dans la Méditerranée (Med Pol): description du programme. Programme des Nations Unies pour l'environnement. http://books.google.com/books?id=uwhsHQAACAAJ**

236. **Poirier L., Berthet B., Amiard J.-C., Jeantet A.-Y., Amiard-Triquet C., 2006. A suitable model for the biomonitoring of trace metal bioavailabilities in estuarine sediments: the annelid polychaete *Nereis diversicolor*. Journal of the Marine Biological Association of the United Kingdom 86, 71-82.**

237. **Porchet-Henneré E., M'Berri M., 1987. Cellular reactions of the polychaete annelid *Nereis diversicolor* against coelomic parasites. Journal of Invertebrate Pathology 50, 58-66.**

238. **Poutiers J.M., 1987. Bivalves (Acéphales, Lamellibranches, Pelecypodes), in: W., Bauchot, Schneider (Eds.), Fiches FAO d'identification des espèces pour les besoins de la pêche. (Révision 1). Méditerranée et mer Noire. Zone de pêche 37. CEE; FAO, Rome, pp. 396-500.**

239. **Poutiers J.M., 1993. Bivalves of the world: an annotated list of marine and brackish water species of interest to fisheriesfisheries. Draft version prepared to be published as a FAO species catalogue., in: FAO (Ed.).**

240. **Prosser C.M., Unger M.A., Vogelbein W.K., 2011. Multistressor interactions in the zebrafish (*Danio rerio*): Concurrent phenanthrene exposure and *Mycobacterium marinum* infection. Aquatic Toxicology 102, 177-185.**

241. **Quiniou F., Damiens G., Gnassia-Barelli M., Geffard A., Mouneyrac C., Budzinski H., Roméo M., 2007. Marine water quality assessment using transplanted oyster larvae. Environment International 33, 27-33.**

242. **Raleigh J., Keegan B.F., 2006. The gametogenic cycle of *Scrobicularia plana* (Mollusca: Bivalvia) in Mweeloon Bay (Galway, west coast of Ireland). Journal of the Marine Biological Association of the United Kingdom 86, 1157-1162.**

243. **Ramos-Gómez J., Coz A., Viguri J.R., Luque Á., Martín-Díaz M.L., DelValls T.Á., 2011a. Biomarker responsiveness in different tissues of caged *Ruditapes philippinarum* and its use within an integrated sediment quality assessment. Environmental Pollution 159, 1914-1922.**

244. **Ramos-Gómez J., Martins M., Raimundo J., Vale C., Martín-Díaz M.L., DelValls T.Á., 2011b. Validation of Arenicola marina in field toxicity bioassays using benthic cages: Biomarkers as tools for assessing sediment quality. Marine Pollution Bulletin 62, 1538-1549.**

245. **Rice C.P., O'Keefe P., Kubiak T., 2002. Sources, Pathways, And Effects Of Pcbs, Dioxins, And Dibenzofurans, Handbook of Ecotoxicology, Second Edition. CRC Press.**

246. **Riera P., Stal L.J., Nieuwenhuize J., Richard P., Blanchard G., Gentil F., 1999. Determination of food sources for benthic invertebrates in a salt marsh (Aiguillon Bay, France) by carbon and nitrogen stable isotopes: importance of locally produced sources. Marine Ecology Progress Series 187, 301-307.**

247. **Riisgård H.U., 1991. Suspension feeding in the polychaete *Nereis diversicolor*. Marine Biology 70, 29-37.**

248. **Ringwood A.H., Hogue J., Keppler C., Gielazyn M., 2004. Linkages between cellular biomarker responses and reproductive success in oysters - *Crassostrea virginica*. Marine environmental research 58, 151-155.**

249. **RNO, 2006. Surveillance du Milieu Marin, Travaux du Réseau National d'Observation de la qualité du milieu marin. Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER). http://envlit.ifremer.fr/content/download/27640/224803/version/1/file/rno06.pdf**

250. **RNO, 2009. Intensité de l'imposex chez *Nucella lapillus* le long des côtes de la manche et de l'atlantique en 2009. Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), Paris, Nantes. 60 pages.**

251. **Rodríguez-Rúa A., Prado M.A., Romero Z., Bruzón M., 2003. The gametogenic cycle of *Scrobicularia plana* (da Costa, 1778) (Mollusc: Bivalve) in Guadalquivir estuary (Cádiz, SW Spain). Aquaculture 217, 157-166.**

252. **Romaña L.-A., 1994. Les grands estuaires Français, Equinoxe. IFREMER, Toulon.**

253. **Roméo M., Giambérini L., 2008. Historique, in: Amiard, Amiard-Triquet (Eds.), Les biomarqueurs dans l'évaluation de l'état écologique des milieux aquatiques. Lavoisier, Paris, pp. 17-54.**

254. **Romero-Ruiz A., Alhama J., Blasco J., Gómez-Ariza J.L., López-Barea J., 2008. New metallothionein assay in *Scrobicularia plana*: Heating effect and correlation with other biomarkers. Environmental Pollution 156, 1340-1347.**

255. **Rosa S., Granadeiro J.P., Vinagre C., França S., Cabral H.N., Palmeirim J.M., 2008. Impact of predation on the polychaete *Hediste diversicolor* in estuarine intertidal flats. Estuarine, Coastal and Shelf Science 78, 655-664.**

256. **Ruiz J.M., Bryan G.W., Wigham G.D., Gibbs P.E., 1995. Effects of tributyltin (TBT) exposure on the reproduction and embryonic development of the bivalve *Scrobicularia plana*. Marine Environmental Research 40, 363-379.**

257. **Ruus A., Schaanning M., Øxnevad S., Hylland K., 2005. Experimental results on bioaccumulation of metals and organic contaminants from marine sediments. Aquatic Toxicology 72, 273-292.**

258. **Ryan J.A., Hightower L.E., 1994. Evaluation of heavy-metal ion toxicity in fish cells using a combined stress protein and cytotoxicity assay. Environmental Toxicology and Chemistry 13, 1231-1240.**

259. **Sabik H., Gagné F., Blaise C., Marcogliese D.J., Jeannot R., 2003. Occurrence of alkylphenol polyethoxylates in the St. Lawrence River and their bioconcentration by mussels (*Elliptio complanata*). Chemosphere 51, 349-356.**

260. **Sammarco P.W., Hallock P., Lang J.C., LeGore R.S., 2007. Roundtable Discussion Groups Summary Papers: Environmental Bio-Indicators in Coral Reef Ecosystems: The Need to Align Research, Monitoring, and Environmental Regulation. Environmental Bioindicators 2, 35-46.**

261. **Sanchez W., Porchez J.-M., 2009. Fish biomarkers for environmental monitoring within the Water Framework Directive of the European Union. TrAC. Trends in Analytical Chemistry 28, 150-158.**

262. **Sandahl J.F., Baldwin D.H., Jenkins J.J., Scholz N.L., 2005. Comparative thresholds for acetylcholinesterase inhibition and behavioral impairment in coho salmon exposed to chlorpyrifos. Environ Toxicol Chem 24, 136-145.**

263. **Santos S., Cardoso J.F.M.F., Carvalho C., Luttikhuizen P.C., van der Veer H.W., 2011a. Seasonal variability in somatic and reproductive investment of the bivalve *Scrobicularia plana* (da Costa, 1778) along a latitudinal gradient. Estuarine, Coastal and Shelf Science 92, 19-26.**

264. **Santos S., Luttikhuizen P.C., Campos J., Heip C.H.R., van der Veer H.W., 2011b. Spatial distribution patterns of the peppery furrow shell *Scrobicularia plana* (da Costa, 1778) along the European coast: A review. Journal of Sea Research 66, 238-247.**

265. **Scaps P., 2002. A review of the biology, ecology and potential use of the common ragworm *Hediste diversicolor* (O.F. Müller) (Annelida: Polychaeta) Hydrobiologia 470, 203-218.**

266. **Scaps P., Borot O., 2000. Acetylcholinesterase activity of the polychaete *Nereis diversicolor*: effects of temperature and salinity. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 125, 377-383.**

267. **Scholz N.L., Truelove N.K., French B.L., Berejikian B.A., Quinn T.P., Casillas E., Collier T.K., 2000. Diazinon disrupts antipredator and homing behaviors in chinook salmon (*Oncorhynchus tshawytscha*). Canadian Journal of Fisheries and Aquatic Sciences 57, 1911-1918.**

268. **Serafim A., Company R., Lopes B., Fonseca V.F., França S., Vasconcelos R.P., Bebianno M.J., Cabral H.N., 2011. Application of an integrated biomarker response index (IBR) to assess temporal variation of environmental quality in two Portuguese aquatic systems. Ecological Indicators *In Press*.**

269. **Siah A., Pellerin J., Amiard J.C., Pelletier E., Viglino L., 2003. Delayed gametogenesis and progesterone levels in soft-shell clams (*Mya arenaria*) in relation to in situ contamination to organotins and heavy metals in the St. Lawrence River (Canada). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 135, 145-156.**

270. **Sigel A., Sigel H., Sigel R.K.O., 2009. Metallothioneins and Related Chelators. Royal Society of Chemistry, Cambridge, UK.**

271. **Smaoui-Damak W., Rebai T., Berthet B., Hamza-Chaffai A., 2006. Does cadmium pollution affect reproduction in the clam *Ruditapes decussatus*? A one-year case study. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 143, 252-261.**

272. **Sola J.C., 1997. Reproduction, population dynamics, growth and production of *Scrobicularia plana* da Costa (pelecypoda) in the Bidasoa estuary, Spain. Aquatic Ecology 30, 283-296.**

273. **Solé M., Kopecka-Pilarczyk J., Blasco J., 2009. Pollution biomarkers in two estuarine invertebrates, *Nereis diversicolor* and *Scrobicularia plana*, from a Marsh ecosystem in SW Spain. Environment International 35, 523-531.**

274. **Tairova Z.M., Giessing A.M.B., Hansen R., Andersen O., 2009. 1-Hydroxypyrene as a biomarker of PAH exposure in the marine polychaete *Nereis diversicolor*. Marine Environmental Research 67, 38-46.**

275. **Tannenbaum L.V., 2005. A Critical Assessment of the Ecological Risk Assessment Process: A Review of Misapplied Concepts. Integrated Environmental Assessment and Management 1, 66-72.**

276. **Teixeira Alves M., 2009. *Lymnaea stagnalis* : modèle biologique d'étude des effets des perturbateurs endocriniens. Rapport bibliographique. Master Santé et populations. 27 pages. http://spiral.univ-lyon1.fr/files_m/M4966/Files/578548_5193.pdf**

277. **Tetreault G.R., Bennett C.J., Shires K., Knight B., Servos M.R., McMaster M.E., 2011. Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges. Aquatic Toxicology 104, 278-290.**

278. **Thirumoorthy N., Manisenthil K., K. T., Shyam Sundar A., Panayappan L., Malay C., 2007. Metallothionein: An overview. World Journal of Gastroenterology 13, 993-996.**

279. **Tiede K., Hassellöv M., Breitbarth E., Chaudhry Q., Boxall A.B.A., 2009. Considerations for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles. Journal of Chromatography A 1216, 503-509.**

280. **Tierney K., Casselman M., Takeda S., Farrell T., Kennedy C., 2007. The relationship between cholinesterase inhibition and two types of swimming performance in chlorpyrifos-exposed coho salmon (*Oncorhynchus kisutch*). Environ Toxicol Chem 26, 998-1004.**

281. **Tolba M.K., El-Kholy O.A., Programme U.N.E., 1992. The World environment 1972-1992: two decades of challenge. Published by Chapman & Hall on behalf of the United Nations Environment Programme. 884 pages.**

282. **Tran D., Ciret P., Ciutat A., Durrieu G., Massabuau J.-C., 2003. Estimation of potential and limits of bivalve closure response to detect contaminants: Application to cadmium. Environmental Toxicology and Chemistry 22, 914-920.**

283. **Tran D., Fournier E., Durrieu G., Massabuau J.-C., 2004. Copper detection in the Asiatic clam *Corbicula fluminea*: optimum valve closure response. Aquatic Toxicology 66, 333-343.**

284. **Tran D., Fournier E., Durrieu G., Massabuau J.-C., 2007. Inorganic mercury detection by valve closure response in the freshwater clam *Corbicula fluminea*: Integration of time and water metal concentration changes. Environmental Toxicology and Chemistry 26, 1545-1551.**

285. **Trebble N., 1976. British bivalve seashells: a handbook for identification, 2 ed. Her Majesty's Stationery Office Edinburgh and the Trustees of the Britsh Museum. 212 pages.**

286. **UNEP/RAMOGE, 1999. Manuel sur les biomarqueurs recommandés pour le programme de biosurveillance du MED POL, Athènes. http://195.97.36.231/acrobatfiles/MAPDocAcrobatfiles/Ramoge/Ramoge_english.pdf**

287. **Van Colen C., Lenoir J., De Backer A., Vanelslander B., Vincx M., Degraer S., Ysebaert T., 2009. Settlement of *Macoma balthica* larvae in response to benthic diatom films. Marine Biology 156, 2161-2171.**

288. **Vasseur P., Cossu-Leguille C., Atienzar F., Rodius F., Lemière S., 2008. Marqueurs de genotoxicite et effets *in situ*, individuels et populationnnels, in: Amiard, Amiard-Triquet (Eds.), Les biomarqueurs dans l'évaluation de l'état écologique des milieux aquatiques. Lavoisier, Paris, pp. 295-330.**

289. **Verdelhos T., Neto J.M., Marques J.C., Pardal M.A., 2005. The effect of eutrophication abatement on the bivalve *Scrobicularia plana*. Estuarine, Coastal and Shelf Science 63, 261-268.**

290. **Viarengo A., Canesi L., Mazzucotelli A., Ponzano E., 1993. Cu, Zn, Cd content in different tissues of the antarctic scallop *Adamussium colbecki* (Smith 1902): Role of metallothionein in the Homeostasis and in the detoxication of heavy metals. Marine Ecology Progress Series 95, 163-168.**

291. **Viarengo A., Lafaurie M., Gabrielides G.P., Fabbri R., Marro A., Roméo M., 2000. Critical evaluation of an intercalibration exercise undertaken in the framework of the MED POL biomonitoring program. Marine Environmental Research 49, 1-18.**

292. **Wanink J.H., Zwarts L., 1993. Environmental effects of the growth rate of intertidal invertebrates and some implications for foraging waders. Netherlands Journal of Sea Research 31, 407-418.**

293. **Weis J.S., Bergey L., Reichmuth J., Candelmo A., 2011. Living in a Contaminated Estuary: Behavioral Changes and Ecological Consequences for Five Species. BioScience 61, 375-385.**

294. **Weis J.S., Smith G., Zhou T., Santiago-Bass C., Weis P., 2001. Effects of Contaminants on Behavior: Biochemical Mechanisms and Ecological Consequences. BioScience 51, 209-217.**

295. **Wilding J., Maltby L., 2006. Relative toxicological importance of aqueous and dietary metal exposure to a freshwater crustacean: Implications for risk assessment. Environmental Toxicology and Chemistry 25, 1795-1801.**

296. **Wilson V.S., Bobseine K., Lambright C.R., Gray L.E., 2002. A Novel Cell Line, MDA-kb2, That Stably Expresses an Androgen- and Glucocorticoid-Responsive Reporter for the Detection of Hormone Receptor Agonists and Antagonists. Toxicological Sciences 66, 69-81.**

297. **Wolff W.J., 1973. The estuary as a habitat an analysis of data on the soft-bottom Macrofauna of the Estuarine area of the rivers rhine, Meuse, and Scheldt. Zoologische Verhandelingen 126, 242.**

298. **Woodling J.D., Lopez E.M., Maldonado T.A., Norris D.O., Vajda A.M., 2006. Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 144, 10-15.**

299. **Worrall C.M., Widdows J., Lowe D.M., 1983. Physiological ecology of three populations of the bivalve *Scrobicularia plana* Marine Ecology Progress Series 12, 267-279.**

300. **Wosniok W., Broeg K., Feist S.W., 2005. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO). Annex 7, La Tremblade, France, pp. 76-84.**

301. **Xuereb B., Bezin L., Chaumot A., Budzinski H., Augagneur S., Tutundjian R., Garric J., Geffard O., 2011. Vitellogenin-like gene expression in freshwater amphipod *Gammarus fossarum* (Koch, 1835): functional characterization in females and potential for use as an endocrine disruption biomarker in males. Ecotoxicology 20, 1286-1299.**

302. **Xuereb B., Chaumot A., Mons R., Garric J., Geffard O., 2009a. Acetylcholinesterase activity in *Gammarus fossarum* (Crustacea Amphipoda): Intrinsic variability, reference levels, and a reliable**

tool for field surveys. Aquatic Toxicology 93, 225-233.

303. Xuereb B., Lefèvre E., Garric J., Geffard O., 2009b. Acetylcholinesterase activity in *Gammarus fossarum* (Crustacea Amphipoda): Linking AChE inhibition and behavioural alteration. Aquatic Toxicology 94, 114-122.

304. Yeom D.-H., Adams S.M., 2007. Assessing effects of stress across levels of biological organization using an aquatic ecosystem health index. Ecotoxicology and Environmental Safety 67, 286-295.

305. Zelikoff J.T., Carlson E., Li Y., Raymond A., Duffy J., Beaman J.R., Anderson M., 2002. Immunotoxicity Biomarkers in Fish: Development, Validation and Application for Field Studies and Risk Assessment. Human and Ecological Risk Assessment: An International Journal 8, 253-263.

306. Zwarts L., 1991. Seasonal variation in body weight of the bivalves *Macoma balthica, Scrobicularia plana, Mya arenaria* and *Cerastoderma edule* in the Dutch Wadden sea. Netherlands Journal of Sea Research 28, 231-245.

307. Zwarts L., Ens B.J., Year. Predation by birds on marine tidal flats. In: *Proceedings of the 22nd international ornithological congress: August 1998 1999; South Africa. Johannesburg,*: Birdlife South Africa; 1999.

308. Zwarts L., Wanink J.H., 1993. How the food supply harvestable by waders in the Wadden Sea depends on the variation in energy density, body weight, biomass, burying depth and behaviour of tidal-flat invertebrates. Netherlands Journal of Sea Research 31, 441-476.

309. Zwarts L., Wannink J.H., Ens B.J., 1996. Predicting seasonal and annual fluctuations in the local exploitation of different prey by Oystercatchers *Haematopus ostralegus*: a ten-year study in the Wadden Sea. ARDEA 84, 401 - 440.

Résumé

L'évolution des recherches écotoxicologiques durant la dernière décennie a conduit à la mise en exergue de biomarqueurs capables de prédire des effets au niveau supra-individuels bien avant que la dégradation de la qualité du milieu n'ait atteint un point non-retour. Ces biomarqueurs « écologiquement significatifs » sont basés sur les processus capables d'affecter le succès de la reproduction et d'induire des conséquences au niveau populationnel. C'est dans ce contexte que s'inscrit cette thèse, dont l'objectif principal était d'établir le lien entre les biomarqueurs mesurés à différents niveaux d'organisation biologique chez deux espèces endogées : *Hediste diversicolor* et *Scrobicularia plana* provenant de milieux estuariens et côtiers. Les réponses biologiques concernant le comportement, la bioénergétique et la reproduction ont été étudiées dans des sites correspondant à un gradient de pollution. Les études comportementales étaient basées sur la capacité d'évitement des organismes à l'égard des prédateurs et des contaminants et le comportement alimentaire, en lien avec l'acquisition de l'énergie. La bioénergétique a été abordée par la prise alimentaire, l'assimilation de la nourriture ingérée, les taux des réserves énergétiques, l'indice de condition des bivalves, la relation taille-poids des annélides et l'indice gonadosomatique. Les atteintes à la reproduction ont été évaluées par la recherche d'intersexe chez *S. plana* au travers d'études histologiques, et par la recherche de la présence de perturbateurs endocriniens dans le sédiment d'origine des bivalves

Mots-clés: Biomarqueurs, Scrobicularia plana, Hediste diversicolor, Bioénergétique, Comportement, reproduction

Abstract

During the last decade, ecotoxicological research has permitted to propose relevant biomarkers to forecast effects at supra-individual levels, well before environmental degradation has reached a hardly manageable degree. These biomarkers with added ecological value are based on processes which can affect the reproductive success and have most probably consequences at the population level. In this context, the main objective of this thesis was to establish the link between the biomarkers measured at different levels of biological organization (sub-individual level at the population level) in two sentinel species: *Hediste diversicolor* and *Scrobicularia plana* originating from estuarine and coastal environments. Biological responses in terms of behaviour, bioenergetics and reproduction have been studied in estuaries corresponding to a pollution gradient. Behavioural studies were done to determining avoidance ability of endobenthic invertebrates towards predators and contaminants as well as feeding behaviour controlling energy input. The study of bioenergetics have investigate food uptake (behaviour); food assimilation (determination of enzyme activities (amylase and cellulase) relevant with regards to food habits), energy reserves (as glycogen, proteins and lipids) and size-weight relationships in polychaetes, condition and gonadosomatic indices of bivalves. Impairments in reproduction were evaluated by looking for intersex in *S. plana* through histological studies and the search for the presence of endocrine disruptors in sediment of origin of the bivalves

Key words: Biomarkers, Scrobicularia plana, Hediste diversicolor, Bioenergetics, Behaviour, reproduction