N° d'ordre: 2315

THESE

PRESENTEE A

L'UNIVERSITE BORDEAUX I

Ecole Doctorale des Sciences du Vivant, Géosciences,
Sciences de l'Environnement

Par Mary-Laure VIDAL

Pour obtenir le grade de

DOCTEUR

Spécialité: ECOTOXICOLOGIE

ETUDE DE MARQUEURS BIOCHIMIQUES DE POLLUTION CHEZ LE MOLLUSQUE BIVALVE D'EAU DOUCE *CORBICULA FLUMINEA* (MÜLLER) - PURIFICATION ET CARACTERISATION DES GLUTATHION *S*-TRANSFERASES

Soutenue le 29 novembre 2001

Après avis de:

Christian BLAISE	Professeur Université de Montréal	Rapporteur
Laurent LAGADIC	Chargé de Recherche INRA Rennes	Rapporteur

Devant la Commission d'examen formée de:

Laurent LAGADIC	Chargé de Recherche INRA Rennes	Rapporteur
Jean-François NARBONNE	Professeur Université Bordeaux I	Examinateur
Anne BASSERES	Ingénieur de Recherche TotalFinaElf	Examinateur
Patrick ROUIMI	Chargé de Recherche INRA Toulouse	Examinateur
Philippe GARRIGUES	Directeur de Recherche CNRS Bordeaux	Président du jury Rap. de soutenance

- 2001 -

A mon fils, Baptiste

A son père, Eric

Ce travail a été effectué dans le Département de Toxicologie Biochimique du Laboratoire de Physico-Toxico-Chimie des Systèmes Naturels (LPTC) de l'Université Bordeaux I grâce au support financier du groupe TotalFinaElf. Je remercie les responsables de cette société et en particulier, Monsieur Bernard Tramier, Directeur Environnement et Sécurité Industrielle, pour l'intérêt porté à ce travail.

Je remercie mon Directeur de Thèse, Monsieur Jean-François Narbonne, responsable du Département de Toxicologie Biochimique (LPTC), Monsieur Philippe Garrigues, Directeur du LPTC et Madame Anne Bassères, Ingénieur de Recherche Environnement du groupe TotalFinaElf pour m'avoir confié la réalisation de cette thèse.

Je remercie également Monsieur Christian Blaise et Monsieur Laurent Lagadic d'avoir accepté de juger ce travail.

Un grand merci à Monsieur Patrick Rouimi pour son aide précieuse, sa disponibilité et son soutien.

Que soient également remerciés les membres du Groupe TotalFinaElf et en particulier du Groupement de Recherches de Lacq, pour leur aide et leurs conseils: Mesdames Muriel Audroin et Nadine Médevielle, Messieurs Alain Ladousse, Alain Castelli et Alain Secq.

Je remercie Madame Michèle Roméo et Pascal Hoarau pour leur accueil dans le Laboratoire de Physiologie et Toxicologie Environnementales de l'Université de Nice-Sophia Antipolis lors des études préliminaires de purification des glutathion *S*-transférases.

Je remercie également Messieurs Jacques Tulliez et Jean-Pierre Cravedi pour m'avoir accueillie au sein du Laboratoire des Xénobiotiques de l'INRA de Toulouse lors des étapes finales de la purification et caractérisation des glutathion *S*-transférases. Merci à Laurent Debrauwer et Estelle Rathahao pour leur aide.

Je tiens à remercier Monsieur Marc Laulier pour m'avoir permis d'effectuer les expériences de génétique biochimique dans son Laboratoire d'Ecophysiologie et Biochimie Métabolique de l'Université du Maine. Merci à Sophie Robillard pour son aide et son amitié.

Merci aux membres du LPTC qui m'ont apporté -chacun à leur manière- leurs conseils et leur soutien: Christelle, Christine, Daniel, Marine, Michelle, Patrice, Olivier,...

Un grand merci à mes amis Manue et Pascal.

Je remercie enfin mes proches pour leur soutien sans faille tout au long de ces années.

Abréviations

1A-l-ARNm	ARNm “CYP1A-like”
1A-l-prot.	Protéine “CYP1A-like”
ACh	acétylcholine
AChE	acétylcholinestérase
ANOVA	analyse de variance
ARO	Aroclor 1254
ATB ou TBA	acide thiobarbiturique ou (thiobarbituric acid)
B[*a*]P	benzo[*a*]pyrène
b5	cytochrome *b5*
BCh	butyrylcholine
BChE	butyrylcholinestérase
BPH	benzo[*a*]pyrène hydroxylase
BSA	bovine serum albumin (albumine bovine sérique)
CA	chromatographie d’affinité
CAT	catalase
CDNB	1-chloro-2,4-dinitrobenzène
CEA ou AEC	chromatographie échangeuse d’anions (ou anion-exchange chromatography)
CF	condition factor (facteur de condition)
ChE	cholinestérase
DA	discriminant analysis (analyse discriminante)
DCNB	1,2-dichloro-4-nitrobenzène ou 3,4-dichloro-1-nitrobenzène
DS1 à DS4	downstream sites (sites en aval)
DTT	dithiothréitol
DTW	dry tissue weight (poids sec des tissus)
EAO ou ROS	espèces activées de l’oxygène (ou reactive oxygen species)
EA ou AE	ethacrynic acid (ou acide éthacrynique)
ECOD	7-éthoxycoumarine-*O*-déséthylase
EDTA	éthylène diamine tétra acétate
EPNP	1,2-époxy-3-(*p*-nitrophénoxy)propane
EROD	7-éthoxyrésorufine-*O*-déséthylase
ESI-MS	electrospray ionization - mass spectrometry (spectrométrie de masse - ionisation par électrospray)
FTW	fresh tissue weight (poids frais des tissus)
GH ou CT	coupe de goudron de houille (ou coal tar fraction)
GI	gills (branchies)
GPX	glutathion peroxydase
GSH	glutathion réduit
GSSG	glutathion oxydé
GST	glutathion *S*-transférase
GST/CDNB	activité glutathion *S*-transférase envers le 1-chloro-2,4-dinitrobenzène
GST/EA	activité glutathion *S*-transférase envers l’acide éthacrynique
GST/STY	activité glutathion *S*-transférase envers le 7-[H^3]-styrène oxyde
HA	hydrocarbure aliphatique
HAP ou PAH	hydrocarbure aromatique polycyclique (ou polycyclic aromatic hydrocarbon)
HCB	2,2’,4,4’,5,5’-hexachlorobiphényl

HCB'	2,2',3,4,4',5'-hexachlorobiphényl
IEF	isoelectrofocusing (focalisation iso-électrique)
L	étude en laboratoire
M	étude en mésocosme
MDA	malonedialdéhyde
MFO	mixed-function oxygenase (monooxygénase à fonctions mixtes)
MTT	thiazolyl blue
NADH-red.	NADH-cytochrome *c* (*b5*) réductase
NADPH-ind. EROD	éthoxyrésorufine-*O*-déséthylase NADPH-indépendante
NADPH-red.	NADPH-cytochrome *c* (P450) réductase
NBT	nitro blue tetrazolium
NP	net peroxidation (peroxydation nette)
OC	insecticides organochlorés
OP	insecticides organophosphorés
P418	cytochrome P418
P450	cytochrome P450
PAGE	polyacrylamide gel electrophoresis (électrophorèse sur gel de polyacrylamide)
PCB	polychlorobiphényl
PChE	propionylcholinestérase
PL ou LP	peroxided lipids (ou lipides peroxydés)
PLI	peroxidizable lipids (ou lipides peroxydables)
PM ou MW	poids moléculaire (ou molecular weight)
PMS	phénazine méthosulfate
PMSF	phényl méthyl sulfonyl fluoride
PROD	7-pentoxyrésorufine-*O*-dépentylase
ROP water	reverse-osmosis purified water (eau purifiée par osmose-inverse)
RP-HPLC	reversed-phase high performance liquid chromatography (chromatographie liquide haute performance en phase inverse)
SDS	sodium dodécyl-sulfate
SOD	superoxyde dismutase
T	étude de terrain
TBARS	thiobarbituric acid reactive species (espèces réagissant avec l'acide thiobarbiturique)
TCB	3,3',4,4'-tétrachlorobiphényl
TCDD	2,3,7,8-tétrachlorodibenzo-*p*-dioxine
TCE	trichloroéthylène
TFA	trifluoroacetic acid (acide trifluoroacétique)
TOL	toluène
UPS	upstream reference site (site de référence amont)
VI	variability index (index de variabilité)
VM	visceral mass (masse viscérale)
IBG(N)	indice biologique global (normalisé)
IPS	indice de pollusensibilité spécifique
IOBS	indice de qualité biologique des sédiments
FTE/FTS	flore totale eau/sédiment
FHE/FHS	flore hydrocarbures eau/sédiment
EPIE/EPIS	flore totale (déterminée par épifluorescence) eau/sédiment
ATP	activité microbiologique (dosage de l'adénosine-tri-phosphate)
MICROE/MICROS	test microtox eau/sédiment

TABLE DES MATIERES

INTRODUCTION GENERALE

De nombreuses substances toxiques générées par les activités humaines sont quotidiennement déversées dans l'environnement aquatique. Bien que les pollutions accidentelles soient mises en exergue par les médias, les pollutions d'origine chronique contribuent majoritairement à la détérioration des écosystèmes aquatiques. Les contaminants majeurs, dont certains sont également d'origine naturelle, sont regroupés en plusieurs familles: les hydrocarbures aromatiques polycycliques (HAPs), les chlorophénols, les polychlorobiphényls (PCBs), les hexachlorohexanes, les hexachlorobenzènes, les dioxines, les organoétains, les pesticides organochlorés, les insecticides organophosphorés et carbamates et les métaux lourds tels que le cadmium, le mercure, l'argent, le plomb, le zinc, le cuivre, le fer. Cette liste n'est évidemment pas exhaustive. De plus, de nouvelles molécules chimiques sont constamment synthétisées. Ces substances étrangères aux organismes vivants (hormis certains métaux essentiels), ou **xénobiotiques**, contaminent tous les compartiments (sédiment, colonne d'eau) et par là même de nombreuses espèces animales aquatiques. Outre la dégradation des écosystèmes et l'instauration progressive d'un déséquilibre écologique, la santé humaine est également menacée par la consommation d'eau souillée et d'animaux directement ou indirectement contaminés. La préoccupation grandissante de la population et des autorités pour les problèmes liés à l'hygiène, la sécurité, l'environnement et de manière plus générale à la qualité de vie a conduit à l'instauration de règlementations de plus en plus strictes concernant les rejets de substances polluantes dans l'environnement. Des outils permettant d'évaluer la qualité des milieux aquatiques se sont ainsi avérés nécessaires, tant pour les organismes de surveillance de la qualité de l'eau (ex.: Agences de l'Eau) que pour les industriels. Ces outils correspondent à deux approches chimique et biologique, certes différentes, mais complémentaires (Amiard *et al.*, 1998).

Les **méthodes d'analyse chimique** permettent la détection, la caractérisation et la quantification de la plupart des contaminants présents dans les différents compartiments aquatiques ainsi que dans les tissus animaux et végétaux. De nombreuses analyses chimiques peuvent ainsi être effectuées en routine pour identifier et quantifier les principales classes de contaminant présentes dans un échantillon pour lequel on ne dispose d'aucune information. Cependant, outre le prix élevé de telles analyses, la détection de certains contaminants et de manière globale de tous les contaminants présents dans un échantillon donné, peut s'avérer difficile, voire impossible. De même, les produits de dégradation ou de transformation peuvent ne pas être pris en compte. Ces limites sont évidemment repoussées d'année en année

grâce à l'évolution des techniques d'analyse permettant notamment de détecter et de quantifier les substances chimiques à des seuils de plus en plus bas. Néanmoins, il est une question essentielle à laquelle les analyses chimiques ne pourront jamais répondre: quel est l'impact des contaminants sur les écosystèmes? Seuls des outils biologiques sont à même d'apporter un ou plusieurs élément(s) de réponse.

Il existe actuellement deux catégories d'outils permettant d'évaluer les effets de la pollution à différents niveaux d'organisation biologique: **les bioindicateurs** (populations et écosystèmes) et les **biomarqueurs** (individus). Les bioindicateurs correspondent à des espèces animales ou végétales qui, de par leur présence (ou leur absence) et/ou leur abondance, nous renseignent sur l'état de santé d'un milieu. On peut ainsi distinguer les espèces «pollusensibles» (bioindicateurs négatifs) et les espèces «pollurésistantes» ou «pollutolérantes» (bioindicateurs positifs) (Lagadic et Caquet, 1996). Des outils tels que les indices biocénotiques reposent sur ce principe et permettent d'évaluer la structure et la santé d'un écosystème à un instant t. Cependant, le recensement des différentes espèces peuplant un écosystème donné présente plusieurs limites: il apporte peu ou pas d'information(s) sur la cause réelle de la disparition des espèces (mortalité ou fuite), sur la nature du ou des contaminant(s) éventuellement présent(s) et éventuellement responsable(s) de la mortalité des individus, sur les mécanismes de toxicité ayant conduit à la mort des individus (en l'absence de fuite). Enfin, il ne permet la mise en place que de programmes correctifs et non préventifs puisque dans le cas d'une mortalité massive, le mal est déjà fait. Seule une approche chimique complémentaire peut pallier certaines de ces déficiences en apportant des informations sur les contaminants présents, avec toutefois les limites inhérentes aux analyses chimiques décrites précédemment. Les biomarqueurs peuvent quant à eux nous renseigner sur les mécanismes de toxicité mis en jeu et leurs effets et, dans certains cas, sur la nature du ou des contaminant(s) responsable(s). Une telle approche présente ainsi un caractère prédictif. Plusieurs définitions concernant les biomarqueurs ont été énoncées au cours de ces dernières années (Schlenk, 1999). Nous retiendrons la suivante: «un biomarqueur est un changement observable et/ou mesurable au niveau moléculaire, biochimique, cellulaire, physiologique ou comportemental révélant l'exposition présente ou passée d'un individu à au moins une substance chimique à caractère polluant» (Amiard *et al.*, 1998). Les biomarqueurs sont mesurés au sein d'individus appartenant à une **espèce sentinelle**. Cette dernière est la plupart du temps une **espèce bioaccumulatrice**, c'est à dire capable d'accumuler les contaminants présents dans

l'environnement jusqu'à des concentrations supérieures à celles du milieu ambiant. Cette bioaccumulation peut s'effectuer directement (bioconcentration) et/ou indirectement *via* les voies trophiques (bioamplification). Les espèces sentinelles sont présentes naturellement dans le milieu étudié ou sont transplantées dans celui-ci par encagement («caging»). Elles doivent posséder des caractéristiques particulières: sessiles, abondantes, présentant une large distribution géographique, adaptées aux variations de divers paramètres environnementaux (température, oxygénation, *etc.*) et relativement résistantes aux différents contaminants présents dans le milieu aquatique. De plus, leur physiologie doit être bien connue (Amiard *et al.*, 1998). Les moules marines *Mytilus edulis* et *Mytilus galloprovincialis* présentent toutes ces caractéristiques. De plus, elles filtrent de grandes quantités d'eau pour satisfaire leurs besoins respiratoires et nutritionnels et de ce fait, peuvent accumuler d'importantes quantités de substances chimiques dissoutes ou en suspension. Elles sont donc largement utilisées comme espèces sentinelles dans de nombreux programmes de biosurveillance (Viarengo et Canesi, 1994).

Les biomarqueurs ont été classifiés en trois catégories: les biomarqueurs d'exposition, d'effet et de susceptibilité (Timbrell *et al.*, 1994). La frontière entre ces trois catégories étant ténue, certains biomarqueurs peuvent être à la fois des biomarqueurs d'exposition et d'effet, voire de susceptibilité. Les **biomarqueurs d'exposition** sont en général impliqués dans les métabolismes de (dé)toxication des xénobiotiques (ex.: cytochromes P450) ou dans les mécanismes de défense cellulaire (ex.: enzymes antioxydantes). Leurs variations peuvent être spécifiques ou non d'une catégorie de contaminants (ex.: induction chez le poisson du cytochrome P450 CYP1A par les HAPs) et n'entraînent pas obligatoirement d'effets délétères. Elles signifient la plupart du temps que l'organisme s'est adapté à une agression extérieure d'ordre chimique. Les **biomarqueurs d'effet** correspondent à des cibles moléculaires qui, lorsqu'elles sont atteintes, signifient que les mécanismes de défense ou de détoxication de l'organisme n'ont pas été suffisamment efficaces pour contrer l'action néfaste d'un xénobiotique (ex.: indicateurs de stress oxydatif, adduits à l'ADN). Les conséquences peuvent être parfois irréversibles, entraînant à terme la mort de l'animal ou bien une incapacité à se reproduire. De tels effets peuvent par la suite altérer la structure même des populations et donc des écosystèmes. Les **biomarqueurs de susceptibilité** peuvent correspondre à des protéines qui, lorsqu'elles sont surexprimées ou sousexprimées, augmentent la sensibilité d'un organisme à un contaminant. Ces variations d'expression peuvent être dues à des différences génétiques interindividuelles ou résulter d'une exposition

précédente à un xénobiotique. Par exemple, un individu présentant une surexpression du cytochrome CYP1A sera plus à même de développer des adduits à l'ADN lorsqu'il sera exposé à des HAPs (synthèse de métabolites hautement réactifs *via* le CYP1A) qu'un individu présentant une expression normale. Par contre, il pourra détoxifier plus efficacement d'autres xénobiotiques (ex.: aflatoxine) et leur sera donc plus résistant (Schlenk, 1999).

Seule l'utilisation simultanée de ces trois catégories de biomarqueurs et ce, à plusieurs niveaux d'organisation biologique (moléculaire, cellulaire, tissulaire, physiologique) peut apporter des informations pertinentes sur le mode d'action et les effets des xénobiotiques présents dans le milieu. Il est également possible d'émettre des hypothèses quant à la nature du ou des contaminant(s) lorsque des biomarqueurs spécifiques sont mesurés. Cependant, cette spécificité est toute relative et concerne surtout des familles de polluants. C'est pourquoi l'analyse chimique peut s'avérer nécessaire pour confirmer la présence d'un contaminant donné ou le quantifier. De plus, les biomarqueurs ne concernent qu'un certain nombre d'individus appartenant à une ou plusieurs espèce(s) sentinelle(s). Ils ne peuvent ainsi présenter une signification écologique que lorsqu'ils sont associés à des études concernant les populations et les communautés. Finalement, **seule une approche globale intégrant des analyses chimiques et biologiques (bioindicateurs et biomarqueurs) permet d'évaluer la qualité d'un écosystème et les risques qu'il encourt** (figure 1) (Lagadic et Caquet, 1996).

Au cours de ces dernières décennies, le milieu marin a fait l'objet d'une multitude d'études environnementales. L'utilisation des biomarqueurs s'est généralisée, essentiellement chez les moules et les poissons (Livingstone, 1993). En comparaison, peu d'études analogues ont été conduites pour évaluer la qualité des milieux dulçaquicoles (d'eau douce), bien que ces derniers soient largement soumis à diverses pollutions d'origine industrielle, agricole et domestique (Réseau de Bassin Adour-Garonne, 1997). Afin de mettre en place des programmes de surveillance et d'évaluation de la qualité de tels milieux, nous avons choisi de travailler sur un composant majeur des communautés benthiques: le mollusque bivalve d'eau douce ***Corbicula fluminea*** **(Müller)**. Cet organisme possède de multiples avantages lui permettant d'être considéré comme une espèce sentinelle potentielle au même titre que *M. edulis* et *M. galloprovincialis* pour le milieu marin (cf. synthèse bibliographique).

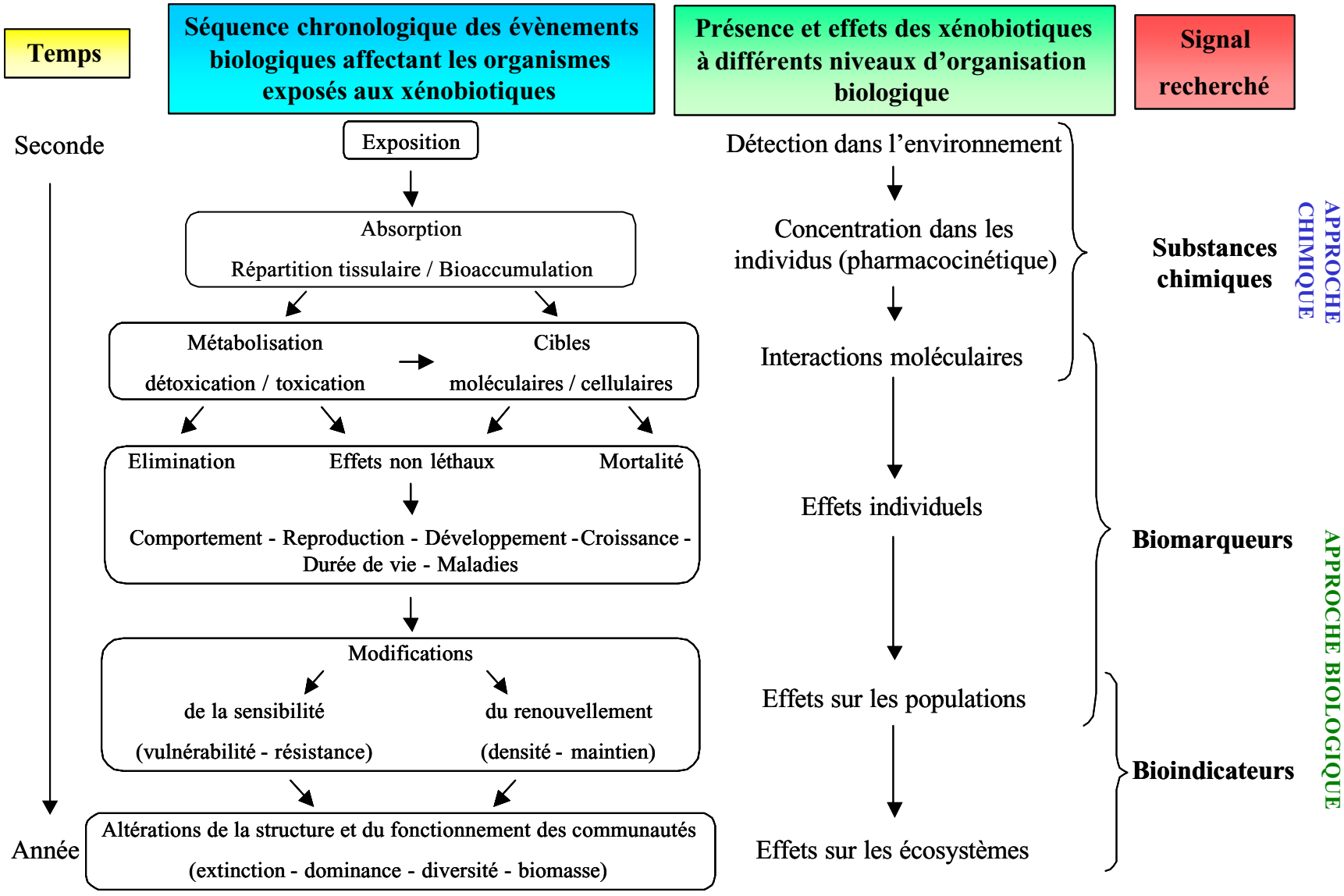


Figure 1. Evaluation de la qualité de l'environnement par une approche globale -chimique et biologique- (d'après Lagadic et Caquet, 1996).

Notre travail s'inscrit ainsi dans une démarche de **validation de *C. fluminea* comme espèce sentinelle. Dans ce but nous avons étudié au sein de cet organisme les variations de plusieurs paramètres biochimiques considérés comme biomarqueurs potentiels de pollution, après exposition à des conditions naturelles ou contrôlées.** Nous avons privilégié une **approche multibiomarqueurs** en sélectionnant les paramètres biochimiques suivants:

- une enzyme impliquée dans les mécanismes de défense antioxydante: la catalase;
- des indicateurs de stress oxydatif: les teneurs en lipides peroxydés et peroxydables;
- des composants du métabolisme de (dé)toxication de phase I: les cytochromes P450, P418 et *b5*; les enzymes NADPH-cytochrome *c* (P450) réductase, NADH-cytochrome *c* (*b*5) réductase et éthoxyrésorufine-*O*-déséthylase NADPH-indépendante;
- des composants du métabolisme de (dé)toxication de phase II: les enzymes glutathion *S*-transférases;
- une enzyme du système nerveux: la propionylcholinestérase.

Une connaissance approfondie de chacun de ces paramètres s'avère fondamentale pour leur utilisation ultérieure en tant que biomarqueur et nécessite la plupart du temps de longues études moléculaires et ce, pour chaque espèce sentinelle considérée. Chez *C. fluminea*, de telles études ont été effectuées pour la propionylcholinestérase (Mora *et al.*, 1999a, 1999b). Les autres paramètres ont été caractérisés, ou sont en cours de caractérisation, chez les moules marines (cf. synthèse bibliographique). Dans le cadre de cette thèse, nous nous sommes particulièrement intéressés aux glutathion *S*-transférases (GSTs), de par le rôle majeur qu'elles exercent dans le métabolisme de (dé)toxication des xénobiotiques (cf. synthèse bibliographique). **Le premier chapitre correspond ainsi à la purification et la caractérisation des différentes isoenzymes de GSTs chez *C. fluminea*.**

Plusieurs études sont nécessaires pour valider l'utilisation de paramètres biochimiques comme biomarqueurs de pollution. En effet, le principal handicap à l'utilisation des biomarqueurs dans le milieu naturel demeure l'interférence avec d'autres facteurs de l'environnement (Amiard *et al.*, 1998). Afin d'interpréter au mieux leurs réponses après exposition à un ou plusieurs contaminant(s), il est donc essentiel de connaître leur variabilité naturelle en fonction de la saison et de divers facteurs biotiques et abiotiques. Nous avons donc dans un

premier temps étudié la variabilité saisonnière de la plupart des paramètres cités précédemment chez deux populations de *C. fluminea* vivant dans des écosystèmes différents: le lac de Cazaux-Sanguinet (département des Landes, notre site de référence) et la rivière Dronne (département de la Gironde). Ces deux sites présentent respectivement une eau de qualité excellente et passable (communication personnelle de la Direction Départementale des Affaires Sanitaires et Sociales -DDASS-; Agence de l'Eau Adour-Garonne, 1996: cf. annexe 1). Les deux populations étudiées ont par ailleurs été caractérisées par des études de génétique biochimique afin d'évaluer leur homogénéité (cf. annexe 2). Par la suite, une étude de laboratoire nous a permis de connaître les effets de plusieurs facteurs abiotiques (température, pH, oxygénation, type d'eau et sédiment) sur les réponses de ces paramètres. **L'ensemble de ces travaux fait l'objet du second chapitre**.

Le milieu naturel est éminemment complexe et peut recéler un grand nombre de contaminants pouvant interagir entre eux selon des actions synergiques, antagonistes ou additives (Seed *et al.*, 1995). Il est donc indispensable d'étudier au préalable les effets individuels de contaminants modèles ou appartenant aux familles les plus fréquemment rencontrées dans les systèmes aquatiques. De telles études s'effectuent tout d'abord en laboratoire en conditions contrôlées. Nous avons ainsi exposé des *C. fluminea* pendant cinq jours à plusieurs doses des contaminants suivants: trichloroéthylène, toluène, chlorure de cadmium et une coupe de goudron de houille riche en HAPs (cf. annexe 3 pour la composition détaillée de ce dernier produit). Les réponses obtenues nous ont permis de mettre en évidence les paramètres biochimiques pouvant *a priori* être utilisés en tant que biomarqueurs pour chacun des contaminants étudiés. Enfin, une étude de terrain sur un site industriel nous a permis d'appréhender l'efficacité d'une approche multibiomarqueurs lors de l'évaluation de la qualité d'un écosystème. **Ces études de laboratoire et de terrain font l'objet du troisième et dernier chapitre**.

SYNTHESE BIBLIOGRAPHIQUE

I. *Corbicula fluminea* (Müller)

I.1. Origine, systématique

Jusqu'au début du 20^{ième} siècle, les espèces vivantes appartenant au genre *Corbicula* étaient présentes sur les continents asiatique, africain, australien et en Nouvelle-Guinée. Par la suite, des *Corbicula* sp. ont été découvertes en Amérique du Nord, en 1924 en Colombie Brittanique au Canada (Counts, 1981) puis en 1938 aux Etats-Unis dans la rivière Columbia, dans l'état de Washington (Britton et Morton, 1979). Ce mollusque bivalve a progressivement colonisé toute l'Amérique du Nord, principalement au sud de la latitude 40° N, puis l'Amérique du Sud et plus récemment l'Europe occidentale (Dubois, 1995). Des *Corbicula* sp. sont désormais présentes au Portugal (Mouthon, 1981; Araujo *et al.*, 1993), en Espagne (Araujo *et al.*, 1993), en Grande-Bretagne (Howlett et Baker, 1999), en France dans la Loire (Gruet, 1992), la Dordogne (Mouthon, 1981), le Canal du Midi (Girardi, 1989-1990), le Canal Latéral à la Garonne (Dubois, 1995), ainsi que dans plusieurs autres rivières du Sud-Ouest (Fontan et Meny, 1996). Plusieurs hypothèses ont été avancées pour tenter d'expliquer la colonisation rapide de nouveaux continents par ce mollusque bivalve. L'intervention humaine, volontaire ou non est la plus probable. Les *Corbicula* sp. sont des mets très appréciés et couramment consommés en Asie. Il est donc possible que des immigrants chinois les aient initialement introduites aux Etats-Unis comme source de nourriture. Par la suite, elles ont pu être disséminées par des pêcheurs les utilisant comme appâts, par des oiseaux ou des poissons bien que ces deux dernières éventualités soient moins probables (Britton et Morton, 1979; Isom, 1986). De plus, les *Corbicula* sp. peuvent dériver en pleine eau selon un processus passif (remous provoqués par des bateaux) ou actif (sécrétion de filaments muqueux par les juvéniles) (Dubois, 1995). La détermination spécifique des *Corbicula* sp. a fait l'objet de nombreuses controverses car elle était initialement basée sur la forme et la couleur de la coquille. Or de tels critères peuvent varier au sein d'une même espèce en fonction de la niche écologique. Deux espèces ont cependant été reconnues: *C. fluminalis* (Müller, 1774), vivant dans les estuaires et n'incubant pas ses larves et *C. fluminea* (Müller, 1774) vivant en eau douce et incubant ses larves (Britton et Morton, 1979). En 1979, Britton et Morton estimaient que seule l'espèce *C. fluminea* avait été introduite en Amérique du Nord. En étudiant par des techniques électrophorétiques des populations de *Corbicula* sp. présentant des différences morphologiques au niveau de la coquille, Hillis et Patton (1982) et Mc Leod

(1986) ont conclu que deux espèces, et non une seule, avaient colonisé les Etats-Unis: la forme «blanche» et la forme «violette» (couleur de la face interne de la coquille). La première correspondrait à *C. fluminea* et la seconde possèderait un statut taxonomique incertain. Concernant les populations européennes, Araujo *et al.* (1993) estiment que la plupart des *Corbicula* sp. décrites appartiennent à l'espèce *C. fluminea* dont la systématique est la suivante:

Embranchement: Mollusca
Classe: Bivalvia
Ordre: Eulamellibranchiata
Sous Ordre: Heterodonta
Super Famille: Corbiculacea
Famille: Corbiculidae
Genre: *Corbicula*
Espèce: *fluminea*

En nous basant sur des critères morphologiques et physiologiques, les deux populations étudiées dans ce travail appartiennent à l'espèce *C. fluminea*.

I.2. Ecologie

C. fluminea est une espèce opportuniste pouvant facilement coloniser différentes niches écologiques. Elle a ainsi été retrouvée dans des cours d'eau, des canaux, des lacs et des retenues d'eau. Elle semble cependant privilégier les environnements lotiques (eaux courantes) aux environnements lentiques (eaux calmes). Sa morphologie et sa physiologie peuvent varier en fonction de l'habitat. Dans un environnement lentique, les individus peuvent présenter une taille importante et une fécondité réduite par rapport à des individus peuplant un cours d'eau où les pressions de sélection sont beaucoup plus sévères (Britton et Morton, 1979). *C. fluminea* peut coloniser différents types de substrats. Les travaux de Belanger *et al.* (1985) ont cependant montré qu'elle préfère le sable fin à du sable composé de grains plus grossiers ou à du gravier. De plus, les densités les plus importantes sont observées au sein de sédiments bien oxygénés (Belanger, 1991) car *C. fluminea* tolère mal les conditions hypoxiques extrêmes (Johnson et McMahon, 1998). La température de l'eau est également un

facteur déterminant. Ainsi, la croissance de *C. fluminea* est optimale vers 20°C (Foe et Knight, 1986). D'après les travaux de Mattice et Dye (1976) les températures léthales inférieure et supérieure seraient de 2°C et 34°C, respectivement. Dans un environnement présentant des conditions optimales, la population croît rapidement et des densités de plusieurs milliers d'individus par m^2 peuvent être atteintes. Malgré tout, une mortalité massive et soudaine est parfois observée. Différents facteurs peuvent expliquer ce phénomène: de fortes températures associées à de faibles teneurs en oxygène dissous ou au contraire des températures très basses lors d'hivers rigoureux, la présence d'importantes quantités de particules de boue ou d'argile lors des crues de printemps, un pH trop acide entraînant une dissolution de la coquille et donc une absence de protection, des contaminations par des bactéries, des virus ou des parasites, la présence de polluants, des prédateurs (poissons, oiseaux, ratons laveurs, écrevisses, vers plats), une compétition inter- ou intraspécifique ainsi que des modifications génétiques (Sickel, 1986).

Depuis son apparition aux Etats-Unis, *C. fluminea* a posé (et pose encore) de nombreux problèmes tant au niveau matériel qu'au niveau écologique. Le fait qu'elle soit une espèce non-indigène opportuniste soulève la question suivante: entre-t-elle en compétition avec les espèces indigènes et si oui, de quel côté penche la balance? Il est indéniable que *C. fluminea* présente des avantages morphologiques et physiologiques par rapport à certaines espèces indigènes de mollusques. Par exemple, son cycle de reproduction ne nécessite pas l'intervention d'un hôte (poisson). Cependant, la nature de l'environnement joue également un grand rôle. Une compétition interspécifique intervient en général lorsque *C. fluminea* rencontre des conditions qui lui sont favorables ou rendues favorables, notamment par l'intervention de l'homme. Il a été ainsi observé que *Corbicula* sp. ne pouvait entrer en compétition avec *Lampsilis* sp., un mollusque indigène d'eau douce, que dans une rivière «modifiée» par l'homme (Kraemer, 1979). En effet, le dragage des fonds pour entretenir les réseaux de navigation et la stabilisation des berges permettent l'apparition de fins sédiments, favorables au développement de *Corbicula* sp. mais ne convenant plus à celui de *Lampsilis* sp. Dans les zones où les densités de *C. fluminea* sont très élevées, une forte diminution du phytoplancton a été constatée (Cohen *et al.*, 1984; Leff *et al.*, 1990). Chaque individu peut en effet filtrer de 109 à 1370 ml d'eau/h ou de 160 à 861 ml d'eau/h selon les études (Buttner et Heidinger, 1981; Lauritsen, 1986). Cependant, cette diminution de phytoplancton n'entraîne pas obligatoirement la disparition des espèces indigènes (Leff *et al.*; 1990). Enfin, la présence de poissons prédateurs peut limiter l'apparition de fortes densités de *C. fluminea*, et par là

même éviter l'instauration de compétitions interspécifiques (Robinson et Wellborn, 1988). Chaque cas est donc particulier.

Outre son impact sur les écosystèmes, *C. fluminea* est la source de nombreux problèmes matériels aux Etats-Unis. Le plus important est le colmatage des circuits de pompage d'eau d'usines, de centrales électriques et nucléaires et de réseaux municipaux. Les dégâts occasionnés par *C. fluminea* ainsi que la mise en oeuvre de moyens de contrôle efficaces entraîneraient un coût estimé à 1 milliard de dollars par an (Isom, 1986; Cairns et Bidwell, 1996).

I.3. Morphologie, physiologie

C. fluminea est un mollusque bivalve dont la durée de vie est de 2 à 3 ans. Ses organes sont enserrés à l'intérieur d'une coquille comprenant deux valves de taille et de forme identiques reliées dorsalement par un épais ligament externe. La **coquille** est formée par du carbonate de calcium et peut atteindre une taille d'environ 3 cm. Sa surface externe est généralement de couleur sombre (marron à vert-olive) et recouverte par le *periostracum* qui la protège de la dissolution dans les eaux acides (figure 2).

Figure 2. *Corbicula fluminea* (Müller) provenant du lac de Cazaux-Sanguinet (photo: P. Mora, 1997).

Différents muscles viennent s'attacher au niveau de sa surface interne. *C. fluminea* peut s'enterrer dans le sédiment grâce à son **pied** et vit ainsi à l'interface sédiment-eau, ne laissant affleurer que ses deux siphons (figure 3) (Britton et Morton, 1979, 1982).

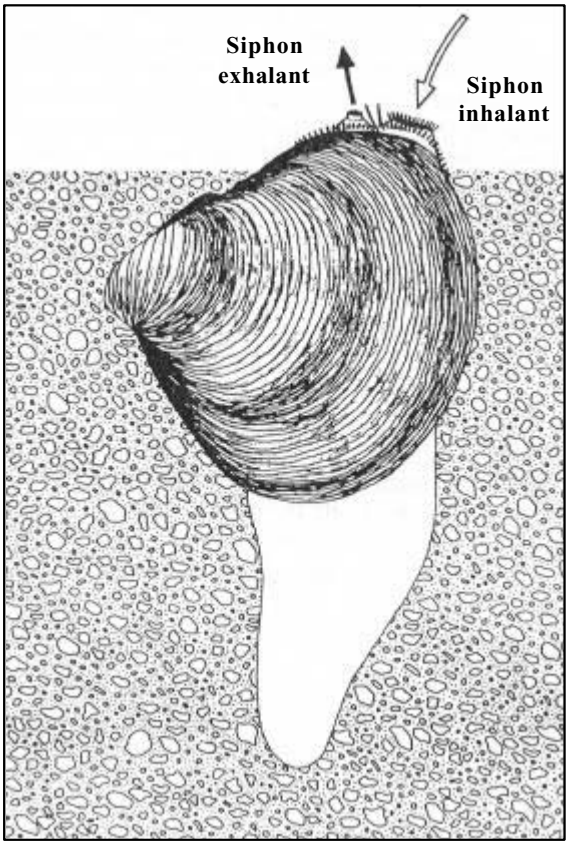


Figure 3. *Corbicula fluminea* dans sa position naturelle, à l'interface sédiment-eau (d'après Britton et Morton, 1982).

Les tissus et organes principaux de *C. fluminea* sont le manteau, les branchies, le coeur, le rein et la masse viscérale comprenant la glande digestive et les gonades (figure 4). Les aspects morphologiques et fonctionnels des branchies et de la masse viscérale seront plus particulièrement développés.

Le **manteau** repose sous la coquille dont il assure la formation. Il est constitué de deux lobes dont la fusion postérieure donne naissance aux **siphons inhalant et exhalant**. La cavité formée par le manteau baigne constamment dans l'eau et renferme le corps de l'animal. Les **branchies** (*ctenidia*) consistent en deux paires de feuillets (hémibranchies) situées de part et d'autre du corps. Chaque paire comprend une hémibranchie externe et une hémibranchie interne, plus importante. Au niveau de leur partie postérieure les branchies séparent le siphon

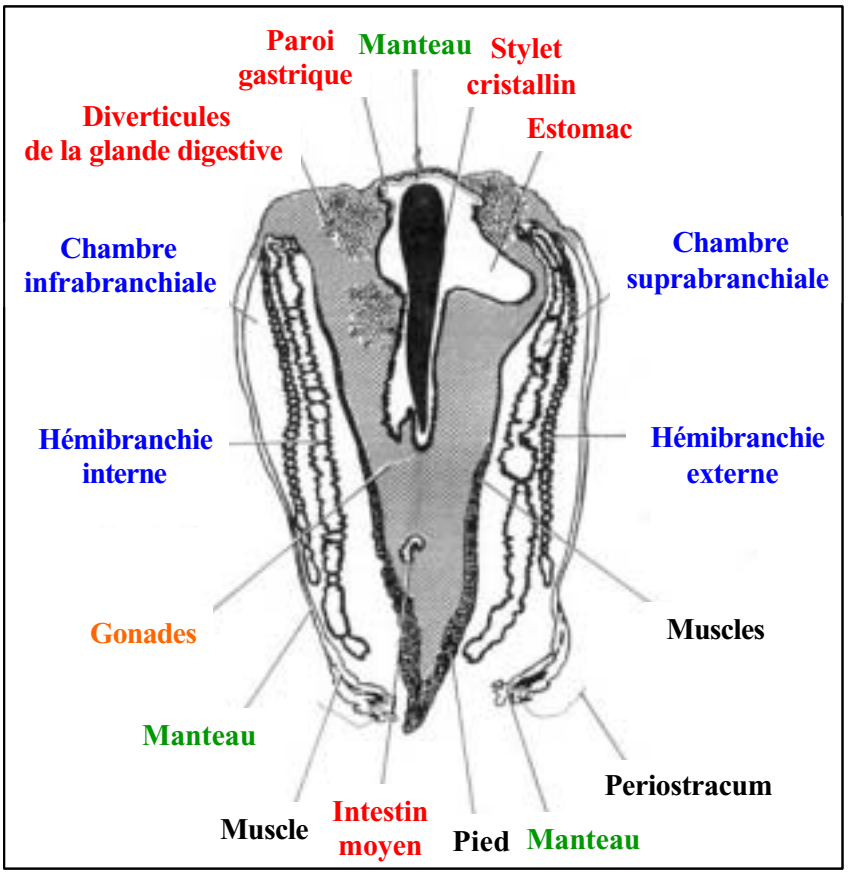


Figure 4. Coupe transversale de *Corbicula fluminea* mettant en évidence les principaux organes (d'après Britton et Morton, 1982).

inhalant du siphon exhalant. Elles sont reliées antérieurement aux palpes labiaux. Elles divisent également la cavité formée par le manteau en deux chambres infrabranchiale et suprabranchiale. Chaque hémibranchie est formée d'une lamelle ascendante et d'une lamelle descendante reliées entre elles par des connections interlamellaires. L'espace entre deux lamelles constitue une chambre interlamellaire communiquant avec la chambre suprabranchiale. Les lamelles descendantes des deux hémibranchies sont reliées entre elles et à la masse viscérale au niveau de l'axe ctenidial. Chaque lamelle comprend une multitude de filaments reliés entre eux par des connections interfilamentaires. Certains filaments sont séparés par des pores (*ostia*) permettant l'accès à la chambre interlamellaire. Un filament consiste en un épithélium divisé en deux zones principales: une zone respiratoire dans la chambre interlamellaire et une zone ciliée vers l'extérieur. Les branchies possèdent un rôle capital dans la respiration, la nutrition mais aussi la reproduction de *C. fluminea*. Les échanges gazeux ont lieu au niveau des vaisseaux sanguins des branchies mais aussi à la surface du manteau. L'eau pénètre dans la chambre infrabranchiale par le siphon inhalant, puis dans les chambres interlamellaires *via* les pores et quitte finalement l'animal par le

siphon exhalant *via* la chambre suprabranchiale. Les particules transportées par l'eau ne peuvent pénétrer dans les chambres interlamellaires. Elles sont donc véhiculées le long de chaque hémibranchie grâce à l'action conjuguée de nombreux cils recouvrant les filaments. Certaines cellules de l'épithélium (mucocytes) sécrètent un mucus facilitant la collecte et la migration de ces particules. Les particules les plus petites peuvent pénétrer dans le sillon ventral de chaque hémibranchie et sont ensuite acheminées vers les palpes labiaux tandis que les plus grosses sont rejetées dans la cavité du manteau. Les **palpes labiaux** sont situés de part et d'autre de la masse viscérale et sont reliés à la fois aux branchies et à la bouche. La présence de sillons et de cils leur permet d'effectuer un tri supplémentaire au sein des particules provenant des branchies. Seules les particules les plus petites et constituant une source de nourriture sont dirigées vers la bouche, les autres sont rejetées à la surface de la masse viscérale, également recouverte de cils, puis dans la cavité du manteau. Finalement, les particules rejetées soit par les branchies, soit par les palpes labiaux sont véhiculées par les cils recouvrant le manteau vers le siphon inhalant au niveau duquel elles sont libérées dans le milieu extérieur sous forme de pseudo-fèces (figure 5) (Britton et Morton, 1982; Lemaire-gony et Boudou, 1997).

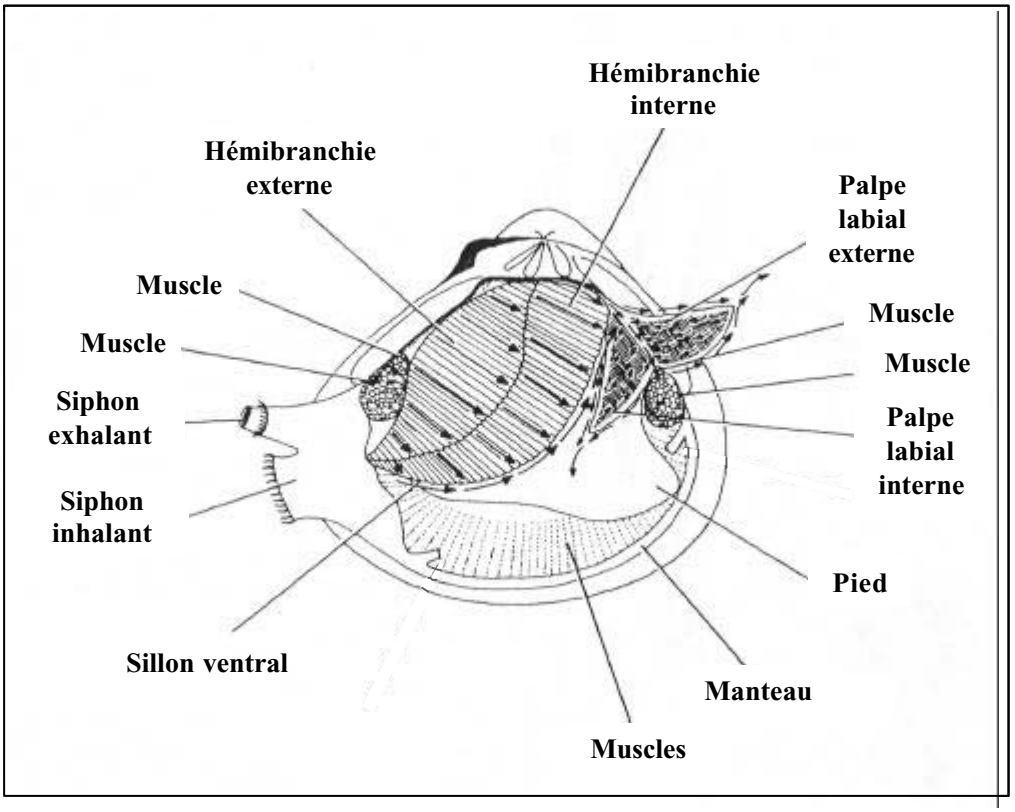


Figure 5. Coupe longitudinale de *Corbicula fluminea* mettant en évidence le trajet des particules à la surface des branchies et des palpes labiaux (d'après Britton et Morton, 1982).

Des particules provenant du sédiment peuvent également être acheminées vers les palpes labiaux grâce aux cils recouvrant le pied. Un tel phénomène pourrait intervenir lorsque *C. fluminea* se développe sur des substrats riches en substances organiques ou lorsque l'eau contient une faible concentration de particules en suspension (Way *et al.*, 1990). Les hémibranchies internes jouent également un rôle majeur dans la reproduction en stockant les oeufs fécondés.

Au sein de la **masse viscérale**, la glande digestive et les gonades sont intimement liées. La **bouche** communique avec l'**œsophage** qui s'ouvre à son tour sur l'**estomac**. Celui-ci est entouré par les diverticules de la **glande digestive** assurant l'absorption et la digestion intracellulaire des particules de nourriture. La paroi interne de l'estomac comprend de multiples zones ciliées assurant un tri entre les petites et les grosses particules. Seules les plus petites sont absorbées. Les autres sont acheminées vers l'**intestin moyen**. Une des caractéristiques de l'estomac est la présence d'un **stylet cristallin**. Ce dernier est enserré dans un sac communiquant avec l'intestin moyen. Il est formé d'une matrice de mucopolysaccharides associée à des enzymes qui sont libérées dans l'estomac afin d'assurer la digestion extracellulaire des particules de nourriture. Le stylet agit également comme un pilon permettant la fragmentation des particules les plus larges. L'intestin moyen est relié à l'**intestin postérieur** puis au **rectum** et à l'**anus** (figure 6). Les fèces sont déversées dans la chambre suprabranchiale et rejetés de l'organisme par le siphon exhalant (Britton et Morton, 1982).

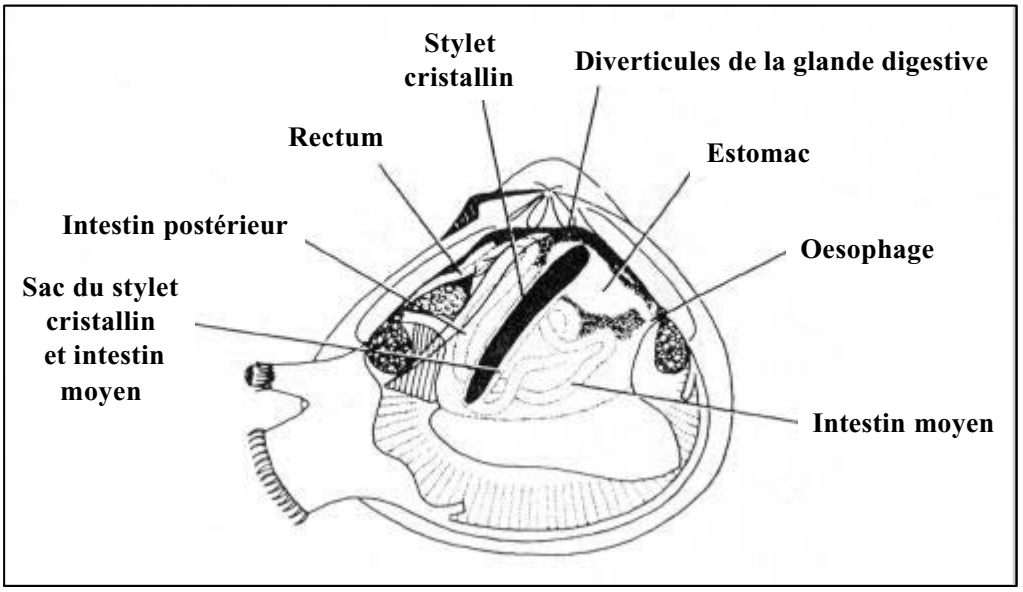


Figure 6. Coupe longitudinale de *Corbicula fluminea* mettant en évidence l'appareil digestif au sein de la masse viscérale (d'après Britton et Morton, 1982).

Le système reproducteur occupe une grande partie de la masse viscérale. *C. fluminea* est une espèce hermaphrodite. Les **gonades** sont constituées par de nombreux follicules ovariens et tubes séminifères dans lesquels ont lieu l'ovogenèse et la spermatogenèse, respectivement. Les oeufs matures sont sphériques et contiennent un noyau entouré par le vitellus puis la membrane vitelline et les spermatozoïdes sont biflagellés. Au cours du développement de *C. fluminea*, les follicules ovariens apparaissent en premier. Lorsqu'ils sont bien différenciés et lorsque l'ovogenèse est suffisamment avancée, la spermatogenèse peut commencer. L'ovogenèse se produit toute l'année avec cependant un ralentissement pendant l'hiver. La spermatogenèse est quant à elle saisonnière et est induite par des variations de température. La plupart des *C. fluminea* présentent deux pics de spermatogenèse dans l'année, le premier au printemps lorsque la température augmente et le second à la fin de l'été et/ou au début de l'automne lorsque la température diminue. Dans le cas d'une fécondation croisée, les spermatozoïdes quittent la masse viscérale par les gonopores situés de part et d'autre de celle-ci et sont libérés de l'organisme *via* la chambre suprabranchiale et le siphon exhalant. Ils viennent féconder une autre *C. fluminea* en pénétrant dans la chambre infrabranchiale. Ils migrent ensuite dans la chambre suprabranchiale des hémibranchies internes pour fertiliser les oeufs (provenant des follicules ovariens *via* les gonopores). La proximité des gamètes males et femelles au sein de la masse viscérale entraîne parfois des autofécondations. Des embryons ont été mis ainsi en évidence dans la masse viscérale. Les différents stades de développement des **larves** sont les suivants: **blastula**, **gastrula**, **trocophore**, **véligère**, **pédivéligère**, **premier stade juvénile** et **second stade juvénile**. Les larves sont libérées dans l'environnement lorsqu'elles ont atteint l'un des deux stades juvéniles ou plus rarement le stade pédivéligère (environ 250 µm), c'est à dire après 6 à 12 jours d'incubation dans les branchies. Lorsque leur taille atteint environ 1 mm, elles sont capables de produire un byssus leur permettant de se fixer sur un substrat solide. Ce byssus est transitoire et disparaît au maximum au bout d'un an (Britton et Morton, 1982; Kraemer et Galloway, 1986; Kraemer *et al.*, 1986).

Le **système nerveux** est constitué par des ganglions reliés entre eux par des nerfs. La cavité cœlomique comprend le **coeur** et les **reins**. L'urine est déversée dans la chambre suprabranchiale et rejetée de l'organisme par le siphon exhalant.

I.4. Utilisation en tant qu'espèce sentinelle

C. fluminea présente plusieurs caractéristiques lui permettant d'être considérée comme une espèce sentinelle potentielle. (1) Son mode de vie à l'interface sédiment-eau lui permet d'être en contact avec ces deux compartiments. Elle doit filtrer de grandes quantités d'eau pour remplir ses besoins respiratoires et nutritionnels mais peut également se nourrir de particules provenant du sédiment. Elle est ainsi susceptible d'accumuler des contaminants présents dans la colonne d'eau et les sédiments. (2) Elle s'est largement répandue aux Etats-Unis depuis son introduction dans les années 30s et constitue dorénavant un composant majeur des communautés benthiques. Sa présence en Europe, et notamment en France est également devenue courante. De plus, ses grandes capacités d'adaptation lui permettent de peupler de multiples habitats, lentiques et lotiques. En particulier, sa croissance rapide, sa durée de vie relativement longue (plusieurs années) et son mode de reproduction particulièrement efficace lui permettent d'atteindre rapidement de fortes densités. Il est donc relativement aisé de trouver un ou plusieurs site(s) peuplé(s) par *C. fluminea* et de prélever un nombre suffisant d'animaux pour la réalisation d'études de laboratoire ou de terrain. (3) Elle est hermaphrodite, ce qui permet d'éliminer les variations inhérentes au sexe dans les études environnementales. (4) Enfin, sa taille est suffisamment importante (jusqu'à 3 cm) pour permettre la dissection et l'étude de ses organes majeurs, et suffisamment réduite pour travailler sur un grand nombre d'individus.

En 1990 Doherty a recensé de nombreuses études environnementales de laboratoire et de terrain ayant été effectuées sur *C. fluminea*. D'après ces études, elle est capable de bioaccumuler des métaux lourds (cuivre, cadmium, zinc, plomb, *etc.*) essentiellement à partir de la colonne d'eau, ainsi que des contaminants organiques (pesticides, polychlorobiphényls). Après exposition des animaux à ces polluants, divers effets concernant des paramètres biochimiques (activités enzymatiques, teneurs en protéines, lipides et glycogène), physiologiques (respiration) ou comportementaux (fermeture/ouverture des valves) ont également été observés. Des études plus récentes ont mis en évidence la bioaccumulation de pentachlorophénol (Basack *et al.*, 1997), de mercure, de cadmium (Inza *et al.*, 1997), de plomb, d'uranium (Labrot *et al.*, 1998) et d'hydrocarbures aromatiques polycycliques (Narbonne *et al.*, 1999) ainsi que des réponses significatives de plusieurs marqueurs biochimiques après exposition au plomb, à l'uranium (Labrot *et al.*, 1996), au cadmium (Baudrimont *et al.*, 1997) et à des insecticides organophosphorés (Basack *et al.*, 1998).

Cependant, malgré tous les avantages présentés par *C. fluminea* pour son utilisation en tant qu'organisme sentinelle, Doherty (1990) souligne qu'il serait inopportun d'introduire cette espèce invasive dans des cours d'eau où elle n'est pas encore présente, lors d'études environnementales.

II. Les marqueurs biochimiques étudiés

II.1. Les indicateurs de stress oxydatif

Bien que la consommation d'oxygène soit indispensable à toute forme de vie aérobie, elle entraîne également la formation de composés réactifs - les espèces activées de l'oxygène (EAO) - pouvant exercer de multiples effets délétères sur les composants cellulaires. Les EAO sont produites naturellement, soit de manière «accidentelle» par les monooxygénases à cytochrome P450, les chaînes mitochondriales transporteuses d'électrons, *via* l'auto-oxydation de certaines molécules (la plupart du temps catalysée par des ions métalliques de transition), soit de manière «délibérée» par des phagocytes activés, des lymphocytes, des fibroblastes, *etc.* (Halliwell et Cross, 1994). Des facteurs exogènes peuvent également initier la synthèse d'EAO: les radiations ionisantes et certains xénobiotiques dont les hydrocarbures aromatiques polycycliques (Lemaire et Livingstone, 1993). L'organisme possède naturellement des systèmes de défense antioxydante lui permettant de piéger ou d'inactiver ces EAO. Cependant, dans certaines conditions ces systèmes ne sont pas suffisants. Ainsi, lorsque l'équilibre entre les forces prooxydantes et les défenses antioxydantes est rompu, les cellules subissent un stress oxydatif.

II.1.1. Les principales espèces activées de l'oxygène (EAO)

Les EAO incluent des formes radicalaires (radicaux libres) correspondant à des structures mono- ou poly-atomiques indépendantes possédant un ou plusieurs électrons non apparié(s) isolé(s) sur une orbitale externe, mais également des formes non radicalaires (Halliwell et Cross, 1994).

L'anion superoxyde ($O_2^{-\bullet}$) résulte de la réduction monoélectronique de l'oxygène moléculaire:

$$O_2 + e^- \rightarrow O_2^{-\bullet}$$

Il est produit au cours de réactions d'autooxydation de molécules telles que les quinones, de réactions enzymatiques (monooxygénases à cytochrome P450, NADPH oxydase, xanthine oxydase, *etc.*), lors du transport d'électrons dans la chaîne respiratoire et sous l'action de facteurs environnementaux tels que les radiations ionisantes (X, gamma) ou certains xénobiotiques (Sies et Cadenas, 1983). En solution aqueuse, l'anion superoxyde se comporte comme un faible agent oxydant capable d'oxyder l'acide ascorbique ou des thiols. Par contre, c'est un fort agent réducteur pouvant réduire certains complexes de métaux. Ces réactions peuvent donner lieu à sa dismutation en générant de l'oxygène moléculaire et une autre espèce réactive (mais non radicalaire): **le peroxyde d'hydrogène** (**H_2O_2**). Il est également capable de se dismuter par voie enzymatique (*via* la superoxyde dismutase) ou spontanément (Aruoma, 1998). Ainsi, tout système générateur d'anion superoxyde produit indirectement du peroxyde d'hydrogène. D'autres oxydases peuvent être directement à l'origine de la formation de celui-ci comme, par exemple, l'acyl-CoA oxydase, la NADH oxydase ou la glutathion oxydase (Sies et Cadenas, 1983).

Exemple de réaction conduisant à la formation de peroxyde d'hydrogène: dismutation spontanée de l'anion superoxyde:

$$O_2^{-\bullet} + H^+ \rightarrow HO_2^{\bullet}$$

$$O_2^{-\bullet} + HO_2^{\bullet} + H^+ \rightarrow H_2O_2 + O_2$$

Le peroxyde d'hydrogène est un faible agent oxydant et réducteur relativement stable en l'absence de métaux de transition libres. Il est faiblement réactif à de faibles concentrations. Par contre, il peut inactiver certaines enzymes telles que la glyceraldéhyde-3-phosphate déshydrogénase à de fortes concentrations. Il peut également conduire à la formation du **radical hydroxyle** (**$^{\bullet}OH$**) en réagissant avec l'anion superoxyde selon la réaction d'Haber-Weiss (Haber et Weiss, 1934):

$$O_2^{-\bullet} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$$

Bien que thermodynamiquement favorable, cette réaction est très lente (Halliwell, 1978). Par contre, la production du radical hydroxyle peut devenir significative en présence de métaux de transition tels que le fer ou le cuivre *via* les deux réactions suivantes:

$$Fe^{3+} + O_2^{-\bullet} \rightarrow Fe^{2+} + O_2 \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-} (2)$$

La seconde réaction est dite réaction de Fenton (Walling, 1975). La somme des deux réactions correspond à la réaction d'Haber-Weiss. Le radical hydroxyle est une espèce hautement réactive pouvant attaquer la plupart des molécules biologiques dont les protéines, les lipides et les acides nucléiques.

II.1.2. Les principaux systèmes de défense antioxydante

Selon Gutteridge (1995), un antioxydant peut être défini comme toute substance qui, lorsqu'elle est présente à de faibles concentrations par rapport à celles d'un substrat oxydable, retarde considérablement ou inhibe l'oxydation de ce dernier. Plusieurs mécanismes de défense existent, enzymatiques et non-enzymatiques.

II.1.2.1. Enzymes antioxydantes

Trois principales activités enzymatiques peuvent détruire les EAO: la **superoxyde dismutase** (**SOD**), la **catalase** (**CAT**) et les **glutathion peroxydases** (**GPX**).

Il existe trois types de **SOD**: une SOD contenant du manganèse, présente dans la matrice mitochondriale des eucaryotes et chez certaines bactéries, une SOD à fer présente dans le périplasme des bactéries anaérobies facultatives et une SOD contenant du cuivre et du zinc, présente dans le cytosol des cellules eucaryotes (Sies et Cadenas, 1983; Pelmont, 1995). La SOD catalyse la dismutation de l'anion superoxyde en oxygène moléculaire et peroxyde d'hydrogène à une vitesse largement supérieure à celle de la dismutation spontanée, selon la réaction (exemple de la SOD à cuivre et à zinc):

$$SOD\text{-}Cu^{2+} + O_2^{-\bullet} \rightarrow SOD\text{-}Cu^{+} + O_2$$

$$SOD\text{-}Cu^{+} + O_2^{-\bullet} + 2H^{+} \rightarrow SOD\text{-}Cu^{2+} + H_2O_2$$

Le peroxyde d'hydrogène est pris en charge par la **CAT**. Cette enzyme est présente chez tous les organismes aérobies. Chez les eucaryotes, elle est principalement située à l'intérieur des peroxysomes (corpuscules cytoplasmiques entourés d'une membrane simple). Ces derniers contiennent également des oxydases, génératrices de peroxyde d'hydrogène. La plupart des CAT sont formées de quatre sous-unités identiques contenant chacune un ion Fe^{3+} situé dans un noyau héminique (Pelmont, 1995). La CAT transforme le peroxyde d'hydrogène en oxygène et eau moléculaires selon la réaction suivante:

$$\mathbf{CAT\text{-}Fe(III) + H_2O_2 \rightarrow CAT\text{-}Fe(V)=O + H_2O}$$
$$\mathbf{CAT\text{-}Fe(V)=O + H_2O_2 \rightarrow CAT\text{-}Fe(III) + O_2 + H_2O}$$

Le peroxyde d'hydrogène peut également être transformé en eau moléculaire par certaines **GPXs**. Il existe principalement deux types de GPXs: sélénium-dépendante et sélénium-indépendante. Elles utilisent toutes deux le glutathion réduit (GSH) comme donneur d'électrons. Par contre, alors que la GPX sélénium-dépendante réduit le peroxyde d'hydrogène et de nombreux hydroperoxydes (ROOH), la GPX sélénium-indépendante ne réduit que les hydroperoxydes (Sies et Cadenas, 1983). Cette dernière activité est attribuée à certaines isoformes de la glutathion *S*-transférase, enzyme impliquée dans le métabolisme de (dé)toxication de phase II (Ketterer *et al.*, 1988). Les deux réactions catalysées par les GPX sont les suivantes:

$$\mathbf{H_2O_2 + 2\ GSH \rightarrow 2\ H_2O + GSSG}$$
$$\mathbf{ROOH + 2\ GSH \rightarrow ROH + H_2O + GSSG}$$

GSSG: glutathion oxydé.
La régénération du GSH est assurée par des glutathion réductases NADPH-dépendantes:

$$\mathbf{GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+}$$

II.1.2.2. Molécules antioxydantes

Ce sont des molécules à faible poids moléculaire, liposolubles ou hydrosolubles. La **vitamine E** (alpha-tocophérol), liposoluble, est le principal composé antioxydant au sein des

membranes. Elle désactive les radicaux peroxyles (ROO $^{\bullet}$) formés au cours de la peroxydation lipidique, inhibant ainsi la propagation de cette dernière. Le radical tocopheryloxyl en résultant est suffisamment stable pour ne pas propager la peroxydation. Il peut au contraire réagir avec un autre radical peroxyle (réaction de terminaison) ou être reconverti en alpha-tocophérol par d'autres molécules antioxydantes, hydrosolubles, comme la **vitamine C** (acide ascorbique). Cette dernière peut également se comporter comme un agent prooxydant, en réduisant le Fe^{3+} en Fe^{2+}, membre actif de la réaction de Fenton (Kaur et Perkins, 1991). De nombreuses autres molécules antioxydantes existent telles que le **bêta-carotène**, le **rétinol** (liposolubles), l'**acide urique** et le **glutathion réduit** (hydrosolubles).

II.1.3. Les effets du stress oxydatif

Les effets du stress oxydatif sont multiples et concernent d'importantes cibles moléculaires telles que les **acides nucléiques**, les **protéines** et les **lipides**. Le principal agent responsable de ces effets délétères est le radical hydroxyle. Il peut notamment réagir avec les bases de l'**ADN**. Malgré l'existence de sytèmes de réparation de l'ADN, ces modifications peuvent jouer un rôle important dans les mécanismes de cancérogenèse (Klaunig *et al.*, 1998). Les **protéines** sont facilement attaquées par les EAO, soit directement, soit indirectement *via* la peroxydation lipidique. L'oxydation des acides aminés peut dans le cas de la cystéine, conduire à la formation de ponts disulfures intra- ou inter-moléculaires. Ces modifications structurales peuvent altérer les fonctions des protéines: inhibition ou stimulation d'activités enzymatiques, altération des mécanismes de transduction des signaux, *etc.* (Klaunig *et al.*, 1998). La **peroxydation lipidique** concerne les acides gras polyinsaturés, particulièrement nombreux dans les membranes biologiques. Elle peut être d'origine enzymatique ou non-enzymatique. Dans ce dernier cas, elle consiste en trois réactions de type radicalaire: initiation, propagation et terminaison (Kappus, 1991). La première étape, dite réaction d'initiation, consiste en l'abstraction d'un atome d'hydrogène au niveau d'un groupement allylique CH_2 d'un acide gras polyinsaturé par une espèce hautement réactive, en l'occurence un radical hydroxyle ($^{\bullet}$OH), alkoxyle (RO $^{\bullet}$) ou peroxyle (ROO $^{\bullet}$) pour donner un radical alkyle (R $^{\bullet}$):

$$\mathbf{RH + X^{\bullet} \rightarrow R^{\bullet} + XH}$$

Il s'ensuit un réarrangement moléculaire permettant l'obtention d'un diène conjugé. Puis ce dernier réagit avec une molécule d'oxygène pour former un radical peroxyle très réactif (ROO $^{\bullet}$):

$$\mathbf{R^{\bullet} + O_2 \rightarrow ROO^{\bullet}}$$

Le radical peroxyle peut à son tour attaquer une nouvelle chaîne d'acide gras polyinsaturé (réaction de propagation) donnant naissance à un nouveau radical alkyle et à un hydroperoxyde lipidique (ROOH):

$$\mathbf{ROO^{\bullet} + R'H \rightarrow ROOH + R'^{\bullet}}$$

Le processus de peroxydation se poursuit jusqu'à ce que deux radicaux réagissent entre eux ou qu'une molécule antioxydante intervienne (ex.: vitamine E) lors de la réaction de terminaison:

$$\mathbf{R^{\bullet} + R^{\bullet} \rightarrow R\text{-}R}$$

$$\mathbf{R^{\bullet} + ROO^{\bullet} \rightarrow ROOR}$$

$$\mathbf{ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + {}^1O_2 \text{ (oxygène singulet)}}$$

Les hydroperoxydes lipidiques peuvent être décomposés par chauffage, irradiation ou *via* l'intervention d'ions métalliques. Cette dernière voie (réaction de Fenton) est la plus courante dans les systèmes biologiques et produit des radicaux alkoxyles très réactifs (RO $^{\bullet}$):

$$\mathbf{Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO^{\bullet} + OH^{-}}$$

Des composés à hème, comme le cytochrome P450, peuvent également catalyser la décomposition des hydroperoxydes lipidiques (Kappus, 1991).

Les radicaux alkoxyles peuvent ensuite donner naissance à de multiples produits de dégradation: alcools, cétones, aldéhydes, éthers, acides et alcanes. Parmi les aldéhydes formés, le malonedialdéhyde (MDA) est souvent pris en considération pour évaluer le degré de peroxydation lipidique. Il se forme à partir d'acides gras polyinsaturés possédant au moins

trois doubles liaisons successives. Le principe du dosage repose sur la réaction du MDA avec l'acide thiobarbiturique (ATB) à chaud. L'adduit en résultant absorbe à 532 nm (Buege et Aust, 1978). Cependant, ce dosage n'est pas spécifique du MDA car d'autres aldéhydes peuvent réagir avec l'ATB d'où le terme anglo-saxon «thiobarbituric acid reactive species» (TBARS). De plus, le MDA est un composé volatile pouvant réagir avec différentes biomolécules dont les protéines et les acides nucléiques (Janero, 1990).

II.1.4. Les indicateurs de stress oxydatif chez les mollusques bivalves et leur utilisation comme biomarqueurs de pollution

Les principaux systèmes de défense antioxydante ont été mis en évidence chez les invertébrés aquatiques (Winston et Di Giulio, 1991; Lemaire et Livingstone, 1993). En particulier, chez la moule *Mytilus edulis*, l'activité CAT est principalement peroxysomiale, l'activité SOD est cytosolique (SOD à cuivre et zinc) et mitochondriale (SOD à manganèse) et les activités GPXs (sélénium-dépendante et -indépendante) sont principalement cytosoliques (Livingstone, 1992). Les activités CAT, SOD et GPX sélénium-dépendante sont plus importantes dans la glande digestive que dans les branchies ou les tissus musculaires (Gamble *et al.*, 1995). Outre les enzymes antioxydantes, les molécules antioxydantes de faible poids moléculaire sont également présentes chez les moules et sont principalement localisées dans la glande digestive et les branchies: vitamines E, C et A, GSH (Ribera *et al.*, 1989). Chez les invertébrés aquatiques, de nombreuses études ont mis en évidence la modulation des activités CAT, SOD et GPX, des concentrations en molécules antioxydantes mais également du niveau de peroxydation lipidique après exposition à certains xénobiotiques dont les métaux lourds et les hydrocarbures aromatiques polycycliques (tableau 1). D'une manière générale, un indicateur de stress oxydatif peut présenter diverses réponses (parfois même contradictoires) après exposition d'un même organisme à un même xénobiotique. Cela peut être dû à des différences concernant le mode, la dose et/ou la durée d'exposition. Cependant, les indicateurs de stress oxydatif sont également sujets à de fortes variations saisonnières, ce qui peut compliquer l'interprétation des résultats (Viarengo *et al.*, 1991; Power et Sheehan, 1996).

Tableau 1. Modulation des indicateurs de stress oxydatif chez les mollusques bivalves après exposition à différents polluants.

Organisme	Polluant [a]	Mode d'exposition [b]	Paramètre [c]	Variation [d]	Référence
Adamussium colbecki	Cu ou Hg	L	CAT GSH	↘ ↘	Regoli *et al.*, 1997
Corbicula sp.	Pb	L	CAT GPX PL	↘ ↘ ↗ ou ↘	Labrot *et al.*, 1996
	U	L	CAT GPX PL	↘ - ↘	Labrot *et al.*, 1996
Mytilus edulis	B[*a*]P	L	SOD CAT GPX PL PLI	- - - - ↘	Livingstone *et al.*, 1990
	B[*a*]P	L	SOD CAT	↗ ou ↘ ↘	Eertman *et al.*, 1995
	Fluoranthène	L	SOD CAT	↗ ou ↘ ↘	Eertman *et al.*, 1995
	HAPs	L	CAT	↗	Cajaraville *et al.*, 1992
	Pétrole	T	SOD PL CAT	- ↗ ↘	Solé *et al.*, 1996
	HAPs, Cu	M	GSH	↘	Suteau *et al.*, 1988
	HAPs, Cu, PCBs	T	GSH	-	Suteau *et al.*, 1988

Tableau 1 (suite).

Organisme	Polluant [a]	Mode d'exposition [b]	Paramètre [c]	Variation [d]	Référence
Mytilus galloprovincialis	Cu	L	PL GSH	↗ ↘	Viarengo *et al.*, 1990
	Cu	L	SOD CAT GPX GSH	↗ ou - ↘ ou - ↗ ou - ↘	Regoli et Principato, 1995
	Cu	L	GSH	↘	Canesi *et al.*, 1999
	Hg	L	GSH	↗ ou -	Canesi *et al.*, 1999
	CH_3Hg	L	GSH	↘	Canesi *et al.*, 1999
	Cd ou Zn	L	PL GSH	- -	Viarengo *et al.*, 1990
	Métaux	T	SOD CAT GPX GSH	- ↗ ou - - ↘	Regoli et Principato, 1995
	B[*a*]P	L	CAT	↘	Cancio *et al.* ,1998
	B[*a*]P	M	CAT	↗	Akcha et *al.*, 2000
	TCB	L	GSH	↗	Michel *et al.*, 1993
	HCB	L	GSH	↗	Michel *et al.*, 1993
	HAPs, PCBs, HAs	T	SOD CAT	- -	Livingstone *et al.*, 1995

Tableau 1 (suite).

Organisme	Polluant [a]	Mode d'exposition [b]	Paramètre [c]	Variation [d]	Référence
Perna viridis	Al, Pb ou Cd	L	SOD CAT PL	↗ ↗ ↗ ou ↘	Tejo Prakash et Jagannatha Rao, 1995
	HAPs	T	SOD CAT GPX GSH	↗ ↗ ou ↘ ↗	Cheung *et al.*, 2001
Ruditapes decussatus	Cu	L	PL PLI	↗ ou - ↘	Roméo et Gnassia-Barelli, 1997

[a] Al, Cd, Cu, Hg, CH_3Hg, Pb, U, B[*a*]P, HAPs, HAs, PCBs, HCB, TCB: aluminium, cadmium, cuivre, mercure inorganique, mercure organique, plomb, uranium, benzo[*a*]pyrène, hydrocarbures aromatiques polycycliques, hydrocarbures aliphatiques, polychlorobiphényls, 2,2',4,4',5,5'-hexachlorobiphényl et 3,3',4,4'-tétrachlorobiphényl, respectivement.

[b] L, M, T: étude en laboratoire, en mésocosme et de terrain, respectivement.

[c] SOD, CAT, GPX, GSH, PL, PLI: activités superoxyde dismutase, catalase et glutathion peroxydase, taux de glutathion réduit, taux de peroxydation lipidique et de peroxydation lipidique induite (mesurés en tant que TBARS), respectivement.

[d] ↗, ↘, -: augmentation, diminution et aucune variation significative, respectivement.

II.2. Les cytochromes P450, composants du métabolisme de (dé)toxication de phase I

Lors d'une agression cellulaire par des composés organiques toxiques des mécanismes de défense s'organisent, essentiellement par le biais de réactions enzymatiques pouvant être regroupées en trois catégories: phases I, II et III. Lors du métabolisme de phase I, le xénobiotique est «fonctionnalisé» afin de le rendre plus réactif et de ce fait, plus facilement métabolisable par les enzymes des phases suivantes. Cette fonctionnalisation correspond la plupart du temps à l'introduction d'un atome d'oxygène. Elle est effectuée essentiellement par des monooxygénases à cytochrome P450 et des monooxygénases à flavine (ces dernières ne seront pas abordées).

Le cytochrome P450 se distingue des cytochromes transporteurs d'électrons par son activité enzymatique qui est celle d'une monooxygénase. Il existe un grand nombre de monooxygénases à cytochrome P450. Plus de 30 familles et 750 séquences ont déjà été identifiées dans le monde animal, notamment chez les vertébrés, les insectes, les mollusques, et les nématodes (Nelson *et al.*, 1996; Nelson, 1998). Ces enzymes sont impliquées dans la biosynthèse et la biodégradation de nombreuses molécules endogènes telles que les stéroï des, les acides gras, les prostaglandines et les leukotriènes mais également dans le métabolisme oxydatif d'innombrables composés exogènes dont les substances médicamenteuses et les contaminants environnementaux (Mansuy *et al.*, 1989). Les cytochromes P450 jouent donc un rôle primordial dans les mécanismes de détoxication. Ils participent au métabolisme de phase I au cours duquel le substrat est oxydé pour ensuite être éventuellement pris en charge par les enzymes du métabolisme de phase II (glutathion *S*-transférases, sulfotransférases, UDP-glucuronosyltransférases, époxydes hydrolases). Cependant, le métabolisme de détoxication conduit parfois à des molécules hautement réactives, plus nocives que celles dont elles sont issues. C'est pourquoi les cytochromes P450 participent largement aux mécanismes de mutagenèse et de cancérogenèse. Il est donc préférable de parler de métabolisme de **(dé)**toxication. Outre les monooxygénases à cytochrome P450, d'autres enzymes participent au métabolisme de phase I comme les monooxygénases à flavine.

II.2.1. Le cycle catalytique des monooxygénases à cytochrome P450

Chez les mammifères, les monooxygénases à cytochrome P450 sont des protéines membranaires enchassées dans le réticulum endoplasmique ou la membrane interne des mitochondries. Leur fonctionnement requiert la présence d'électrons, fournis généralement par le NADPH *via* une NADPH-cytochrome P450 réductase, dans le cas du réticulum endoplasmique. Certains cytochromes P450 acceptent des électrons provenant du NADH *via* le cytochrome *b5* et la NADH-cytochrome *b5* réductase (Nebert et Gonzalez, 1987). Les cytochromes P450 contiennent un groupement héminique (protoporphyrine IX ayant lié du fer). Leur cycle catalytique est décrit dans la figure 7. Au repos le cytochrome P450 existe sous deux formes, une forme dans laquelle le fer à l'état ferrique (Fe^{3+}) est rélié à la structure porphyrinique par six liaisons de coordinance (situation bas-spin: les électrons célibataires du fer sont regroupés par paires) et une forme dans laquelle le fer (Fe^{3+}) est relié à la porphyrine par cinq liaisons de coordinance (état haut-spin: les électrons célibataires sont non-appariés). La liaison du substrat au niveau d'un groupement hydrophobe proche de l'hème modifie cet équilibre en faveur de la forme à l'état haut-spin. Ce complexe est alors réduit par un électron provenant du NADPH, *via* la NADPH-cytochrome P450 réductase. Le complexe ferreux obtenu (Fe^{2+}) est capable de lier plusieurs ligands dont le monoxyde de carbone CO (permet le dosage spectrophotométrique du cytochrome P450) et l'oxygène moléculaire. La liaison de ce dernier conduit à un complexe à l'état bas-spin qui est ensuite réduit par un second électron provenant du NADPH *via* la NADPH-cytochrome P450 réductase ou du NADH *via* la NADH-cytochrome *b5* réductase et le cytochrome *b5* à l'état ferreux (réduit). Si cet électron n'est pas délivré assez tôt, le complexe ferreux peut s'autooxyder et donner naissance à une espèce activée de l'oxygène (EAO) radicalaire, l'anion superoxyde, pouvant ensuite se dismuter pour former de l'oxygène moléculaire et une EAO non radicalaire, le peroxyde d'hydrogène (cf. paragraphe II.1.1.). Celui-ci peut également résulter de la réduction du complexe oxygéné par le second électron. Cependant, lorsque le substrat possède une structure complémentaire de celle du site actif de l'enzyme, cette étape n'a pas lieu. Il est important de préciser que ces EAO peuvent avoir des effets délétères multiples (stress oxydatif) si leur production dépasse les capacités de piégeage ou de destruction des systèmes de défense antioxydante. Dans un cycle normal, la liaison entre les deux atomes d'oxygène est rompue, libérant une molécule d'eau. La structure $[FeO]^{3+}$ en résultant (et possédant plusieurs formes de résonance) arrache un atome d'hydrogène au substrat. Celui-ci est ensuite hydroxylé au cours d'un réarrangement radicalaire. Le substrat hydroxylé possède moins

d'affinité pour le site actif que le substrat initial. Il s'en détache donc pour laisser la place à un nouveau substrat (Mansuy *et al.*, 1989; Halkier, 1996). La réaction générale est la suivante:

$$RH + O_2 + 2e^- + 2H^+ \text{ (NADPH ou NADH)} \rightarrow ROH + H_2O$$

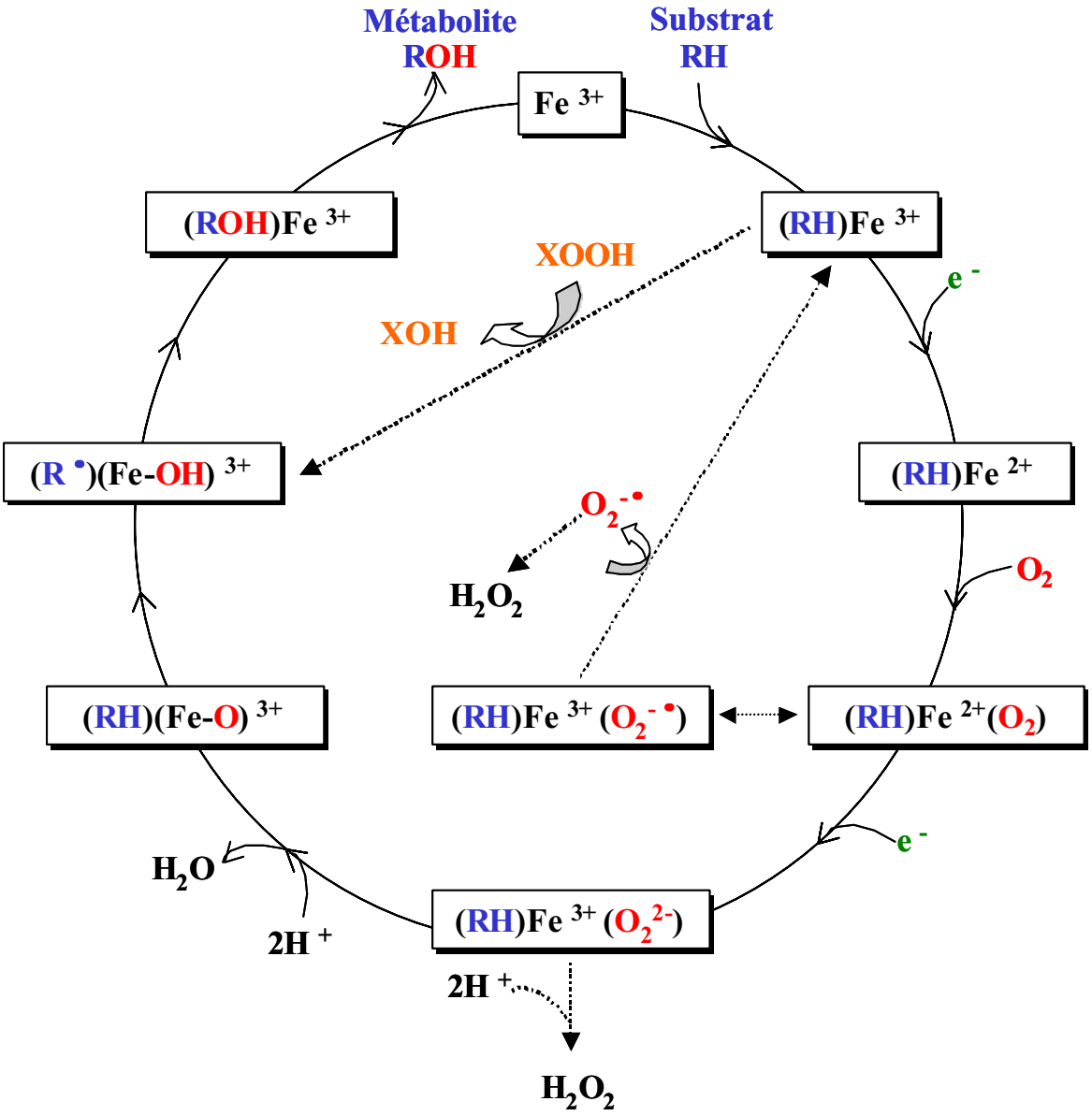


Figure 7. Cycle catalytique du cytochrome P450 (d'après Halkier, 1996).

Lors de certaines réactions un peroxyde (XOOH) peut se substituer à l'oxygène moléculaire. Dans ce cas, l'obtention du complexe à $[FeO]^{3+}$ ne fait pas intervenir de transfert d'électrons *via* la ou les réductases.

II.2.2. La diversité des réactions catalysées par les cytochromes P450

Les réactions de monooxygénation ne sont pas les seules réactions catalysées par les cytochromes P450. Ces derniers sont également à l'origine de réactions de réduction, déshydratation, déshydrogénation ou même d'isomérisation. C'est pourquoi le point commun des cytochromes P450 n'est pas l'activation de l'oxygène moléculaire et le transfert de l'un des deux atomes de ce dernier vers un substrat, mais labsorption du complexe Fe(II)-CO à 450 nm, produisant le pic de Soret (Mansuy, 1998). Cette propriété est d'ailleurs utilisée pour doser les cytochromes P450 (Omura et Sato, 1964). Les nombreuses réactions catalysées par les cytochromes P450 sont présentées dans le tableau 2.

Tableau 2. Réactions catalysées par les cytochromes P450 (d'après Mansuy, 1998).

Types de réaction	
Monooxygénations	*C-H Hydroxylations* *N- Hydroxylations* *S- Oxydations* *O- Désalkylations oxydatives* *N- Désalkylations oxydatives* *Epoxydations*
Autres réactions d'oxydation	*Déshydrogénations* *Activités oxydases* *Activités de type peroxydase* *Déformylations oxydatives* *NO Oxydations de type synthase*
Autres réactions	*Isomérisations* *Réductions* *Déshydratations* *NO Réductions*

En particulier, les réactions de type oxydase générant des EAO (anion superoxyde, peroxyde d'hydrogène) et de l'eau moléculaire sont importantes lors de la métabolisation des xénobiotiques qui, n'étant pas des substrats naturels des cytochromes P450, ne sont pas parfaitement positionnés au sein du site actif.

II.2.3. La diversité des familles de cytochrome P450

En 1998, Nelson a recensé 37 familles de cytochrome P450 dont 16 chez les mammifères (certaines sont également présentes chez les poissons, les oiseaux et les reptiles) et 21 exclusivement chez les insectes, les mollusques et les nématodes. De nouvelles familles sont découvertes régulièrement et les mises à jour peuvent être consultées sur le site internet créé par Nelson (http://drnelson.utmem.edu/homepage.html) (Nelson *et al.*, 1996). Chaque famille possède ses propres caractéristiques. La plupart sont impliquées dans le métabolisme de substances endogènes (ex: CYP4: métabolisme des acides gras; CYP11, 17, 19, 21 et 24: biosynthèse des stéroïdes). Certaines n'ont pas de substrats endogènes connus et ne métabolisent que des xénobiotiques (familles CYP1 et CYP3). Quant à la famille CYP2, elle est à la fois impliquée dans le métabolisme des xénobiotiques et celui des stéroïdes (Nelson *et al.*, 1996). L'expression de certains cytochromes P450 est inductible par des médicaments et/ou des contaminants environnementaux. C'est le cas des cytochromes appartenant à la sous-famille CYP1A, notamment le CYP1A1. Chez les vertébrés, l'expression de ce dernier est induite par les dioxines, les hydrocarbures aromatiques polycycliques (HAPs) et les polychlorobiphényls (PCBs) *via* un récepteur spécifique (l'AhR, pour «Aryl hydrocarbon Receptor») (Hahn, 1998). Les activités dépendantes des cytochromes P450 peuvent également être inhibées par certaines substances exogènes (Murray et Reidy, 1990). Le dosage de ces activités est possible grâce à des substrats spécifiques de certaines sous-familles de cytochrome P450, par exemple la 7-éthoxyrésorufine pour le CYP1A1 (Burke et Mayer, 1974).

II.2.4. Les cytochromes P450 chez les mollusques bivalves et leur utilisation comme biomarqueurs de pollution

La présence d'un métabolisme de phase I a été découverte relativement tôt chez les invertébrés aquatiques tels que les crustacés, les annélidés, les échinodermes et les mollusques (Lee, 1981). Chez la moule marine *Mytilus edulis*, des mesures spectrophotométriques ont permis de mettre en évidence la présence des cytochromes P450 et *b5* mais aussi des activités NADPH-cytochrome P450 réductase (NADPH-red.), NADH-cytochrome *b5* réductase (NADH-red.) et benzo[*a*]pyrène hydroxylase (BPH), cette dernière activité étant impliquée dans la métabolisation du benzo[*a*]pyrène, un HAP majeur (Livingstone et Farrar, 1984). En

particulier, le cytochrome P450 et les activités NADPH-red. et BPH ont été détectés principalement dans les microsomes de glande digestive alors que le cytochrome *b5* a été localisé dans les microsomes et le cytosol de la glande digestive, des branchies et du manteau. Quant à l'activité NADH-red., elle a été détectée dans les microsomes de tous les tissus. En plus du pic de Soret absorbant à 450 nm, un autre pic a été observé à une longueur d'onde de 416 à 424 nm, selon les études (Livingstone *et al.*, 1989). Ce pic additionnel, souvent nommé P418 ou P420, pourrait correspondre à un produit de dénaturation du cytochrome P450 ou bien à une autre protéine héminique (Livingstone et Farrar, 1984). Plus récemment, des études moléculaires ont permis de confirmer la présence de cytochrome(s) P450 chez les moules *Mytilus* sp. Une préparation de cytochrome P450 partiellement purifié à partir de microsomes de glande digestive a réagi avec un anticorps polyclonal dirigé contre du CYP1A de perche (*Perca fluviatilis*) indiquant la présence d'un épitope ou d'une protéine possédant des similitudes avec celui-ci (Porte *et al.*, 1995). Des expériences de Northern et de Southern Blots effectuées avec des sondes d'acides nucléiques de vertébrés correspondant aux sous-familles CYP1A1, CYP3A, CYP4A1 et CYP11A1 ont mis en évidence la présence et l'expression de gènes similaires chez *Mytilus* sp. (Wootton, 1995). De plus, des expériences de RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) et de Southern Blot ont confirmé la présence dans la glande digestive de *M. edulis* d'un gène présentant de fortes similitudes avec celui codant pour le CYP1A1 de la truite arc-en-ciel, *Oncorhynchus mykiss* (Wootton *et al.*, 1996). Enfin, des Western Blots mis en oeuvre avec des anticorps polyclonaux dirigés contre plusieurs familles de cytochromes P450 de vertébrés ont révélé la présence de protéines apparentées aux sous-familles CYP1A, CYP2B, CYP2E, CYP3A et CYP4A (Peters *et al.*, 1998a). A ce jour, seules quatre séquences de gènes codant pour des cytochromes P450 ont été identifiées chez les mollusques (Snyder, 2000) dont deux chez des bivalves: CYP4Y1 chez *M. galloprovincialis* (Snyder, 1998) et CYP30 chez *Mercenaria mercenaria* (Brown *et al.*, 1998).

Outre la BPH, d'autres activités pouvant être catalysées par les monooxygénases à cytochrome P450 ont été mises en évidence chez les invertébrés aquatiques telles que l'activité 7-éthoxycoumarine-*O*-déséthylase (ECOD) et 7-éthoxyrésorufine-*O*-déséthylase (EROD) (Lee, 1981; Livingstone *et al.*, 1989). Chez la moule marine, l'activité EROD n'a été que rarement détectée (Stegeman, 1985). Ces activités sont en général bien plus faibles que celles mesurées chez les vertébrés, dont les poissons (Bucheli et Fent, 1995). Elles présentent la singularité d'être catalysées en l'absence de NADPH, voire d'être inhibées par celui-ci

(Livingstone *et al.*, 1989; Michel *et al.*, 1992; Dauberschmidt *et al.*, 1997). En particulier, la métabolisation *in vitro* du B[*a*]P par la glande digestive de *M. galloprovincialis* peut s'effectuer en présence de NADPH avec une production préférentielle de diols et de phénols mais aussi en son absence avec cette fois une production préférentielle de quinones (Michel *et al.*, 1992). Ce dernier phénomène pourrait être dû à la présence d'une source endogène d'équivalents réducteurs ou à l'apport d'oxygène moléculaire sous la forme de peroxydes endogènes tels que les hydroperoxydes lipidiques. L'effet inhibiteur du NADPH et d'autres agents réducteurs pourrait indiquer l'intervention préférentielle d'un mécanisme d'oxydation à un seul électron, au lieu de deux (Livingstone *et al.*, 1989). Ainsi dans le cas du B[*a*]P, deux types de métabolisation catalysés par différents cytochromes P450 pourraient coexister: (1) *via* une oxydation à un seul électron catalysée par une forme constitutive et non-inductible de cytochrome P450, (2) *via* une monooxygénation à deux électrons catalysée par une forme inductible de cytochrome P450 de type CYP1A (Livingstone *et al.*, 1997). Les composants du métabolisme de phase I et en particulier l'activité EROD (associée à la famille CYP1A) inductible par les HAPs, sont largement utilisés comme biomarqueurs de pollution chez les poissons (Bucheli et Fent, 1995). Le tableau 3 donne un aperçu des réponses obtenues chez les mollusques bivalves après exposition à divers contaminants. D'une manière générale, le cytochrome P450 et des activités telles que la BPH ou la NADPH-red. sont induits en présence de HAPs et de PCBs. De plus, l'utilisation récente de la biologie moléculaire dans de telles études environnementales semble confirmer l'intérêt de ces paramètres biochimiques comme biomarqueurs de pollution, bien qu'ils soient sensibles à d'autres facteurs tels que le sexe, l'âge, la température, le cycle reproducteur et la saison (Sheehan et Power, 1999).

Tableau 3. Modulation des composants du métabolisme de phase I chez les mollusques bivalves après exposition à différents polluants.

Organisme	Polluant [a]	Mode d'exposition [b]	Paramètre [c]	Variation [d]	Référence
Mytilus edulis	Pétrole	T	P450 P418 1A-l-prot. BPH NADPH-red.	↗ - ↗ - -	Solé *et al.*, 1996
	HAPs, Cu	M	BPH	↗	Suteau *et al.*, 1988
	HAPs, Cu, PCBs	T	BPH	-	Suteau *et al.*, 1988
Mytilus galloprovincialis	B[*a*]P	L	P450 NADPH-red.	↗ ↗	Michel *et al.*, 1993
	B[*a*]P	M	1A-l-prot. BPH	- ↗	Akcha *et al.*, 2000
	3MC	L (injection)	P450 P420 b5	↗ ↗ ↗	Gilewicz *et al.*, 1984
	HAPs	T	P450 P420 b5	↗ ↗ ↗	Gilewicz *et al.*, 1984
	HCB	L	P450 NADPH-red.	↗ -	Michel *et al.*, 1993
	TCB	L	P450 NADPH-red.	↗ ↗	Michel *et al.*, 1993
	HCB'	L (injection)	1A-l-prot. 1A-l-ARNm	↗ -	Livingstone *et al.*, 1997
	ARO	L	1A-l-prot. 1A-l-ARNm	↗ -	Livingstone *et al.*, 1997
	HAPs, OP, PCBs	T	P450 b5 NADPH-red.	↗ - ↗	Solé *et al.*, 1994

Tableau 3 (suite).

Organisme	Polluant [a]	Mode d'exposition [b]	Paramètre [c]	Variation [d]	Référence
Mytilus galloprovincialis	HAPs, HAs, PCBs	T	P450 P418 1A-l-prot. 1A-l-ARNm NADPH-red.	- - ↗ ↗ -	Livingstone *et al.*, 1995
	HAPs, PCBs	T	1A-l-prot.	↗	Peters *et al.*, 1998
	HAPs, HAs, PCBs, OC	T	P450 P418 1A-l-prot. BPH	↘ ↘ - ↗	Solé *et al.*, 2000
	HAPs, HAs	T	P450 P418 BPH	- - -	Porte *et al.*, 2001

[a] Cu, B[*a*]P, 3MC, HAPs, HAs, PCBs, HCB, HCB', TCB, ARO, OP, OC: cuivre, benzo[*a*]pyrène, 3-méthylcholanthrène, hydrocarbures aromatiques polycycliques, hydrocarbures aliphatiques, polychlorobiphényls, 2,2',4,4',5,5'-hexachlorobiphényl, 2,2',3,4,4',5'-hexachlorobiphényl, 3,3',4,4'-tétrachlorobiphényl, Aroclor 1254, insecticides organophosphorés et organochlorés, respectivement.

[b] L, M, T: étude en laboratoire, en mésocosme et de terrain, respectivement.

[c] P450, P420, P418, b5, 1A-l-prot., 1A-l-ARNm, NADPH-red., BPH: cytochromes P450, P420, P418 et *b5*, protéine «CYP1A-like», ARNm «CYP1A-like», activité NADPH-cytochrome P450 réductase et activité benzo[*a*]pyrène hydroxylase, respectivement.

[d] ↗, ↘, -: augmentation, diminution et aucune variation significative, respectivement.

II.3. Les glutathion *S*-transférases, composants du métabolisme de (dé)toxication de phase II

Dans les mécanismes de (dé)toxication, les enzymes de phase II ont pour rôle essentiel de coupler un groupement hydrosoluble à un xénobiotique préalablement métabolisé ou non par les enzymes de phase I afin de le rendre lui-même plus hydrosoluble et donc plus facilement excrétable par l'organisme. On trouve principalement dans cette catégorie les glutathion *S*-transférases (GSTs), les UDP-glucuronosyle-transférases, les sulfotransférases et les époxydes hydrolases. Seule les GSTs seront abordées dans ce chapitre.

Les GSTs catalysent la conjugaison d'un tripeptide, le glutathion réduit (γ-glutamyl-cystéinyl-glycine: GSH) à une multitude de substrats hydrophobes présentant un centre électrophile. Elles ont été mises en évidence dans la plupart des êtres vivants tels que la levure (Foley et Sheehan, 1998), les mollusques (Fitzpatrick et Sheehan, 1993; Fitzpatrick *et al.*, 1995a; Blanchette et Singh, 1999), les vers de terre (Stenersen *et al.*, 1979, Borgeraas *et al.*, 1996), les crustacés (Keeran et Lee, 1987; Leblanc et Cochrane, 1987), les insectes (Stenersen *et al.*, 1987; Prapanthadara *et al.*, 1996), les poissons (George et Young, 1988; Martínez-Lara *et al.*, 1997; Pérez-López *et al.*, 2000), les mammifères (Habig *et al.*, 1974; Kamisaka *et al.*, 1975; Rouimi *et al.*, 1996; Bolton et Ahokas, 1997) et les plantes (Pascal *et al.*, 1998; Hong *et al.*, 1999). Elles constituent une large famille d'isoenzymes homo- et hétérodimériques principalement cytosoliques bien que certaines formes membranaires aient été identifiées. Les sous-unités possèdent une masse moléculaire de 20 à 30 kDa et sont codées par des familles multigéniques. A l'origine, l'intérêt pour les GSTs provient de leur participation dans la voie de biosynthèse des acides mercapturiques, molécules solubles dans l'eau et facilement excrétées par l'organisme. La réaction de conjugaison entre le GSH et les xénobiotiques représente en effet la première des quatre étapes menant à leur formation (Beckett et Hayes, 1993). Les GSTs ont été depuis largement étudiées chez les mammifères, plus particulièrement chez le rat et l'humain et de nombreux rôles leur ont été attribués.

II.3.1. Les différentes classes de glutathion *S*-transférases

De nombreuses nomenclatures ont été utilisées pour classifier les GSTs cytosoliques de mammifères. Elles étaient initialement basées sur les propriétés chromatographiques des

GSTs, l'ordre chronologique de leur caractérisation ou leur point isoélectrique. Par exemple, trois polypeptides séparés par SDS-PAGE à partir d'un éluat issu d'une chromatographie par exclusion d'un cytosol de foie de rat ont été nommés Ya, Yb, Yc. Il a ensuite été démontré que les sous-unités de type Ya et Yc pouvaient s'associer entre elles mais non avec des sous-unités de type Yb. Deux familles différentes ont donc été mises en évidence dans le foie de rat. Une troisième famille, absente dans un foie normal mais présente dans le placenta et des tumeurs du foie a par la suite été identifiée et nommée Yp ou Yf. Quant aux GSTs humaines, elles étaient à l'origine divisées en trois groupes: les formes basiques (foie), neutres (foie) et acides (placenta) (Beckett et Hayes, 1993). Mannervik *et al.* (1985) ont uniformisé la nomenclature en s'appuyant sur les séquences d'acides aminés NH_2-terminales des GSTs, leurs substrats et inhibiteurs spécifiques et leurs propriétés immunologiques. Ils ont ainsi proposé trois classes de GSTs communes aux rats, aux souris et aux humains (les sous-unités d'une classe donnée ne pouvant s'apparier avec celles d'une autre classe): Alpha (sous-unités Ya et Yc de rat; 1, 2, 8 et 10 de rat; GSTs basiques d'humain), Mu (sous-unités Yb de rat; 3, 4, 6, 9 et 11 de rat; GSTs neutres d'humain) et Pi (Yp ou Yf de rat; 7 de rat; GSTs acides d'humain). Depuis, de nouvelles classes de GST ont été identifiées: Thêta (Meyer *et al.*, 1991), Sigma (Meyer et Thomas, 1995), Zéta (Board *et al.*, 1997). D'autres classes ont été mises en évidence chez les bactéries, les insectes et les plantes: classes Bêta (Rossjohn *et al.*, 1998), Delta (Board *et al.*, 1997) et Phi (Board *et al.*, 1997), respectivement. Plus récemment, une nouvelle classe a été découverte chez les mammifères et le nématode *Caenorhabditis elegans*: la classe Oméga (Board *et al.*, 2000). Les GSTs sont principalement cytosoliques mais certaines GSTs sont localisées dans la matrice mitochondriale (classe Kappa, Pemble *et al.*, 1996) ou la membrane (Morgenstern et DePierre, 1983). Les structures tertiaires des différentes classes de GSTs sont présentées dans la figure 8.

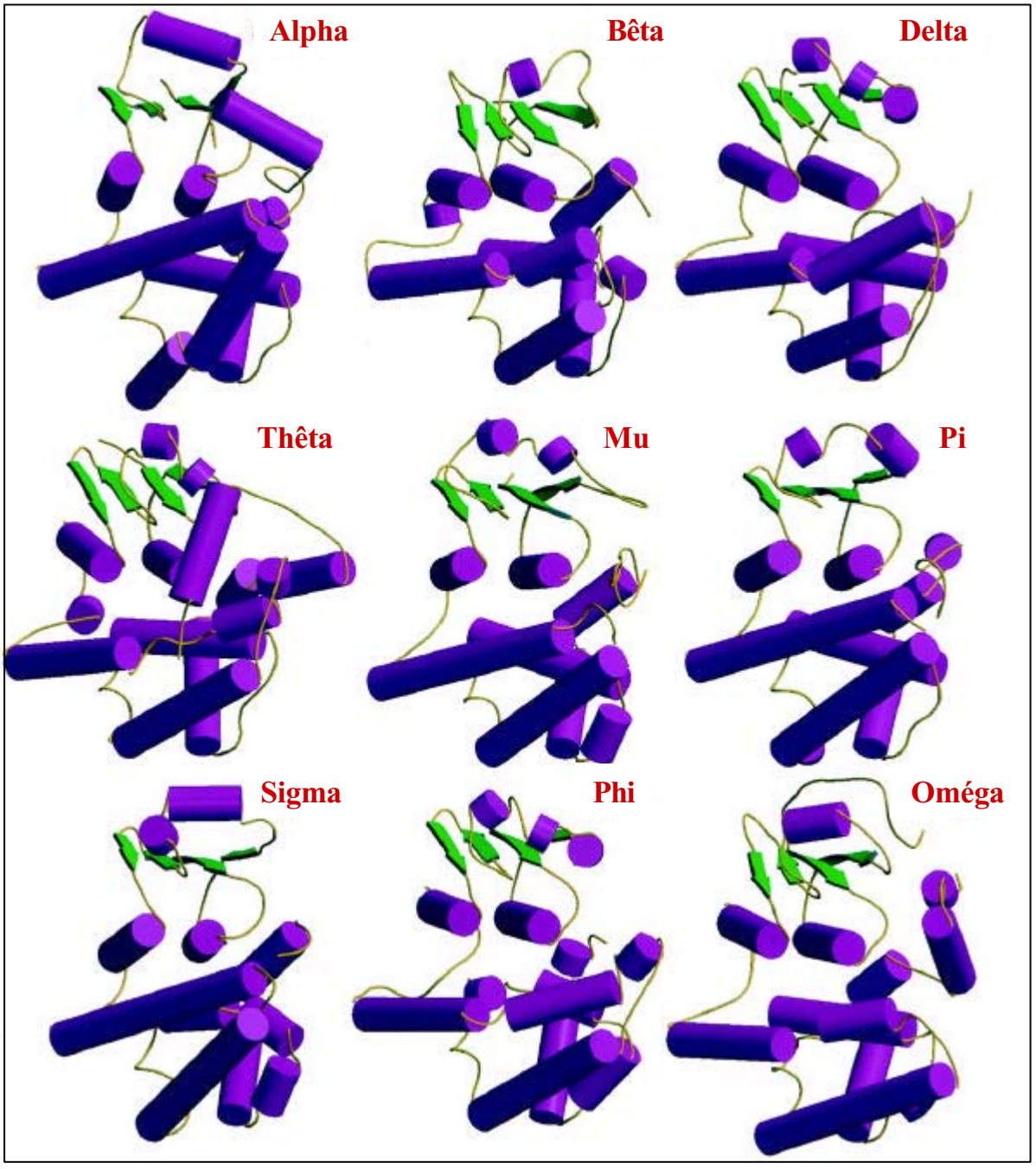


Figure 8. Structure tertiaire des classes Alpha, Bêta, Delta, Thêta, Mu, Pi, Sigma, Phi et Oméga de GSTs. Les hélices alpha sont représentées par des cylindres et les feuillets bêta par des flèches (Board *et al.*, 2000).

II.3.2. Mécanisme catalytique des glutathion *S*-transférases

Les GSTs catalysent de nombreuses réactions au cours desquelles le GSH (figure 9) sous la forme d'anion thiolate (GS^-) joue le rôle d'un substrat nucléophile.

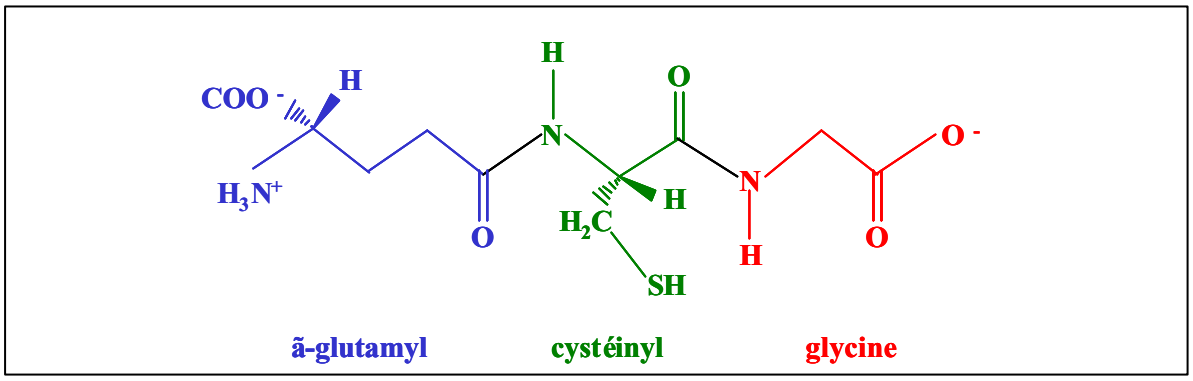


Figure 9. Glutathion réduit ou ã-glutamyl-cystéinyl-glycine (d'après Armstrong, 1991).

La principale activité catalytique des GSTs est de faciliter la formation ou la stabilisation de l'anion thiolate. Celui-ci peut ensuite attaquer le centre électrophile (atome de carbone, d'oxygène, de soufre ou d'azote) d'un autre substrat. Le composé en résultant est un conjugué thioéther (Beckett et Hayes, 1993). La réaction générale est la suivante (Armstrong, 1991):

$$E + GSH \leftrightarrows E.GS^- + H^+ \overset{R\text{-}X}{\leftrightarrows} E.GS^-.R\text{-}X \leftrightarrows E.GSR.X^- \leftrightarrows E + GSR + X^-$$

E: enzyme

R-X: substrat possédant un centre électrophile

Les enzymes cytosoliques possèdent deux sites actifs par dimère se comportant indépendamment l'un de l'autre. Chaque site actif comprend au moins deux sites de fixation: le site de fixation du GSH, très spécifique, et celui du substrat électrophile, beaucoup moins spécifique, ce qui permet aux GSTs de réagir avec une large gamme de composés (Wilce et Parker, 1994).

Différents substrats permettent de mesurer l'activité des GSTs grâce à l'obtention de conjugués absorbant à certaines longueurs d'onde (Habig *et al.*, 1974). Le substrat le plus utilisé est le 1-chloro-2,4-dinitrobenzène (CDNB). Le CDNB est souvent considéré comme le

substrat «universel» des GSTs car il est métabolisé par la majorité des isoenzymes. D'autres substrats considérés comme plus spécifiques de telle ou telle classe de GSTs sont également couramment utilisés, par exemple le 1,2-dichloro-4-nitrobenzène (DCNB), l'acide éthacrynique (AE) et le 1,2-époxy-3-(*p*-nitrophénoxy)propane (EPNP), spécifiques des classes Mu, Pi et Thêta, respectivement (figure 10). Cependant, cette spécificité n'est que relative car certains substrats peuvent être métabolisés par plusieurs classes de GSTs (Beckett et Hayes, 1993).

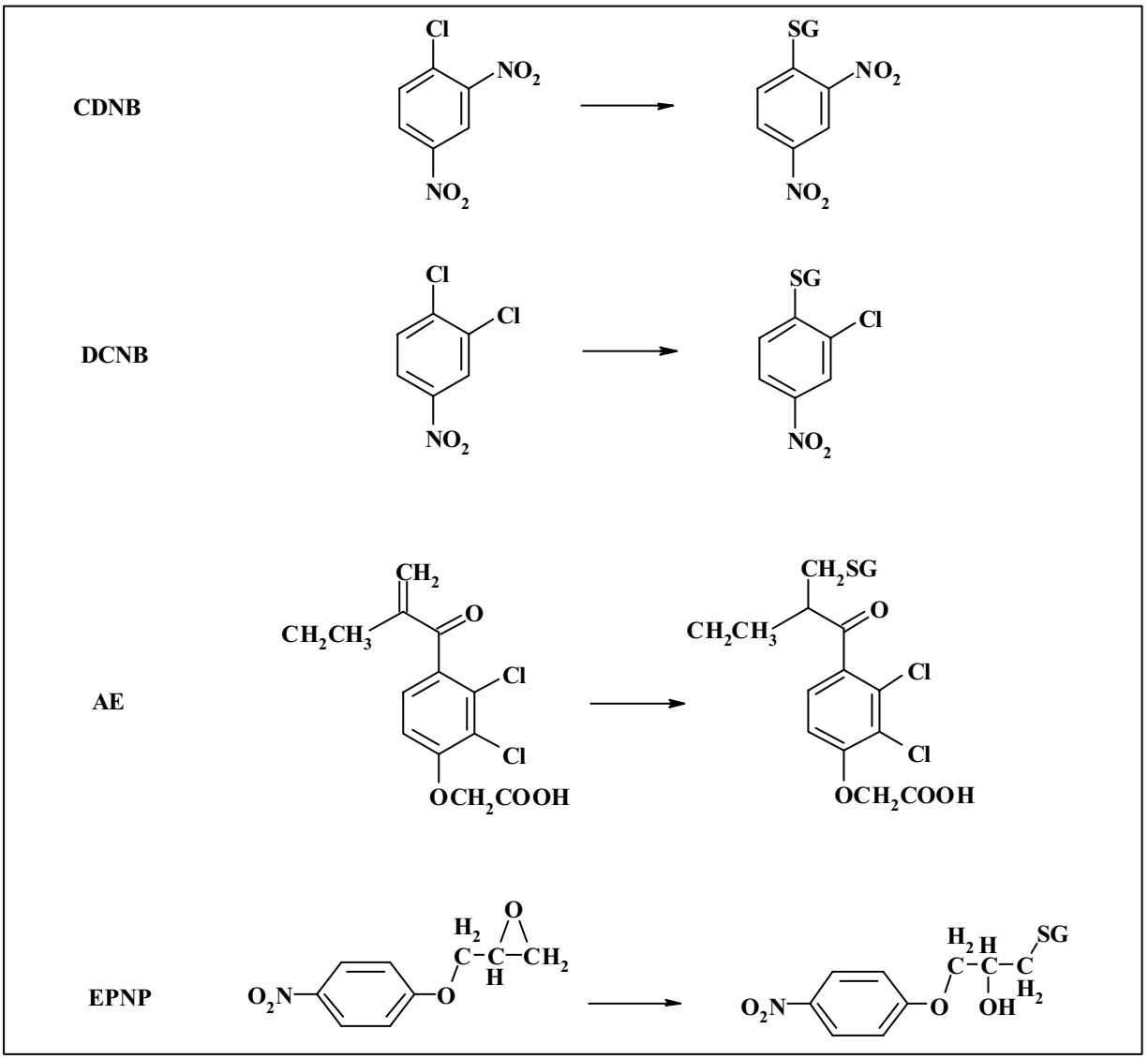


Figure 10. Substrats modèles métabolisés par les glutathion *S*-transférases: le 1-chloro-2,4-dinitrobenzène (CDNB), le 1,2-dichloro-4-nitrobenzène (DCNB), l'acide éthacrynique (AE) et le 1,2-époxy-3-(*p*-nitrophénoxy)propane (EPNP) (d'après Beckett et Hayes, 1993).

Outre leur capacité à former des conjugués thioéthers avec le GSH, les GSTs peuvent également catalyser d'autres réactions telles que la lyse de groupements thiols, la réduction d'hydroperoxydes organiques (réaction de type peroxydase) ou l'isomérisation de certains stéroï des (figure 11).

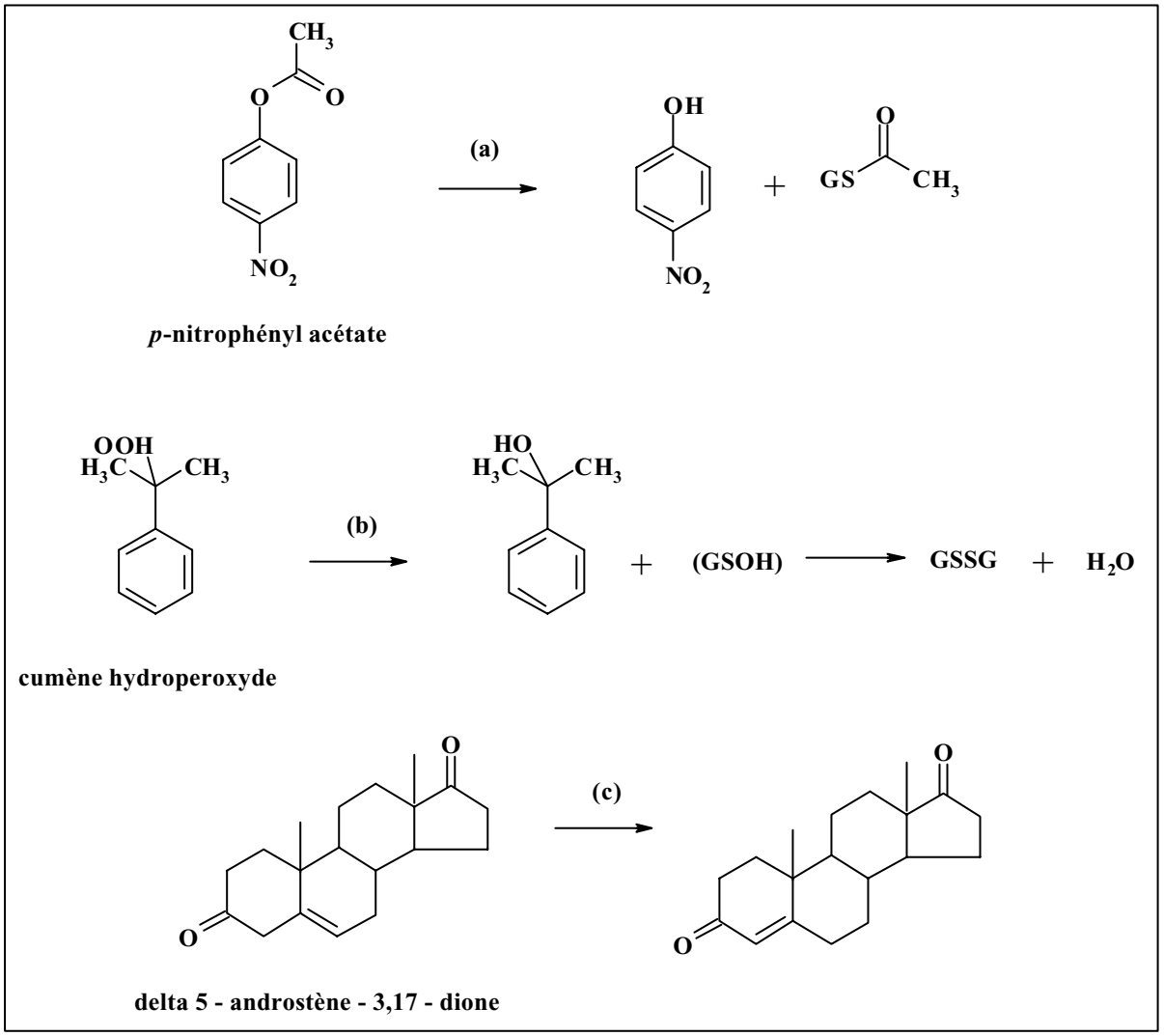


Figure 11. Autres réactions catalysées par les glutathion *S*-transférases: (a) thiolyse, (b) réaction de type peroxydase, (c) isomérisation.

II.3.3. Rôles des glutathion *S*-transférases

II.3.3.1.Métabolisation de substrats exogènes

Les GSTs peuvent détoxifier un large spectre de xénobiotiques initialement électrophiles ou devenus électrophiles après métabolisation. Ces composés peuvent être des herbicides ou des pesticides (thiocarbamates, organophosphates), des amines aromatiques, des hydrocarbures aromatiques polycycliques (benzo[*a*]pyrène -B[*a*]P-), des toxines (aflatoxine B1) ou des subtances pharmaceutiques (paracétamol, nitroglycérine, bromosulfophthaléine). Parmi ces xénobiotiques, certains peuvent être à l'origine de composés cancérigènes comme le B[*a*]P. Ce dernier est métabolisé par des monooxygénases à cytochrome P450 et des époxydes hydrolases en composés hautement réactifs tels que des époxydes, des phénols, des diols et des quinones. En particulier, le métabolite le plus cancérigène du B[*a*]P, le 7,8-diol-9,10-époxyde peut se fixer de manière covalente à l'ADN. Les GSTs sont capables de conjuguer les époxydes et leurs dérivés et constituent donc un système essentiel de protection (Hinson et Kadlubar, 1988). Cependant, la conjugaison de certains substrats avec le GSH peut parfois conduire à la formation de composés encore plus réactifs et toxiques que le composé initial. Soit le conjugué peut exercer son effet en interagissant avec un récepteur, soit la conjugaison transforme (ou constitue une étape dans la transformation) un composé relativement inactif en un composé électrophile pouvant réagir de manière irréversible avec des macromolécules cellulaires. Dans ce dernier cas, les conjugués pouvant s'avérer toxiques sont divisés en trois groupes: (1) les conjugués constituant eux-mêmes des espèces réactives (exemple de substrat: le 1,2-dibromoéthane), (2) les conjugués préalablement métabolisés pour donner naissance à des espèces réactives (exemples de substrats: hydroquinone, hexachlorobutadiène), (3) les conjugués pouvant «stocker» l'espèce réactive puis la libérer dans certaines conditions (exemple de substrat: benzyl isothiocyanate) (Van Bladeren, 1988).

Certaines isoenzymes de GSTs peuvent être induites par des xénobiotiques tels que le phénobarbital, le 3-méthylcholanthrène, l'hexachlorobenzène et les antioxydants phénoliques (Vos et Van Bladeren, 1990).

Les xénobiotiques modifiés par les enzymes de phase II (et/ou de phase I) peuvent ensuite être expulsés de la cellule par les enzymes de phase III. Ces dernières regroupent des protéines membranaires dont le mode d'action repose sur un mécanisme ATP-dépendant.

Chez les mammifères, la protéine de phase III la plus étudiée est la protéine MRP (multidrug resistance related protein) (Parant, 1998). Cette dernière phase n'a pas été abordée lors de notre travail.

II.3.3.2. Métabolisation de substrats endogènes

Les GSTs possèdent un rôle capital dans les mécanismes de protection de l'organisme vis à vis des effets néfastes du stress oxydatif. Certaines GSTs peuvent en effet métaboliser les hydroperoxydes lipidiques résultant de la peroxydation lipidique. Contrairement aux glutathion peroxydases dépendantes du sélénium, ces GSTs ne possèdent pas de sélénium dans leur structure et ne métabolisent pas le peroxyde d'hydrogène. Les produits finaux de la peroxydation lipidique tels que les aldéhydes, le malonedialdéhyde, les hydroxyalkénals peuvent également être métabolisés par certaines isoenzymes. Enfin, les GSTs participeraient aux mécanismes de réparation des molécules d'ADN oxydées, en association avec d'autres enzymes (Ketterer et Meyer, 1989). Outre leurs fonctions de protection, les GSTs participent également à la biosynthèse de molécules biologiquement actives telles que les prostaglandines et les leukotriènes (Beckett et Hayes, 1993).

II.3.3.3. Autres rôles

Les GSTs sont capables de lier de manière covalente ou non de nombreux composés hydrophobes qu'elles ne métabolisent pas, ceci à des fins de stockage, de séquestration ou de transport (certains xénobiotiques ou leurs métabolites, des acides biliaires, des hormones stéroïdes et thyroïdiennes ainsi que des neurotransmetteurs).

II.3.4. Les glutathion *S*-transférases chez les mollusques bivalves et leur utilisation comme biomarqueurs de pollution

Les GSTs sont des enzymes ubiquitaires présentes aussi bien chez les vertébrés que les invertébrés. De nombreuses études de purification et de caractérisation des isoenzymes de GST ont été ainsi réalisées chez les insectes, les arachnides, les crustacés, les échinodermes, les cnidaires, les helminthes et les mollusques (Stenersen *et al.*, 1987; Clark, 1989). Les GSTs

présentes chez les moules marines ont été purifiées par chromatographie d'affinité (résine couplée à du GSH) et par chromatographie échangeuse d'anions. Fitzpatrick et Sheehan (1993) ont mis ainsi en évidence quatre isoenzymes cytosoliques dans la glande digestive de *Mytilus galloprovincialis* et dans des *Mytilus edulis* entières, ainsi qu'une cinquième dans les branchies de cette dernière espèce. Ces isoenzymes sont vraisemblablement des homodimères constitués de sous-unités de 24,5 à 27,3 kDa. Parmi les isoenzymes présentes dans les branchies de *M. edulis*, deux ont été particulièrement bien caractérisées. La première est un homodimère présentant une activité GST envers plusieurs substrats dont le CDNB et possède des similitudes avec les GSTs de classe Pi. La seconde est un homodimère ne possèdant pas d'activité GST envers les susbstrats usuels et ne réagit pas avec des anticorps dirigés contre des GSTs de rat des classes Alpha, Pi et Mu et ce, bien qu'elle présente des homologies avec cette dernière classe au niveau de sa séquence d'acides aminés NH_2-terminale. Ces deux isoenzymes ont respectivement été nommées GST 1 et «GSH-binding protein» (Fitzpatrick *et al.*, 1995a; Power *et al.*, 1996). L'activité GST a souvent été utilisée comme marqueur biochimique, le plus souvent en utilisant le substrat «universel» CDNB. Les réponses obtenues chez plusieurs espèces de mollusques bivalves exposées à différentes catégories de contaminants sont présentées dans le tableau 4. Ces réponses sont la plupart du temps contradictoires: induction, inhibition ou aucune variation après exposition à une même famille de contaminants (les hydrocarbures aromatiques polycycliques, par exemple). De plus, elles peuvent varier en fonction de la dose, du temps d'exposition et du tissu considéré (glande digestive ou branchies). Cependant, il est possible que l'induction ou l'inhibition d'une isoenzyme particulière passe inaperçue lors du dosage de l'activité GST envers le CDNB. Sur ce point, l'utilisation de substrats plus spécifiques tels que l'AE ou l'EPNP peut s'avérer plus satisfaisante, bien que la spécificité des isoenzymes envers différents substrats ne soit pas stricte. L'étude de l'expression des différentes isoenzymes ou des sous-unités de GST peut seule confirmer ou infirmer les résultats obtenus après un dosage d'activité enzymatique. Une telle démarche a été effectuée par Fitzpatrick *et al.* (1995b, 1997) chez *M. galloprovincialis* et *M. edulis* lors d'études de terrain mais aucune variation du profil des différentes isoenzymes de GST n'a été détectée. Cependant, de telles études moléculaires chez les mollusques bivalves doivent être poursuivies car elles se sont avérées efficaces chez les poissons (Martínez-Lara *et al.*, 1996; Pérez-López *et al.*, 2000). De plus, les GSTs sont beaucoup moins sensibles aux variations saisonnières que les indicateurs de stress oxydatif et les composants du métabolisme de phase I (Power et Sheehan, 1996).

Tableau 4. Modulation des activités glutathion *S*-transférases chez les mollusques bivalves après exposition à différents polluants.

Organisme	Polluant [a]	Mode d'exposition [b]	Paramètre [c]	Variation [d]	Référence
Modiolus modiolus	B[*a*]P	L (injection)	GST/CDNB	-	Willett *et al.*, 1999
	TCDD	L (injection)	GST/CDNB	-	Willett *et al.*, 1999
	Pétrole	T	GST/CDNB	-	Solé *et al.*, 1996
	Pétrole	T	GST/CDNB	↗ ou -	Willett *et al.*, 1999
Mytilus Edulis	HAPs, Cu	M	GST/STY	-	Suteau *et al.*, 1988
	HAPs, Cu	M	GST/CDNB	-	Lee, 1988
	HAPs, Cu, PCBs	T	GST/STY	↗	Suteau *et al.*, 1988
	HAPs, Cu, PCBs	T	GST/CDNB	-	Lee, 1988
Mytilus galloprovincialis	B[*a*]P	L	GST/STY	↘	Michel *et al.*, 1993
	B[*a*]P	M	GST/CDNB	↘ ou -	Akcha *et al.*, 2000
	HCB	L	GST/STY	↗	Michel *et al.*, 1993
	TCB	L	GST/STY	↗ ou ↘	Michel *et al.*, 1993
	HAPs, HAs,PCBs	T	GST/CDNB	-	Livingstone *et al.*, 1995
	Cu	L	GST/CDNB GST/DCNB GST/EPNP	- ↘ ou - -	Regoli et Principato, 1995
	Cu	L	GST/CDNB	↗	Canesi *et al.*, 1999
	Hg	L	GST/CDNB	↗	Canesi *et al.*, 1999
	CH_3Hg	L	GST/CDNB	↗	Canesi *et al.*, 1999
	Métaux	T	GST/CDNB GST/DCNB GST/EPNP	↘ ou - ↘ ou - -	Regoli et Principato, 1995

Tableau 4 (suite).

Organisme	Polluant [a]	Mode d'exposition [b]	Paramètre [c]	Variation [d]	Référence
Perna viridis	HAPs	T	GST/CDNB	↗	Cheung *et al.*, 2001
	Al, Pb ou Cd	L	GST/CDNB	↗	Tejo Prakash et Jagannatha Rao, 1995
Sphaerium corneum	Dieldrine	L	GST/CDNB	↗	Boryslawskyj *et al.*, 1988
	Lindane	L	GST/CDNB	↗	Boryslawskyj *et al.*, 1988

[a] Al, Cd, Cu, Hg, CH_3Hg, Pb, B[*a*]P, HAPs, HAs, PCBs, HCB, TCB, TCDD: aluminium, cadmium, cuivre, mercure inorganique, mercure organique, plomb, benzo[*a*]pyrène, hydrocarbures aromatiques polycycliques, hydrocarbures aliphatiques, polychlorobiphényls, 2,2',4,4',5,5'-hexachlorobiphényl, 3,3',4,4'-tétrachlorobiphényl et 2,3,7,8-tétrachlorodibenzo-*p*-dioxine, respectivement.

[b] L, M, T: étude en laboratoire, en mésocosme et de terrain, respectivement.

[c] GST/CDNB, GST/DCNB, GST/EPNP et GST/STY: activité GST envers le 1-chloro-2,4-dinitrobenzène, le 1,2-dichloro-4-nitrobenzène, le 1,2-époxy-3-(*p*-nitrophénoxy)propane et le 7-[H^3]-styrène oxyde, respectivement.

[d] ↗, ↘, -: augmentation, diminution et aucune variation significative, respectivement.

II.4. Les cholinestérases

Les cholinestérases (ChE) sont des enzymes pouvant hydrolyser les esters de choline plus rapidement que d'autres substrats. Les vertébrés possèdent deux ChE correspondant à deux gènes distincts, l'acétylcholinestérase (AChE) et la butyrylcholinestérase (BChE). Ces deux enzymes se distinguent par leur spécificité envers différents esters de choline (l'acétylcholine -ACh- et la butyrylcholine -BCh- pour l'AChE et la BChE, respectivement) et leur réactivité envers divers inhibiteurs.

II.4.1. Les différentes formes de cholinestérases

Les AChE existent sous diverses formes moléculaires, ancrées dans les membranes pré- ou post-synaptiques, associées aux lames basales situées dans l'espace synaptique, ancrées dans les membranes des érythrocytes et des lymphocytes ou sécrétées à l'état soluble dans le milieu extracellulaire et présentes dans le plasma sanguin (figure 12). Deux formes diffèrent par leur structure quaternaire, la forme asymétrique (A) et la forme globulaire (G). Les formes A sont caractérisées par la présence d'une triple hélice collagénique (queue collagénique) dont chaque brin (sous-unité Q) peut être attaché à un tétramère de sous-unités catalytiques. Les formes A peuvent comporter un, deux ou trois tétramère(s) (formes A_4, A_8 et A_{12}). Elles n'existent que chez les vertébrés et se trouvent uniquement dans le système nerveux et les muscles. Elles se caractérisent par leur capacité à interagir à fa ible force ionique avec des composants polyanioniques présents dans les lames basales, tels que les glycosaminoglycanes, et ne sont solubles qu'à force ionique élevée. Les formes G ne possèdent pas de queue collagénique. Elles existent sous forme de monomères (G_1), dimères (G_2) ou tétramères (G_4) de sous-unités catalytiques. Certaines sont amphiphiles (G^a) et d'autres non (G^{na}). Les formes G^a présentent un domaine hydrophobe, distinct du domaine catalytique, leur permettant de s'ancrer dans les membranes: soit un groupement glycophosphatidylinositol -GPI- (G^a de type I), soit un groupement de nature non glycolipidique (G^a de type II), soit une sous-unité hydrophobe non catalytique de 20 kDa -sous-unité P- (Massoulié *et al.*, 1993). Les AChE possèdent différentes sous-unités catalytiques codées par des ARNm obtenus par épissage différentiel à partir d'un même gène. Ces sous-unités présentent le même domaine catalytique mais diffèrent par leurs peptides C-terminaux. On distingue les sous-unités de type H (présence de séquences hydrophobes), T

(nécessaire à l'assemblage d'une queue collagénique ou de nature hydrophobe), S (trouvé uniquement dans le venin de certains serpents) et R (pourrait résulter d'une erreur d'épissage). Les formes asymétriques ne sont constituées que de sous-unités T (Massoulié *et al.,* 1999).

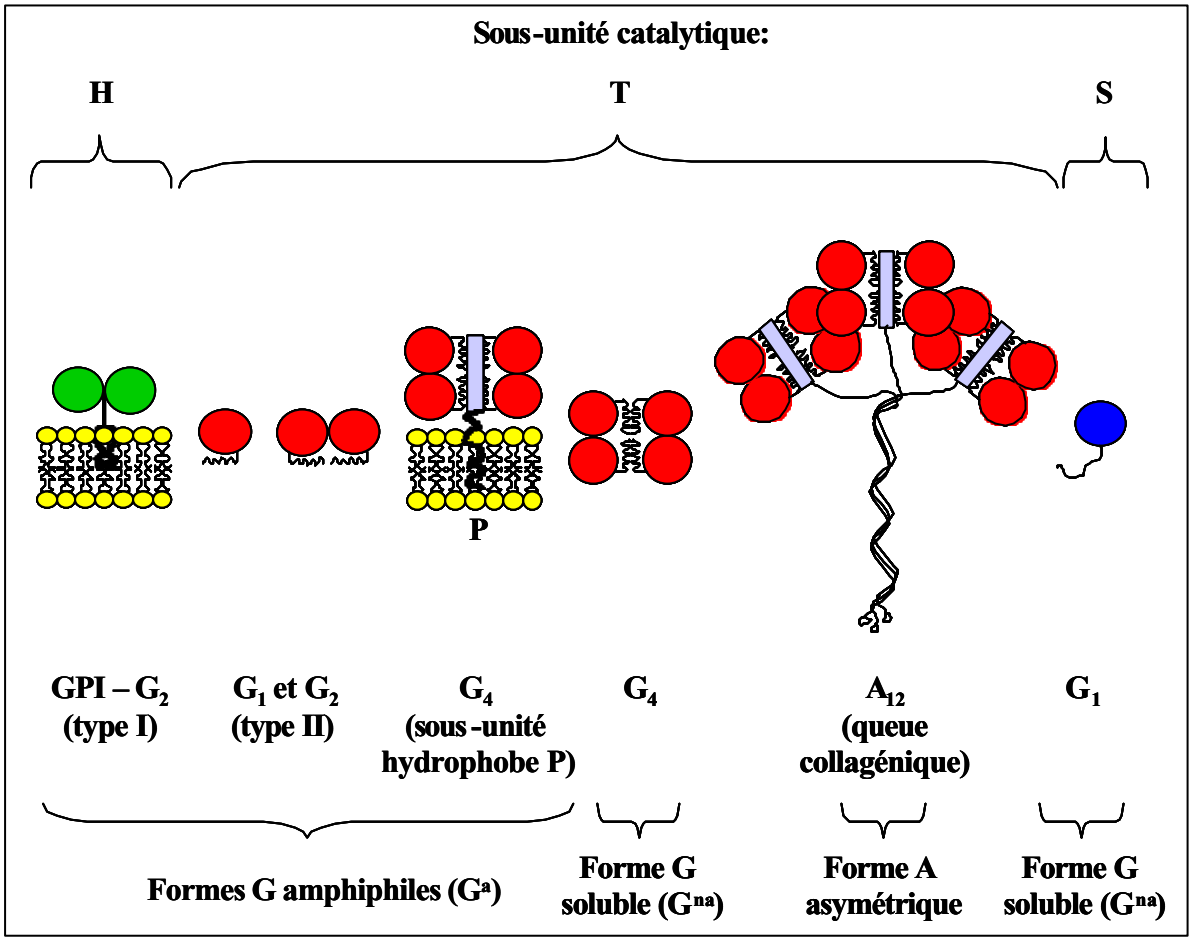


Figure 12. Les différentes formes moléculaires de l'acétylcholinestérase chez les vertébrés (d'après Massoulié *et al.*, 1999).

II.4.2. Rôles de l'acétylcholinestérase

Chez les vertébrés, l'AChE est une enzyme vitale dont le rôle majeur est d'hydrolyser un neuromédiateur, l'ACh, au niveau des synapses cholinergiques (nerveuses et neuromusculaires). A l'arrivée de l'influx nerveux, la terminaison présynaptique libère l'ACh qui diffuse à travers l'espace synaptique et vient activer des récepteurs spécifiques situés dans la membrane postsynaptique. Dans les jonctions neuromusculaires, les fibres musculaires postsynaptiques présentent au contact de la terminaison nerveuse une région membranaire spécialisée riche en récepteurs nicotiniques qui sont également des canaux ioniques. La

fixation de l'ACh sur ces derniers en provoque l'ouverture. L'entrée consécutive d'ions sodium entraîne une dépolarisation locale qui déclenche un potentiel d'action propagé dans toute la fibre musculaire, la libération d'ions calcium et finalement la contraction. Pour que la repolarisation soit possible, l'ACh est très rapidement hydrolysée en acétate et choline par l'AChE (figure 13), localisée dans l'espace synaptique. Le site actif de l'AChE comprend deux sous-sites: un sous-site estérasique comportant une sérine active et le groupe imidazole d'une histidine et un sous-site anionique liant le groupement ammonium quaternaire de l'ACh ou des inhibiteurs. La fixation de l'ACh entraîne un changement conformationnel de l'enzyme, puis une réaction d'échange entre l'ACh et la sérine activée par le groupe imidazole. Au cours de cette réaction, une molécule de choline est libérée et la sérine acétylée. L'intervention d'une molécule d'eau permet de désacétyler cette dernière en libérant de l'acétate afin de régénérer l'enzyme libre (Massoulié et Bon, 1993).

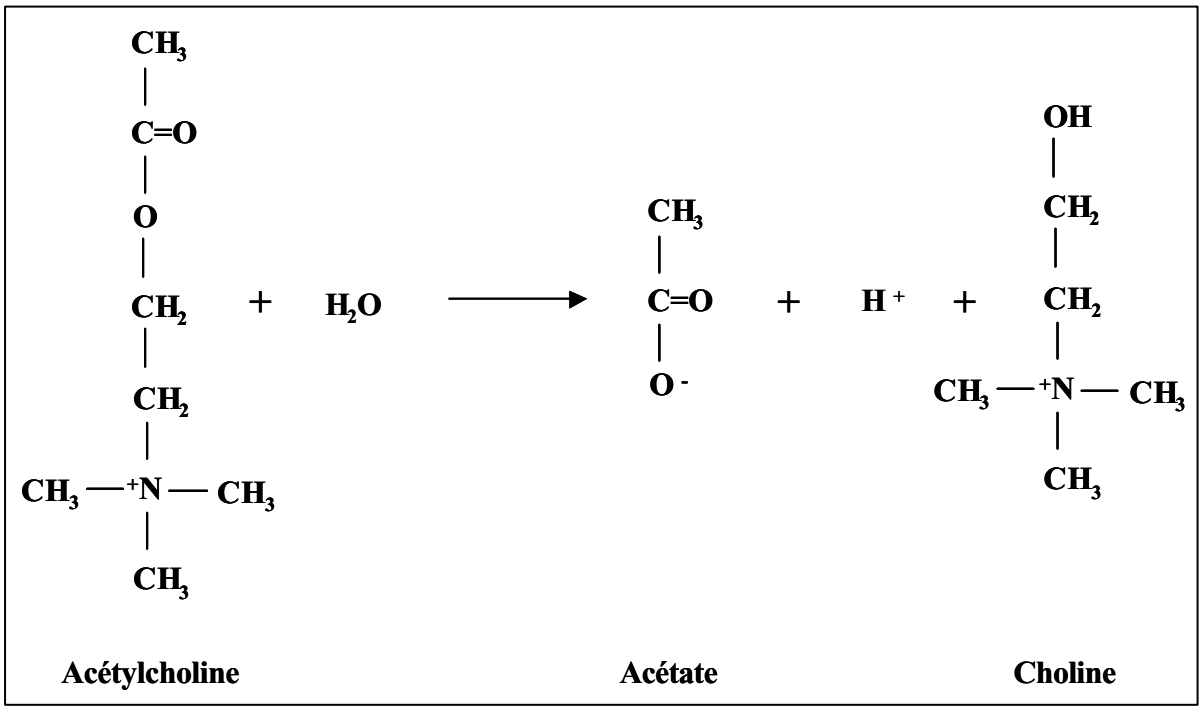


Figure 13. Hydrolyse de l'acétylcholine par l'acétylcholinestérase
(d'après Trundle et Marcial, 1988).

L'inhibition de l'AChE peut avoir des conséquences catastrophiques pouvant entraîner la mort. Parmi les inhibiteurs de l'AChE, les plus connus sont certains gaz de combat et les insecticides oganophosphorés et carbamates. Le mécanisme d'inhibition par ces derniers est basé sur la formation d'un complexe carbamylé ou phosphorylé qui est plus stable que la forme acétylée. Cependant la forme carbamylée s'hydrolyse rapidement (inhibition

réversible) alors que la réactivation de la forme phosphorylée est beaucoup plus lente (inhibition réversible) voire impossible (inhibition irréversible) selon la nature de l'organophosphoré (Lotti, 1995).

Le rôle de la BChE est moins connu que celui de l'AChE. Elle est capable d'hydrolyser certaines drogues telle que l'héroï ne et pourait être impliquée dans la détoxication de certains composés naturels. De manière générale, les ChE pourraient également être associées à des fonctions non cholinergiques telles que l'embryogenèse, les mécanismes de reconnaissance, de signalisation, d'adhérence, de prolifération et de différenciation cellulaires (Massoulié et Bon, 1993).

II.4.3. Les cholinestérases chez les mollusques bivalves et leur utilisation comme biomarqueurs de pollution

La présence de différentes formes de ChE a été mise en évidence chez plusieurs invertébrés aquatiques tels que l'huître *Crassostrea gigas* (Bocquené *et al.*, 1997), les céphalopodes *Octopus vulgaris* (Talesa *et al.*, 1995) et *Eledone moschata* (Talesa *et al.*, 1998), les moules marines *Mytilus edulis* et *Mytilus galloprovincialis* (Von Wachtendonk et Neef, 1979; Mora *et al.*, 1999a; Talesa *et al.*, 2001) et la palourde d'eau douce *Corbicula fluminea* (Mora *et al.*, 1999a). En ce qui concerne plus précisément les mollusques bivalves, Von Wachtendonk et Neef (1979) ont purifié une AChE globulaire non-membranaire dans l'hémolymphe de *M. edulis*. Mora *et al.* (1999a) ont mis en évidence dans les branchies de *M. edulis et M. galloprovincialis* une forme globulaire de ChE hydrolysant préférentiellement l'acétylthiocholine (substrat utilisé pour doser l'activité AChE *in vitro* selon Ellman *et al.*, 1961). De par son caractère amphiphile et la présence d'un groupement GPI, elle pourrait être similaire aux formes G^a de type I présentes chez les vertébrés. De plus, les auteurs n'ont pas exclu la présence d'autres ChE solubles similaires aux formes G^a de type II (amphiphiles mais facilement solubilisées) ou G^{na} (non-amphiphiles, pleinement solubles). Plus récemment, Talesa *et al.* (2001) ont purifié deux AChE globulaires hydrophiles similaires aux formes G_2^{na} et G_4^{na} dans l'hémolymphe de *M. galloprovincialis* ainsi qu'une AChE globulaire amphiphile à groupement GPI similaire à la forme G_2^a de type I dans les tissus. Cette dernière AChE correspond vraisemblablement à celle purifiée par Mora *et al.* (1999a). Une seule forme de ChE a été mise en évidence chez *C. fluminea* à partir d'extraits d'animaux entiers par Mora *et*

al. (1999a). Elle hydrolyse préférentiellement la propionylthiocholine (d'où son nom de propionylcholinestérase -PChE-) et serait similaire aux formes G^a de type I.

Dans les études environnementales, l'AChE et la PChE sont essentiellement utilisées comme biomarqueurs d'exposition aux pesticides organophosphorés et carbamates. Cependant, les métaux lourds et plus rarement les hydrocarbures aromatiques polycycliques peuvent également moduler l'activité des cholinestérases (en l'inhibant la plupart du temps), selon un mode d'action moins connu que celui des pesticides (tableau 5).

Tableau 5. Modulation des activités cholinestérases chez les mollusques bivalves après exposition à différents polluants.

Organisme	Polluant [a]	Mode d'exposition [b]	Paramètre [c]	Variation [d]	Référence
Corbicula fluminea	Pb	L (*in vitro*)	AChE	↘	Labrot *et al.*, 1996
		L	AChE	-	Labrot *et al.*, 1996
	U	L (*in vitro*)	AChE	↗ ou ↘	Labrot *et al.*, 1996
		L	AChE	↘	Labrot *et al.*, 1996
	Fénitrothion	L	AChE	↘	Basack *et al.*,1998
	Parathion	L	AChE	↘	Basack *et al.*,1998
	Paraoxon	L	AChE	↘	Basack *et al.*,1998
	Carbaryl	L (*in vitro*)	AChE	↘	Mora *et al.*, 1999b
		L	AChE	↘	Mora *et al.*, 1999b
	CH_3 parathion	L (*in vitro*)	AChE	-	Mora *et al.*, 1999b
		L	AChE	-	Mora *et al.*, 1999b
	CH_3 paraoxon	L (*in vitro*)	AChE	↘	Mora *et al.*, 1999b
Dreissena polymorpha	Déméton-*S*-methyl	L (*in vitro*)	AChE	-	Dauberschmidt *et al.*, 1997
		L	AChE	-	Dauberschmidt *et al.*, 1997
	Disulfoton	L	AChE	-	Dauberschmidt *et al.*, 1997
	Thiométon	L	AChE	-	Dauberschmidt *et al.*, 1997
Mytilus galloprovincialis	Fe	L (*in vitro*)	AChE	↘	Najimi *et al.*, 1997
	Zn	L (*in vitro*)	AChE	↘	Najimi *et al.*, 1997
	Cd	L (*in vitro*)	AChE	↘	Najimi *et al.*, 1997
	Cu	L (*in vitro*)	AChE	↘	Najimi *et al.*, 1997
	Cu	L	PChE	↘ ou -	Regoli et Principato, 1995
	Métaux	T	PChE	-	Regoli et Principato, 1995
	Carbaryl	L (*in vitro*)	AChE	↘	Mora *et al.*, 1999b
		L	AChE	↘	Mora *et al.*, 1999b
	CH_3 parathion	L (*in vitro*)	AChE	-	Mora *et al.*, 1999b
		L	AChE	↘	Mora *et al.*, 1999b
	CH_3 paraoxon	L (*in vitro*)	AChE	↘	Mora *et al.*, 1999b
	B[*a*]P	M	AChE	↘	Akcha *et al.* , 2000

Tableau 5 (suite).

Organisme	Polluant [a]	Mode d'exposition [b]	Paramètre [c]	Variation [d]	Référence
Perna perna	Fe	L (*in vitro*)	AChE	↘	Najimi *et al.*, 1997
	Zn	L (*in vitro*)	AChE	↘	Najimi *et al.*, 1997
		L	AChE	↘ ou ↗	Najimi *et al.*, 1997
	Cd	L (*in vitro*)	AChE	↘	Najimi *et al.*, 1997
		L	AChE	↘ ou ↗	Najimi *et al.*, 1997
	Cu	L (*in vitro*)	AChE	↘	Najimi *et al.*, 1997

[a] Cd, Cu, Pb, U, Fe, Zn, B[*a*]P: cadmium, cuivre, plomb, uranium, fer, zinc et benzo[*a*]pyrène, respectivement.

[b] L, M, T: étude en laboratoire, en mésocosme et de terrain, respectivement.

[c] AChE, PChE: activités acétylcholinestérase et propionylcholinestérase, respectivement.

[d] ↗, ↘, -: augmentation, diminution et aucune variation significative, respectivement.

CHAPITRE I

Purification et caractérisation des glutathion *S*-transférases de *Corbicula fluminea*

Les glutathion *S*-transférases (GSTs) sont des enzymes du métabolisme de (dé)toxication de phase II capables de conjuguer une molécule de glutathion réduit (GSH) au centre électrophile d'un xénobiotique afin de rendre celui-ci plus soluble et donc plus facilement excrétable par l'organisme. Leur expression peut être induite ou inhibée par certains contaminants, ce qui leur confère un grand intérêt en tant que biomarqueurs potentiels de pollution. Notre étude s'inscrit dans une démarche de validation du mollusque bivalve *Corbicula fluminea* comme espèce sentinelle des milieux dulçaquicoles, à l'instar des moules pour le milieu marin. Afin d'intégrer les GSTs dans une approche multibiomarqueurs chez cet organisme nous avons optimisé et caractérisé l'activité GST envers plusieurs substrats. Cette étude fait l'objet de l'**article n°1**. Nous avons sélectionné les substrats suivants:

- le 1-chloro-2,4-dinitrobenzène (CDNB): substrat non spécifique métabolisé par la majeure partie des classes de GSTs,
- l'acide éthacrynique (AE): spécifique des GSTs de classe Pi,
- le 3,4-dichloro-1-nitrobenzène (DCNB): spécifique des GSTs de classe Mu,
- le 1,2-époxy-3-(*p*-nitrophénoxy)propane (EPNP): spécifique des GSTs de classe Thêta.

Le choix de plusieurs substrats présentant chacun une spécificité envers une classe donnée de GST s'est appuyé sur la volonté de mettre en évidence les variations d'une classe particulière de GST en réponse à une contamination. Une telle variation pourrait en effet passer inaperçue lors du dosage de l'activité avec le substrat non spécifique CDNB. Il convient néanmoins de préciser que cette spécificité n'est pas stricte. Les activités GST envers le CDNB et l'AE ont été localisées principalement dans la fraction cytosolique de masse viscérale (720,9 et 71,0 nmoles/min/mg protéine, respectivement) et de branchies (206,9 et 45,7 nmoles/min/mg protéine, respectivement). Une faible activité, hautement variable, a été mise en évidence avec l'EPNP, quel que soit le tissu ou la fraction considéré(e). Une faible activité GST envers le DCNB a été détectée uniquement dans les microsomes de masse viscérale. Les conditions optimales de dosage (concentration en protéines, pH, température, concentrations en substrats) ainsi que les paramètres cinétiques apparents ont été déterminés pour le CDNB, l'AE et le GSH dans le cytosol de masse viscérale. Un K_m et un V_{max} apparents de 0,59 mM et 64,4 nmoles/min et de 0,14 mM et 8,16 nmoles/min ont ainsi été obtenus pour le CDNB et l'AE, respectivement, soulignant une plus grande affinité de la ou des GST(s) non purifiée(s)

pour l'AE. L'obtention d'une activité GST envers l'AE nous a conduit à pressentir des GSTs de classe Pi comme les GSTs majeures présentes dans le cytosol de masse viscérale et de branchies de *Corbicula fluminea*. Afin de mettre en évidence une éventuelle variation spécifique de la ou des GST(s) de cette classe, nous avons utilisé conjointement le CDNB et l'AE dans nos expériences ultérieures. De plus, pour confirmer ou infirmer la nature des GSTs présentes dans la fraction cytosolique de masse viscérale et de branchies nous avons conduit par la suite des études de purification et de caractérisation des isoenzymes de GSTs dans ces tissus. Ces résultats seront présentés dans l'**article n°2**.

ARTICLE 1

Characterization of glutathione *S*-transferase activity in the Asiatic clam *Corbicula fluminea*

Vidal, M-L., Narbonne J-F.

***Bulletin of Environmental Contamination and Toxicology*, 2000, 64(3), 455-462.**

Bull. Environ. Contam. Toxicol. (2000) 64:455–462
© 2000 Springer-Verlag New York Inc.
DOI: 10.1007/s001280000022

Environmental Contamination and Toxicology

Characterization of Glutathione *S*-Transferase Activity in the Asiatic Clam *Corbicula fluminea*

M. L. Vidal, J. F. Narbonne

Environmental and Toxicological Chemistry Laboratory, UPRES-A 5472 CNRS, Department of Biochemical Toxicology, University of Bordeaux 1, Avenue des Facultés, 33405 Talence, France

Received: 29 September 1999/Accepted: 6 January 2000

The glutathione *S*-transferases (GSTs; EC 2.5.1.18) form a group of multifunctional enzymes catalyzing the conjugation of a broad range of electrophilic substrates to the tripeptide glutathione (Wilce and Parker 1994). They are involved in the cellular detoxification and excretion of many physiological and xenobiotic substances. Moreover, cells may respond to xenobiotic exposure by induction of particular isoenzymes (Ketterer et al. 1988). They have been mainly studied in mammals (Wilce and Parker 1994) but numerous studies also report their presence in invertebrates (Dierickx 1984, Stenersen et al. 1987, Livingstone 1991), yeasts (Foley and Sheehan 1998) and plants (Schrenk et al. 1998). Because GSTs can be induced or inhibited by certain xenobiotics, they have been proposed as potential pollution biomarkers for several aquatic species such as fishes, crustaceans or mollusks. Thus, increase of GST activity as a result of exposure to certain compounds has already been demonstrated in these species (Lee et al. 1988; Boryslawskyj et al. 1988; Tejo Prakash and Jagannatha Rao 1995).

Corbicula fluminea is a freshwater bivalve originating from Asia that has become a major component of many benthic communities in the United States and more recently in Europe (Araujo et al. 1993). It is known to accumulate pollutants such as heavy metals (Baudrimont et al. 1997) or polycyclic aromatic hydrocarbons (Narbonne et al. 1999). In our laboratory we study the possible use of this species as a sentinel organism of freshwater ecosystems. As we previously observed the presence of GST activity in *C. fluminea*, it must be now characterized in order to include it in a multibiomarkers profile. As a preliminary study of purification procedure, this paper reports GST enzymatic characterization and assay optimization on crude fractions using several substrates.

MATERIALS AND METHODS

Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (EA), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) and 3,4-dichloro-1-nitrobenzene (DCNB) were obtained from Sigma-Aldrich Chemical, Saint Quentin Fallavier, France. Other chemicals were of the best technical grade available.

Adult *Corbicula fluminea*, anterioposterior length of which ranging from 15 to 20 mm, were collected during the spring 1998 from the banks of the non-polluted freshwater Cazaux-Sanguinet lake (Aquitaine, France). They were placed into a plastic tank containing lake water for the transport back to the laboratory. They were maintained in stabulation for seven days prior the experiment in glass aquariums containing dechlorinated tap water. Temperature was maintained at 18.5 °C and aeration was provided by air bubbling. Clams were not fed during this period and were held under a natural light cycle. No sexual differences were taken in account for *C. fluminea* are

Correspondence to: M. L. Vidal

hermaphroditic.

All steps of homogenization were achieved at 4°C. After the shell and the crystalline style were removed, gill, foot, visceral mass, and mantle were dissected-out from a pool of 100 animals. Bodies samples were rinsed in 100 m*M* phosphate buffer, pH 7.4, dried on absorbent paper sheets, weighed and homogenized in the same phosphate buffer (1:4 weight:volume ratio) using an Ultra-Turrax®Antrieb T25 (IKA® Labortechnik). Homogenates were then centrifuged at 9000 g for 30 min in a Sigma 3 MK centrifuge. Parts of the supernatants, consisting in the submitochondrial fractions (S9), were collected and stored at –80°C. Microsomes were obtained by centrifuging fractions of the S9 set apart at 105000 g for 1 hr in a Beckman LE-80 ultracentrifuge. The supernatants (cytosolic fractions) were collected and stored at –80°C. Microsomal pellets were resuspended in a 100 m*M* phosphate buffer, pH 7.4, containing 1 m*M* EDTA and 20 % glycerol, then collected and stored at –80°C.

GST activities were measured on a temperature-controlled Kontron Uvikon 932 spectrophotometer. Assays were run in triplicate. Four substrates were assayed (CDNB, EA, EPNP, DCNB) according to the method of Habig et al. (1974) following the apparition of glutathione conjugates at 340 nm (CDNB), 270 nm (EA), 360 nm (EPNP) and 345 nm (DCNB). A 100 m*M* phosphate buffer pH 7.4, a sample protein concentration of 0.1 mg/mL and a temperature of 25 °C were used for all substrates as initial standard assay conditions. Particular assay conditions for each substrate were as follows. CDNB : 1 m*M* GSH and 1 m*M* CDNB; EA : 0.25 m*M* GSH and 0.2 m*M* EA; EPNP : 5 m*M* GSH and 0.5 m*M* EPNP; DCNB : 5 m*M* GSH and 1 m*M* DCNB. Total assay volume was of 1 mL. As we progressed in the study, optimal parameters were determined for each substrate. CDNB, EA, EPNP and DCNB were diluted in ethanol. The concentration of this latter did not exceed 2% in assay. GSH was diluted in phosphate buffer. Sample concentration, buffer pH, buffer concentration, temperature and substrate concentration varied in different experiments, details of which are described in relevant sections of the "Results and Discussion". GST activities were expressed as specific activities (in nmoles/min/mg protein) or as reaction rates (nmoles/min). The enzymatic rate was obtained by subtracting the chemical rate (without sample) to the total rate. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Assuming Michaelis-Menten kinetics, apparent K_m and V_{max} values of the GST activities were calculated using the computer program Statistica (5.1 release, Statsoft®). In a reaction rate vs. substrate plot this program fitted a non-linear regression curve by using least squares for minimizing the deviations in reaction rate on the given substrate-values. For each set of observations, the program presented the values for the measured and calculated reaction rate and finally it provided the apparent K_m and V_{max} values as calculated from the fitted curve.

RESULTS AND DISCUSSION

In *Corbicula fluminea*, the subcellular distribution of GST activities was quite similar using CDNB or EA as substrates (cf. table 1). They were mainly localized in cytosolic fractions as expected (53 % and 52 % for CDNB and EA respectively) for most of GST isoenzymes are cytosolic ones. They were localized in a minor extent in submitochondrial ones (40 % and 38 % for CDNB and EA respectively). Low but substantial GST activity towards CDNB was also observed in microsomal fractions (7 %) and could not only be explained by a contamination of these fractions by submitochondrial particles. Such an activity was also observed in microsomal fraction of hepatopancreas of the freshwater crayfish *Astacus astacus* (Lindström-Seppä et al. 1983).

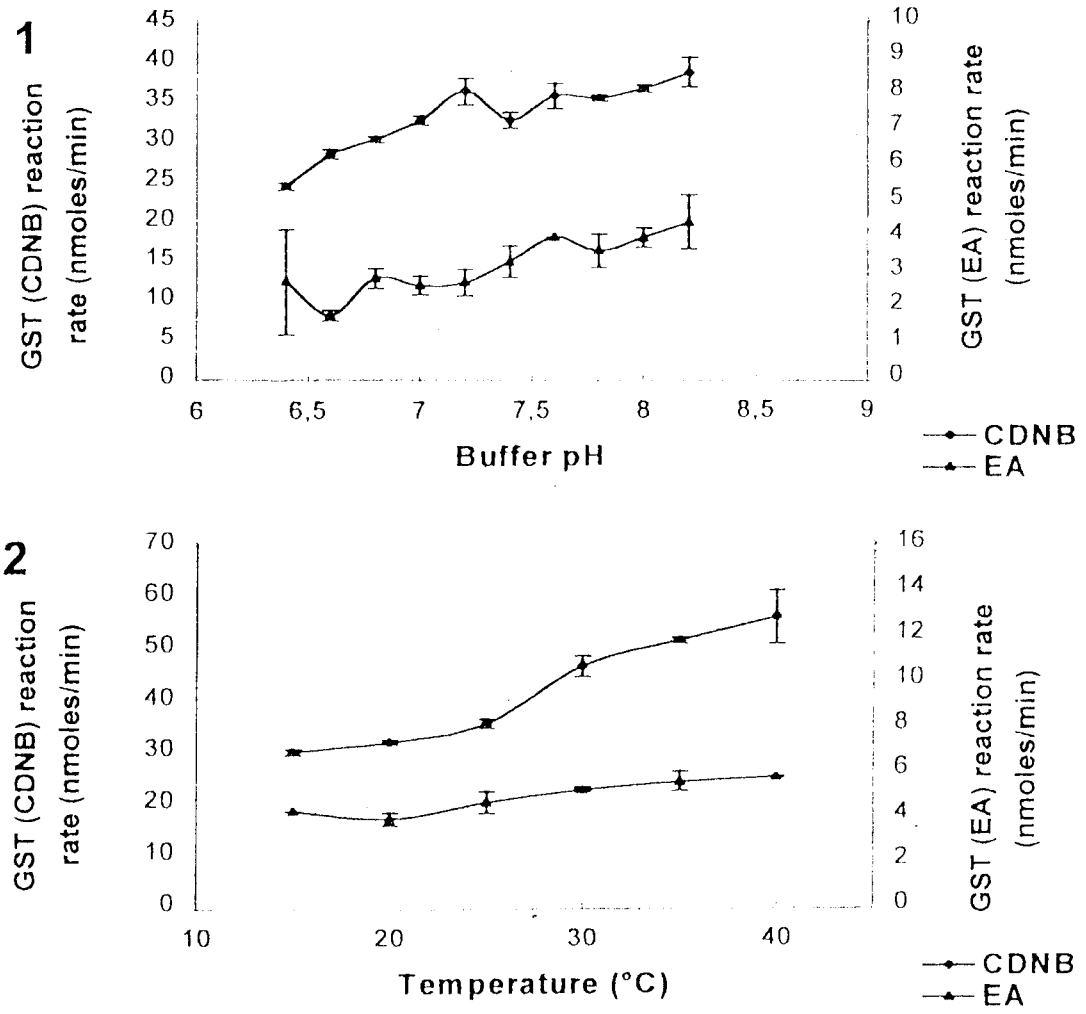
Table 1 . Subcellular and tissular distributions of GST specific activities towards CDNB and EA. Results are expressed in nmoles/min/mg protein. Values are means ± SD (n=3).

Substrate / Fraction	Tissue			
	Foot	Gill	Mantle	Visceral mass
CDNB				
Submitochondrial	53.7 (± 1.5)	149.8 (± 8.1)	107.5 (± 2.7)	554.7 (± 4.8)
Cytosolic	98.5 (± 1.6)	206.9 (± 4.2)	118.8 (± 5.8)	720.9 (± 10.5)
Microsomal	18.7 (± 1.4)	20.7 (± 0.3)	27.5 (± 2.2)	85.9 (± 2.9)
EA				
Submitochondrial	18.5 (± 1.6)	32.2 (± 3.4)	30.6 (± 4.8)	54.7 (± 1.6)
Cytosolic	32.1 (± 4.1)	45.7 (± 1.8)	35.7 (± 4.1)	71.0 (± 1.2)
Microsomal	3.7 (± 3.7)	6.9 (± 0.4)	8.2 (± 3.2)	15.2 (± 3.7)

Regarding tissue distribution, GST activity of *C. fluminea* towards CDNB was found to be ubiquitous in tissular fractions as already observed for *Mytilus edulis* or *A. astacus* (Fitzpatrick and Sheehan 1993 ; Lindström-Seppä et al. 1983). It was predominant in visceral mass and in a lesser extent in gill (63 % and 18 % respectively in cytosolic fractions) whereas in the case of *M. edulis*, it was mainly localized in the gill (Fitzpatrick and Sheehan 1993). In mollusks, digestive gland presents functions analogous to that of vertebrate liver in which high GST activity is encountered. The visceral mass of *C. fluminea* includes two organs impossible to dissociate without breaking their integrity, namely the digestive gland and gonads. Thus the high activity observed in the visceral mass could be considered as the sum of activities of both organs. *C. fluminea* also exhibited GST activities towards more specific substrates as EA, EPNP and DCNB but they were lower than that observed with CDNB (results not shown for EPNP and DNCB). When using EA as a substrate *C. fluminea* presented a relatively high activity (up to 10 % of activity towards CDNB) and the tissular distribution was close to that observed for CDNB (39 % and 25 % in cytosol of visceral mass and gill respectively). In the study of Stenersen et al. (1987) *M. edulis* exhibited one of the greatest GST activity towards EA among aquatic and terrestrial animals studied. We observed GST activity towards EPNP in almost every subcellular and tissular fraction but at a low level (4 % of that with CDNB in cytosol of visceral mass). Moreover it exhibited a high variability that could not be reduced despite steps of assay optimization. Such a variability was already observed in the yeast (Foley and Sheehan 1998). Among invertebrates, activity towards EPNP was neither detected in the purified isoenzyme GST 1 of *M. edulis* (Fitzpatrick et al. 1995) nor in several species of earthworms (Stenersen et al. 1979). In the case of DCNB, GST activity was not observed in cytosolic fractions of *C. fluminea*. As it was assayed on cytosolic crude fractions, it was perhaps too low to be detected. Thus, Stenersen et al. (1987) did not observe activity in most of invertebrates studied (*M. edulis* included) whereas it was detected in the purified cytosolic isoenzyme GST 1 of *M. edulis* gill (Fitzpatrick et al. 1995). On the other hand, we detected activity in microsomes of visceral mass (3.06 nmoles/min/mg protein ± 1.01). For the following studies, GST activities were measured in cytosol of visceral mass using CDNB and EA as substrates.

Under standard assay conditions, GST reaction rate was found to be linear with protein concentration in the range from 0.01 to 0.15 mg protein/mL for CDNB and from 0.01 to 0.2 mg protein/mL for EA (results not shown). For the following steps of the study, a protein concentration of 0.05 mg protein/mL was selected for both substrates.

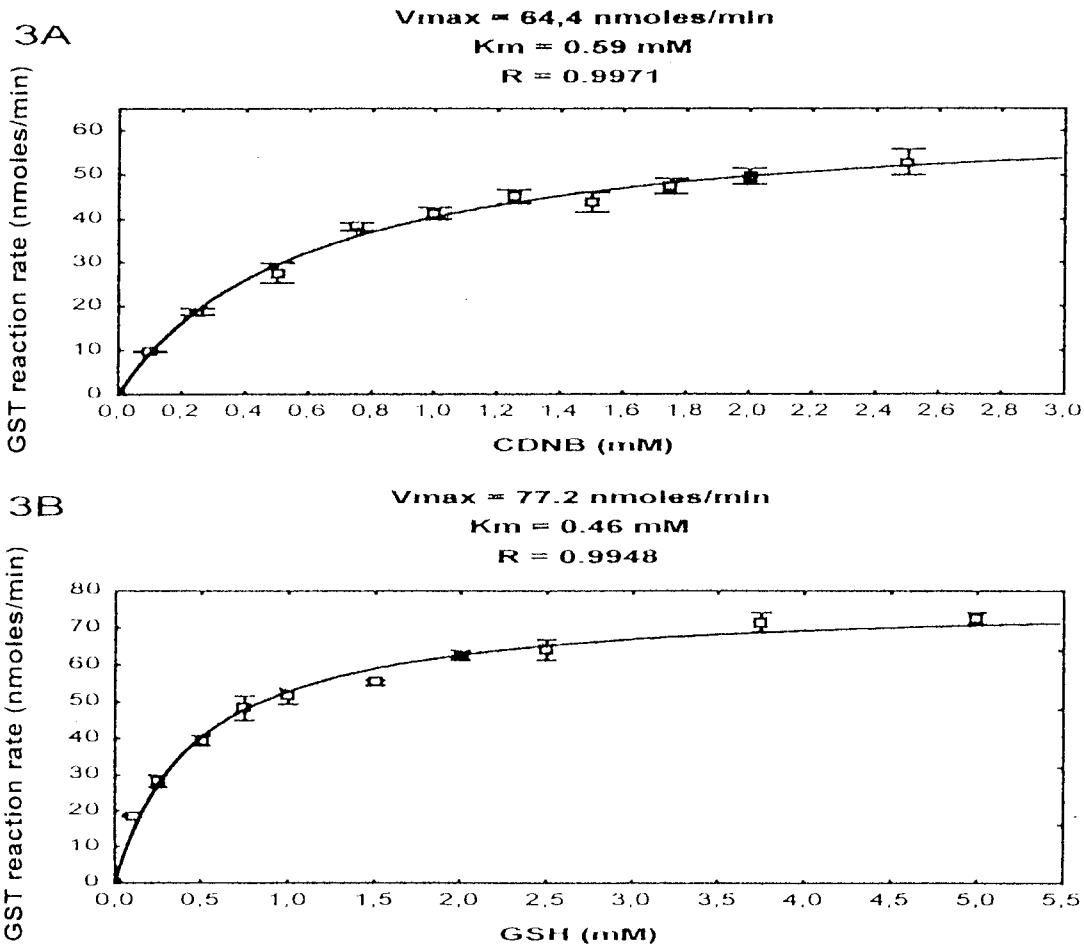
To study the dependence of GST reaction rate on buffer pH, a range of phosphate buffer pH from 6.4 to 8.2 was assayed. The presence of at least two pH optima for CDNB and EA conjugation was observed for *C. fluminea* (pH 7.2 and 7.6 for CDNB, pH 6.8 and 7.6



Figures 1 & 2 . Dependence of GST reaction rate on buffer pH (figure 1) or temperature (figure 2) using CDNB or EA as a substrate. Values are means ± SD (n=3).

for EA) (cf. figure 1). Such results let us foretell for the possible presence of at least two major GST isoenzymes in the cytosol of visceral mass. Few studies are available on the pH optimum of GST activities among invertebrates. Balabaskaran et al. (1986) observed pH optima varying between pH 7 and 9 for CDNB conjugation in three gastropod species. More generally, pH optima with a variety of substrates range from 6.5 to 9.5 for non-vertebrate organisms with an optimum in the vicinity of pH 8 for CDNB (Clark 1989). In the same review, the author reported that the distribution of pH optima for CDNB could be bimodal, indicating the possible existence of two general classes of enzyme. Three buffer concentrations were also evaluated : 25, 50 and 100 m*M*. A maximal GST reaction rate was obtained for a concentration of 50 and 25 m*M* using CDNB and EA respectively (results not shown). For further studies, we selected a 50 m*M* buffer, pH 7.2 and a 25 m*M* buffer, pH 7.6 in the case of CDNB and EA respectively.

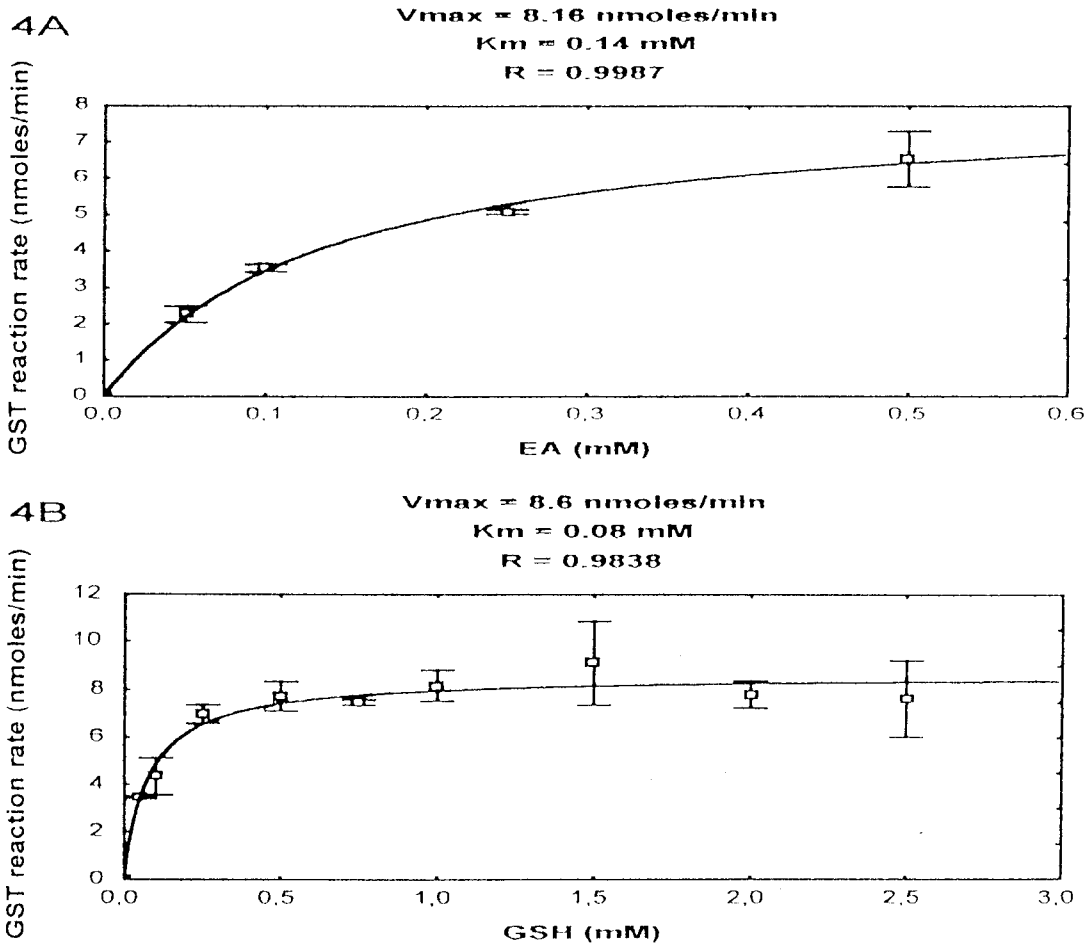
To study the dependence of GST reaction rate on temperature, a range of temperatures from 15 to 40°C was assayed. Using CDNB as a substrate, the reaction rate increased along with temperature with an inflexion between 25 and 30°C (cf. figure 2). In the case of EA, it slightly decreased between 15 and 20°C and then increased slowly up to 40°C. Whatever the substrate, no denaturation point was detected in the range assayed. Arrhenius graphs obtained for CDNB and EA were discontinuous (results not shown) as already



Figures 3A & 3B . Dependence of GST reaction rate on CDNB (figure 3A) and GSH (figure 3B) concentrations. Apparent kinetic parameters and regression coefficient of the Michaelis-Menten model fitted curve are given for each substrate.

observed among insects (Clark 1989). It could be due to the presence of several isoenzymes in the crude extract. Activation energy of *C. fluminea* GST using CDNB was close to these observed in other non-vertebrate species as reported by Clark (1989), namely in the vicinity of 30 Kj/mole (26 Kj/mole for *C. fluminea* above 25°C). That obtained for EA was dramatically lower (1.4 KJ/mole above 20°C) suggesting a different mechanism of conjugation with this substrate. A temperature of 30°C for CDNB and EA was selected for the following assays.

Results of dependence of the reaction rate on the substrate concentration are presented on figures 3A and 4A. For the determination of kinetic parameters, only substrate concentration varied. Ethanol concentration was maintained constant (2%). Other parameters were as determined before. Using CDNB, ten concentrations of this substrate were assayed, ranging from 0.1 to 2.5 m*M*. We were limited for the choice of the upper concentration in that CDNB solubility in assay did not exceed 2.5 m*M* at 30°C. When using EA, eight concentrations were assayed ranging from 0.05 to 2 m*M*. No problem of solubility in the assay was encountered. Curves of GST reaction rate vs. substrate concentration fitted the Michaelis-Menten model with a regression coefficient R of 0.997 and 0.998 for CDNB (despite slightly discontinuities) and EA respectively. In the case of EA, only four plots were used for kinetic parameters determination as GST reaction rate rapidly decreased from a substrate concentration located between 0.5 and 0.75 m*M* (cf. figure 5). No more activity was detected from a concentration of 1.5 m*M*. The same effect



Figures 4A & 4B . Dependence of GST reaction rate on EA (figure 4A) and GSH (figure 4B) concentrations. Apparent kinetic parameters and regression coefficient of the Michaelis-Menten model fitted curve are given for each substrate.

was observed when using a higher GSH concentration. EA and especially its GSH-conjugate are known as inhibitors of GSTs in humans using CDNB as a substrate (Ploemen et al. 1993). The same phenomenon seems also occur in *C. fluminea* except that in our case, EA is both substrate and inhibitor. Apparent K_m of GST activity towards CDNB (0.59 m*M*) was higher than that of EA (0.14 m*M*) suggesting an higher affinity of GST for EA than for CDNB. For following studies using CDNB, we could not be at the V_{max} because of the limited solubility of this substrate. Nevertheless, we selected a CDNB concentration of 1.75 m*M* higher than the apparent K_m. In the case of EA, a concentration of 0.5 m*M* was selected.

Results of dependence of the reaction rate on the GSH concentration are presented on figures 3B and 4B. For the determination of kinetic parameters, only GSH concentration varied. Other parameters were as determined before. Using CDNB, ten concentrations of GSH were assayed, ranging from 0.1 to 5 m*M* whereas for EA, eight concentrations of GSH were assayed, ranging from 0.05 to 2.5 m*M*. Despite discontinuities, curves of GST reaction rate vs. GSH concentration fitted the Michaelis-Menten model with a regression coefficient R of 0.995 and 0.984 for CDNB and EA respectively. Apparent K_m^{GSH} using CDNB as a substrate was higher than that observed with EA suggesting an higher affinity of GST using EA for GSH than GST using CDNB (K_m^{GSH} of 0.46 m*M* and 0.08 m*M* for CDNB and EA respectively). As a general manner, GST activity had an higher affinity for GSH than for the conjugating substrate. Thus, GST in *Corbicula* seems to be constituted

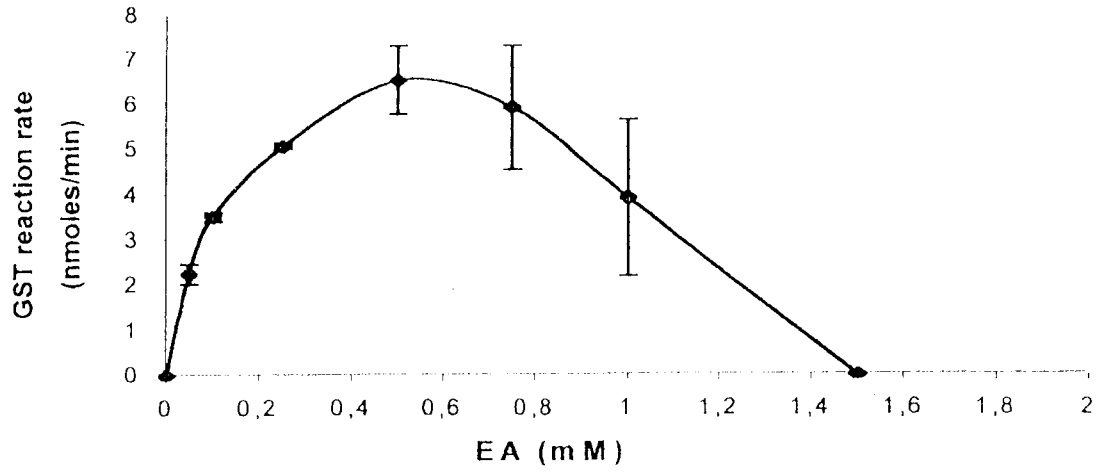


Figure 5. Dependence of GST reaction rate on EA concentration. Values are means ± SD (n=3).

by a very specific GSH binding site and a less specific electrophilic substrate one as it is the case in mammals (Wilce and Parker 1994).

It is difficult to compare these apparent kinetic parameters with those found in literature in different assay conditions. Nevertheless, our values are close of those obtained by Keeran and Lee (1987) for two purified isoenzymes of the blue crab *Callinectes sapidus* (K_m^{CDNB} of 0.28 and 0.31 m*M*, K_m^{GSH} of 0.14 and 0.43 m*M* for isoenzyme I and II respectively). For the purified isoenzyme GST 1 of *M. edulis* gill, K_m^{CDNB} (3.7 m*M*) was much higher whereas K_m^{GSH} (0.5 m*M*) was close of that observed for *C. fluminea* (Fitzpatrick et al. 1995). The slight discontinuities observed on curves of GST reaction rate vs. CDNB or GSH concentration could be explained by the possible presence of several isoenzymes in the crude fractions, as hypothesized before. For further assays, GSH concentrations of 5 m*M* and 0.25 m*M* for CDNB and EA respectively were selected to be close of V_{max} conditions.

As other mollusks, cytosolic fractions of *C. fluminea* exhibit high GST activity towards CDNB and in a lesser extent, EA. Several isoenzymes may be present and must be further characterized.

Acknowledgments. This study was supported by a grant of the Environment Department of the Petroleum Society Elf Aquitaine. The authors thank Dr Pascal Mora for helpful discussions and results processing.

REFERENCES

Araujo R, Moreno D, Ramos MA (1993) The Asiatic clam *Corbicula fluminea* (Müller, 1774) (Bivalvia : Corbiculidae) in Europe. American Malacol Bull 10 : 39-49

Balabaskaran SCS, Chew S, Segaran M (1986) Studies on glutathione *S*-transferase in molluscs. Comp Biochem Physiol 85B : 183-192

Baudrimont M, Metivaud J, Maury-Brachet R, Ribeyre F, Boudou A (1997) Bioaccumulation and metallothionein response in the Asiatic clam (*Corbicula fluminea*) after experimental exposure to cadmium and inorganic mercury. Environ Toxicol Chem 16 : 2096-2105

Boryslawskyj M, Garrood AC, Pearson JT (1988) Elevation of glutathione-*S*-transferase activity as a stress response to organochlorine compounds, in the freshwater mussel,

Sphaericum corneum. Mar Environ Res 24 : 101-104
Clark AG (1989) The comparative enzymology of the glutathione *S*-transferases from non-vertebrate organisms. Comp Biochem Physiol 92B : 419-446
Dierickx PJ (1984) Glutathione *S*-transferase in aquatic macro-invertebrates and its interaction with different organic micropollutants. Sci Tot Environ 40 : 93-102
Fitzpatrick PJ, Sheehan D (1993) Separation of multiple forms of glutathione *S*-transferase from the blue mussel, *Mytilus edulis*. Xenobiotica 23 : 851-861
Fitzpatrick PJ, Krag TOB, Højrup P, Sheehan D (1995) Characterization of a glutathione *S*-transferase and a related glutathione-binding protein from gill of the blue mussel, *Mytilus edulis*. Biochem J 305 : 145-150
Foley V, Sheehan D (1998) Glutathione *S*-transferases of the yeast *Yarrowia lipolytica* have unusually large molecular mass. Biochem J 333 : 839-845
Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione *S*-transferases. J Biochem Chem 249 : 7130-7139
Keeran WS, Lee RF (1987) The purification and characterization of glutathione *S*-transferase from the hepatopancreas of the blue crab, *Callinectes sapidus*. Arch Biochem Biophys 255 : 233-243
Ketterer B, Meyer DJ, Clark AG (1988) Soluble glutathione transferase isozymes. In : Sies H, Ketterer B (ed) Glutathione conjugation, mechanisms and biological significance, Academic Press Limited, London, p73
Lee RF, Sheehan WS, Pickwell GV (1988) Marine invertebrate glutathione-*S*-transferases : purification, characterization and induction. Mar Environ Res 24 : 97-100
Lindström-Seppä P, Koivusaari U, Hänninen O (1983) Metabolism of foreign compounds in freshwater crayfish (*Astacus astacus* L.) tissues. Aquat Toxicol 3 : 35-46
Livingstone DR (1991) Organic xenobiotic metabolism in marine invertebrates. In : Gilles R (ed) Advances in Comparative and Environmental Physiology, Springer-Verlag, Berlin, p 45
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193 : 265-275
Narbonne JF, Djomo JE, Ribera D, Ferrier V, Garrigues P (1999) Accumulation kinetics of polycyclic aromatic hydrocarbons adsorbed to sediment by the mollusk *Corbicula fluminea*. Ecotoxicol Environ Saf 42 : 1-8
Paulson KE (1989) Xenobiotic regulation of glutathione *S*-transferase Ya gene expression. Mol Toxicol 2 : 215-235
Ploemen JHTM, Van Ommen B, Bogaards JJP, Van Bladeren PJ (1993) Ethacrynic acid and its glutathione conjugate as inhibitors of glutathione *S*-transferases. Xenobiotica 23 : 913-923
Schrenk C, Pflugmacher S, Brüggemann R, Sandermann H Jr., Steinberg CEW, Kettrup A (1998) Glutathione *S*-transferase activity in aquatic macrophytes with emphasis on habitat dependence. Ecotoxicol Environ Saf 40 : 226-233
Stenersen J, Guthenberg C, Mannervik B (1979) Glutathione *S*-transferases in earthworms (Lumbricidae). Biochem J 181 : 47-50
Stenersen J, Kobro S, Bjerke M, Arend U (1987) Glutathione transferases in aquatic and terrestrial animals from nine phyla. Comp Biochem Physiol 86C : 73-82
Tejo Prakash NT, Jagannatha Rao KS (1995) Modulations in antioxidant enzymes in different tissues of marine bivalve *Perna viridis* during heavy metal exposure. Mol Cell Biochem 146 : 107-113
Wilce MCJ, Parker MW (1994) Structure and function of glutathione *S*-transferases. Biochem Biophys Acta 1205 : 1-18

Dans notre article précédent nous avons émis l'hypothèse de la présence de GST(s) de classe Pi dans le cytosol de masse viscérale et de branchies de *Corbicula fluminea*. Afin de valider cette hypothèse et d'une manière plus générale d'acquérir des bases moléculaires en vue de l'utilisation des GSTs comme biomarqueurs de pollution, nous avons conduit des études de purification et de caractérisation des isoformes de GST, présentées dans l'**article n°2**. La fraction cytosolique de masse viscérale a fait l'objet d'une étude approfondie car, comme nous l'avons démontré précédemment, elle est le siège de la plus forte activité GST, que ce soit avec le 1-chloro-2,4-dinitrobenzène (CDNB) ou l'acide éthacrynique. Cependant, nous nous sommes également intéressés aux branchies à titre comparatif car ils recèlent une activité GST non négligeable et, par leur fonction de filtration, sont constamment exposés aux phénomènes de contamination. Les GSTs cytosoliques de *Corbicula fluminea* ont été purifiées par chromatographie d'affinité -CA- (matrice glutathion-sépharose), chromatographie échangeuse d'anions (CEA) et chromatographie liquide haute performance en phase inverse (RP-HPLC). Des électrophorèses sur gel de polyacrylamide en conditions dénaturantes (SDS-PAGE) d'un extrait de masse viscérale purifié par CA ont mis en évidence quatre sous-unités d'un poids moléculaire (PM) apparent de 30,2, 29,2, 28,5 et 27,2 kDa. Des électrophorèses en conditions non dénaturantes ont révélé l'existence de trois protéines dimériques acides présentant un PM apparent de 64, 55 and 45 kDa, nommées GSTc1, GSTc2 et GSTc3, respectivement, en se basant sur leur ordre d'élution par CEA. Seules GSTc2 et GSTc3 ont présenté une activité envers le CDNB. Les profils des sous-unités de GST obtenus par RP-HPLC à partir d'extraits de masse viscérale et de branchies purifiés par CA se sont avérés différents: trois pics majeurs ont été résolus, dont un commun aux deux tissus. Le PM de chaque sous-unité a été determiné par spectrométrie de masse par électrospray: 23602 ± 1 Da pour la sous-unité majeure et 23289 ± 1 Da pour les sous-unités mineures. Des analyses d'immunoblotting ont mis en évidence l'appartenance des sous-unités de masse viscérale et de branchies à la classe Pi, confirmant ainsi notre hypothèse. De plus, les sous-unités mineures de masse viscérale ont légèrement réagi avec des anti-corps dirigés contre des GSTs de classe Mu.

ARTICLE 2

Purification and characterisation of glutathione *S*-transferases from the freshwater clam *Corbicula fluminea* (Müller)

Vidal, M-L., Rouimi, P., Debrauwer, L., Narbonne, J-F.

Soumis à *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology*

Abstract

Glutathione *S*-transferases (GSTs) are involved in the (de)toxification metabolism of phase II. To provide molecular bases for their use as biomarkers of pollution, cytosolic GSTs from the freshwater clam *Corbicula fluminea* have been purified by glutathione-sepharose affinity chromatography, anion-exchange chromatography (AEC) and reversed-phase (RP) HPLC. SDS-PAGE of visceral mass (VM) affinity-purified extract revealed four subunits with apparent molecular mass (MW) of 30.2, 29.2, 28.5 and 27.2 kDa. Non-denaturing electrophoretic analyses revealed three acidic dimeric proteins with apparent MW of 64, 55 and 45 kDa, named GSTc1, GSTc2 and GSTc3, respectively, basing on their elution order by AEC. Only GSTc2 and GSTc3 exhibited GST activity towards 1-chloro-2,4-dinitrobenzene. A tissue-specific subunit pattern was obtained by RP-HPLC of affinity-purified extract from VM and gills (GI): three major peaks were resolved, one of which was common to both tissues. MW of each VM subunit was determined by electrospray ionisation - mass spectrometry: 23602 ± 1 Da for the major subunit and 23289 ± 1 Da for the minor ones. Immunoblot analyses revealed all subunits from both tissues were related to the Pi-class GSTs. In addition, minor VM subunits were slightly related to the Mu-class ones. The interest of such molecular studies in biomonitoring programs is discussed.

Keywords. Biomarkers, *Corbicula fluminea*, freshwater clam, glutathione *S*-transferases, Mu-class, Pi-class, purification

Introduction

Bivalve molluscs filtrate large amounts of water to cope with nutritional and respiratory needs and therefore are able to bioaccumulate environmental contaminants, thus reflecting the degree of pollution of their ecosystem. That particular feature has been widely exploited in most biomonitoring programs. However, because bioaccumulation of xenobiotics alone does not provide information about the detrimental effects exerted by those contaminants on living organisms, a complementary approach has been developed, based on responses of biological parameters or biomarkers. Until now, most of the studies have focused on seawater ecosystems using mussels and fishes as major bioindicator species (Viarengo and Canesi, 1991; Livingstone, 1993). A minor but increasing attention has been paid to freshwater ecosystems as well. During the last years some freshwater bivalves have been proposed as potential bioindicator species, one of which is *Corbicula fluminea* (Müller). This clam originating from Asia has first colonised the United States before settling in Europe (Britton and Morton, 1979; Araujo et al., 1993). Like marine mussels, *C. fluminea* is able to bioaccumulate several contaminants (Inza et al., 1997; Narbonne et al., 1999) and interesting results have been obtained by measuring biomarker responses after exposure to heavy metals (Labrot et al., 1996), organophosphorus pesticides (Basack et al., 1998) or solvents (Vidal et al., 2001). Among biomarkers assessed in environmental studies, components related to (de)toxification metabolism of phases I and II are often considered. Components of phase I metabolism, namely cytochrome P450 and P450-related activities, are considered as good biomarkers of pollution in fishes because their responses are most of the time significantly related to contaminant levels (Bucheli and Fent, 1995). In mussels, some components of the mixed function oxygenase (MFO) system have been evidenced but induction levels are lower than those observed in fishes and not always correlated with the degree of contamination (Livingstone et al., 2000). Furthermore, phase I components are known to be affected by several abiotic and biotic factors such as temperature, season, sex or age in both fishes and mussels (Kirchin et al., 1992; Bucheli and Fent, 1995) which can interfere with xenobiotic effects. In mussels, glutathione *S*-transferases (GSTs), which belong to the phase II (de)toxification metabolism, are not as affected by those factors as MFO components are (Power and Sheehan, 1996). Thus that could confer an advantage to GSTs as biomarkers of pollution (Sheehan and Power, 1999). GSTs form a family of dimeric mainly cytosolic isoenzymes assuming important physiological functions, one of which is to catalyse the

conjugation of the tripeptide glutathione to xenobiotic compounds to increase their hydrophilicity and then favour their excretion by the organism. They are ubiquitous enzymes, reported in most animal phyla, e.g. molluscs (Fitzpatrick and Sheehan, 1993; Fitzpatrick et al., 1995a; Blanchette and Singh, 1999), earthworms (Stenersen et al., 1979), crustaceans (Keeran and Lee, 1987; Leblanc and Cochrane, 1987), insects (Stenersen et al., 1987), fishes (George and Young, 1988; Martínez-Lara et al., 1997; Pérez-López et al., 2000) and mammalians (Habig et al., 1974; Kamisaka et al., 1975; Rouimi et al., 1996). In mammalians, GSTs have been assigned to at least seven classes basing on substrate specificity, immunological properties and primary structure: Alpha, Mu, Pi, Theta, Sigma, Kappa and Zeta (Mannervik et al., 1985; Meyer et al., 1991; Meyer and Thomas, 1995; Pemble et al., 1996; Board et al., 1997). GST activity has been measured for years in aquatic species as a biomarker in environmental studies using the universal substrate 1-chloro-2,4-dinitrobenzene (CDNB) (Lee, 1988; Garrigues et al., 1990; Livingstone et al., 1995; Solé et al., 1996; Willett et al., 1999) which is conjugated by number of GST isoenzymes. In addition, other substrates initially considered as specific of certain GST classes are also employed despite overlapping substrate specificity, e.g. ethacrynic acid (EA), 3,4-dichloro-1-nitrobenzene (DCNB) and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) for Pi-, Mu- and Theta- classes, respectively (e.g. Regoli and Principato, 1995; Beyer et al., 1996; Lenártová et al., 1997). In *C. fluminea* GST activity has been measured using both CDNB and EA as it was found to be practically inactive towards DCNB and EPNP (Vidal and Narbonne, 2000). However, responses obtained in *C. fluminea* after exposure to several contaminants such as trichloroethylene, toluene and polycyclic aromatic hydrocarbons are not significant (Vidal et al., 2001; Vidal et al., unpublished results) and as a general manner, results obtained with GST activity in environmental studies are conflicting. Because the induction or inhibition of a specific GST isoenzyme or subunit can not be evidenced by the measurement of global GST activity, a molecular approach is more relevant. Thus the study of the isoenzyme or subunit pattern following exposition of the bioindicator species to pollution has been performed in mussels (Fitzpatrick et al., 1995b; 1997) and fishes (Martínez-Lara et al., 1996; Egaas et al., 1999; Pérez-López et al., 2000). As we intend to follow the same approach in *C. fluminea*, we have first carried out the purification and characterisation of its GST isoenzymes and subunits, results of which are presented in this paper. Purification was performed from both visceral mass and gill with a particular attention to GSTs from visceral mass for which the highest GST activity in *C. fluminea* is observed (Vidal and Narbonne, 2000).

Materials and methods

Chemicals

Ethylenediaminetetraacetate (EDTA), dithiothreitol (DTT), phenylmethylsulfonylfluoride (PMSF), reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), nitroblue tetrazolium (NBT), phenazine methosulfate, bovine serum albumin (BSA), lysine, arginine, glycine, sodium dodecyl sulfate (SDS), â-mercaptoethanol, acrylamide, N,N'-methylene-bis-acrylamide, trifluoroacetic acid (TFA), Sephadex G-25 were purchased from Sigma-Aldrich (St. Quentin Fallavier, France) and GSH-sepharose 4B from Pharmacia-Biotech (Orsay, France). All other chemicals were of the best analytical grade. Ultra-pure water from Milli-Q system (Millipore, Bedford, MA, USA) was used in all experiments.

Purification procedure

C. fluminea were collected in the banks of the non-polluted freshwater Cazaux-Sanguinet lake (Aquitaine, France). They were sexually mature and exhibited an anterior-posterior shell length of 25-30 mm. Sex was not taken into account for *C. fluminea* are hermaphroditic. In the laboratory they were maintained at 20°C in the lake water aerated by constant bubbling using a Shego M2K3 pump. They were not fed and were submitted to a natural light cycle. Dissection occurred two days after collection. All purification steps were performed at 4°C. Visceral mass (VM) and gills (GI) were dissected out, rinsed in 10 mM Tris HCl buffer (pH 7.3) and dried on absorbent paper towels. 13-15 g of fresh tissue were homogenised in two volumes of 10 mM Tris HCl buffer (pH 7.3), 0.15 M KCl, 0.25 M sucrose, 1 mM EDTA, 1 mM DTT and 1 mM PMSF using an Ultra-Turrax Antrieb T25 (IKA Labortechnik). Homogenate was centrifuged 30 min at 9000 g in a Sigma 3MK centrifuge and the resulting supernatant was filtered through gauze before centrifugation at 105000 g for 1 hr in a Beckman LE-80 ultracentrifuge. The cytosolic fraction (supernatant) was then filtered again and stored at -80°C. Affinity purification was performed on 15-20 ml of cytosol corresponding to 13-15 g of VM or GI. Low molecular weight substances were removed previously from the filtrate by gel filtration using a Sephadex G-25 column (100 × 1.6 cm) equilibrated with 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, pH 7.8 (buffer A). Fractions active towards CDNB were pooled and loaded on a GSH-Sepharose affinity column (10 × 1.6 cm) equilibrated with buffer A plus 140 mM NaCl (buffer B, pH 7.5). Extensive

wash was performed with buffer B until no protein was detected in the effluent (absorbance at 280 nm). Bound material was then eluted with 50 mM Tris HCl, 10 mM GSH, pH 8.6. Active fractions were either pooled and stored at -80°C until HPLC analysis (VM and GI) or passed through the Sephadex G-25 column equilibrated with 10 mM Tris HCl, pH 8.6 (buffer C) to remove GSH and submitted to anion-exchange chromatography -AEC- (VM). AEC was performed on a 5 ml Econo-Pac Q cartridge (Bio-Rad, Marnes-la-Coquette, France) equilibrated with buffer C. After washing with the same buffer, the column was eluted with a linear salt gradient, from 0 to 0.3 M NaCl in buffer C, in 70 min at 1 ml/min. Fractions were stored at -80°C.

Separation and identification of GST subunits

The affinity-purified extracts and AEC fractions were submitted to reversed-phase (RP) HPLC analysis for determination of subunit composition. Analysis was carried out on a Vydac 218TP54 C18 reversed-phase column (250 × 4.6 mm), using a Waters 625 LC system equipped with UV detector 486 (Millipore). Elutants were mixtures of acetonitrile in water (D: 3/7, E: 7/3; v/v) containing 0.1% (v/v) TFA. Separation of GST subunits was performed at a constant flow rate of 1 ml/min using successive linear gradients of E in D: isocratic 20% of E for 5 min, 35% at 10 min, 100% at 45 min and isocratic 100% of E for 15 min. The elution profile was monitored by UV absorption at 214 nm. Fractions containing subunits were collected manually, dried in a Speed Vac evaporator (Savant Instruments, Farmingdale, NY, USA) and stored at -80°C.

Samples of affinity-purified extracts and AEC fractions were desalted and concentrated using a Microcon® centrifugal filter with a 10 kDa cut-off membrane (Millipore) and submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), native-PAGE and isoelectric focusing (IEF) -PAGE using a Bio-Rad Mini-Protean® 3 Cell. For SDS-PAGE, a discontinuous buffer system was used as described by Laemmli (1970). The stacking and the resolving gels contained 4 and 12% (w/v) of acrylamide, respectively, and 2.67% (w/v) of cross-linker. Protein samples were heated previously at 95°C for 4 min in the presence of 2% (w/v) SDS and 5% (v/v) â-mercaptoethanol. Each well was loaded with 0.15 - 0.2 µg of proteins. The gel was stained for proteins with the Bio-Rad Silver Stain Plus kit. Native-PAGE was performed on a 4-15% (w/v) acrylamide gradient gel, using a continuous buffer system (50 mM Tris, 25

mM borate, pH 8.7) as described by McLellan (1982). Gel staining was carried out with either Bio-Rad Coomassie Brilliant Blue R-250 staining kit or glutathione-NBT (Ricci et al., 1984) to detect GST CDNB conjugating activity directly on gel. This method allowed detection of GST activity as low as 0.005 unit (1 unit corresponds to 1 µmole of CDNB conjugated/min). Based on total GST activity, at least 0.03 unit was loaded in each well. IEF-PAGE was performed on a Bio-Rad ready gel, pH of which ranging from 3 to 10 with 7 mM phosphoric acid as anode buffer and 20 mM lysine, 20 mM arginine as cathode buffer. Approximately 0.15-0.3 µg of proteins was loaded. Gel was stained with the Bio-Rad Silver Stain kit. Subunits resolved by RP-HPLC from affinity-purified extracts were submitted to SDS-PAGE to check their homogeneity and determine their apparent molecular weight (MW). Separation was performed on a PhastGel homogeneous media using a PhastSystem apparatus from Pharmacia Biotech, according to the method of Laemmli (1970). The stacking and resolving gels contained 6 and 12.5% (w/v) of acrylamide, respectively, and 3 and 2% (w/v) of cross-linker, respectively. Protein samples were heated previously at 100°C for 5 min in the presence of 2.5% (w/v) SDS and 5% (v/v) â-mercaptoethanol. Approximately 2-3 µg of proteins were loaded. Gel was Coomassie stained.

Analysis of RP-HPLC fractions by electrospray ionization - mass spectrometry (ESI-MS) was performed using a Finnigan LCQ quadrupole ion-trap mass spectrometer (Thermoquest, Orsay, France) as described by Rouimi et al. (1995). Briefly, dried protein samples were solubilized in 50% (v/v) aqueous methanol containing 0.5% (v/v) acetic acid to a final concentration of 20-40 µM before infusion into the electrospray interface. Myoglobin was used for the instrument calibration. MW determination of each subunit was deduced from at least three measurements.

Dot-blot analyses were performed on protein fractions obtained by RP-HPLC from affinity-purified extracts. Antisera against A2 (Alpha-class), M1 (Mu-class), P1 (Pi-class) and T1 (Theta-class) rat GSTs were generous gifts from Dr. D. Sheehan. Trout GST P1 (Pérez-López et al., 2000) and affinity-purified extract from rat liver and intestine (Rouimi et al., 1995) were used as controls except for Theta-class GSTs which are not retained on GSH-affinity matrix (Meyer et al., 1991). About 1-3 µg of proteins previously resuspended in SDS-PAGE sample buffer (Laemmli, 1970) were deposited onto Biodyne B nitrocellulose membranes (Pall, France). After membranes were rinsed with 25 mM Tris HCl (pH 8.3) containing 20% (v/v) methanol and 192 mM glycine, they were placed in 10 mM Tris HCl (pH 7.4) plus 150

mM NaCl (buffer F), 0.05% (v/v) Tween-20 (buffer G), 1% (w/v) BSA and 1% (w/v) non-fat dry milk (buffer H). They were incubated overnight at 4°C with antiserum diluted 1/400 (except antiserum against GST T1: 1/1000) in buffer G, then washed with buffers G and H and incubated for 1.5 hr with a goat anti-rabbit secondary antibody coupled to peroxidase (Sigma-Aldrich) diluted 1/500 in buffer G. The ECL Plus substrate was used to reveal the blots (Amersham Pharmacia Biotech).

Enzyme assay and protein measurement

GST activity was assayed spectrophotometrically following the conjugation of CDNB with GSH at 340 nm as described in Vidal and Narbonne (2000) by a method adapted from Habig et al. (1974). Protein concentration was determined according to the method of Lowry et al. (1951). Amido black staining (Schaffner and Weissmann, 1973) was performed to avoid GSH interference.

Results

Purification

A typical purification procedure of GSTs from *C. fluminea* VM and GI is presented in table 1. GST activity in VM was about three-fold higher than in GI. GST specific activity was practically unchanged in GI after the first gel filtration whereas it was increased for VM, possibly due to removing of low MW inhibitory substances. 44% and 50% of activity were retained on GSH-Sepharose matrix for VM and GI, respectively. Furthermore, only 14% and 13% of activity were present in the corresponding affinity flow-through fraction, showing that a partial enzyme inactivation has occurred during the affinity chromatography step. Proteins retained on the GSH-Sepharose matrix represented 0.59% and 0.42% of total proteins in VM and GI cytosols, respectively. Subsequent AEC of the affinity-retained fraction from VM was performed (fig. 1). All proteins bound to the column at pH 8.6. The first protein peak (peak I) eluted by 0.04-0.06 M NaCl was related to low GST total activity towards CDNB. Two peaks containing high GST activity (peaks II and III) were obtained at a salt concentration of 0.065-0.08 M and 0.085-0.1 M, respectively. The largest (peak III) displayed the highest total GST activity and the smallest (peak II), the highest purification factor (154-fold instead of 92-fold).

Reversed-phase (RP) HPLC analysis

Potential GST subunits from VM and GI extracts were separated by RP-HPLC (Fig. 2). Elution profiles were qualitatively but not quantitatively similar for VM and GI. Three major peaks were resolved for each extract, with retention times of 19.2 (peak a), 19.6 (peak b) and 29.5 (peak c) min for VM and 20.9 (peak d), 21.6 (peak e) and 29.5 (peak f) min for GI. Peaks c and f were the most abundant, based on their absorbance at 214 nm, and were eluted at the same retention time. Very small peaks a and b and d and e were detected in GI and VM, respectively, which could not be further analysed. Similar RP-HPLC analysis was performed on peaks I, II and III resolved by AEC from VM affinity-purified extract. Thus, peaks a and b prevailed in peaks I - II and were minor in peak III whereas peak c was minor in peak I - II and largely predominant in peak III (data not shown).

Molecular masses

Table 2 shows results of SDS-PAGE of the affinity-purified extract and AEC fractions from VM (also depicted in fig. 3) as well as SDS-PAGE and ESI-MS analysis of RP-HPLC fractions from affinity-purified extracts from VM and GI. Apparent MWs obtained were similar to those of GST subunits encountered in other species (Stenersen et al., 1987), except for 68.2, 50.8 and 44 kDa that could correspond to non-denatured GSTs (dimeric form) or other GSH-binding proteins. Peak c resolved by RP-HPLC gave a minor band of 15.2 kDa when a large amount of protein was loaded. Determination of MW of GI subunits d, e and f by ESI-MS was unsuccessful, probably because of the low level and/or solubility of these proteins. Native-PAGE revealed the presence of proteins between 45 and 64 kDa in the VM affinity-purified extract, the majority of which exhibiting GST activity towards CDNB (fig. 4). The analysis of each peak obtained by AEC allowed a more accurate characterisation of these native proteins. Based on both Coomassie blue and glutathione-NBT staining, three proteins of approximately 64, 55 and 45 kDa were detected and referred as GSTc1, GSTc2 and GSTc3, respectively. GSTc1 was present in peaks I and II and displayed no GST activity. Although GSTc2 was not stained with Coomassie blue - probably because of its low amount - its GST activity was detected. This protein was mainly present in peak II. GSTc3 was principally detected in peak III. GSTc1, GSTc2 and GSTc3 could correspond to the three proteins of high MW observed previously by SDS-PAGE of the affinity-purified extract. Based on RP-HPLC results performed on peaks resolved by AEC, GSTc3 could be a

homodimer of subunit c, for it is the major subunit expressed in peak III. As the bulk of subunits a and b was detected in peaks I and II, they could enter in the composition of GSTc1 and GSTc2. Since peak a was higher than peak b we could consider several possible subunit combinations such as a-a and b-b or a-a and a-b.

Isoelectric points (pIs)

IEF-PAGE performed on affinity-purified extract from VM revealed three major bands with acidic pI of 5.1, 4.8 and 4.7 and two minor bands with pI inferior to 4.45 (fig. 5). Three minor bands with basic pI of 7.8, 7.3 and 7.2 were also observed. The three major acidic proteins were recovered in peaks I and II from AEC and those with a pI of 5.1 and 4.7 displayed the strongest signal. In peak III, the band with a pI of 4.7 was the most intense whereas that focusing at pI 5.1 was very weak. In addition, two proteins focused at a pI inferior to 4.45. Thus, considering the elution order of GSTc1, GSTc2 and GSTc3 during AEC as well as the relative intensity of bands on IEF-PAGE for each peak, these proteins could be characterised by a pI of 5.1, 4.7-4.8 and inferior to 4.45, respectively.

Immunological properties

In order to establish affiliation of each GST subunit purified from VM and GI to the major GST classes Alpha, Mu, Pi and Theta, dot-blot analyses were performed on RP-HPLC fractions (fig. 6). A cross-reaction was considered positive when stronger than that obtained with the negative control (background signal). All subunits were strongly immunorecognised by antiserum to the Pi (P1) rat GST subunit but not by antisera to the Alpha (A2) and Theta (T1) rat GST subunits. Subunits a and b from VM slightly cross-reacted with antiserum to Mu (M1) rat GST subunit.

Discussion

The bulk of GST activity towards CDNB was localised in VM of *C. fluminea* and to a lesser extent in GI as previously reported by Vidal and Narbonne (2000). This tissue distribution contrasts with that reported in *M. edulis* by Fitzpatrick and Sheehan (1993). Indeed, GI was the main organ displaying GST activity in that mussel, followed by the digestive gland (hepatopancreas). Such a discrepancy could be due to the nature of VM in *C. fluminea*, for

this tissue consists of both digestive gland and gonads, these organs being tightly connected. GI have to filtrate large amounts of water, hence they can be exposed to important levels of contaminants and thus possess some (de)toxification capacities. Nevertheless, the digestive gland constitutes the major organ of (de)toxification and its functions are analogous to that encountered in the liver of vertebrates. That is why most of GST purification studies have been performed, when possible, from digestive gland or hepatopancreas in invertebrates (e.g. Keeran and Lee, 1987; Tang et al., 1994) or from liver in vertebrates such as fishes (e.g. George and Young, 1988; Martínez-Lara et al., 1997; Angelucci et al., 2000; Pérez-López et al., 2000) or mammalians (e.g. Habig et al., 1974; Rouimi et al., 1996). In mussels (Fitzpatrick and Sheehan, 1993), the affinity step resulted in a far higher yield for digestive gland (up to 101%) whereas that obtained for GI was similar to ours (47%). As a general manner, the use of a GSH-affinity column for the purification of invertebrate GSTs led to relatively low yields: 12, 27, 40 and 46% for *Mercinaria mercinaria* (Blanchette and Singh, 1999), *Gammarus italicus* (Aceto et al., 1991), *Daphnia magna* (LeBlanc and Cochrane, 1987) and *Octopus vulgaris* (Tang et al., 1994), respectively. In our study, such a low yield could be explained by the inactivation of GST activity during chromatography as only 14 and 13% of activity were recovered in the flow-through fraction for VM and GI, respectively. To avoid the loss of enzymatic activity upon oxidation in further experiments, the loading buffer could be supplemented with a thiol agent such as DTT. Unretained fractions were not characterised in the present study. However, it is likely they contained some GSTs retained initially on the affinity matrix as the yield was found to increase slightly when passing again those fractions through the affinity column. We could also envisage the presence of some particular GST(s) not retained on the GSH-affinity matrix, as it is the case for Theta-class rat GSTs (Meyer et al., 1991). In addition, a dimeric GSH-binding protein not catalytically active towards GST substrates but possessing a similar amino acid composition to mammalian Mu class GSTs was identified in *M. edulis* (Fitzpatrick et al., 1995a; Power et al., 1996). Up to fourteen GSTs have been separated from the grey mullet liver by AEC (Martínez-Lara et al., 1997). In our study it allowed the separation of three proteins from the VM affinity-purified extract of *C. fluminea*, two of which displayed activity towards CDNB (GSTc2 and GSTc3). GSTc2 was expected to have a high specific activity, as only detected by the very sensitive method of Ricci et al. (1984). As GSTc1 (64kDa) displayed no activity towards CDNB, other substrates should be assayed in further studies. Considering their apparent MW, these three proteins are probably dimeric. RP-HPLC is a reliable tool to separate GST subunits (Ostlund Farrants et al., 1987) and thus it has been used successfully in several studies on aquatic

species (e.g. Fitzpatrick and Sheehan, 1993; Martínez-Lara et al., 1997; Pérez-López et al., 2000). Particularly, it allows the resolution of very similar subunits being electrophoretically indistinguishable. Quantitative differences in elution profiles were obtained from *C. fluminea* different tissues. One major subunit (c or f) was common to both tissues, whereas among the four others, two were more specific of VM (a and b) and the two others of GI (d and e). Similar tissue-specific patterns were also reported in mussels (Fitzpatrick and Sheehan, 1993) and the rainbow trout (Pérez-López et al., 2000). Thus differences in GST isoenzyme distribution from one tissue to another occurring in rat and human (Ketterer et al., 1988) seem to be conserved in different animal phyla. Some subunits almost indistinguishable by SDS-PAGE were resolved by RP-HPLC. Therefore it is evident that SDS-PAGE analysis can imply misinterpretations although it has been used for decades to separate and estimate apparent MW of GST subunits. Indeed, the electrophoretic mobility of some mammalian subunits was found to depend on the amount of cross-linker in the resolving gel (Hayes and Mantle, 1986). RP-HPLC is now preferred to SDS-PAGE to separate GST subunits and ESI-MS increasingly used for the accurate determination of MW, with a precision as good as ± 1 Da. Indeed, the protein of about 27 kDa detected in peak b by SDS-PAGE which exhibited a MW of 23289 ± 1 Da by ESI-MS could have caused misinterpretations. Based on RP-HPLC results and MWs, GST homo and heterodimers could be present in *C. fluminea*. Despite the identification of three proteins by native-PAGE, more than three bands were obtained by IEF-PAGE. Likewise, previous studies in rat (Habig et al., 1974), blue crab (Keeran and Lee, 1987) and giant African snail (Ajele and Afolayan, 1992) reported that apparently homogenous GSTs could display several bands on electrofocusing. As mentioned by Keeran and Lee (1987), that band multiplicity might be caused by the presence of charge variants and/or intramolecular sulfhydryl oxidation. Dot-blot analyses performed with antisera raised against rat GST subunits from the four major classes Alpha, Mu, Pi and Theta revealed that all subunits were related to the Pi-class. This is in accordance with the acidic pI of the three isoenzymes and the relatively low MW of identified subunits which are features encountered in Pi-class GSTs. Furthermore, we have reported previously that non-purified extracts from different tissues of *C. fluminea* displayed GST activity towards EA (Vidal and Narbonne, 2000), a substrate specific for Pi-class GSTs (Beckett and Hayes, 1993). Four GST isoenzymes were purified from the digestive gland of *M. galloprovincialis* and an additional one from the GI of *M. edulis*, subunit MWs of which ranging from 24.5 up to 27.3 kDa by SDS-PAGE. The first peak eluted by AEC displayed the bulk of GST activity towards CDNB whereas the second one displayed practically no activity, especially in GI, but represented the

largest amount of protein of the affinity extract (Fitzpatrick and Sheehan, 1993). These two proteins, GST 1 and the so-called "GSH-binding protein" were subsequently well characterised in the studies of Fitzpatrick et al. (1995a) and Power et al. (1996). Both were of dimeric nature. GST 1 was related to the Pi-class whereas the "GSH-binding protein" displayed similarities to Mu class GSTs. In *C. fluminea* GSTc1 and GSTc3 could be compared to the "GSH-binding protein" and to GST 1, respectively, despite some dissimilarities. In particular, GSTc1 appears to be related to the Pi-class and to a lesser extent to the Mu-class, if composed of subunits a and/or b. Furthermore, MWs of subunits a, b and especially c (common to both VM and GI) are lower compared to those of the mussel ones. Some similarities also exist when comparing our results with those of an other bivalve mollusc, the Northern quahog *M. mercinaria* (Blanchette and Singh, 1999). Indeed, affinity-purified extracts from whole animals yielded two major and two minor subunit bands from 22 to 27 KDa by SDS-PAGE, and three isoenzymes showing an acidic pI of 5.1, 4.9 and 4.6 by IEF-PAGE. These isoenzymes were supposed to belong to the Pi-class because of their acidic pI. Among crustaceans, *D. magna* was shown to express at least seven GSH-binding proteins, four of which were acidic (pI of 4.6-5.6) and exhibited GST activity towards CDNB (Baldwin and Leblanc, 1996). Two acidic GSTs were also isolated from *G. italicus*, one of which was related to the Pi-class (Aceto et al., 1991). Thus, GSTs related to the Pi-class are encountered in most aquatic invertebrate species but also in vertebrate ones such as fishes (Dominey et al., 1991; Pérez-López et al., 2000).

Previous biomonitoring studies involving GST response as a biomarker focused mainly on GST specific activity towards the non-specific substrate CDNB. Substrates such as EA, DCNB or EPNP were sometimes considered for they are supposed to be specific of GST from Pi-, Mu- and Theta-classes in mammalians, respectively, despite a certain degree of overlapping. However, laboratory and field studies performed on aquatic species, and particularly invertebrate ones, led to conflicting results. In *C. fluminea* GST activity towards CDNB and EA was unaffected by a laboratory exposure to trichloroethylene, toluene (Vidal et al., 2001) or a coal tar fraction consisting of a complex mixture of polycyclic aromatic hydrocarbons (Vidal et al., unpublished results). On the other hand, *C. fluminea* displayed a reduced GST activity towards CDNB when exposed to cadmium (Vidal et al., unpublished results). Laboratory exposure of *M. galloprovincialis* to benzo(*a*)pyrene yielded to a decrease of GST activity whereas horse mussels (*Modiolus modiolus*) displayed no significant change (Michel et al, 1993; Willett et al., 1999). In field studies, mussels living in sites contaminated

by oil or mixtures of polycyclic aromatic hydrocarbons, polychlorinated biphenyls and/or metals exhibited no significant change of GST activity compared to that from non- or less polluted sites (Lee, 1988; Livingstone et al., 1995; Solé et al., 1996), except for the studies of Suteau et al. (1988) and Willett et al (1999) in which an increase was reported. Response of GST activity was sometimes tissue-dependent, that could be explained by the tissue-specific expression of GST isoenzymes. Thus, exposure of *M. galloprovincialis* to copper (laboratory) or a mixture of metals (field) resulted in a decrease of GST activity in GI whereas no change was observed in the digestive gland (Regoli and Principato, 1995). On the contrary, horse mussels living in oily ecosystems showed a higher GST activity in digestive gland and an unaffected one in GI compared to mussels living in a non-oily environment (Willett et al., 1999). In fishes, such contradictory results were commonly reported. Particularly, some differences in GST responses were observed depending on the species. Thus, in the study of Lemaire et al. (1996), exposition to 3-methylcholanthrene led to a decrease of GST activity in the sea bass *Dicentrarchus labrax* whereas no change was observed in the dab *Limanda limanda*. On the other hand, P450-related activities such as ethoxyresorufin *O*-deethylase activity displayed most of the time responses of higher magnitude, often correlated with the amount of contaminant, contrary to GST responses (e.g. Raoux et al., 1989; Garrigues et al., 1990). However, because GST activity is less sensitive than MFO components to seasonal variation, it has been proposed as a better indicator of xenobiotic exposure (Sheehan and Power, 1999). Hence, considering all the contradictory results reported in literature concerning GST specific activity responses after exposure to different classes of contaminants, it was necessary to apprehend a molecular approach in environmental studies. In particular, GST subunit classes can be induced or repressed in a differential manner depending on the contaminant. Therefore, the measurement of a global GST activity may not be representative of the actual molecular events. Differential induction of GST subunits is well documented for vertebrate species but less is known for invertebrates. To our knowledge, such studies in bivalve molluscs have been only performed in marine mussels (Fitzpatrick et al., 1995b; 1997). Unfortunately, no significant differences between the relative levels of the different GST isoenzymes were observed between contaminated and non-contaminated sites. On the contrary, fishes exposed to various xenobiotics gave rise to a characteristic isoenzyme pattern compared to that of the control ones (Martínez-Lara et al., 1996; Pérez-López et al., 2000). That could be explained by the fact that fishes possess several GST subunit classes whereas active GSTs of mussels (and also *C. fluminea*) are only related to the Pi-class, which has been proven not to be inducible by certain chemicals such as phenobarbital, 3-

methylcholanthrene or polychlorinated biphenyls in rats (Vos and Van Bladeren, 1990). In the present study, we have purified and characterised for the first time three major isoenzymes and several subunits of GST in *C. fluminea*. We must now study their expression pattern after exposure to model contaminants in order to validate their use as biomarkers of contamination in freshwater ecosystems.

Acknowledgements

This study was supported by a PhD grant from the TotalFinaElf Company. We are grateful to Dr. D. Sheehan (Department of Biochemistry, University College Cork, Cork, Ireland) for providing us with antisera to rat GSTs; to Drs. J. Tulliez and J.P. Cravedi for their collaboration; to Dr. E. Rathahao for her assistance in ESI-MS; to P. Hoarau and Dr. M. Roméo (Laboratoire de Physiologie et Toxicologie Environnementales, Université de Nice-Sophia Antipolis, Nice, France) for their contribution to the preliminary steps of this study and to Drs. E. Darrouzet and P. Mora for their precious advice.

References

Aceto, A., Di Ilio, C., Bucciarelli, T., Pantani, C., Dell'Agata, M., Pannunzio, G., Federici, G., 1991. Characterization of glutathione transferase from Gammarus italicus. Comp. Biochem. Physiol. 99B(3), 523-527.

Ajele, J.O., Afolayan, A., 1992. Purification and characterisation of glutathione transferase from the giant African snail, Archachatina marginata. Comp. Biochem. Physiol. 103B(1), 47-55.

Angelucci, S., Sacchetta, P., Moio, P., Melino, S., Petruzzelli, R., Gervasi, P., Di Ilio, C., 2000. Purification and characterization of glutathione transferases from the sea bass (Dicentrarchus labrax) liver. Arch. Biochem. Biophys. 373(2), 435-441.

Araujo, R., Moreno, D., Ramos, M.A., 1993. The Asiatic clam Corbicula fluminea (Müller, 1774) (Bivalvia: Corbiculidae) in Europe. American Malacol. Bull. 10, 39-49.

Baldwin, W.S., LeBlanc, G.A., 1996. Expression and induction of an immunochemically related class of glutathione S-transferases in Daphnia magna. Comp. Biochem. Physiol. 113B(2), 261-267.

Basack, S.B., Oneto, M.L., Fuchs, J.S., Wood, E.J., Kesten, E.M., 1998. Esterases of Corbicula fluminea as biomarkers of exposure to organophosphorus pesticides. Bull. Environ. Contam. Toxicol. 61, 569-576.

Beckett, G.J., Hayes, J.D., 1993. Glutathione S-transferases: biomedical applications. Adv. Clin. Chem. 30, 281-380.

Beyer, J., Sandvik, M., Hylland, K., Fjeld, E., Egaas, E., Aas, E., Skåre, J.U., Goksøyr, A., 1996. Contaminant accumulation and biomarker responses in flounder (Platichthys flesus L.) and Atlantic cod (Gadus morhua L.) exposed by caging to polluted sediments in Sørfjorden, Norway. Aquat. Toxicol. 36, 75-98.

Blanchette, B.N., Singh, B.R., 1999. Purification and characterization of the glutathione-S-transferases from the northern quahog Mercinaria mercinaria. Mar. Biotechnol. 1, 74-80.

Board, P.G., Baker, R.T., Chelvanayagam, G., Jermiin, L.S., 1997. Zeta, a novel class of glutathione transferases in a range of species from plants to humans. Biochem. J. 328, 929-935.

Britton, J.C., Morton, B., 1979. Corbicula in north America: the evidence reviewed and evaluated. In: Britton, J.C., Mattice, J.S., Murphy, C.E., Newland, L.W. (Eds.), Proceedings, First International Corbicula Symposium. Texas Christian University Research Foundation Publication, Fort Worth, Texas, pp. 249-287.

Bucheli, T.D., Fent, K., 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Crit. Rev. Environ. Sci. Technol. 25(3), 201-268.

Dominey, R.J., Nimmo, I.A., Cronshaw, A.D., Hayes, J.D., 1991. The major glutathione S-transferase in salmonid fish livers is homologous to the mammalian pi-class GST. Comp. Biochem. Physiol. 100B, 93-98.

Egaas, E., Sandvik, M., Fjeld, E., Källqvist, T., Goksøyr, A., Svensen, A., 1999. Some effects of the fungicide propiconazole on cytochrome P450 and glutathione S-transferase in brown trout (Salmo trutta). Comp. Biochem. Physiol. 122C, 337-344.

Fitzpatrick, P.J., Sheehan, D., 1993. Separation of multiple forms of glutathione S-transferase from the blue mussel, Mytilus edulis. Xenobiotica 23, 851-861.

Fitzpatrick, P.J., Krag, T.O.B., Højrup, P., Sheehan, D., 1995a. Characterization of a glutathione S-transferase and a related glutathione-binding protein from gill of the blue mussel, Mytilus edulis. Biochem. J. 305, 145-150.

Fitzpatrick, P.J., Sheehan, D., Livingstone, D.R., 1995b. Studies on isoenzymes of glutathione S-transferase in the digestive gland of Mytilus galloprovincialis with exposure to pollution. Mar. Environ. Res. 39, 241-244.

Fitzpatrick, P.J., O'Halloran, J., Sheehan, D., Walsh, A.R., 1997. Assessment of glutathione S-transferase and related proteins in the gill and digestive gland of Mytilus edulis (L.), as potential organic pollution biomarkers. Biomarkers 2, 51-56.

Garrigues, P., Raoux, C., Lemaire, P., Mathieu, A., Ribera, D., Narbonne, J.F., Lafaurie, M., 1990. In situ correlations between polycyclic aromatic hydrocarbons (PAH) and PAH metabolizing system activities in mussels and fish in the Mediterranean Sea: preliminary results. Intern. J. Environ. Anal. Chem., 38, 379-387.

George, S.G., Young, P., 1988. Purification and properties of plaice liver cytosolic glutathione-S-transferases. Mar. Environ. Res. 24, 93-96.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249(22), 7130-7139.

Hayes, J.D., Mantle, T.J., 1986. Anomalous electrophoretic behaviour of the glutathione S-transferase Ya and Yk subunits isolated from man and rodents. Biochem. J. 237, 731-740.

Inza, B., Ribeyre, F., Maury-Brachet, R., Boudou, A., 1997. Tissue distribution of inorganic mercury, methylmercury and cadmium in the Asiatic clam (Corbicula fluminea) in relation to the contamination levels of the water column and sediment. Chemosphere 35(12), 2817-2836.

Kamisaka, K., Habig, W.H., Ketley, J.N., Arias, M., Jakoby, W.B., 1975. Multiple forms of human glutathione S-transferase and their affinity for bilirubin. Eur. J. Biochem. 60(1), 153-161.

Keeran, W.S., Lee, R.F., 1987. The purification and characterization of glutathione S-transferase from the hepatopancreas of the blue crab, Callinectes sapidus. Arch. Biochem. Biophys. 255(2), 233-243.

Ketterer, B., Meyer, D.J., Clark, A.G., 1988. Soluble glutathione transferase isozymes. In: Ketterer, B., Sies, H. (Eds.), Glutathione Conjugation, Mechanisms and Biological Significance. Academic Press, London, pp. 73-135.

Kirchin, M.A., Wiseman, A., Livingstone, D.R., 1992. Seasonal and sex variation in the mixed-function oxygenase system of digestive gland microsomes of the common mussel, Mytilus edulis L. Comp. Biochem. Physiol. 101C, 81-91.

Labrot, F., Ribera, D., Saint-Denis, M., Narbonne, J-F., 1996. In vitro and in vivo studies of potential biomarkers of lead and uranium contamination: lipid peroxidation, acetyl-cholinesterase, catalase and glutathione peroxidase activities in three non-mammalian species. Biomarkers 1, 23-30.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

LeBlanc, G.A., Cochrane, B.J., 1987. Identification of multiple glutathione S-transferases from Daphnia magna. Comp. Biochem. Physiol. 88B(1), 39-45.

Lee, R.F., 1988. Glutathione S-transferase in marine invertebrates from Langesundfjord. Mar. Ecol. Prog. Ser. 46, 33-36.

Lemaire, P., Förlin, L., Livingstone, D.R., 1996. Responses of hepatic biotransformation and antioxidant enzymes to CYP1A-inducers (3-methylcholanthrene, â-naphtoflavone) in sea bass (Dicentrarchus labrax), dab (Limanda limanda) and rainbow trout (Oncorhynchus mykiss). Aquat. Toxicol. 36, 141-160.

Lenártová, V., Holovská, K., Pedrajas, J-R., Martínez-Lara, E., Peinado, J., López-Barea, J., Rosival, I., Košúth, P., 1997. Antioxidant and detoxifying fish enzymes as biomarkers of river pollution. Biomarkers 2, 247-252.

Livingstone, D.R., 1993. Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. J. Chem. Technol. Biotechnol. 57, 195-211.

Livingstone, D.R., Lemaire, P., Matthews, A., Peters, L.D., Porte, C., Fitzpatrick, P.J., Förlin, L., Nasci, C., Fossato, V., Wootton, N., Goldfarb, P., 1995. Assessment of the impact of organic pollutants on goby (Zosterisessor ophiocephalus) and mussel (Mytilus galloprovincialis) from the Venice lagoon, Italy: biochemical studies. Mar. Environ. Res. 39, 235-240.

Livingstone, D.R., Chipman, J.K., Lowe, D.M., Minier, C., Mitchelmore, C.L., Moore, M.N., Peters, L.D., Pipe, R.K., 2000. Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates: recent molecular, genotoxic, cellular and immunological studies on the common mussel (Mytilus edulis L.) and other mytilids. Int. J. Environ. Pollut. 13(1-6), 56-91.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M.K., Warholm, M., Jornvall, H., 1985. Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. Proc. Natl. Acad. Sci. U.S.A. 82(21), 7202-7206.

Martínez-Lara, E., Toribio, F., López-Barea, J., Bárcena, J.A., 1996. Glutathione-S-transferase isoenzyme patterns in the gilthead seabream (Sparus aurata) exposed to environmental contaminants. Comp. Biochem. Physiol. 113C(2), 215-220.

Martínez-Lara, E., George, S.G., López-Barea, J., Bárcena, J.A., 1997. Purification and characterization of multiple glutathione transferase isoenzymes from grey mullet liver. Cell. Mol. Life Sci. 53, 759-768.

McLellan, T., 1982. Electrophoresis buffers for polyacrylamide gels at various pH. Anal. Biochem. 126(1), 94-99.

Meyer, D.J., Coles, B., Pemble, S.E., Gilmore, K.S., Fraser, G.M., Ketterer, B., 1991. Theta, a new class of glutathione transferases purified from rat and man. Biochem. J. 274, 409-414.

Meyer, D.J., Thomas, M., 1995. Characterization of rat spleen prostaglandin H D-isomerase as a sigma-class GSH transferase. Biochem. J. 311, 739–742.

Michel, X.R., Suteau, P., Robertson, L.W., Narbonne, J-F., 1993. Effects of benzo(a)pyrene, 3,3',4,4'-tetrachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl on the xenobiotic-metabolizing enzymes in the mussel (Mytilus galloprovincialis). Aquat. Toxicol. 27, 335-344.

Narbonne, J.F., Djomo, J.E., Ribera, D., Ferrier, V., Garrigues, P., 1999. Accumulation kinetics of polycyclic aromatic hydrocarbons adsorbed to sediment by the mollusk Corbicula fluminea. Ecotoxicol. Environ. Saf. 42, 1-8.

Ostlund Farrants, A-K., Meyer, D., Coles, B., Southan, C., Aitken, A., Johnson, P.J., Ketterer, B., 1987. The separation of glutathione transferase subunits by using reverse-phase high-pressure liquid chromatography. Biochem. J. 245, 423-428.

Pemble, S.E., Wardle, A.F., Taylor, J.B., 1996. Glutathione S-transferase class Kappa: characterization by the cloning of rat mitochondrial GST and identification of a human homologue. Biochem. J. 319, 749-754.

Pérez-López, M., Anglade, P., Bec-Ferté, M.P., Debrauwer, L., Perdu, E., Cravedi, J.P., Rouimi, P., 2000. Characterization of hepatic and extrahepatic glutathione S-transferases in rainbow trout (Oncorhynchus mykiss) and their induction by 3,3',4,4'-tetrachlorobiphenyl. Fish Physiol. Biochem. 22, 21-32.

Power, A., Sheehan, D., 1996. Seasonal variation in the antioxidant defence systems of gill and digestive gland of the blue mussel, Mytilus edulis. Comp. Biochem. Physiol. 114C(2), 99-103.

Power, A., McCarthy, R., Raggett, E., Sheehan, D., 1996. Ligand-binding properties of the glutathione-binding protein of the mussel, Mytilus edulis. Comp. Biochem. Physiol. 115B(4), 439-443.

Raoux, C., Lemaire, P., Mathieu, A., Garrigues, P., Lafaurie, M., 1989. Bioprotective system activities in Serranus scriba from the Mediterranean Sea : relation between pollutant content and enzymatic activities. Océanis, 15(4), 623-627.

Regoli, F., Principato, G., 1995. Glutathione, glutathione-dependent and antioxidant enzymes in mussel, Mytilus galloprovincialis, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. Aquat. Toxicol. 31, 143-164.

Ricci, G., Lo Bello, M., Caccuri, A.M., Galiazzo, F., Federici, G., 1984. Detection of glutathione transferase activity on polyacrylamide gels. Anal. Biochem. 143, 226-230.

Rouimi, P., Debrauwer, L., Tulliez, J., 1995. Electrospray ionization-mass spectrometry as a tool for characterization of glutathione S-transferase isozymes. Anal. Biochem. 229, 304-312.

Rouimi, P., Anglade, P., Debrauwer, L., Tulliez; J., 1996. Characterization of pig liver glutathione S-transferases using HPLC-electrospray-ionization mass spectrometry. Biochem. J. 317, 879-884.

Schaffner, W., Weissmann, C., 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. Anal. Biochem. 56, 502-514.

Sheehan, D., Power, A., 1999. Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. Comp. Biochem. Physiol. 123C, 193-199.

Solé, M., Porte, C., Biosca, X., Mitchelmore, C.L., Chipman, J.K., Livingstone, D.R., Albaigés, J., 1996. Effects of the "Aegan Sea" oil spill on biotransformation enzymes,

oxidative stress and DNA-adducts in digestive gland of the mussel (Mytilus edulis L.). Comp. Biochem. Physiol. 113C(2), 257-265.

Stenersen, J., Guthenberg, C., Mannervik, B., 1979. Glutathione S-transferases in earthworms (Lumbricidae). Biochem. J. 181, 47-50.

Stenersen, J., Kobro, S., Bjerke, M., Arend, U., 1987. Glutathione transferases in aquatic and terrestrial animals from nine phyla. Comp. Biochem. Physiol. 86C(1), 73-82.

Suteau, P., Daubeze, M., Migaud, M.L., Narbonne, J.F., 1988. PAH-metabolizing enzymes in whole mussels as biochemical tests for chemical pollution monitoring. Mar. Ecol. Prog. Ser. 46, 45-49.

Tang, S-S., Lin, C-C., Chang, G-G., 1994. Isolation and characterization of octopus hepatopancreatic glutathione S-transferase. Comparison of digestive gland enzyme with lens S-crystallin. J. Protein Chem. 13(7), 609-618.

Viarengo, A., Canesi, L., 1991. Mussels as biological indicators of pollution. Aquaculture 94, 225-243.

Vidal, M-L., Narbonne, J.F., 2000. Characterization of glutathione S-transferase activity in the Asiatic clam Corbicula fluminea. Bull. Environ. Contam. Toxicol. 64, 455-462.

Vidal, M-L., Bassères, A., Narbonne, J-F., 2001. Potential biomarkers of trichloroethylene and toluene exposure in Corbicula fluminea. Environ. Toxicol. Pharmacol. 9, 87-97.

Vos, R.M.E., Van Bladeren, P.J., 1990. Glutathione S-transferases in relation to their role in the biotransformation of xenobiotics. Chem.-Biol. Interact. 75, 241-265.

Willett, K.L., Wilson, C., Thomsen, J., Porter, W., 1999. Evidence for and against the presence of polynuclear aromatic hydrocarbon and 2,3,7,8-tetrachloro-p-dioxin binding proteins in the marine mussels, Bathymodiolus and Modiolus modiolus. Aquat. Toxicol. 48, 51-64.

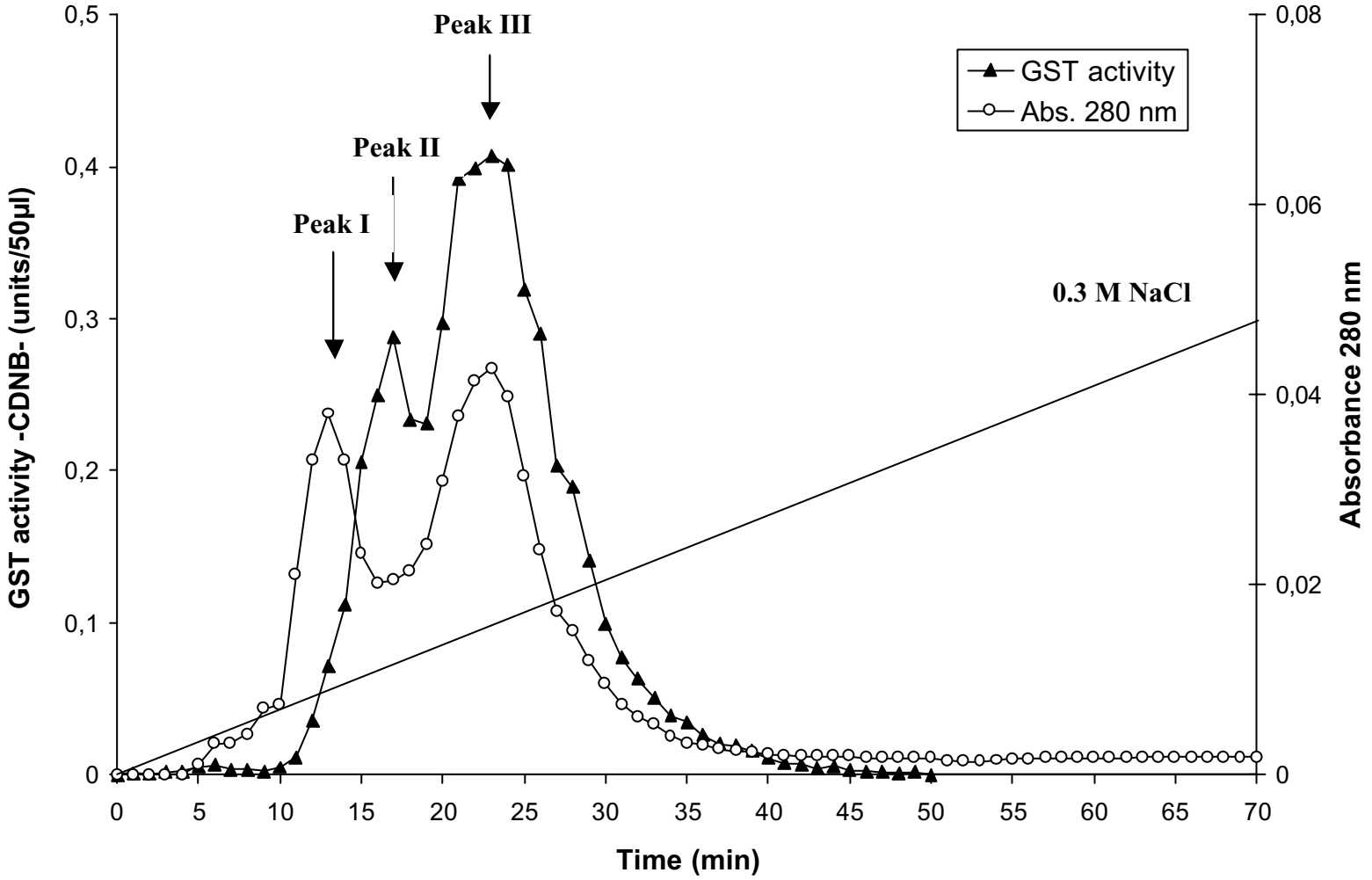


Figure 1. Anion-exchange chromatography of affinity-purified extract from visceral mass. 32.1 ml of sample were loaded on a Bio-Rad Econo-Pac Q cartridge at a flow rate of 1 ml/min. Elution was performed with a linear gradient of NaCl (0-0.3 M) in 70 min. Fractions corresponding to GST (CDNB) activity and/or protein peaks were pooled and numbered as indicated on the graph.

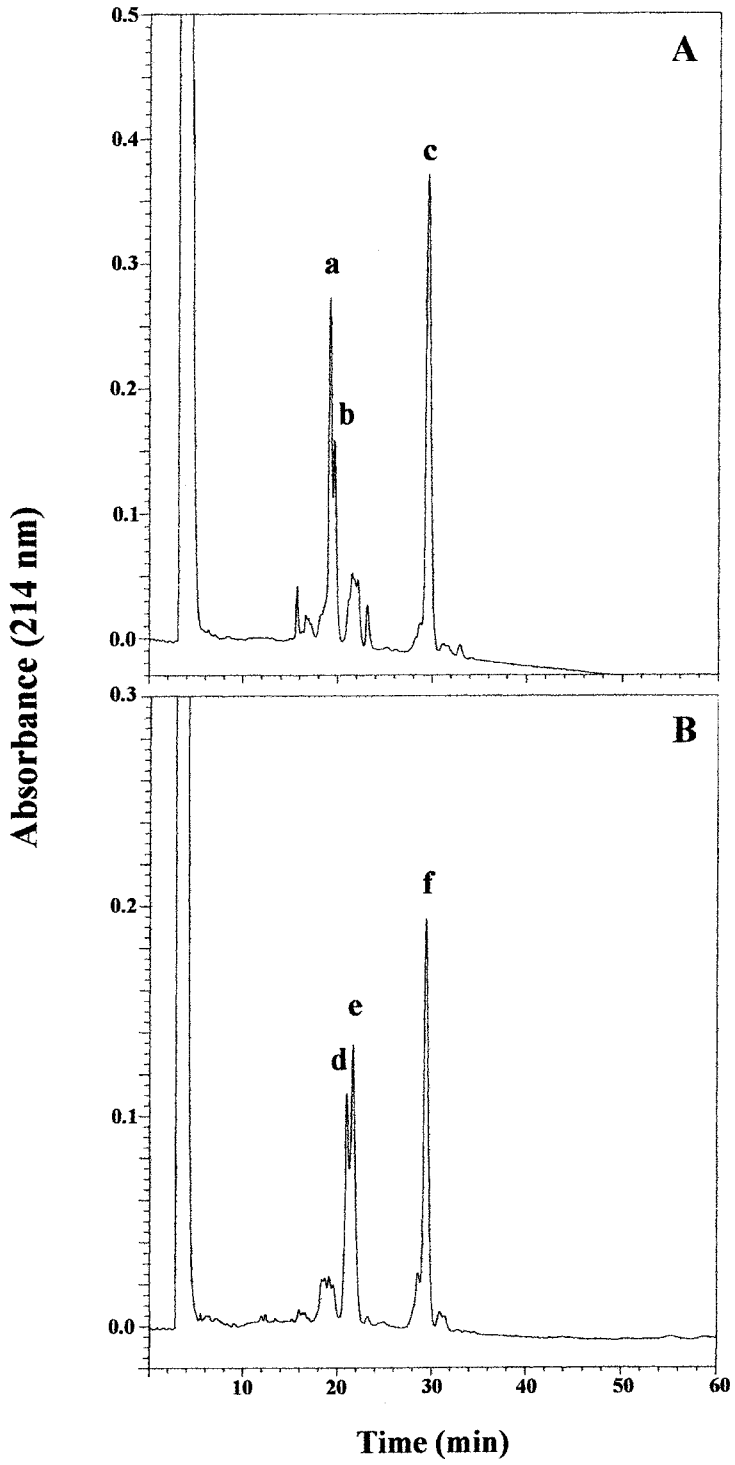


Figure 2. Reversed-phase HPLC profiles of affinity-purified extracts from visceral mass (A) and gills (B). 600 µl of extract were loaded on a Vydac 218TP54 C18 column (250 × 4.6 mm) at a flow rate of 1 ml/min. Elution conditions are described in the materials and methods section. Peaks further analysed are indicated on the graphs.

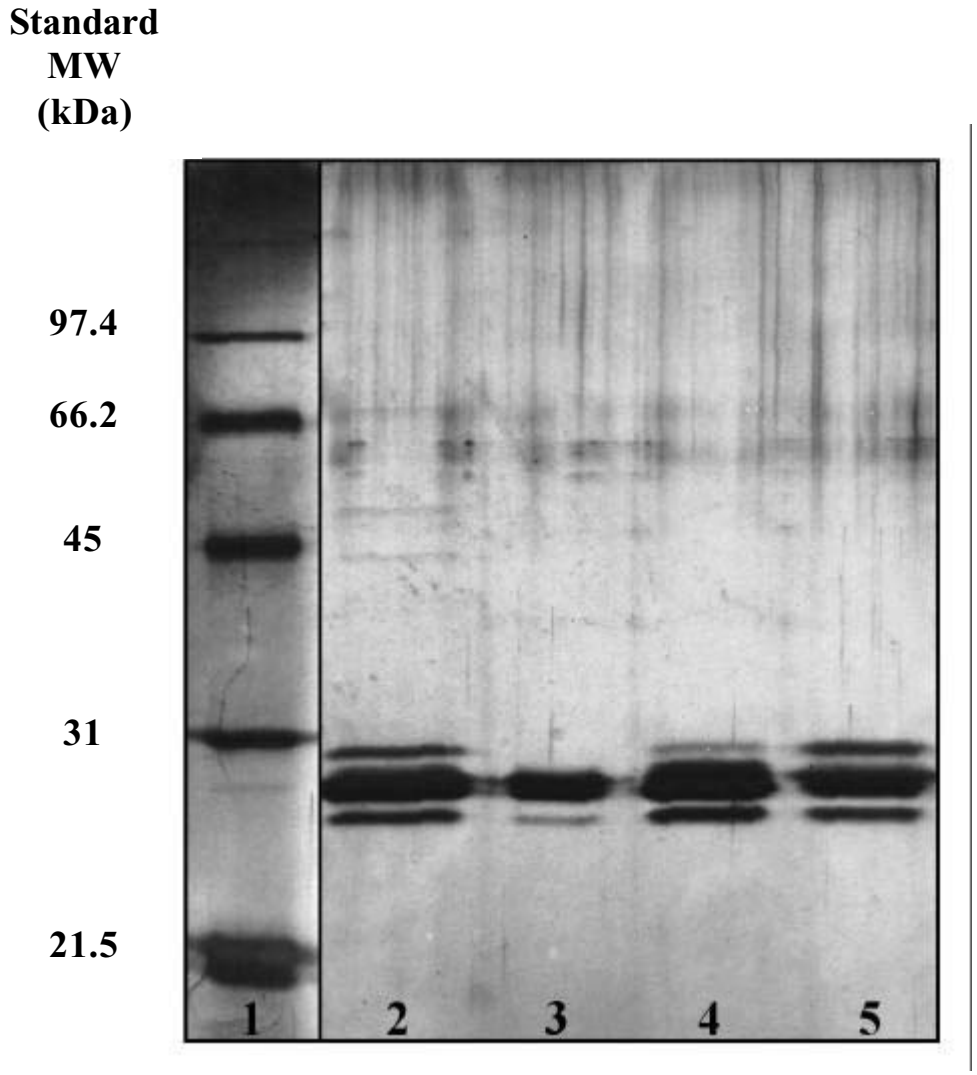


Figure 3. SDS-PAGE of the affinity-purified extract from visceral mass and peaks obtained subsequently by anion-exchange chromatography. Lane 1: Bio-Rad Silver Stain SDS-PAGE standards (low range), from top to bottom rabbit muscle phosphorylase b (97.4 kDa), BSA (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa); lane 2: affinity-purified extract; lane 3: peak I; lane 4: peak II; lane 5: peak III.

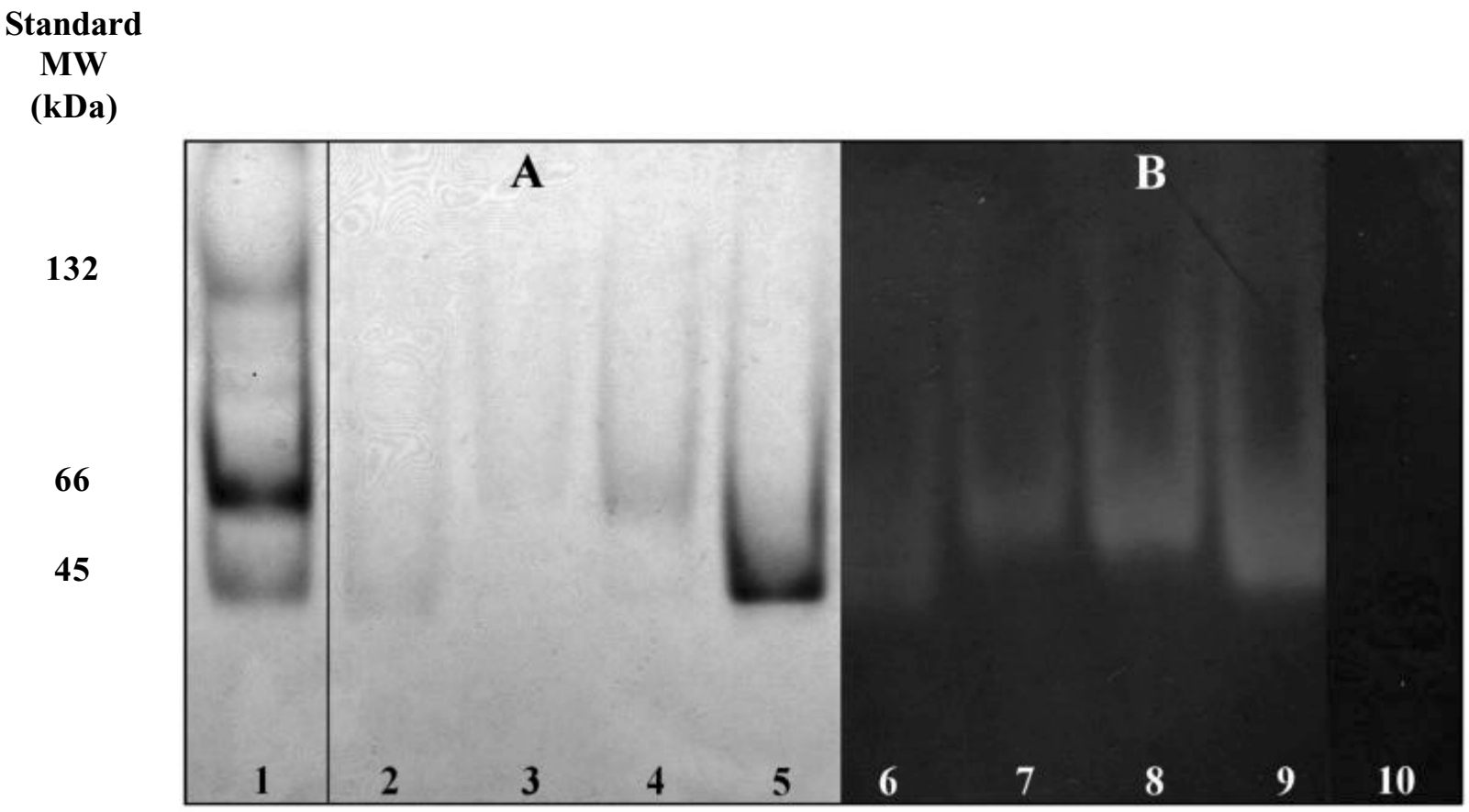


Figure 4. Native-PAGE of the affinity-purified extract from visceral mass and peaks obtained subsequently by anion-exchange chromatography. A: gel stained with Coomassie blue; B: gel stained for GST activity towards CDNB. Lane 1: Sigma-Aldrich standards, from top to bottom BSA dimer (132 kDa), BSA monomer (66 kDa) and chicken egg albumin (45 kDa); lanes 2 and 6: affinity-purified extract; lanes 3 and 7: peak I; lanes 4 and 8: peak II; lanes 5 and 9: peak III; lane 10: blank without CDNB.

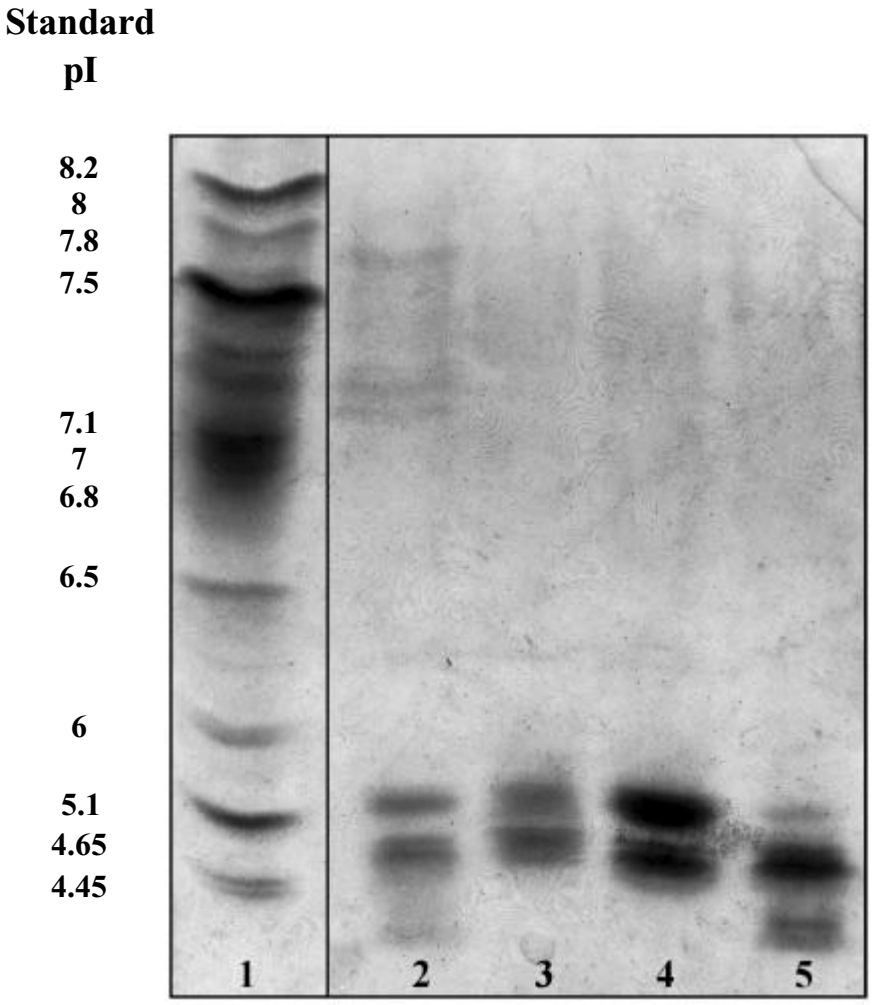


Figure 5. IEF-PAGE of the affinity-purified extract from visceral mass and peaks obtained subsequently by anion-exchange chromatography. Lane 1: Bio-Rad IEF standards, from top to bottom lentil lectin (3 bands of pI 7.8, 8.0 and 8.2), human hemoglobin C (pI 7.5), human hemoglobin A (pI 7.1), equine myoglobin (2 bands of pI 6.8 and 7.0), human carbonic anhydrase (pI 6.5), bovine carbonic anhydrase (pI 6.0), β-lactoglobulin B (pI 5.1) and phycocyanin (2 bands of pI 4.45 and 4.65); lane 2: affinity-purified extract; lane 3: peak I; lane 4: peak II; lane 5: peak III.

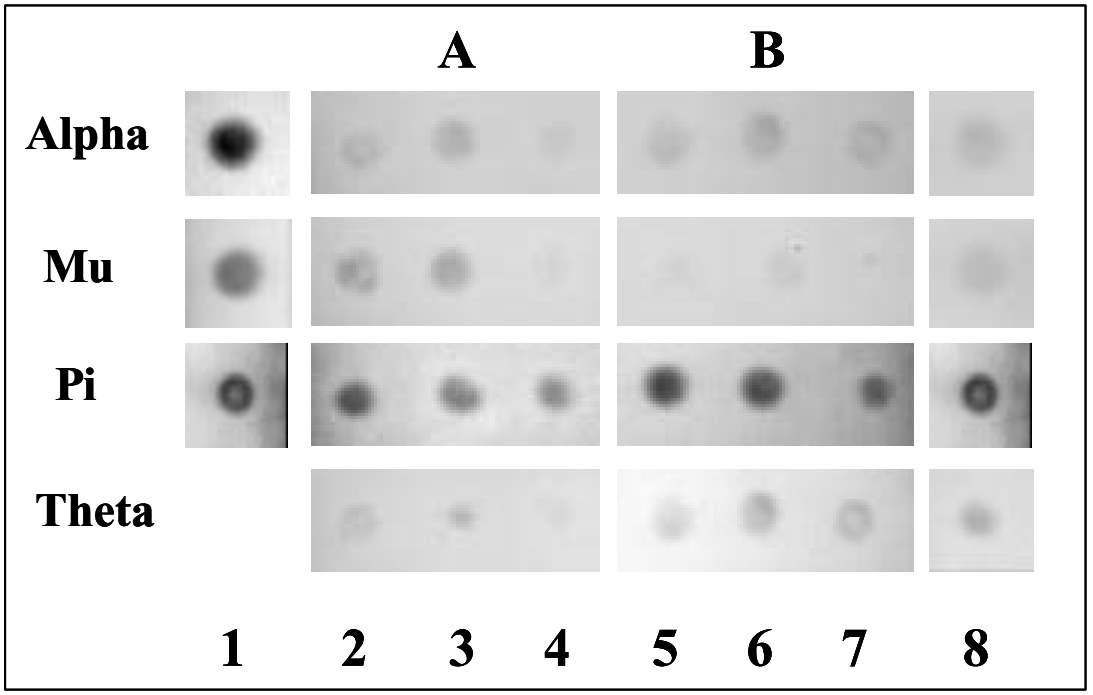


Figure 6. Dot-blot analyses of GST subunits resolved by RP-HPLC, from affinity-purified extracts from visceral mass (A) and gills (B) using antisera to rat Alpha-class GSTA2, Mu-class GSTM1, Pi-class GSTP1 and Theta-class GSTT1. Lane 1: rat proteins eluted from GSH-Sepharose as positive control (except for Theta-class); lane 2: subunit a; lane 3: subunit b; lane 4: subunit c; lane 5: subunit d; lane 6: subunit e; lane 7: subunit f; lane 8: control (trout GSTP1).

Table 1. Purification of GSTs from visceral mass and gills of *Corbicula fluminea.*

Organ	Purification step	Volume (ml)	Proteins		GST (CDNB) activity			
			(mg)	%	Total units[a]	Yield (%)	Specific (units/mg)	Purification (fold)
Visceral mass	Cytosol	16.0	150.18	100.00	248.15	100.00	1.65	1.00
	Sephadex G-25 n°1	38.5	123.28	82.09	251.82	101.48	2.04	1.24
	Not-retained affinity	51.0	80.84	53.83	35.33	14.24	0.44	nd
	Retained affinity	21.0	0.88	0.59	109.88	44.28	124.86	75.67
	Sephadex G-25 n°2	32.1	0.51	0.34	105.13	42.37	206.14	124.93
	Anion-exchange							
	Peak I	2.9	nd	nd	3.05	1.23	nd	nd
	Peak II	2.4	0.05	0.03	12.67	5.11	253.40	153.58
	Peak III	3.1	0.15	0.10	22.81	9.19	152.07	92.16
Gills	Cytosol	16.5	57.57	100.00	31.44	100.00	0.55	1.00
	Sephadex G-25	31.5	46.27	80.37	24.77	78.78	0.54	0.98
	Not-retained affinity	33.0	37.59	65.29	4.08	12.98	0.11	nd
	Retained affinity	15.2	0.24	0.42	15.85	50.41	66.04	120.07

[a] A unit of GST activity corresponds to 1 µmole of CDNB conjugated/min

nd: not determined

Table 2. Determination of *Corbicula fluminea* GST subunit molecular masses (Da) by SDS-PAGE and ESI-MS.

Organ	Fraction	SDS-PAGE	ESI-MS
Visceral mass	Affinity-purified extract	68,200 [a]	nd
		50,800 [a]	nd
		44,000 [a]	nd
		30,200 [b]	nd
		29,200 [c]	nd
		28,500 [c]	nd
		27,200 [b]	nd
	Anion-exchange chromatography		
	Peak I	29,200 [c]	nd
		28,500 [c]	nd
		27,200 [a]	nd
	Peak II	30,200 [a]	nd
		29,200 [c]	nd
		28,500 [c]	nd
		27,200 [b]	nd
	Peak III	30,200 [b]	nd
		29,200 [c]	nd
		28,500 [c]	nd
		27,200 [b]	nd
	RP-HPLC		
	a	29,500 [c]	23,289 ± 1
	b	29,500 [c]	23,289 ± 1
		27,500 [b]	
	c	29,500 [c]	23,602 ± 1
		15,200 [a]	
Gills	RP-HPLC		
	d	29,500 [c]	nd
	e	29,000 [c]	nd
	f	29,500 [c]	nd

[a, b, c] Scarcely detectable band, minor band and major band, respectively.

nd: not determined.

CHAPITRE II

Variabilité saisonnière des paramètres biochimiques

&

Etude en laboratoire des effets de facteurs abiotiques

Afin de mettre en évidence les paramètres biochimiques présentant la plus forte variabilité naturelle et donc susceptibles d'apporter des réponses érronées après exposition à un ou des contaminant(s), nous avons étudié les variations saisonnières des activités catalase, NADPH-cytochrome *c* réductase, NADH-cytochrome *c* réductase, glutathion *S*-transférase (substrats 1-chloro-2,4-dinitrobenzène et acide éthacrynique) et propionylcholinestérase. Cette étude de deux ans a été conduite sur deux populations de *Corbicula fluminea* peuplant des écosystèmes différents peu ou pas pollués: la rivière Dronne (département de la Gironde) et le lac de Sanguinet (département des Landes) et ce, afin de souligner les éventuelles disparités de réponses inhérentes à la population et à l'habitat. Parallèlement à l'étude des biomarqueurs potentiels nous avons mesuré des indicateurs de condition et de reproduction reflétant l'état général des animaux (teneurs en protéines, glucides, lipides et cendres, poids frais et sec, index de condition, quantité d'ovocytes présents dans les gonades, animaux incubant des larves dans les branchies) ainsi que des paramètres environnementaux (température de l'eau, pH, oxygène dissous, concentration en chlorophylle *a*,...). Cette étude est présentée dans l'**article n°3**. Les effets du site, de la date de prélèvement et de la saison (printemps, été, automne et hiver) mais également les relations entre les facteurs biotiques et abiotiques ont été étudiés. Finalement, un index de variabilité a été calculé pour chacun des biomarqueurs. Les activités catalase, propionylcholinestérase, NADH-cytochrome *c* réductase et la teneur en lipides ont présenté les plus fortes variations saisonnières alors que les activités glutathion *S*-transférase, NADPH-cytochrome *c* réductase et la teneur en protéines ont présenté les variations les plus faibles. Pour certains paramètres, les résultats se sont avérés différents en fonction du site. La plupart des variations saisonnières ont été associées aux différentes phases du cycle de reproduction et/ou à la température de l'eau. Ces résultats nous ont conduit à mettre en oeuvre une étude expérimentale en laboratoire (présentée dans l'**article n°4**) destinée à analyser plus finement les effets de plusieurs facteurs abiotiques, tels que la température et la qualité de l'eau, sur les réponses des paramètres biochimiques.

ARTICLE 3

Seasonal variations of pollution biomarkers in two populations of *Corbicula fluminea* (Müller)

Vidal, M-L., Bassères, A., Narbonne J-F.

Accepté dans *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology*

Abstract

Seasonal variations of pollution biomarkers have been studied in two populations of *Corbicula fluminea* (Müller) during two years. Clams were collected from the Sanguinet lake and the Dronne river (Southwest France), sites characterised by a water of excellent and passable quality, respectively. Biological parameters studied were enzymatic activities (catalase, propionylcholinesterase, glutathione *S*-transferase, NADPH-cytochrome *c* reductase and NADH-cytochrome *c* reductase activities) as well as indicators of physiological (protein, carbohydrate, lipid and ash contents, fresh and dry tissue weights, condition factor) and reproductive (amount of: oocytes present in gonads, clams incubating larvae in gills) status. Environmental parameters (e.g. water temperature, chlorophyll *a* concentration) were also monitored. Effects of site, sampling date and season (from spring to winter) but also relationships between abiotic and biotic factors were investigated. Finally, an index of variability was calculated for each biomarker. Catalase, propionylcholinesterase, NADH-cytochrome *c* reductase and lipids experienced the most pronounced seasonal fluctuations whereas glutathione *S*-transferase, NADPH-cytochrome *c* reductase and proteins suffered the less pronounced ones. For some parameters, results differed depending on the site. Most of seasonal changes were related to the reproductive cycle and/or water temperature. Implications of such variations in the use of pollution biomarkers in environmental surveys were discussed.

Keywords: Biomarkers; *Corbicula fluminea*; fresh water; lake; physiological status; reproductive cycle; river; seasonality

Introduction

Mussels are filter-feeding invertebrates able to bioconcentrate many chemical pollutants. Beside estimation of xenobiotic concentrations within their tissues, responses of biochemical parameters - biomarkers - have been measured to assess early effects of pollution on seawater ecosystems in biomonitoring programs (Viarengo and Canesi, 1991). Henceforth, there is a need for applying such programs to freshwater ecosystems. In this sense, several freshwater bivalves such as *Unio tumidus* (Cossu et al., 1997) or *Dreissena polymorpha* (De Lafontaine et al., 2000) have proven to be potential bioindicators. Among them, *Corbicula fluminea* (Müller) is a freshwater clam originating from Asia, which has progressively colonised the United States and more recently Europe (Britton and Morton, 1979; Araujo et al., 1993). Its presence was reported for the first time in France in the eighties (Mouthon, 1981) and it is now a major component of many benthic communities. This species is known to accumulate various pollutants (e.g. Basack et al., 1997; Inza et al., 1997; Narbonne et al., 1999a). Furthermore, responses of several biochemical parameters usually measured in mussels as pollution biomarkers have been studied in that clam after experimental exposure to heavy metals (Labrot et al., 1996), organophosphorus pesticides (Basack et al., 1998) or solvents (Vidal et al., 2001). Thus *C. fluminea* can be considered as an interesting biological monitor (for a review: Doherty, 1990). Although laboratory studies make up prerequisite steps in the validation of biochemical parameters as biomarkers, they do not reflect realistic environmental conditions for they do not take into account all biotic (e.g. age, reproductive cycle) and abiotic factors (e.g. water temperature, pH) which can influence fundamentally parameter responses. Particular attention has been paid to seasonal fluctuations of the most commonly measured biochemical parameters such as components of metabolism of phases I and II or antioxidant defences, specially in marine mussels (for a review: Sheehan and Power, 1999). Aim of our study was to assess the importance of seasonal variations of such indicators in two populations of *C. fluminea* during two years to provide background information for further environmental surveys in freshwater ecosystems using this species as a sentinel organism. We selected two populations of clams inhabiting two completely different ecosystems - a lake and a river, with an excellent and passable water quality, respectively - to check if the basal level as well as the seasonal changes of the whole parameters studied were depending on the population and/or the site of animal collection. Indeed, such a dependence on the population, the site and/or the season could be detrimental in most environmental studies for it could occult variations induced by contaminants thus leading to

misinterpretations. This is particularly true in field studies performed on populations inhabiting different sites or in caging experiments. We studied two categories of biological parameters: (1) enzymatic activities measured usually in many species to assess the quality of freshwater or marine ecosystems: catalase (CAT), an antioxidant enzyme; propionylcholinesterase (PChE), involved in nervous functions; NADPH-cytochrome *c* reductase (NADPH-red) and NADH-cytochrome *c* reductase (NADH-red), involved in the detoxification metabolism of phase I; glutathione *S*-transferase, a conjugation enzyme of phase II metabolism, using either 1-chloro-2,4-dinitrobenzene (GST/CDNB) or ethacrynic acid (GST/EA) as a substrate; (2) indicators related to the physiological and reproductive status of clams: fresh and dry tissue weights (FTW and DTW, respectively), condition factor (CF), protein, carbohydrate, lipid and ash contents as well as the percentage of clams incubating larvae in gills and the amount of oocytes present in gonadal tissue. Indicators of general condition are interesting because they can contribute to the interpretation of variations of "more specific" biochemical parameters such as CAT, PChE, NAD(P)H-red and GST(CDNB/EA). Furthermore they can be considered as indices of general stress likely to undergo fluctuations induced by contaminants (Cantelmo-Cristini et al., 1985; Sujatha et al., 1995; Black and Belin, 1998). In parallel, indicators of water quality were monitored (e.g. temperature, pH, conductivity) as well as biomass production in terms of chlorophyll *a*.

Materials and methods

Sampling sites

The first sampling site is located on the eastern shore of the Sanguinet freshwater lake in the department of Landes (Aquitaine, France). This is an oligotrophic lake with low phytoplanktonic biomass and sediments poor in nutritive elements (Beuffe, 1995). Its water is considered of an excellent quality (personal communication from the Direction Départementale des Affaires Sanitaires et Sociales - DDASS, France). *C. fluminea* colonised banks of that lake 15-20 years ago and are now extremely abundant. Combined with the good quality of water, this abundance makes the Sanguinet lake a reference site allowing a good supplying in clams throughout the year. The second sampling site is located in the Dronne river, just downstream of the village Coutras in the department of Gironde (Aquitaine, France), about 150 km from the Sanguinet lake. Water is considered of passable quality according to the quality criteria of the French Water Board (Agence de l'Eau Adour-Garonne,

1996). *C. fluminea* have been detected in this river at least since 1985, in relative abundance depending on the year (Fontan and Meny, 1996).

Animal sampling

Clams were collected every two or three months in the Sanguinet lake and the Dronne river for two years, from July 1998 to July 2000. As the Dronne river was in spate and thus inaccessible from January 2000 to May 2000, data corresponding to these sampling dates are unavailable. As a general manner, sampling was carried out on the same day for both sites. *C. fluminea* from Sanguinet were collected in the sand of an area located 10-20 m from the beach, at a depth of about 1 m. Clams from the Dronne were sampled in a sediment made up of coarse sand and gravel, in an area situated 1-2 m from the banks, at a depth of about 1 m. *C. fluminea* from these two sites displayed morphological differences. Shells of *C. Fluminea* from the lake had a maximum anterior-posterior length of around 30 mm with a black colour whereas shells of the river clams had a maximum anterior-posterior length of about 20-25 mm with a yellow-green colour tending to darken for the largest and oldest specimens. To assess eventual genotypic differences between these two populations and among each population, electrophoretic investigations were performed as described in McLeod (1986). They revealed genetic homogeneity within each population but dissimilarities between the two populations concerning the esterase enzymatic system among the several enzymatic systems studied (results not shown). To obtain animal samples exhibiting size and age homogeneity, we collected adult sexually mature animals with an anterior-posterior shell length of 20-25 mm and 15-20 mm for the lake and the river clams, respectively. For each site, 90 clams were collected. 75 clams were dissected-out immediately and the soft parts (entire bodies or visceral masses, depending on the analysis) were stored in liquid nitrogen until further biochemical analyses (enzymatic assays; protein, carbohydrate and lipid contents). The crystalline style was removed in clams undergoing further enzymatic measurements. The 15 remaining clams were transported in lake or river water in plastic tanks. In the laboratory, aeration was continuously provided by a diffuser connected to an air pump (Shego M2K3) and temperature was maintained at 20 ± 0.1°C. Clams were not fed to clear out gut content and were held under a natural light cycle. Two days after their arrival, clams were sacrificed for measurement of FTW, DTW, CF and ash content as well as for the evaluation of their reproductive status.

Environmental parameters

For each sampling date and each site, physico-chemical parameters as well as the biomass production (in terms of chlorophyll *a*) of water were checked. Temperature, pH, conductivity and oxygen saturation were measured *in situ* using a Universal Pocket Meter Multiline P4, WTW. Water hardness, concentrations of potassium, chlorides, nitrates, nitrites, phosphates and ammonium were estimated *in situ* by the mean of semi-quantitative tests (Quantofix, Prolabo, France). To quantify chlorophyll *a*, a 1-l sample of lake or river water was collected and stored in an amber glass flask filled to avoid air bubbles at 4°C during the transport back to the laboratory. Then, the assay procedure followed that described in the standard method AFNOR T90-117 (Association Française de Normalisation -AFNOR-, 1984).

Reproductive status

To assess seasonal changes in reproductive status of *C. fluminea*, 10 clams were dissected-out in the laboratory. Maturation level of the gonads (inner part of the visceral mass) and presence or absence of larvae in the gills (ctenidia) were checked as follows. A gonadal smear and a ctenidial one were made by excising a small portion of gonadal tissue and inner demibranchs, respectively, and examining them under microscope as described in Britton and Morton (1982). Development of gonads was evaluated by an estimation of the percentage of developing oocytes in the gonadal tissue. In parallel, the percentage of clams incubating larvae in gills was determined.

Determination of fresh tissue weight (FTW), dry tissue weight (DTW), ash content and condition factor (CF)

Among the clams transported from each site back to the laboratory, five clams were dissected-out. Soft entire bodies were weighed individually for determination of FTW and afterwards dried to constant weight at 100°C for 72 hr to determine their DTW. Ash content was assessed by placing the weighed dried material for each clam in a furnace at 560°C for 12 hr and expressed as a percentage of DTW. For each clam, the anterior-posterior shell length was measured with a Vernier calliper in order to specify a CF as follows:

$$CF = (\text{ash-free DTW -}\textit{in mg}\text{-})/(\text{ anterior-posterior shell length -}\textit{in mm}\text{-}) \times 10$$

Determination of protein, carbohydrate and lipid contents

Five pools of five clams (soft entire bodies) were dried to constant weight at 100°C for 72 hr, subsequently ground to a fine homogeneous powder with a mortar and a pestle and stored in closed vessels. Fresh and dry tissue weights of each pool were measured. This dry material was used for the determination of protein, carbohydrate and lipid contents. 100 mg of each dry powdered pool were placed for 3 hr in 5 ml of 1N NaOH at 60°C for protein and carbohydrate extraction. Protein content was estimated in NaOH extract according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. Carbohydrate content was measured in NaOH extract as described in Dubois et al. (1956) in presence of 5% phenol and concentrated H_2SO_4 and deduced from a glucose calibration curve. Lipid content was determined according to the method of Bligh and Dyer (1959) by extracting lipids from 100 mg of each dry powdered pool in a water-chloroform-methanol mixture and by weighing after evaporation to dryness of the chloroform layer. Protein, carbohydrate and lipid contents were expressed as a percentage of dry tissue weight. To verify the eventual loss of proteins and carbohydrates during heating at 100°C resulting in possible misleading results, determination of protein and carbohydrate contents was performed in fresh homogenised tissue. No significant differences were found comparing to contents measured in dry homogenised tissue (data not shown).

Enzymatic assays

Five pools of five *C. fluminea* were homogenised at 4°C according to the following procedure. Body samples were rinsed in 100 mM phosphate buffer (K_2HPO_4/KH_2PO_4), pH 7.4, dried on absorbent paper towels, weighed and homogenised in the same phosphate buffer (1:4 weight : volume ratio) using an Ultra-Turrax Antrieb T25. Homogenates were then centrifuged at 9000 g for 30 min in a Sigma 3MK centrifuge at 4°C. The resulting supernatants (S9) were filtered through two layers of gauze to remove suspended lipids. S9 of soft entire bodies were directly stored at -80°C whereas those of visceral masses were centrifuged for additional 60 min at 105000 g in a Beckman LE-80 ultracentrifuge at 4°C. Cytosolic fractions were then stored at -80°C and microsomal pellets were resuspended in a 100 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 20% glycerol before storage at -80°C. Measurements of enzymatic activities were performed in duplicates or in triplicates for each pool on a dual-beam temperature-controlled Kontron Uvikon 932 spectrophotometer, at

30°C except for CAT and PChE activities (25°C). CAT and PChE activities were measured in S9 of soft entire bodies whereas GST and NAD(P)H-red activities were measured in cytosol and microsomes of visceral masses, respectively. CAT activity was determined as described in Clairborne (1985). Cholinesterase activity was assayed using propionylthiocholine as a substrate as in Mora et al. (1999a) according to the method of Ellman et al. (1961). GST activities were assayed as in Vidal and Narbonne (2000) by a method adapted from Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (GST/CDNB) or ethacrynic acid (GST/EA) as a substrate. NADPH-red activity was measured as described in Guengerich (1994). NADH-red activity was determined likewise, replacing NADPH by NADH. All activities were expressed in relation to protein concentration measured according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Statistical analysis

All statistical calculations were performed with Statistica software (5.1 release, Statsoft). Data were checked for normal distribution and homogeneity of variance (Brown-Forsythe test). A univariate analysis of variance (one-way ANOVA) was performed with the site, the sampling date (for each site) or the season - spring, summer, autumn, winter - (for each site) as an independent variable and biological parameters as dependent variables. Whenever a significant ($p \leq 0.05$) effect was established on a parameter response, a Tukey HSD test for multiple comparison between paired means was applied to detect significant ($p \leq 0.05$) differences between sites, sampling dates (for each site) or seasons (for each site). Non-parametric tests (Kruskall-Wallis ANOVA and Mann-Whitney test) were performed when data were not distributed normally or when they exhibited heterogeneous variances. Correlations among biological parameter responses and with environmental parameters were determined for each site with the Pearson correlation coefficient (significant for $p \leq 0.05$). As an additional study, discriminant analysis (DA), often referred to as canonical variate analysis, was performed, displaying the advantage of an integrated approach (Adams et al., 1994). Aim of such a multivariate analysis was to try to discriminate seasons from each other for each site and to show up biological parameters allowing the best discrimination between seasons. Finally, an index of variability (VI) was calculated for each biological parameter for both clam populations to assess the extent of seasonal variability. This index is the ratio between the maximal value and the minimum value observed during the two-year study.

Results

Environmental parameters

Seasonal variations of water temperature, pH, conductivity, oxygen saturation and chlorophyll *a* concentration are presented in Table 1. Throughout the two-year study, the lake water was of good quality with constant hardness and concentrations of chlorides, potassium, nitrates, nitrites, phosphates and ammonium inferior to 90 mg $CaCO_3$/l, 500, 200, 10, 1, 3 and 10 mg/l, respectively. However, as these results arose from semi-quantitative assays and because concentration ranges were important for some components (e.g. chlorides), only high variations could have been evidenced. Chlorophyll *a* concentrations were around 3-4 µg/l in summer 1998 and dramatically low or equal to zero for the other sampling dates. Highest and lowest water temperatures were registered in summer (maximum of 27.4°C in July 2000) and in autumn-winter (minimum of 7.7°C in January 1999), respectively. Water pH was of 6-7 and nearly constant throughout the study. Conductivity was low, with highest values in winter (207 µS/cm in January 2000). Oxygen saturation was high except in January 2000 (57.7%). The Dronne river displayed some differences compared to the lake, except for concentrations of chlorides, potassium, nitrites and ammonium. Water hardness was slightly higher (90-180 mg $CaCO_3$/l) and nitrates were generally more abundant (10-25 mg/l), except in July-September 1998 and January 1999 (0-10 mg/l). A peak of 25-50 mg/l was observed in November 1998. Concentration of phosphates exhibited one peak of 3-10 mg/l in September 1998 and July 2000 otherwise it was inferior to 3 mg/l. Chlorophyll *a* concentration was generally lower than that of the lake with the greatest value obtained in July 1998. For both sites, levels reported in 2000 were lower than those obtained in 1998 and 1999. Water temperature was slightly lower than that of the lake with maxima in summer (23-24°C) and minima in autumn-winter (near 4°C). Water pH and conductivity were higher in the river: pH was slightly alkaline and almost constant whereas conductivity was generally above 400 µS/cm with highest levels in summer and autumn. The river was well oxygenated (90-100% of oxygen saturation).

Reproductive status

Seasonal variations of percentage of oocytes in gonads and of clams incubating larvae in gills are presented in Fig. 1. Though samplings were not carried out monthly, some important

trends in the reproductive cycle of both clam populations were showed up. Oocytes were always present in gonads throughout the study but at different levels depending on the site. A marked trend was observed for the lake clams with an increasing number of oocytes from November 1998 to May 1999 followed by a decrease until October 1999. The same trend was observed the year after with a maximum of oocytes in May 2000. In parallel, larvae were detected in spring and summer. Oocytes were present in greater amount in the river clams compared to the lake ones (90-92% oocytes from July to November 1998 versus less than 50%). Furthermore, two peaks of oogenesis were observed: in April and September 1999. The absence of some data the year after did not enable us to confirm this trend. However, a high amount of oocytes was observed in July 2000, equivalent to that observed in July 1998. Larvae were present in gills from May to September, to a greater extent compared to the Sanguinet clams (except in September 1999).

Fresh tissue weight (FTW), dry tissue weight (DTW) and condition factor (CF)

Seasonal variations of FTW, DTW and CF are given in Fig. 2. One-way ANOVA results showing effects of site, sampling date and season on these parameters are presented in Table 2. FTW was generally higher for the lake clams than for the river ones. Only FTW of these latter displayed a particular seasonal trend with higher values in late summer compared to that observed in spring and winter. DTW of both populations were almost the same with no significant seasonal trend. An important DTW was just observed in September 1998 for the river clams. CF was significantly lower for clams inhabiting the lake. No seasonal trend was evidenced for both populations. Only a higher value was reported in September 1998 for the river clams, as already mentioned for FTW and DTW.

Biochemical composition

Seasonal variations of proteins, carbohydrates, lipids and ashes are described in Fig. 3. One-way ANOVA results showing effects of site, sampling date and season on these parameters are presented in Table 2. Protein and ash contents were significantly higher in the lake clams than in the river ones. The contrary was observed for carbohydrate and lipid levels. Clams inhabiting the lake displayed significant lower protein values in winter (45.1% of DTW in January 2000) compared to that observed in spring and summer whereas clams from the river exhibited a lower level in spring (41.5% of DTW in May 1999). The highest carbohydrate

levels reported in the lake clams were in summer-autumn (20.2 and 19.5% of DTW in November 1998 and early September 1999, respectively) and the lowest ones in winter-spring. An opposite trend was observed in the case of the river clams with the highest value in January 1999 and the lowest one in July 2000 (35.5 and 11.2% of DTW, respectively). A more distinct pattern was observed for lipid content of the lake clams with an increase from winter to spring (maxima in May 1999 and 2000) and a subsequent decrease to winter. It was correlated positively and negatively with the oocyte percentage in gonads and carbohydrates, respectively. Compared to the lipid level of the lake clams, that of the river ones increased from November 1998 until September 1999. However, no significant seasonal trend was evidenced though lowest and highest values were observed in winter and spring, respectively. Lipids were correlated negatively with carbohydrates. Ash content exhibited significant higher values in winter for the lake clams (up to 16.40% of DTW in January 2000). The opposite was observed for the river clams. Summation of protein, carbohydrate, lipid and ash contents always yielded totals inferior to 100% of DTW for both populations.

Enzymatic activities

Seasonal variations of CAT, PChE, NADPH-red, NADH-red, GST/CDNB and GST/EA activities are given in Fig. 4. One-way ANOVA results showing effects of site, sampling date and season on these parameters are presented in Table 2. No site-effect was observed for CAT activity. *C. fluminea* from the lake displayed the highest levels in summer (July 1998, early September 1999 and July 2000). For CAT activity of the river clams, no significant season-effect was observed despite higher responses in April and October 1999. PChE activity was slightly lower for the river clams than for the lake ones. These latter showed no significant seasonal trend in PChE activity contrary to that of the river clams which displayed the lowest and highest values in winter and summer, respectively. NADPH-red activity was significantly lower for the river clams than for the lake ones. Despite significant variations with sampling dates for both populations, no significant seasonal trend came out because of high variability. NADH-red activity was also significantly lower for the river clams. Activity displayed similar significant seasonal trends for both populations with the highest and lowest responses obtained in summer-autumn and winter-spring, respectively. This trend was sensibly the same during the second part of the study. NADH-red activity of the river clams was correlated positively and negatively with lipids and carbohydrates, respectively. No site-effect was observed for GST/CDNB activity. For *C. fluminea* from the lake, it displayed significant

fluctuations according to the sampling date but no significant seasonal trend. From May 1999 to July 2000, responses were higher than those obtained during the first part of the study. As the same was observed for the river clams, it could be imputable to assay variability (though assay procedure was always the same). Although a significant season-effect was reported for GST/CDNB activity of the river clams, a seasonal trend was not really obvious. GST/EA activity was significantly lower for the river clams than for the lake ones. These latter showed higher activities in autumn than in winter mainly during the second part of the study. On the other hand, season did not affect significantly GST/EA activity of the river clams.

Discriminant analysis (DA)

Synthesis of DA for each population of *C. fluminea* is presented in Table 3. NADH-red activity, lipids, CAT, GST/CDNB and PChE activities contributed the most to discrimination between seasons (from spring to winter) for the lake clams. For clams inhabiting the river, PChE and GST/CDNB activities, ashes, CF and proteins were the most discriminating parameters. Seasons can be considered as groups, separation of which depends on discriminating functions. Those functions are linear combinations of the whole parameters. Each group can be considered as an entity with a centroid (gravity centre) located in a space which comprises as many dimensions as significant functions. The more the distance between two groups (the Mahalanobis distance) is high and significant, the more those groups are discriminated. These results are given in Table 4. For both clam populations, all seasons were significantly discriminated from each other. For the lake clams, the greatest distance (discrimination) was between summer and winter and the lowest one was between summer and autumn. In the case of clams inhabiting the river, spring and summer were the best discriminated seasons whereas spring and autumn were the less discriminated ones.

Index of variability (VI)

VI of enzymatic activities, proteins, carbohydrates, lipids, ashes, FTW, DTW and CF of both clam populations are presented in Table 5. Considering the whole parameters for the lake clams, ashes, CAT and PChE activities exhibited the greatest seasonal variations and DTW, proteins and FTW, the less pronounced ones. For the river clams, NADH-red and PChE activities followed by carbohydrates displayed the highest VI and NADPH-red activity, proteins and lipids the lowest ones. Considering only enzymatic activities, CAT and NADH-

red activities showed the greatest VI for the lake and the river clams, respectively whereas NADPH-red activity displayed the lowest one for both populations. When taking into account only other parameters, ashes and carbohydrates showed the highest VI whereas FTW and lipids exhibited the lowest ones for the lake and the river clams, respectively. Among all parameters assessed, only GST and NADPH-red activities as well as proteins displayed similar VI between populations. We must be slightly cautious however because three samplings could not have been carried out for the river clams.

Discussion

Aim of this study was to investigate seasonal variations of several biological parameters proposed as biomarkers of pollution in two populations of *C. fluminea* inhabiting a lake and a river. These two populations exhibit morphological differences possibly imputable to different ecological niches and/or genotypic differences. Electrophoretic studies revealed genotypic differences between them within the esterase enzymatic system (data not shown) but it is not sufficient to affirm they belong to different species. Furthermore, shell growth and colour are highly influenced by physico-chemical characteristics of the water (Gruet, 1992; Tan Tiu and Prezant, 1992). The river exhibited chronic and punctual pollution by nitrates and phosphates, probably due to fertilisers used in intensive agricultural activities in the Dronne valley (Agence de l'Eau Adour-Garonne, 1996). However, no significant correlation with responses of biological parameters was evidenced. The very low concentration of chlorophyll *a* seems normal for an oligotrophic lake such as the Sanguinet lake (Beuffe, 1995) but it is surprising for the Dronne river, especially with regard to its nitrate level. Nevertheless, the sampling site was slightly sunlit, which could explain a reduced production of phytoplankton. As tissue growth of *C. fluminea* is known to become independent of food at chlorophyll *a* concentrations of about 20 µg/l in the spring and 17.3 µg/l in the summer (Foe and Knight, 1985), both clam populations could be considered as food limited. However, as neither FTW nor DTW were correlated with chlorophyll *a* concentration, an other source of food could be expected. Thus, Way et al. (1990) proposed that *C. fluminea* could increase their food intake by deposit-feeding in addition to filter-feeding. The high CF of the Dronne clams could result from the presence of rich organic substrates in the riverbed.

It is admitted that reproduction of *C. fluminea* is seasonal and correlated with cyclical changes in the environment (Britton and Morton, 1982). Clams inhabiting the lake showed only one

oogenesis peak in spring as reported by Baudrimont et al. (1997) whereas clams from the river displayed two peaks, the first one in spring and the second one in late summer. Indeed, such a bimodal reproductive cycle is commonly encountered in *C. fluminea* living in running water and related to water temperature changes (Kraemer and Galloway, 1986). The decrease of FTW, DTW and CF observed in the river clams after September 1998 could correspond to the post-spawning period when all juveniles have been released and oocyte percentage in gonads is reduced. This is not consistent with other studies on *C. fluminea* in which an increase of the tissue biomass was generally observed during late autumn and early winter (Williams and Mc Mahon, 1989). Such discrepancies could be caused by different environmental conditions as well as a lower dependence of FTW, DTW and CF to the reproductive cycle. On the other hand, carbohydrate and lipid contents were modulated by reproductive events. Particularly, lipids from the lake clams increased from winter to spring in parallel to increasing oogenesis in gonads. The subsequent decrease until January was related to the release of juveniles in late spring - summer and to the decrease of oogenesis. The opposite trend was observed for carbohydrates. In their study on *C. fluminea*, Cantelmo-Cristini et al. (1985) reported that glycogen, an energy source derived from glucose, may be used directly or converted into lipids and then incorporated into the developing gonads. This phenomenon was less pronounced in the river clams, possibly because of the important amount of oocytes in gonads throughout the year. Pattern of protein variations was different depending on the year. Peaks in September 1999 and May 2000 for the lake clams and in September 1999 for the river ones were related to active gametogenesis as observed in *C. gigas* by Deslous-Paoli et al. (1982). High levels of ashes were noticed in winter in *C. fluminea* from the lake as observed in the tellinid bivalve *Macoma balthica* (Beukema and De Bruin, 1977) and could be related partially to the presence of some inorganic material in the mantle cavity or in the visceral mass.

CAT, PChE, NADPH-red, NADH-red and GST activities are biochemical parameters used frequently as pollution biomarkers in many biomonitoring programs (Livingstone, 1993; Van der Oost et al., 1997; Narbonne et al., 1999b; Cajaraville et al., 2000). Their seasonal variations have been widely studied in marine bivalves (for a review: Sheehan and Power, 1999) but never in the freshwater bivalve *C. fluminea*. Clams inhabiting the lake displayed a significant seasonal trend in CAT activity with highest and lowest levels in summer and winter, respectively. Comparable results were reported in the digestive gland of mussels (Viarengo et al., 1991; Power and Sheehan, 1996; Regoli, 1998; Cancio et al., 1999). An

inverse relationship between antioxidant defence systems and peroxidation level was described by Viarengo et al. (1991), suggesting that the reduction of such defences was directly responsible for an increased susceptibility to oxidative stress during the winter. An increase of antioxidant enzyme activities could also be related to reproductive events such as spawning in mussels or, in our study, larvae incubation in *C. fluminea*. Regoli (1998) reported that such an increase could rather be related to a higher metabolic activity of the organism during the warmer season. The absence of a significant seasonal trend in CAT activity of the river clams may be explained partly by less pronounced phases of oogenesis compared to the lake clams as well as lower water temperature maxima. Cholinesterase (ChE) is a useful biomarker for monitoring effects of pesticides (Livingstone, 1993). One form of ChE hydrolysing preferentially propionylthiocholine (PChE activity) was reported in *C. fluminea* (Mora et al., 1999a; 1999b). PChE of the river clams displayed a significant seasonal trend with highest values in late summer corresponding to the warmest temperatures. Escartín and Porte (1997) observed important seasonal variations in AChE activity of mussels collected from the Ebro Delta in relation to pesticide concentration but noted that an increase in ChE activity with increasing temperature could be anticipated in a non - contaminated environment. PChE activity of the lake clams did not seem to be temperature dependent. As presence of pesticides may be discarded because of the great quality of water, environmental factors other than temperature could influence PChE activity as well as possible discrepancies in esterase forms between the two clam populations. NADPH-red and NADH-red activities are related to the mixed function oxygenase (MFO) system, main components of which have been evidenced in mussels (Livingstone and Farrar, 1984; Porte et al., 1995). It is well known that biotic (e.g. sex, age) and abiotic (e.g. temperature, season) factors can cause variations in P450 levels and related activities in both fishes and mussels (Kirchin et al., 1992; Bucheli and Fent, 1995; Wootton et al., 1996). In our study, only NADH-red activity displayed a significant seasonal pattern, which was the same for both populations, with highest levels in summer - autumn and lowest ones in winter - early spring. This trend was closely related to that of the lipid content, especially for the river clams and probably linked to the reproductive cycle. That could be explained by the functions of NADH-red (representative of NADH-cytochrome b_5 reductase) in the synthesis of fatty acids beside other functions, one of which is drug metabolism (Klotz et al., 1986). GSTs are conjugation enzymes of phase II metabolism which have been purified in mussels (Fitzpatrick and Sheehan, 1993; Fitzpatrick et al., 1995) and more recently in *C. fluminea* (Vidal et al., in preparation). We measured GST activity towards CDNB, the universal substrate for unfractionated GSTs and EA, which is more

specific for the Pi-class GSTs (Ketterer et al., 1988). GST/CDNB activity was similar for both populations and no seasonal trend was evidenced (though significant for the river clams) mainly because of the high basal level in the second year of the study. Power and Sheehan (1996) observed no significant seasonal changes in GST/CDNB activity in the digestive gland from mussels contrary to that in gills, which was higher in winter. On the other hand, Regoli (1998) observed a higher GST/CDNB level in digestive gland of mussels during the warmest months corresponding probably to an increase in metabolic activity as pointed out previously for CAT and PChE activities. GST/EA activity was significantly higher in the lake clams compared to that of the river clams, possibly indicative of a dissimilar expression of particular isoenzymes. Furthermore, whereas variations depending on the sampling date of GST/EA of the river clams were similar to those of GST/CDNB activity, clams inhabiting the lake displayed different and more pronounced variations. Therefore, the study of seasonal variations of GST isoenzymes' expression would be of a great interest.

DA is an integrated approach used in environmental studies mainly to discriminate polluted sites from control ones and from each other (Adams et al., 1994; Van Der Oost et al., 1997). DA provided a synthesis of the whole results obtained in univariate ANOVAs. Seasons were well discriminated for both populations, mainly due to NADH-red, CAT and PChE activities as well as lipids, depending on the population. Calculation of VI allowed a better comparison between seasonal variations of each parameter. Most of parameters displayed different VI but also basal levels depending on the clam population/site. Such discrepancies may be detrimental in environmental studies involving populations living in different areas because biomarker responses would not be comparable. In general, these discrepancies can be related to: (1) differences between populations at a genetic and/or biochemical level; (2) different ecosystems characterised by distinctive physico-chemical features exhibiting specific seasonal trends; (3) an absence of synchrony between reproductive cycles; (4) presence of contaminants. If we only consider one population, parameters displaying important VI may respond differently to pollution depending on the time of the year and thus on physiological and reproductive status of the sentinel species as evidenced for ethoxyresorufin-*O*-deethylase activity in the gizzard shad (Levine et al., 1995). In our study, some parameters showed modest seasonal variations within each population (e.g. GST/EA and NADPH-red activities, proteins) and displayed nearly the same VI whatever the population/site was. Thus they could be considered as more reliable biomarkers than the other parameters. Among them, GST activity might be superior to MFO components as a biomarker of exposure as noticed by

Sheehan and Power (1999). Nevertheless, a suitable biomarker must also display significant variations when animals are exposed to contaminants. On this point, contradictory results exist about modulation of GST/CDNB activity by pollutants (Bucheli and Fent, 1995; Parant, 1998). Therefore, in a multibiomarker approach it would be inadvisable to discard bioindicators displaying high seasonal changes such as indicators of oxidative stress or MFO components because they are generally responsive to pollution (Livingstone et al., 1990; Bucheli and Fent, 1995). The only mean to cope with seasonal variations is to know them when studying responses of a biomarker. Furthermore, the choice of a suitable reference site for each environmental study is essential as pointed out by Sheehan and Power (1999). Most of the time, enzymatic activities displayed higher VI compared to physiological parameters. As evidenced previously through DA results, non-specific parameters such as proteins, carbohydrates, lipids, ashes, FTW, DTW and CF should be included in biomonitoring programs for they allow a better interpretation of variations of more specific pollution biomarkers such as NADH-red activity. Furthermore, previous studies have underlined their interest in assessing the impact of contaminants on sentinel species (Cantelmo-Cristini et al., 1985; Doherty, 1990; Sujatha et al., 1995; Black and Belin, 1998).

Our study in *C. fluminea* combined with those mentioned previously in other species underlined the fact that it is essential in environmental biomonitoring to distinguish effects induced by pollutants from those induced by the natural biological cycle and intrinsic characteristics of animals as well as features of the considered ecosystem. Thus it should be advisable to know: (1) the population of sentinel organisms inhabiting each studied site from a genetic point of view; in particular genetic homogeneity is required within the same population and if possible between populations from different sites; (2) the reproductive cycle and the physiological status of each population as well as the physico-chemical characteristics and the biomass production of each site, throughout the year; (3) the seasonal variations of pollution biomarkers for each population. The knowledge of these factors could greatly reduce erroneous results but also improve data interpretation in further environmental surveys.

Acknowledgements

This study was supported by a PhD grant from the TotalFinaElf company. The authors are grateful to Dr. Darrouzet for animal collection, Dr. Robin for determination of ash content as

well as Prof. Laulier and Mrs Robillard (Laboratoire d'Ecophysiologie et Biochimie Métabolique, Université du Maine, France) for electrophoretic studies.

References

Adams, S.M., Ham, K.D., Beauchamp, J.J., 1994. Application of canonical variate analysis in the evaluation and presentation of multivariate biological response data. Environ. Toxicol. Chem. 13(10), 1673-1683.

Agence de l'Eau Adour-Garonne, 1996. Cahier géographique du bassin Adour-Garonne: Dordogne. Agence de l'Eau Adour-Garonne publ., Toulouse.

Araujo, R., Moreno, D., Ramos, M.A., 1993. The Asiatic clam Corbicula fluminea (Müller, 1774) (Bivalvia: Corbiculidae) in Europe. American Malacol. Bull. 10, 39-49.

Association Française de Normalisation -AFNOR-, 1984. Dosage de la chlorophylle a et des phéopigments par spectrométrie d'absorption moléculaire. AFNOR publ., Paris.

Basack, S.B., Oneto, M.L., Verrengia Guerrero, N.R., Kesten, E.M., 1997. Accumulation and elimination of pentachlorophenol in the freshwater bivalve Corbicula fluminea. Bull. Environ. Contam. Toxicol. 58, 497-503.

Basack, S.B., Oneto, M.L., Fuchs, J.S., Wood, E.J., Kesten, E.M., 1998. Esterases of Corbicula fluminea as biomarkers of exposure to organophosphorus pesticides. Bull. Environ. Contam. Toxicol. 61, 569-576.

Baudrimont, M., Lemaire-Gony, S., Ribeyre, F., Métivaud, J., Boudou, A., 1997. Seasonal variations of metallothionein concentrations in the Asiatic clam (Corbicula fluminea). Comp. Biochem. Physiol. 118C(3), 361-367.

Beuffe, H., 1995. Bilan trophique des plans d'eau landais et suivi des effets des aménagements sur le bassin versant du lac de Parentis-Biscarosse. CEMAGREF publ., Bordeaux.

Beukema, J.J., De Bruin, W., 1977. Seasonal changes in dry weight and chemical composition of the soft parts of the tellinid bivalve Macoma balthica in the Dutch Wadden sea. Neth. J. Sea Res. 11(1), 42-55.

Black, M.C., Belin, J.I., 1998. Evaluating sublethal indicators of stress in Asiatic clams (Corbicula fluminea) caged in urban stream. Environ. Toxicol. Risk Assess. 7, 76-91.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37(8), 911-917.

Britton, J.C., Morton, B., 1979. Corbicula in north America: the evidence reviewed and evaluated. In: Britton, J.C., Mattice, J.S., Murphy, C.E., Newland, L.W. (Eds.), Proceedings, First International Corbicula Symposium. Texas Christian University Research Foundation Publication, Fort Worth, Texas, pp. 249-287.

Britton, J.C., Morton, B., 1982. A dissection guide, field and laboratory manual for the introduced bivalve Corbicula fluminea. Malacol. Rev. Suppl. 3, 1-82.

Bucheli, T.D., Fent, K., 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Crit. Rev. Environ. Sci. Technol. 25(3), 201-268.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci. Total Environ. 247, 295-311.

Cancio, I., Ibabe, A., Cajaraville, M.P., 1999. Seasonal variation of peroxisomal enzyme activities and peroxisomal structure in mussels Mytilus galloprovincialis and its relationship with the lipid content. Comp. Biochem. Physiol. 123C, 135-144.

Cantelmo-Cristini, A., Hospod, F.E., Lazell, R.J., 1985. An in situ study on the adenylate energy charge of Corbicula fluminea in a freshwater system. In: Calabrese, A., Thurberg, F.P., Vernberg, F.J., Vernberg, W. (eds.), Marine Pollution and Physiology: Recent Advances. University South Carolina Press, Columbia, pp. 45-62.

Clairborne, A., 1985. Catalase activity. In: Greenwald R.A. (Ed.), Handbook of Methods for Oxygen Radical Research. CRC press, Boca Raton, Florida, pp. 283-284.

Cossu, C., Doyotte, A., Jacquin, M.C., Babut, M., Exinger, A., Vasseur, P., 1997. Glutathione reductase, selenium-dependent glutathione peroxidase, glutathione levels, and lipid peroxidation in freshwater bivalves, Unio tumidus, as biomarkers of aquatic contamination in field studies. Ecotoxicol. Environ. Saf. 38, 122-131.

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). Aquat. Toxicol. 50, 51-71.

Deslous-Paoli, J-M., Zanette, Y., Heral, M., Massé, H., Garnier, J., 1982. Amélioration de la forme et de la qualité de l'huître Crassostrea gigas Thunberg dans les claires de Marennes-Oléron. Rev. Trav. Inst. Pêches Marit. 45(3), 181-194.

Doherty, F.G., 1990. The Asiatic clam, Corbicula spp., as a biological monitor in freshwater environments. Environ. Monit. Assess. 15, 143-181.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28(3), 350-356.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95.

Escartín, E., Porte, C., 1997. The use of cholinesterase and carboxylesterase activities from Mytilus galloprovincialis in pollution monitoring. Environ. Toxicol. Chem. 16(10), 2090-2095.

Fitzpatrick, P.J., Sheehan, D., 1993. Separation of multiple forms of glutathione S-transferase from the blue mussel, Mytilus edulis. Xenobiotica 23, 851-861.

Fitzpatrick, P.J., Krag, T.O.B., Højrup, P., Sheehan, D., 1995. Characterization of a glutathione S-transferase and a related glutathione-binding protein from gill of the blue mussel, Mytilus edulis. Biochem. J. 305, 145-150.

Foe, C., Knight, A., 1985. The effect of phytoplankton and suspended sediment on the growth of Corbicula fluminea (Bivalvia). Hydrobiologia 127, 105-115.

Fontan, B., Meny, J., 1996. Note sur l'invasion de Corbicula fluminea dans le réseau hydrographique de la région Aquitaine et précision sur son spectre écologique. Préfecture de la région Aquitaine, Direction Régionale de l'Environnement Aquitaine, Service de l'Eau et des Milieux Aquatiques Publ., Aquitaine.

Gruet, Y., 1992. Un nouveau mollusque bivalve pour notre région: Corbicula sp. (Heterodonta sphaeriacea). Bull. Soc. Sci. Nat. Ouest Fr. 14(2), 37-43.

Guengerich, F.P., 1994. Analysis and characterization of enzymes. In: Hayes A.W. (Ed.), Principles and Methods of Toxicology, third edition. Raven Press, New-York, pp. 1269-1270.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249(22), 7130-7139.

Inza, B., Ribeyre, F., Maury-Brachet, R., Boudou, A., 1997. Tissue distribution of inorganic mercury, methylmercury and cadmium in the Asiatic clam (Corbicula fluminea) in relation to the contamination levels of the water column and sediment. Chemosphere 35(12), 2817-2836.

Ketterer, B., Meyer, D.J., Clark, A.G., 1988. Soluble glutathione transferase isozymes. In: Ketterer, B., Sies, H. (Eds.), Glutathione Conjugation, Mechanisms and Biological Significance. Academic Press, London, pp. 73-135.

Kirchin, M.A., Wiseman, A., Livingstone, D.R., 1992. Seasonal and sex variation in the mixed-function oxygenase system of digestive gland microsomes of the common mussel, Mytilus edulis L. Comp. Biochem. Physiol. 101C, 81-91.

Klotz, A.V., Stegeman, J.J., Woodin, B.R., Snowberger, E.A., Thomas, P.E., Walsh, C., 1986. Cytochrome P-450 isozymes from the marine teleost Stenotomus chrysops: their roles in steroid hydroxylation and the influence of cytochrome b_5. Arch. Biochem. Biophys. 249, 326-338.

Kraemer, L.R., Galloway, M.L., 1986. Larval development of Corbicula fluminea (Müller) (Bivalvia: Corbiculacea): an appraisal of its heterochrony. Am. Malacol. Bull. 4(1), 61-79.

Labrot, F., Ribera, D., Saint-Denis, M., Narbonne, J-F., 1996. In vitro and in vivo studies of potential biomarkers of lead and uranium contamination: lipid peroxidation, acetyl-cholinesterase, catalase and glutathione peroxidase activities in three non-mammalian species. Biomarkers 1, 23-30.

Levine, S.L., Oris, J.T., Wissing, T.E., 1995. Influence of environmental factors on the physiological condition and hepatic ethoxyresorufin O-deethylase (EROD) activity of gizzard shad (Dorosoma cepedianum). Environ. Toxicol. Chem. 14(1), 123-128.

Livingstone, D.R., 1993. Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. J. Chem. Technol. Biotechnol. 57, 195-211.

Livingstone, D.R., Farrar, S.V., 1984. Tissue and subcellular distribution of enzyme activities of mixed-function oxygenase and benzo[a]pyrene metabolism in the common mussel Mytilus edulis L. Sci. Total Environ. 39, 209-235.

Livingstone, D.R., Garcia Martinez, P., Michel, X., Narbonne, J.F., O'Hara, S., Ribera, D., Winston, G.W., 1990. Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, Mytilus edulis L., and other molluscs. Funct. Ecol. 4, 415-424.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

McLeod, M.J., 1986. Electrophoretic variation in North American Corbicula. Am. Malacol. Bull. Special edn 2, 125-132.

Mora P., Michel, X., Narbonne, J.F., 1999a. Cholinesterase activity as potential biomarker in two bivalves. Environ. Toxicol. Pharmacol. 7, 253-260.

Mora, P., Fournier, D., Narbonne, J.F., 1999b. Cholinesterases from the marine mussels Mytilus galloprovincialis Lmk. and M. edulis L. and from the freshwater bivalve Corbicula fluminea Müller. Comp. Biochem. Physiol. 122C, 353-361.

Mouthon, J., 1981. Sur la présence en France et au Portugal de Corbicula (Bivalvia, Corbiculidae) originaire d'Asie. Basteria 45, 109-116.

Narbonne, J.F., Djomo, J.E., Ribera, D., Ferrier, V., Garrigues, P., 1999a. Accumulation kinetics of polycyclic aromatic hydrocarbons adsorbed to sediment by the mollusk Corbicula fluminea. Ecotoxicol. Environ. Saf. 42, 1-8.

Narbonne, J.F., Daubèze, M., Clérandeau, C., Garrigues, P., 1999b. Scale of classification based on biochemical markers in mussels: application to pollution monitoring in European coasts. Biomarkers 4(6), 415-424.

Parant, M., 1998. Le métabolisme des xénobiotiques chez les bivalves. C. R. Acad. Sci. Paris, Sciences de la vie 321, 485-494.

Porte, C., Lemaire, P., Peters, L.D., Livingstone, D.R., 1995. Partial purification and properties of cytochrome P450 from digestive gland microsomes of the common mussel, Mytilus edulis L. Mar. Environ. Res. 39, 27-31.

Power, A., Sheehan, D., 1996. Seasonal variation in the antioxidant defence systems of gill and digestive gland of the blue mussel, Mytilus edulis. Comp. Biochem. Physiol. 114C(2), 99-103.

Regoli, F., 1998. Trace metals and antioxidant enzymes in gills and digestive gland of the Mediterranean mussel Mytilus Galloprovincialis. Arch. Environ. Contam. Toxicol. 34, 48-63.

Sheehan, D., Power, A., 1999. Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. Comp. Biochem. Physiol. 123C, 193-199.

Sujatha, C.H., Nair, S.M., Chacko, J., 1995. Tissue lipid levels of the clam, Villorita cyprenoides var. cochinensis, following exposure to endosulfan, malathion, and methyl parathion. Environ. Toxicol. Water Qual. 10, 231-235.

Tan Tiu, A., Prezant, R.S., 1992. The role of environment in shell growth dynamics of the Asian clam Corbicula fluminea (Mollusca: Bivalvia). Malacol. Rev. 25, 109-117.

Van Der Oost, R., Vindimian, E., Van Den Brink, P.J., Satumalay, K., Heida, H., Vermeulen, N.P.E., 1997. Biomonitoring aquatic pollution with feral eel (Anguilla anguilla). III. Statistical analyses of relationships between contaminant exposure and biomarkers. Aquat. Toxicol. 39, 45-75.

Viarengo, A., Canesi, L., 1991. Mussels as biological indicators of pollution. Aquaculture 94, 225-243.

Viarengo, A., Canesi, L., Pertica, M., Livingstone, D.R., 1991. Seasonal variations in the antioxidant defence systems and lipid peroxidation of the digestive gland of mussels. Comp. Biochem. Physiol. 100C (1/2), 187-190.

Vidal, M-L., Narbonne, J-F., 2000. Characterization of glutathione S-transferase activity in the Asiatic clam Corbicula fluminea. Bull. Environ. Contam. Toxicol. 64, 455-462.

Vidal, M-L., Bassères, A., Narbonne, J-F., 2001. Potential biomarkers of trichloroethylene and toluene exposure in Corbicula fluminea. Environ. Toxicol. Pharmacol. 9, 87-97.

Way, C.M., Hornbach, D.J., Miller-Way, C.A., Payne, B.S., Miller, A.C., 1990. Dynamics of filter feeding in Corbicula fluminea (Bivalvia: Corbiculidae). Can. J. Zool. 68, 115-120.

Williams, C.J., McMahon, R.F., 1989. Annual variation of tissue biomass and carbon and nitrogen content in the freshwater bivalve Corbicula fluminea relative to downstream dispersal. Can. J. Zool. 67, 82-90.

Wootton, A.N., Goldfarb, P.S., Lemaire, P., O'Hara, S.C.M., Livingstone, D.R., 1996. Characterization of the presence and seasonal variation of a CYP1A-like enzyme in digestive gland of the common mussel, Mytilus edulis. Mar. Environ. Res. 42(1-4), 297-301.

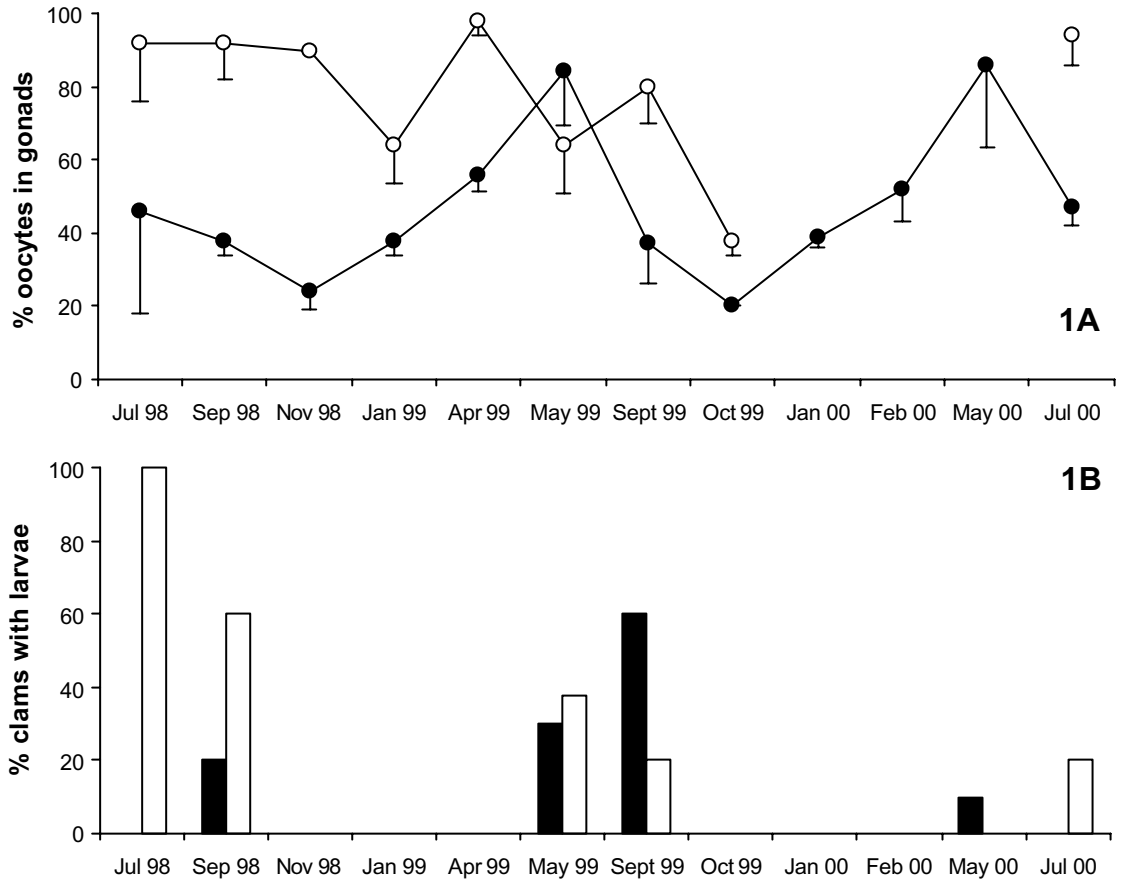


Figure 1. Seasonal variations of the reproductive status of *Corbicula fluminea* from the Sanguinet lake (-●- / ■) and the Dronne river (-❀- / ▫): percentage of oocytes in gonads (1A) and percentage of clams with larvae in gills (1B). In 1A, values are mean ± S.D. (n=10). Data not available from January 2000 to May 2000 for the river clams.

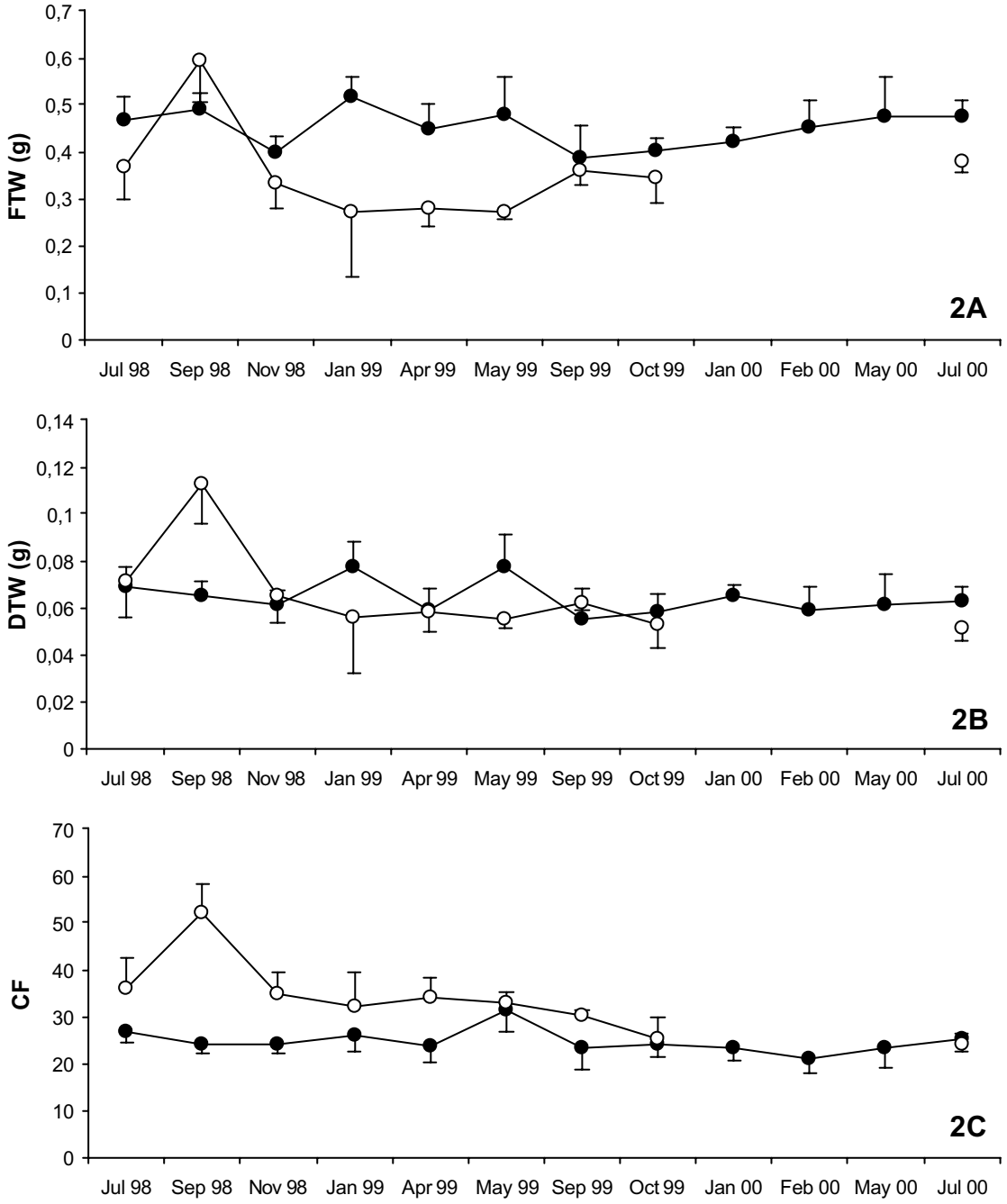


Figure 2. Seasonal variations of fresh tissue weight - FTW - (2A), dry tissue weight - DTW - (2B) and condition factor - CF - (2C) of *Corbicula fluminea* from the Sanguinet lake (-●-) and the Dronne river (-❀-). Values are mean ± S.D. (n=5). Data not available from January 2000 to May 2000 for the river clams.

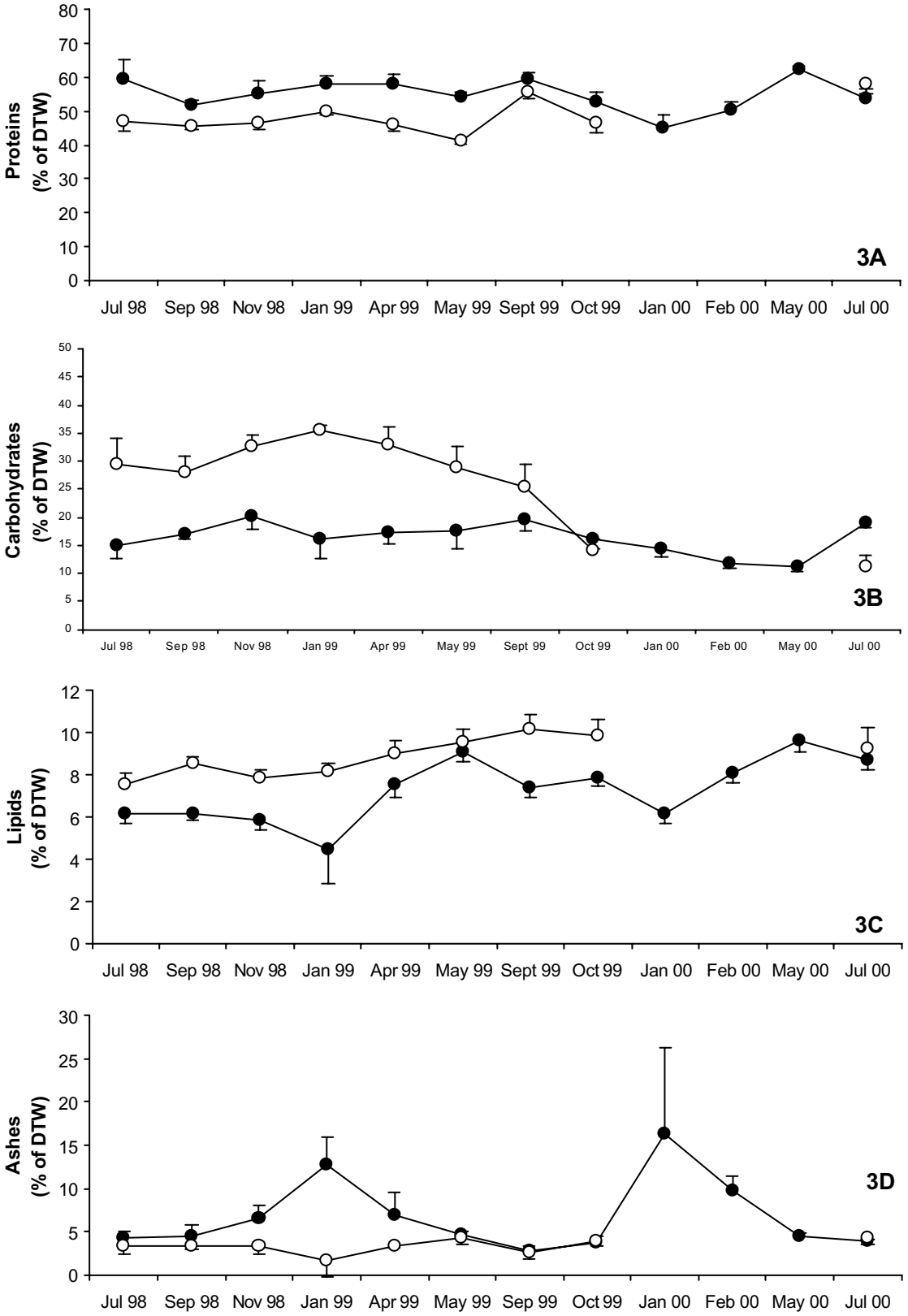


Figure 3. Seasonal variations of proteins (3A), carbohydrates (3B), lipids (3C) and ashes (3D) of *Corbicula fluminea* from the Sanguinet lake (-●-) and the Dronne river (-❀-). Values are mean ± S.D. (n=5). Data not available from January 2000 to May 2000 for the river clams.

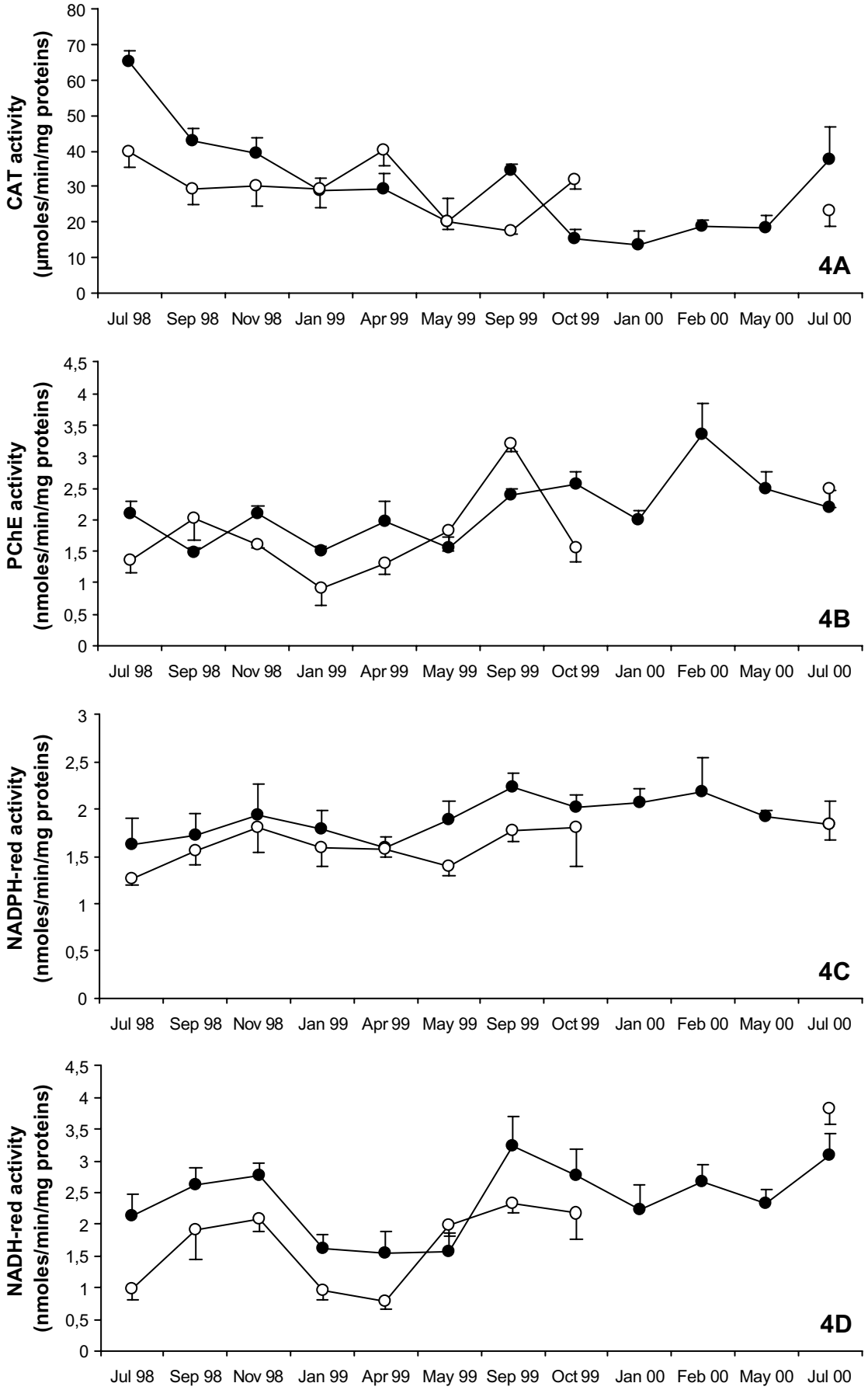


Figure 4 (to be continued).

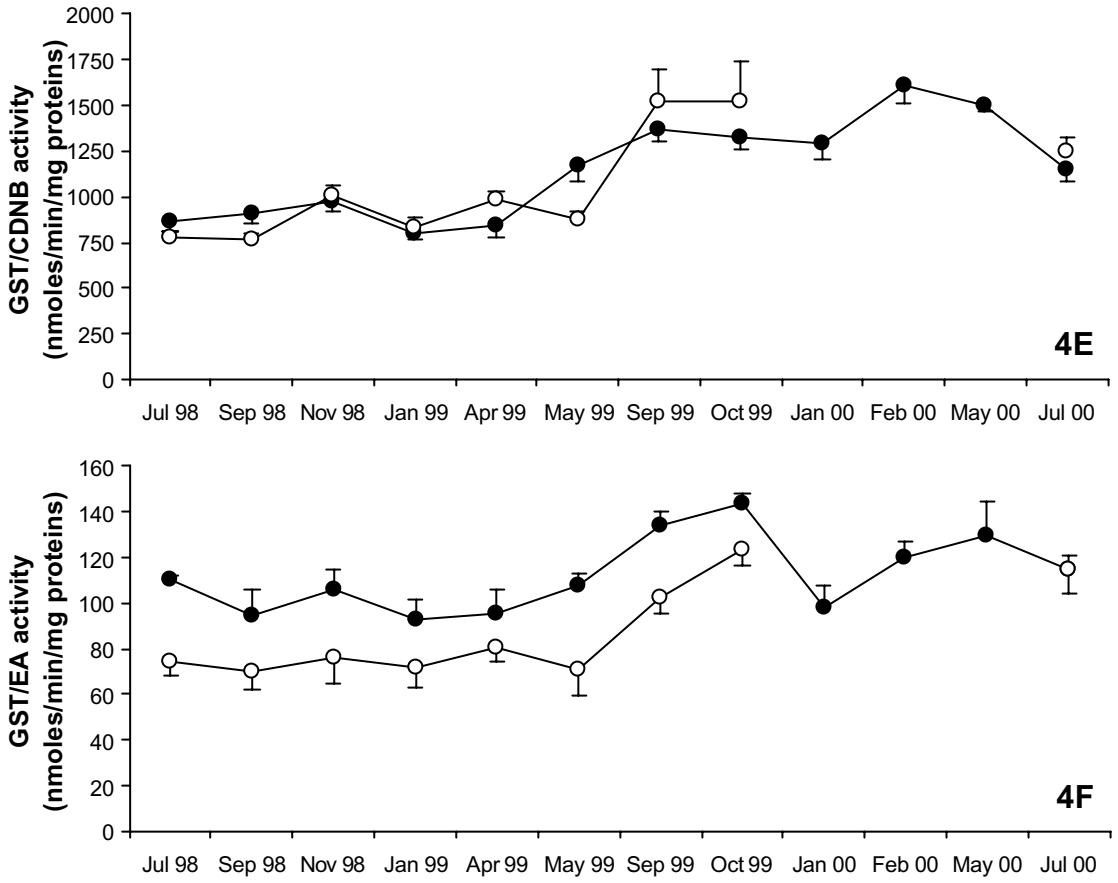


Figure 4 (continuation). Seasonal variations of enzymatic activities of *Corbicula fluminea* from the Sanguinet lake (-●-) and the Dronne river (-֍-): catalase - CAT - (4A), propionylcholinesterase - PChE - (4B), NADPH-cytochome *c* reductase - NADPH-red - (4C), NADH-cytochome *c* reductase - NADH-red - (4D) and glutathione *S*-transferase - GST/CDNB - (4E), - GST/EA - (4F) activities. Values are mean ± S.D. (n=5). Data not available from January 2000 to May 2000 for the river clams.

Table 1. Seasonal variations of temperature, pH, conductivity, oxygen saturation and chlorophyll *a* concentration of the Sanguinet lake (S) and the Dronne (D) river from July 1998 to July 2000. Data not available (NA) from January 2000 to May 2000 for the river.

Environmental parameter	Site	Sampling date											
		Jul 98	Sep 98	Nov 98	Jan 99	Apr 99	May 99	Sep 99	Oct 99	Jan 00	Feb 00	May 00	Jul 00
Temperature (°C)	S	24,8	18,9	8,7	7,7	14,9	18,0	27,0	17,7	8,8	13,1	20,5	27,4
	D	23,5	18,6	4,3	8,3	13,9	15,3	23,7	14,5	NA	NA	NA	22,5
pH	S	7,28	7,01	6,71	6,97	6,42	6,52	6,98	6,56	6,08	6,45	6,77	6,29
	D	8,06	8,10	8,30	8,29	8,31	8,01	8,26	8,27	NA	NA	NA	8,05
Conductivity (µS/cm)	S	145	188	195	192	185	177	188	187	207	195	186	183
	D	453	413	501	467	434	347	434	502	NA	NA	NA	497
Oxygen saturation (%)	S	112,0	96,9	79,4	81,2	94,4	95,1	90,4	89,1	57,7	84,1	117,0	97,1
	D	100,1	99,2	90,5	91,8	96,0	95,0	92,5	90,7	NA	NA	NA	95,4
Chlorophyll *a* (µg/l)	S	2,611	3,607	0,375	NA	0,000	0,309	0,000	0,138	0,095	0,043	0,071	0,029
	D	1,185	0,420	0,285	NA	0,477	0,666	0,142	0,000	NA	NA	NA	0,044

Table 2. Synthesis of the one-way ANOVAs displaying the effects of site, sampling date and season (from spring to winter) on catalase (CAT), propionylcholinesterase (PChE), NADPH-cytochrome *c* reductase (NADPH-red), NADH-cytochrome *c* reductase (NADH-red) and glutathione *S*-transferase (GST/CDNB, GST/EA) activities; proteins, carbohydrates, lipids and ashes; fresh tissue weight (FTW), dry tissue weight (DTW) and condition factor (CF) of *Corbicula fluminea* from the Sanguinet lake and the Dronne river.

Biological parameter	Effect of site [a]			Effect of sampling date [a]						Effect of season [a]					
				Sanguinet			Dronne			Sanguinet			Dronne		
	F	df	*p*	*F*	df	*p*	*F*	df	*p*	*F*	df	*p*	*F*	df	*p*
CAT	0.06161	1	ns	43.7424	11	***	15.1649	8	***	29.0876	3	***	1.5902	3	ns
PChE	5.7326	1	*	18.7351	11	***	30.3914	8	***	3.3491	3	ns	14.3683	3	**
NADPH-red	20.4752	1	***	2.8467	11	**	3.6962	8	**	1.5730	3	ns	2.5925	3	ns
NADH-red	9.6329	1	**	11.3845	11	***	44.9900	8	***	11.4225	3	***	12.5627	3	**
GST/CDNB	3.5904	1	ns	57.0010	11	***	36.0926	8	***	1.3206	3	ns	8.6467	3	*
GST/EA	39.1989	1	***	14.0900	11	***	21.7657	8	***	3.0198	3	*	5.7433	3	ns
Proteins	34.8594	1	***	9.9072	11	***	21.8466	8	***	5.0881	3	**	14.6730	3	**
Carbohydrates	31.0202	1	***	7.9655	11	***	29.8677	8	***	5.7947	3	**	14.2076	3	**
Lipids	24.3281	1	***	23.2389	11	***	8.7413	8	***	8.2804	3	***	1.3149	3	ns
Ashes	27.3604	1	***	38.1125	11	***	3.0751	8	**	35.2125	3	***	4.5665	3	**
FTW	28.1928	1	***	2.3497	11	*	8.5462	8	***	2.5592	3	ns	6.9637	3	***
DTW	1.5780	1	ns	2.3449	11	*	9.1922	8	***	0.9662	3	ns	2.2883	3	ns
CF	32.8396	1	***	2.3845	11	*	11.0658	8	***	1.1966	3	ns	0.9067	3	ns

[a] *F*: *F* ratio; df: degrees of freedom; *p*: probability of *F*. Data displaying non-normal distribution or variance heterogeneity were submitted to non-parametric tests. *, **, ***: significant effect at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively; ns: non-significant effect.

Table 3. Synthesis of discriminant analysis. Biological parameters contributing significantly to discrimination between seasons (from spring to winter) for *Corbicula fluminea* from the Sanguinet lake and the Dronne river.

Biological parameter [a]	Wilks Lambda	Partial Lambda	*F* of exclusion	*P* [b]
	Sanguinet			
NADH-red	0.04330	0.42806	20.9329	***
Lipids	0.03849	0.48162	16.8626	***
CAT	0.03689	0.50238	15.5184	***
GST/CDNB	0.02861	0.64796	8.5119	***
PChE	0.02681	0.69136	6.9939	***
GST/EA	0.02571	0.72081	6.0682	**
Proteins	0.02420	0.76590	4.7885	**
FTW	0.02303	0.80493	3.7966	*
Ashes	0.02224	0.83328	3.1346	*
DTW	0.02197	0.84374	2.9014	*
	Dronne			
PChE	0.06306	0.43851	14.0850	***
GST/CDNB	0.05561	0.49729	11.1198	***
Ashes	0.05449	0.50748	10.6759	***
CF	0.05071	0.54529	9.1729	***
Proteins	0.04731	0.58448	7.8201	***
DTW	0.03962	0.69797	4.7599	**
CAT	0.03885	0.71186	4.4524	**
NADPH-red	0.03834	0.72124	4.2515	*

[a] catalase (CAT), propionylcholinesterase (PChE), NADPH-cytochrome *c* reductase (NADPH-red), NADH-cytochrome *c* reductase (NADH-red) and glutathione *S*-transferase (GST/CDNB, GST/EA) activities; fresh tissue weight (FTW), dry tissue weight (DTW) and condition factor (CF).

[b] *p*: probability of *F* of exclusion; *s*ignificant contribution to discrimination at *: $p \leq 0.05$, **: p 0.01, ***: p 0.001.

Table 4. Discriminant analysis: distances between seasons (from spring to winter) for *Corbicula fluminea* from the Sanguinet lake and the Dronne river, expressed as square of Mahalanobis distances between group centroids [a].

Season	Spring	Summer	Autumn	Winter
	Sanguinet			
Spring	-	28.25 ***	27.79 ***	24.49 ***
Summer	-	-	9.65 ***	45.07 ***
Autumn	-	-	-	30.74 ***
Winter	-	-	-	-
	Dronne			
Spring	-	34.28 ***	9.69 **	28.64 ***
Summer	-	-	22.83 ***	25.82 ***
Autumn	-	-	-	20.88 ***
Winter	-	-	-	-

[a] significant discrimination at *: $p \leq 0.05$, **: p 0.01, ***: p 0.001.

Table 5. Index of variability (VI) of catalase (CAT), propionylcholinesterase (PChE), NADPH-cytochrome *c* reductase (NADPH-red), NADH-cytochrome *c* reductase (NADH-red) and glutathione *S*-transferase (GST/CDNB, GST/EA) activities; proteins, carbohydrates, lipids and ashes; fresh tissue weight (FTW), dry tissue weight (DTW) and condition factor (CF) of *Corbicula fluminea* from the Sanguinet lake and the Dronne river during the two-year experiment.

Biological parameter	Sanguinet						Dronne					
	Maximum value [a]	Minimum value [a]	VI [b]	Ranking in decreasing order			Maximum Value [a]	Minimum value [a]	VI [b]	Ranking in decreasing order		
				All parameters	Enzymes	Others				All parameters	Enzymes	Others
CAT	65,1	13,6	4,79	2	1		40,2	17,6	2,28	5	3	
PChE	3,35	1,47	2,28	3	2		3,2	0,9	3,56	2	2	
NADPH-red	2,24	1,60	1,40	10	6		1,84	1,27	1,45	11	6	
NADH-red	3,23	1,53	2,11	5	3		3,81	0,79	4,82	1	1	
GST/CDNB	1612,8	803,4	2,01	6	4		1526,7	766,1	1,99	9	4	
GST/EA	143,8	92,6	1,55	8	5		123,7	69,7	1,77	10	5	
Proteins	62,4	45,1	1,38	12		6	58,3	41,5	1,40	12		6
Carbohydrates	20,2	11,3	1,79	7		3	35,5	11,2	3,17	3		1
Lipids	9,61	4,48	2,15	4		2	10,19	7,58	1,34	13		7
Ashes	16,40	2,74	5,99	1		1	4,23	1,68	2,52	4		2
FTW	0,519	0,385	1,35	13		7	0,594	0,271	2,19	7		4
DTW	0,077	0,055	1,40	11		5	0,113	0,051	2,22	6		3
CF	31,5	20,9	1,51	9		4	52,0	24,2	2,15	8		5

[a] in nmoles/min/mg proteins for enzymatic activities, except for CAT (µmoles/min/mg proteins); in % of DTW for proteins, carbohydrates, lipids and ashes; in g for FTW and DTW.

[b] variability index (VI) calculated as VI = (maximum value / minimum value).

Comme nous l'avons précédemment mis en évidence, certains paramètres biochimiques ont présenté de fortes variations saisonnières et ce, avec des différences marquées d'un site à l'autre. La température, la qualité de l'eau et de manière plus générale les facteurs abiotiques pourraient être à l'origine de telles variations. La connaissance de ces dernières nous apparaît essentielle et ce, afin d'éviter toute confusion lors de l'interprétation des réponses obtenues en présence de contaminants. Le but de ce travail était d'étudier l'influence de plusieurs facteurs abiotiques sur les réponses de composants du métabolisme de (dé)toxication des phases I et II, de paramètres associés au stress oxydatif et de la propionylcholinestérase, tous proposés comme biomarqueurs de pollution chez *Corbicula fluminea*. Les effets combinés de la température (10 et 20°C), du type d'eau (eau purifiée par osmose inverse, eau provenant du site de prélèvement -lac de Sanguinet- et eau du robinet) et du substrat (présence et absence de sable) ont été évalués après une exposition de cinq jours. Les animaux ont également été exposés pendant cinq jours à des conditions hypoxiques ou des conditions acides, neutres ou basiques, à 20°C, dans de l'eau du lac, en l'absence de sable. Cette étude est présentée dans l'**article n°4**. De manière générale, les réponses se sont avérées plus élevées lorsque les animaux ont été placés dans l'eau du robinet, sauf pour la teneur en lipides peroxydés (LP). En l'absence de sable, les activités catalase (CAT) et NADH-cytochrome *c* réductase (NADH-red.) ont augmenté alors que l'activité propionylcholinestérase (PChE) a diminué. Une température plus faible a entraîné une diminution des activités PChE et NADPH-cytochrome *c* réductase (NADPH-red.). Des conditions hypoxiques ont induit une augmentation de l'activité CAT et de l'activité glutathion *S*-transférase envers l'acide éthacrynique ainsi qu'une diminution de la teneur en LP. Les activités CAT et NADH-red. et la teneur en LP ont diminué lorsque les animaux ont été exposés à des conditions acides et/ou basiques. Si nous considérons l'ensemble des résultats obtenus lors de ces trois études, les paramètres ayant présenté la plus forte sensibilité aux facteurs abiotiques sont ceux possédant la variabilité saisonnière la plus importante: les activités CAT, PChE et NADH-red. (la variabilité saisonnière de la teneur en LP n'a pas été évaluée). Ces résultats démontrent que les effets des facteurs abiotiques doivent être contrôlés ou à défaut pris en compte dans toute étude environnementale de laboratoire et de terrain.

ARTICLE 4

Influence of abiotic factors (temperature, pH, oxygenation, water-type and substrate) on biomarker responses in the freshwater clam *Corbicula fluminea* (Müller)

Vidal, M-L., Bassères, A., Narbonne J-F.

Soumis à *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology*

Abstract

Aim of this study was to investigate the influence of abiotic factors on responses of components of (de)toxification metabolism of phases I and II, parameters related to oxidative stress and propionylcholinesterase, proposed as biomarkers of pollution in *Corbicula fluminea*. Combined effects of temperature (10 and 20°C), water-type (reverse-osmosis purified water, lake water from the collection site and tap water) and substrate (presence and absence of sand) were assessed in a five-day experiment. Additionally, clams were exposed to hypoxia or submitted to acidic, neutral or alkaline conditions at 20°C, in lake water, without sand, for five days. Responses were generally higher when clams were placed in tap water, except for level of peroxided lipids (PL). Catalase (CAT) and NADH-cytochrome *c* reductase (NADH-red.) activities increased whereas propionylcholinesterase (PChE) activity decreased in absence of substrate. Decreasing temperature resulted in depressing PChE and NADPH-cytochrome *c* reductase (NADPH-red.) activities. Hypoxia induced an increase of CAT activity and glutathione *S*-transferase activity towards ethacrynic acid and a decrease of PL level. CAT and NADH-red. activities as well as PL level were reduced when clams were exposed to acidic and/or alkaline conditions. These results indicate that effects of abiotic factors should be taken into account in environmental studies.

Keywords. Biomarkers, *Corbicula fluminea*, fresh water, oxygenation, pH, substrate, temperature

Introduction

Corbicula fluminea is a freshwater bivalve mollusc originating from Asia. During the 20th century, it has progressively colonised America and more recently Europe (Britton and Morton, 1979; Mouthon, 1981; Araujo et al., 1993). It is an opportunist species adaptable to a wide range of lentic and lotic habitats, thus explaining its considerable spread during the last decades. This major component of benthic communities is both a filter- and a deposit-feeding species (Way et al., 1990). This particular feature makes *C. fluminea* a good candidate as a freshwater sentinel organism for environmental survey programs. Like *Mytilus edulis* and *M. galloprovincialis*, its counterparts in marine ecosystems, *C. fluminea* is able to bioaccumulate organic pollutants and heavy metals (e.g. Doherty, 1990; Basack et al., 1997; Inza et al., 1997; Narbonne et al., 1999) and interesting results have already been obtained by measuring biochemical parameter responses after exposure to such xenobiotics (e.g. Labrot et al., 1996; Baudrimont et al., 1997; Basack et al., 1998; Vidal et al., 2001). Among the various parameters considered as biomarkers of pollution, activities belonging to the (de)toxification metabolism of phases I and II have focused particular attention. In molluscs, cytochrome P-450 and the associated mixed-function oxidase (MFO) components have been mainly localised in microsomes of the digestive gland (Livingstone et al., 1989). More recently, a putative cytochrome P450 has partially been purified in *M. edulis* by Porte et al. (1995). Glutathione *S*-transferases (GSTs) form a family of phase II isoenzymes that catalyse the conjugation of glutathione to both endobiotic and xenobiotic compounds. Several isoenzymes have previously been purified and characterised in mussels (Fitzpatrick and Sheehan, 1993; Fitzpatrick et al., 1995) but also in *C. fluminea* (Vidal et al., unpublished results). Both components of metabolism of phases I and II have displayed significant responses after mollusc exposure to several contaminants, despite some mitigated results (Bucheli and Fent, 1995; Livingstone et al., 2000). Antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase as well as indicators of oxidative stress such as peroxidation level are also widely used as biomarkers of pollution in molluscs (Livingstone et al., 1990; Cossu et al., 1997). Cholinesterase, involved in nervous functions, has been purified in both mussels and *C. fluminea* by Mora et al. (1999a) and has proven to be a useful biomarker for monitoring effects of pesticides (Livingstone, 1993). A biomarker of value is supposed to be only modulated by contaminants and not by other environmental and/or biotic factors. It is hardly the case in reality. For instance, biotic (e.g. sex, age, nutrition) and abiotic (e.g. temperature, season) parameters can cause variations of P450 levels and related activities in both fishes and

mussels (Kirchin et al., 1992; Bucheli and Fent, 1995). Likewise, antioxidant defence systems and peroxidation level are strongly influenced by season via the combined actions of temperature and reproductive cycle (Viarengo et al., 1991; Power and Sheehan, 1996; Regoli, 1998). Cholinesterase activity could also be function of temperature (Escartín and Porte, 1997). Contrastingly, GST activity has been reported to be less or not influenced by season (Power and Sheehan, 1996). As such (a)biotic factors are susceptible to interact with contaminants on biomarker responses, it is primordial to know and understand their effects to avoid misinterpretations of results in environmental studies. In this aim, we have studied previously seasonal variations of a panel of biochemical parameters in *C. fluminea* (Vidal et al., submitted). Our objective in the present work was to investigate in laboratory the possible effects of abiotic variables such as temperature, pH, oxygenation, water-type as well as presence and absence of substrate on responses of the same parameters, namely catalase (CAT), peroxided lipids estimated as thiobarbituric acid reactive species (PL), cholinesterase using propionylthiocholine as a substrate (PChE), NADPH-cytochrome *c* (P450) reductase (NADPH-red.), NADH-cytochrome *c* (b5) reductase (NADH-red.) and glutathione *S*-transferase conjugating the non-specific substrate 1-chloro-2,4-dinitrobenzene (GST/CDNB) or ethacrynic acid (GST/EA), more specific to the Pi-class GSTs in mammalians (Ketterer et al., 1988).

Materials and methods

Animals

Sexually mature *C. fluminea*, anterior-posterior shell length of which ranging from 25 to 30 mm, were collected in late March 2000 in the sandy banks of the freshwater Cazaux-Sanguinet lake (Aquitaine, France). The excellent quality of water (personal communication from the Direction Départementale des Affaires Sanitaires et Sociales - DDASS, France) makes this lake a reference site in most environmental studies performed by our laboratory. At this period of the year, gonads from *C. fluminea* were characterised by an increasing oogenesis activity but larvae were not encountered yet in gills. Sex was not taken into account for these clams are hermaphroditic. Animals were brought back to the laboratory in plastic tanks filled with lake water. They were kept in that water aerated by a constant air bubbling (air pump Shego M2K3) at a regulated temperature of 20°C during an acclimatisation period

of ten days. They were not fed during this period to clear out gut content and were submitted to a natural light cycle.

Experimental design

Study of temperature, water-type and substrate effects

Twelve experimental conditions were obtained by combining three sorts of water (reverse-osmosis purified -ROP- water, lake water from the collection site and dechlorinated tap water from the city of Bordeaux, France), two temperatures (10 and 20°C) and the presence or absence of substrate. ROP water (Le Lann, Gradignan, France), lake water and tap water were initially characterised by a pH of 7.2, 7 and 8.2, respectively and by a conductivity of 64, 188 and 453 µS/cm, respectively (Universal Pocket Meter Multiline P4, WTW). Chlorides, nitrates, nitrites, phosphates, ammonium and potassium, measured with semi-quantitative tests (Quantofix, Prolabo, France), were not detected. Hardness of tap water was about 90-180 mg $CaCO_3$/l whereas that of both ROP water and lake water was inferior to 90 mg $CaCO_3$/l (Quantofix, Prolabo). Before experiment, the lake water was filtered through a 0.45 µm polypropylene membrane filter (Gelman Sciences, Michigan, USA) to discard nutritive particles. The substrate consisted of sand collected in the banks of the lake, previously washed several times in ROP water, filtered lake water or tap water depending on the experimental condition. The study was performed in twelve 1-l glass flasks filled with 800 ml of the appropriate water, supplemented or not with 300 g of washed sand. Flasks were placed into a thermostated bath regulated at 10 or 20°C. Aeration was steadily provided by a diffuser connected to an air pump (Shego M2K3) at a flow allowing an oxygen saturation of 85-95% (Universal Pocket Meter Multiline P4, WTW). Once all physico-chemical conditions were stabilised, fifteen clams were placed into each flask for a five-day exposure. They were not fed and were submitted to a natural light cycle during all the experiment. Water was not changed and temperature, pH, conductivity and oxygen saturation were daily checked. Dead clams were removed to avoid water fouling.

Study of oxygenation effects

The study was performed in two 1-l glass flasks filled with 800 ml of filtered lake water, as previously described. No substrate was added. Flasks were placed in a thermostated bath

regulated at 20°C. Two experimental conditions were assayed: normoxia (control) and hypoxia. Normoxic conditions were obtained by a constant aeration provided by air bubbling (air pump Shego M2K3) allowing an oxygen saturation of 85-95%. Hypoxia was reached by flushing with 100% nitrogen gas at the beginning of the experiment. An oxygen saturation of 15-25% was maintained during all the experiment. Fifteen clams were placed into each flask for a five-day exposure. As in the previous study, they were not fed and were submitted to a natural light cycle. During the experiment, flasks were capped and water was not changed. Temperature, pH, conductivity and oxygen saturation were daily checked. Dead clams were removed to avoid water fouling.

Study of pH effects

The study was performed in three 1-l glass flasks filled with 800 ml of filtered lake water, as previously described. No substrate was added. Flasks were placed in an environmental chamber held at a temperature of 20°C. Three experimental conditions were defined, namely acidic, neutral (control) and alkaline conditions. Control consisted of filtered lake water with a pH of 7. Acidic (pH 4-5) and alkaline (pH 8-9) conditions were obtained by injecting periodically 0.05 N HCl or 0.2 N NaOH in filtered lake water through a peristaltic pump (2132 Microperpex®, LKB Bromma) to compensate for the natural water buffering capacity. Water was renewed every day and temperature, pH, conductivity and oxygen saturation were daily checked. Constant aeration was provided by air bubbling (air pump Shego M2K3) to maintain an oxygen saturation of 85-95%. Fifteen clams were placed into each flask for a five-day exposure. They were not fed and were submitted to a natural light cycle. No mortality occurred during the experiment, thus no dead clam could have fouled the water.

Biochemical measurements

At the end of the five-day exposure, *C. fluminea* were dissected out. The entire soft bodies were immediately frozen in liquid nitrogen and subsequently stored at -80°C. All homogenisation steps were then performed at 4°C. For each experimental condition, five pools of three *C. fluminea* were prepared. Body samples were rinsed in 100 mM phosphate buffer (K_2HPO_4/KH_2PO_4), pH 7.4, dried on absorbent paper towels, weighed and homogenised in the same phosphate buffer (1:4 weight:volume ratio) using an Ultra-Turrax Antrieb T25. Homogenates were subsequently centrifuged at 9000 g for 30 min in a Sigma

3MK centrifuge. The resulting supernatants (S9) were divided in two fractions. The first one was stored at -80°C whereas the second one was centrifuged at 105000 g for 1 hr in a Beckman LE-80 ultracentrifuge. Cytosolic fractions were then stored at -80°C. Microsomal pellets were resuspended in a 100 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 20% glycerol and stored at -80°C. All biochemical measurements were performed in duplicates or in triplicates for each pool on a dual-beam temperature-controlled Kontron Uvikon 932 spectrophotometer. CAT and PChE activities as well as PL level were assayed in S9 fractions whereas GST activity towards CDNB or EA and NADPH- and NADH-red. activities were measured in cytosolic and microsomal ones, respectively. CAT activity was determined as described in Clairborne (1985). PL level was estimated according to Buege and Aust (1978) following the appearance of thiobarbituric acid reactive species (TBARS) at 532 nm. Cholinesterase activity was assayed using propionylthiocholine as a substrate as in Mora et al. (1999b) according to the method of Ellman et al. (1961). Measurement of GST activity towards CDNB or EA was performed as in Vidal and Narbonne (2000) by a method adapted from Habig et al. (1974). NADPH-red. activity was measured as described in Guengerich (1994). NADH-red. activity was determined likewise, replacing NADPH by NADH. All biochemical parameter responses were expressed in relation to protein concentration measured according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Statistical analyses

Statistica software (5.1 release, Statsoft) was used for all statistical evaluations. Data checked previously for normal distribution and homogeneity of variance (Brown-Forsythe or Levene test) were submitted to a univariate (ANOVA; study of oxygenation and pH effects) or multivariate (MANOVA; study of temperature, water-type and substrate effects) analysis of variance. Whenever a significant ($p \leq 0.05$) effect was established on a biochemical parameter response, a Tukey HSD multiple comparison test was used to check significant ($p \leq 0.05$) differences between independent variables (temperature, water-type, substrate, oxygenation or pH). Finally, to assess which biochemical parameter was the most affected by abiotic conditions, an index of variability (VI), defined as the ratio between the maximal and minimal mean responses, was calculated for each biochemical and abiotic parameter.

Results

Study of temperature, water-type, and substrate effects

During the study, oxygen saturation was of 90.2 ± 1.5% whatever the experimental condition was. Temperature was maintained at 10.2 ± 0.2 °C or 20.6 ± 0.6 °C. Conductivity was of 123.8 ± 59.6, 258.0 ± 36.4 and 443.5 ± 17.4 µS/cm and pH of 7.8 ± 0.5, 7.7 ± 0.4 and 8.5 ± 0.3 for ROP, lake and tap water, respectively. As a general manner, conductivity was slightly less important in the presence of sand. Both pH and conductivity increased steadily during the experiment. The first day of exposure, one dead clam was removed from the ROP water thermostated at 20°C, in the presence of sand. Responses of biochemical parameters to the different experimental conditions of temperature, water-type and substrate are presented in Table 1. Effects of each factor as well as significant interactions obtained from MANOVA analysis are showed in Table 2. *C. fluminea* placed in ROP or tap water displayed a higher CAT activity than those immersed in the lake water. This water-type effect was more pronounced at 20°C than at 10°C. Furthermore, CAT activity was higher in the absence than in the presence of sand, except for tap water. Contrary to CAT activity, PL level was lower when clams were exposed to ROP or tap water, especially at 20°C. At 10°C, PL level was higher in the case of ROP or tap water compared to that of lake water, depending on the presence or absence of sand, respectively. Using the non-specific substrate CDNB, GST activity was not affected by any parameters. On the other hand, GST activity towards EA was modulated by water-type. As for CAT activity, it was higher for clams placed in ROP or tap water than for those exposed to lake water, but mainly at 10°C instead of 20°C. A special case in which activity was higher for both ROP and lake waters, compared to tap water, was observed at 20°C in presence of sand. Contrary to previous biochemical parameters, temperature significantly affected NADPH-red. activity. It was higher at 20°C than at 10°C, especially when clams were exposed to ROP water, in presence of sand. The tap water induced a higher activity than ROP and lake waters did, particularly at 20°C. A higher NADH-red. activity was observed for clams exposed to tap water compared to those placed in ROP or lake water, whatever the other experimental conditions were. Likewise, activity was more important in absence than in presence of sand. No significant interaction was observed between the different factors. PChE activity was the only parameter affected simultaneously by temperature, water-type and substrate. It was higher for clams exposed to tap water, especially at 20°C, in the presence of sand.

Study of oxygenation effects

During the study, oxygen saturation was maintained at 89 ± 0.9 and 20.2 ± 3.7 % in normoxic and hypoxic conditions, respectively. Temperature, pH and conductivity were of 20.6 ± 0.6 °C, 7.5 ± 0.5 and 294.3 ± 40.0 µS/cm, respectively. Conductivity and pH became more important at the end of exposure. One clam died the second day of exposure under hypoxia. Biochemical parameters exhibiting significant responses compared to control (normoxia) after exposure to hypoxia are presented in Figure 1. Effects of oxygenation basing on ANOVA analysis are showed in Table 2. CAT activity, PL level and GST activity towards EA were affected significantly by hypoxia. CAT and GST/EA activities increased of 37 and 11%, respectively whereas PL level decreased of 22% compared to normoxia.

Study of pH effects

During all the experiment, temperature and oxygen saturation were of 19.4 ± 0.8 °C and 85.0 ± 1.9 %, respectively. Conductivity was of 252.2 ± 4.31, 207.0 ± 14.3 and 308.6 ± 102.3 µS/cm and pH of 4.5 ± 0.3, 7.0 ± 0.2 and 8.9 ± 0.4 for acidic, neutral and alkaline conditions, respectively. Conductivity increased from the beginning to the end of each day, because of the periodical addition of HCl or NaOH (about 1.4 and 2.3 fold for acidic and alkaline conditions, respectively). No mortality occurred during the exposure. Biochemical parameters exhibiting significant responses compared to control (pH 7) after exposure to acidic or alkaline conditions are presented in Figure 2. Effects of pH basing on ANOVA analysis are showed in Table 2. CAT activity, PL level and NADH-red. activity varied significantly according to the pH. CAT activity was reduced at pH 8-9 (-21%). PL level decreased when clams were exposed to both acidic and alkaline pH (-20% and -17%, respectively). The same trend was observed for NADH-red. activity: -18% and -29% for acidic and alkaline pH, respectively.

Index of variability (VI)

VI of biochemical parameters for each experimental condition are presented in Table 3. Globally, abiotic factors inducing the highest variations of biochemical parameter responses were water-type followed by pH, oxygenation, temperature and substrate. Biochemical parameters that were the most affected by abiotic factors were CAT activity followed by PChE activity, PL level, NADH-red., NADPH-red., GST/EA and GST/CDNB activities.

Particularly, CAT and PChE activities displayed the highest VI when changing water-type or substrate condition. PChE and NADPH-red. activities, CAT activity and PL level, NADH-red. activity, PL level and CAT activity exhibited the greatest VI considering temperature, oxygenation and pH, respectively.

Discussion

In the present study, all abiotic variables exerted a significant influence on biochemical parameter responses. Combining (M)ANOVA results and VI, water-type was the factor which determined responses of most parameters, followed by pH, oxygenation, temperature and substrate. Significant interactions between temperature, water-type and/or substrate were always encountered, thus increasing complexity of data interpretation. Nevertheless, general trends were evidenced.

Non-contaminated waters are characterised by their own pH, conductivity and hardness. Those parameters are highly dependent on the geologic properties of the river or lake bottom, as well as the related basin. They have an important influence on the health and diversity of aquatic species. For example, a certain amount of calcium must be available for the shell formation of bivalve molluscs such as *C. fluminea*. In some cases, effects of contaminants on aquatic organisms can be altered by the amount of dissolved salts in the water. In the study of Andrews et al. (1983), the molluscicide niclosamide was reported to be less toxic in hard water than in soft one, due to its reduced solubility in harder waters. On the other hand, Oliveira-Filho et al. (1999) did not observe a significant effect of water hardness on the molluscicidal activity of *Euphorbia milii* latex on *Biomphalaria glabrata*. ROP, lake and tap waters differed mainly by their pH, conductivity and hardness. It appeared that such differences in properties of water significantly affected all responses of biochemical markers, except that of GST/CDNB. As a general manner, responses were higher when clams were placed in tap water, except for PL level. Thus, it seems that *C. fluminea* adapted to the low conductivity and hardness of the lake water have been stressed when placed in the tap water. Other factors than conductivity and hardness could be involved as well. However, it is unlikely that the higher pH of tap water compared to that of ROP and lake waters is responsible for these changes because fewer parameters were affected by alkaline pH in our pH-effect study. Furthermore, lower and not higher CAT and NADH-red. activities were induced by a pH of 8-9.

As reported above, pH of non-polluted waters relies on the geologic properties of the river bottom and basin. A relatively large panel of pH can be encountered but in their majority, rivers are characterised by a pH superior to 8. Bioavailability, bioaccumulation and toxicity of some contaminants can be affected by pH. For instance, accumulation of cadmium at 21°C by *C. fluminea* was reduced significantly at pH 5 compared to pH 7.8 (Graney et al., 1984). Toxicity of several metals to upper water-column and benthic species was reported to be pH-dependent (Schubauer-Berigan et al., 1993). Likewise, toxicity of pentachlorophenol to *Dreissena polymorpha* increased with decreasing pH (Fisher et al., 1999). *C. fluminea* can tolerate pH ranging from about 5.5 up to 8.3, depending on their natural ecological niche (e.g. Araujo et al., 1993; Fontan and Meny, 1996). In the Sanguinet lake, pH varies between 6 and 7. CAT and NADH-red. activities as well as PL level were reduced when clams were exposed to acidic and/or alkaline conditions, suggesting a depression of the metabolic rate. Such a phenomenon, indicated by reduced oxygen consumption, has been observed previously in the freshwater mussels *Lamellidens marginalis* and *Perreysia rugosa* when transferred from pH 7.5 to pH 4 or 10 for several days (Masthanamma et al., 1987). However, the oxygen consumption returned progressively towards the control level suggesting an adaptive mechanism. As the decrease of CAT activity, NADH-red. activity and PL level did not exceed 30% in our study, such a recovery could also occur in *C. fluminea* when increasing the exposure time.

The dissolved oxygen level of an aquatic ecosystem can be modulated by several factors, such as water depth, temperature, algae concentration or pollution. *C. fluminea* are poorly adapted to high temperatures and hypoxia compared to other freshwater bivalve species (Mc Mahon, 1979; Johnson and Mc Mahon, 1998) and the combination of these two factors could contribute to the population regulation (Sickel, 1986). Thus, *C. fluminea* prefer well-oxygenated sandy substrates (Belanger et al., 1985) and can experience substantial mortality when living near sewage treatment plant discharges that depress the dissolved oxygen level (Belanger, 1991). Xenobiotic bioaccumulation can be affected by the dissolved oxygen level too. For instance, Heinonen et al. (1997) reported a lower bioaccumulation of 2,4,5-trichlorophenol in hypoxic than in normoxic water in the freshwater clam *Sphaerium corneum*. Furthermore, biomarker responses can be modulated by hyperoxic (Blanco et al., 1987) or anoxic (Pannunzio and Storey, 1998) conditions. In our study, CAT and GST/EA activities increased and PL level decreased when clams were submitted to hypoxia. EA is a substrate conjugated preferentially by GSTs related to the Pi-class in mammalians (Ketterer et

al., 1988). A Pi-class GST has been identified in mussels (Fitzpatrick et al., 1995) and more recently in *C. fluminea* (Vidal et al., unpublished results). As a GSH-peroxidase activity was related to the mussel Pi-class GST, it could be the same for *C. fluminea*, though not investigated, thus explaining the increase of GST activity specifically towards EA. Higher CAT and GST/EA activities could let us expecting adaptive responses to oxidative stress. However, such an event is rather induced by hyperoxia (Blanco et al., 1987) rather than hypoxia or anoxia. CAT and GST/CDNB activities were partially suppressed in the hepatopancreas of the marine gastropod *Littorina littorea* after a 6-day anoxia exposure (Pannunzio and Storey, 1998) but no change in TBARS level was noticed. According to these authors, the generation of oxygen free radicals as well as the general metabolic rate could decrease in marine molluscs under anoxic conditions. Although we worked in hypoxia rather than in anoxia, a decrease in CAT and GST/EA activities should have been more coherent in *C. fluminea*. Nevertheless, siphons from clams exposed to hypoxic conditions were extended out to the limit, possibly indicating an increased ventilation rate to cope with the low oxygen concentration as previously observed for *S. corneum* (Heinonen et al., 1997). Increase of CAT and GST/EA activities could result from this phenomenon.

Water temperature exerts a considerable influence on aquatic ecosystems. Each aquatic species is related to a particular range of temperature, apart from which it can not survive. Temperature can directly act on contaminants' bioaccumulation and toxicity. For example, Graney et al. (1984) reported a higher Cd accumulation in *C. fluminea* at 21°C than at 9°C. Oliveira-Filho et al. (1999) observed an increase in the molluscicidal activity of *E. milii* latex on the freshwater snail *B. glabrata* with increasing temperature. The same observation was made for the toxicity of pentachlorophenol on *D. polymorpha* (Fisher et al., 1999). Those phenomena were mainly attributed to a higher metabolic rate at important temperatures. In the absence of pollutants, temperature alone was also found to have a profound influence on biomarker responses, especially those related to the MFO system (Bucheli and Fent, 1995). Thus the study of temperature-effects on responses of potential biomarkers is of prime interest and must be based on the knowledge of the living temperature range of the considered sentinel organism. For *C. fluminea*, the lower and upper incipient lethal temperatures are of 2 and 34°C, respectively, basing on the experiments of Mattice and Dye (1976). We work on clams collected in a lake, water temperature of which ranges from about 7 up to 27°C. At the sampling time (end of March), temperature of the lake water was of about 15°C and clams were acclimated in laboratory at 20°C during ten days. The two test temperatures of 10 and

20°C were representative of average values encountered in the lake in winter and spring-fall, respectively. Temperature directly influenced responses of NADPH-red. and PChE activities and indirectly acted upon other biochemical parameters (except GST/CDNB and NADH-red. activities) via significant interactions with water-type and/or substrate. Decreasing temperature resulted in lowering PChE and NADPH-red. activities which could be explained by a general slackening of the clams' metabolic rate. As reported by Graney et al. (1984), *C. fluminea* is an ectothermal poikilotherm, i.e. within certain temperature ranges, each 10 °C rise in temperature causes an approximate doubling in metabolic rate (though such a drastic rise was not observed for PChE and NADPH-red. activities when shifting from 10 to 20°C: +16 and 13%, respectively, for all water-type and substrate conditions). Modulation of ChE activity is a well-known phenomenon and thus an increase of activity according to the temperature can be expected in aquatic organisms inhabiting an environment devoid of organophosphorus pesticides and carbamates (Escartín and Porte, 1997). Furthermore, the study of seasonal variations of PChE activity in *C. fluminea* from the Dronne River (Southwest France) revealed higher responses during the warmest months of the year (Vidal et al., submitted). NADPH-red. activity is related to the MFO system, most components of which are strongly influenced by biotic (e.g. sex, age) and abiotic (e.g. temperature, season) factors in both fishes and mussels (Kirchin et al., 1992; Bucheli and Fent, 1995). For instance, basal level of 7-ethoxyresorufin *O*-deethylase and 7-pentoxyresorufin *O*-dependentylase but not 7-ethoxycoumarin *O*-deethylase (EROD, PROD and ECOD, respectively) were reduced significantly in carp at lower ambient temperatures in field environmental studies (Machala et al., 1997). Contrary to NADPH-red. activity, NADH-red. one was not affected significantly by temperature in our study. Thus, the strong seasonal variations observed previously by Vidal et al. (submitted) for this latter activity in *C. fluminea* could be related to the reproductive cycle rather than the temperature. It is also noteworthy to underline the importance of temperature in the study of contaminant effects on MFO activities. Thus, Stegeman (1979) showed an induction of NADPH-red. and benzo(*a*)pyrene hydroxylase activities in the fish *Fundulus heteroclitus* exposed to benzo(*a*)pyrene at 16.5°C, but no effect at 6.5°C. Likewise, Jimenez and Burtis (1989) reported the delay and the suppression of the induction of EROD activity in the bluegill sunfish exposed to the same contaminant at a low acclimatisation temperature (4°C instead of 18°C). Surprisingly, no direct temperature-effect was observed for CAT activity and PL level, both indicators of a modified oxidative state. Generally, a decrease of the antioxidant defence systems associated with a higher level of lipid peroxidation is reported in mussels during winter whereas the opposite phenomenon

takes place in spring-summer (Viarengo et al., 1991; Regoli, 1998; Cancio et al., 1999). However, the reproductive cycle rather than the temperature could be the main explanatory factor. GST activity was not affected by temperature as well, whatever the substrate was. A 40% decrease of GST/CDNB activity from the marine sponge *Suberites domuncula* was reported after a heat stress of 30 min at 31°C after an acclimatisation period at 21°C (Bachinski et al., 1997). However, no such severe temperature was tested in our case. Furthermore, conflicting results concerning seasonal fluctuations of this activity in mussels have been reported in literature (Power and Sheehan, 1996; Regoli, 1998).

Sediment or substrate constitutes an other important factor in environmental studies. Indeed, non-polar organic micropollutants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls and some pesticides are adsorbed to sediments and thus represent an important source of contamination for aquatic species, beside chemicals dissolved in the water column (Neff, 1984). It is particularly true for the benthic bivalve mollusc *C. fluminea* that is both a filter- and a deposit-feeding species (Way et al., 1990). The nature of the sediment (e.g. grain size, organic carbon concentration) is determinant in the adsorption and thus the bioavailability of pollutants (Neff, 1984). The presence or lack of sediment is primordial, as well. Thus, a greater accumulation of cadmium in *C. fluminea* has been reported in the absence rather than in the presence of substrate (Graney et al., 1984). This clam lives naturally buried and laboratory studies have demonstrated its preference for the presence of substrate, especially fine sand (Belanger et al., 1985). In our study CAT and NADH-red. activities increased generally whereas PChE activity was lowered. Thus, clams prevented from burying in a substrate undergo a non-negligible stress that could interfere with effects of contaminants on biochemical parameter responses. Therefore, this factor should be taken into account in environmental studies privileging water-column contamination.

When combining results of those three studies it becomes evident that abiotic factors exert non-negligible effects on biochemical parameter responses used commonly as biomarkers of pollution. In further experiments, it would be pertinent to study the effects of model contaminants on these responses under the same conditions to appraise possible interactions with abiotic factors. As an ideal biomarker should respond to environmental contamination solely, and not to temperature, presence or absence of substrate, water hardness, conductivity, pH, oxygenation, age, food or season, we could wonder about the validity of the majority of biological parameters assessed in environmental studies. Nevertheless, it seems unlikely that

such an ideal biomarker does exist, assuming that living organisms have to adapt to any change continually, imputable or not to pollution, if they want to survive. Among the seven biochemical parameters considered in our study, only GST activity towards the non-specific substrate CDNB displayed no significant variations, whatever the abiotic factor was. Furthermore, it exhibited the lowest global VI. As little or no seasonal variation of GST response was reported in mussels (Power and Sheehan, 1996) but also in *C. fluminea* (Vidal et al., submitted), that parameter could be superior to MFO components on this point, as proposed by Sheehan and Power (1999). Nevertheless, most studies performed on aquatic species led to conflicting results about modulation of GST/CDNB activity by pollutants (Bucheli and Fent, 1995; Parant, 1998). On the contrary, CAT, PChE, NADH-red. activities and PL level were the parameters the most affected by abiotic factors but also by seasons (Vidal et al., submitted), basing on their VI. However, as they are generally responsive to pollution, it would be inadvisable to discard or underestimate them in environmental studies. Hence, to limit interactions between abiotic factors and contaminants on biomarker responses, some precautions should be taken. The study of contaminant effects in laboratory should be carried out in water from the collection site to avoid changes in basic water features (e.g. pH, conductivity, hardness). Animals should be acclimated previously to the experimental temperature, if different from that encountered at the time of sampling. Maintaining a good oxygenation level is also important. The presence or absence of substrate must be considered when using *C. fluminea* or an other species living buried in the sediment. In field studies (including caging studies), it is not evident to control all these parameters. Thus the choice of an appropriate reference point or site is primordial. Of course, these precautions are not exhaustive as other factors can also interfere such as season or food, for example. Hence, it is of prime importance to acquire a good knowledge on the possible effects of (a)biotic factors on biochemical parameter responses to avoid or at least take into account interactions when studying xenobiotic effects.

Aknowledgements

This study was supported by a PhD grant from the TotalFinaElf Company.

References

Andrews, P., Thyssen, J., Lorke, D., 1982. The biology and toxicology of molluscicide, Bayluscide. Pharmac. Ther. 19(2), 245-295.

Araujo, R., Moreno, D., Ramos, M.A., 1993. The Asiatic clam Corbicula fluminea (Müller, 1774) (Bivalvia: Corbiculidae) in Europe. American Malacol. Bull. 10, 39-49.

Bachinski, N., Koziol, C., Batel, R., Labura, Z., Schröder, H.C., Müller, W.E.G., 1997. Immediate and early response of the marine sponge Suberites domuncula to heat stress: reduction of trehalose and glutathione concentrations and glutathione S-transferase activity. J. Exp. Mar. Biol. Ecol. 210, 129-141.

Basack, S.B., Oneto, M.L., Verrengia Guerrero, N.R., Kesten, E.M., 1997. Accumulation and elimination of pentachlorophenol in the freshwater bivalve Corbicula fluminea. Bull. Environ. Contam. Toxicol. 58, 497-503.

Basack, S.B., Oneto, M.L., Fuchs, J.S., Wood, E.J., Kesten, E.M., 1998. Esterases of Corbicula fluminea as biomarkers of exposure to organophosphorus pesticides. Bull. Environ. Contam. Toxicol. 61, 569-576.

Baudrimont, M., Metivaud, J., Maury-Brachet, R., Ribeyre, F., Boudou, A., 1997. Bioaccumulation and metallothionein response in the Asiatic clam (Corbicula fluminea) after experimental exposure to cadmium and inorganic mercury. Environ. Toxicol. Chem. 16, 2096-2105.

Belanger, S.E., 1991. The effect of dissolved oxygen, sediment, and sewage treatment plant discharges upon growth, survival and density of Asiatic clams. Hydrobiologia 218, 113-126.

Belanger, S.E., Farris, J.L., Cherry, D.S., Cairns, J.Jr., 1985. Sediment preference of the freshwater Asiatic clam, Corbicula fluminea. Nautilus 99(2-3), 66-73.

Blanco, P., Machado, A., Satrustegui, J., 1987. Variations due to hyperoxia and ageing in the activities of glutathione S-transferase and NADPH-cytochrome c reductase. Mech. Ageing Dev. 39, 11-19.

Britton, J.C., Morton, B., 1979. Corbicula in North America: the evidence reviewed and evaluated. In: Britton, J.C., Mattice, J.S., Murphy, C.E., Newland, L.W. (Eds.), Proceedings, First International Corbicula Symposium. Texas Christian University Research Foundation Publication, Fort Worth, Texas, pp. 249-287.

Bucheli, T.D., Fent, K., 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Crit. Rev. Environ. Sci. Technol. 25(3), 201-268.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 50, 302-310.

Cancio, I., Ibabe, A., Cajaraville, M.P., 1999. Seasonal variation of peroxisomal enzyme activities and peroxisomal structure in mussels Mytilus galloprovincialis and its relationship with the lipid content. Comp. Biochem. Physiol. 123C, 135-144.

Clairborne, A., 1985. Catalase activity. In: Greenwald R.A. (Ed.), Handbook of Methods for Oxygen Radical Research. CRC press, Boca Raton, Florida, pp. 283-284.

Cossu, C., Doyotte, A., Jacquin, M.C., Babut, M., Exinger, A., Vasseur, P., 1997. Glutathione reductase, selenium-dependent glutathione peroxidase, glutathione levels, and lipid peroxidation in freshwater bivalves, Unio tumidus, as biomarkers of aquatic contamination in field studies. Ecotoxicol. Environ. Saf. 38, 122-131.

Doherty, F.G., 1990. The Asiatic clam, Corbicula spp., as a biological monitor in freshwater environments. Environ. Monit. Assess. 15, 143-181.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95.

Escartín, E., Porte, C., 1997. The use of cholinesterase and carboxylesterase activities from Mytilus galloprovincialis in pollution monitoring. Environ. Toxicol. Chem. 16(10), 2090-2095.

Fisher, S.W., Hwang, H., Atanasoff, M., Landrum, P.F., 1999. Lethal body residues for pentachlorophenol in zebra mussels (Dreissena polymorpha) under varying conditions of temperature and pH. Ecotoxicol. Environ. Saf. 43, 274-283.

Fitzpatrick, P.J., Sheehan, D., 1993. Separation of multiple forms of glutathione S-transferase from the blue mussel, Mytilus edulis. Xenobiotica 23, 851-861.

Fitzpatrick, P.J., Krag, T.O.B., Højrup, P., Sheehan, D., 1995. Characterization of a glutathione S-transferase and a related glutathione-binding protein from gill of the blue mussel, Mytilus edulis. Biochem. J. 305, 145-150.

Fontan, B., Meny, J., 1996. Note sur l'invasion de Corbicula fluminea dans le réseau hydrographique de la région Aquitaine et précision sur son spectre écologique. Préfecture de la région Aquitaine, Direction Régionale de l'Environnement Aquitaine, Service de l'Eau et des Milieux Aquatiques Publ., Aquitaine.

Graney, R.L.Jr., Cherry, D.S., Cairns, J.Jr., 1984. The influence of substrate, pH, diet and temperature upon cadmium accumulation in the Asiatic clam (Corbicula fluminea) in laboratory artificial streams. Water Res. 18(7), 833-842.

Guengerich, F.P., 1994. Analysis and characterization of enzymes. In: Hayes A.W. (Ed.), Principles and Methods of Toxicology, third edition. Raven Press, New-York, pp. 1269-1270.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249(22), 7130-7139.

Heinonen, J., Kukkonen, J., Penttinen, O-P., Holopainen, I.J., 1997. Effects of hypoxia on valve-closure time and bioaccumulation of 2,4,5-trichlorophenol by the freshwater clam Sphaerium corneum [L.]. Ecotoxicol. Environ. Saf. 36, 49-56.

Inza, B., Ribeyre, F., Maury-Brachet, R., Boudou, A., 1997. Tissue distribution of inorganic mercury, methylmercury and cadmium in the Asiatic clam (Corbicula fluminea) in relation to the contamination levels of the water column and sediment. Chemosphere 35(12), 2817-2836.

Jimenez, B.D., Burtis, L.S., 1989. Influence of environmental variables on the hepatic mixed-function oxidase system in bluegill sunfish, Lepomis macrochirus. Comp. Biochem. Physiol. 93C(1), 11-21.

Johnson, P.D., McMahon, R.F., 1998. Effects of temperature and chronic hypoxia on survivorship of the zebra mussel (Dreissena polymorpha) and Asian clam (Corbicula fluminea). Can. J. Fish. Aquat. Sci. 55, 1564-1572.

Ketterer, B., Meyer, D.J., Clark, A.G., 1988. Soluble glutathione transferase isozymes. In: Ketterer, B., Sies, H. (Eds.), Glutathione Conjugation, Mechanisms and Biological Significance. Academic Press, London, pp. 73-135.

Kirchin, M.A., Wiseman, A., Livingstone, D.R., 1992. Seasonal and sex variation in the mixed-function oxygenase system of digestive gland microsomes of the common mussel, Mytilus edulis L. Comp. Biochem. Physiol. 101C, 81-91.

Labrot, F., Ribera, D., Saint-Denis, M., Narbonne, J-F., 1996. In vitro and in vivo studies of potential biomarkers of lead and uranium contamination: lipid peroxidation, acetyl-cholinesterase, catalase and glutathione peroxidase activities in three non-mammalian species. Biomarkers 1, 23-30.

Livingstone, D.R., 1993. Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. J. Chem. Technol. Biotechnol. 57, 195-211.

Livingstone, D.R., Kirchin, M.A., Wiseman, A., 1989. Cytochrome P-450 and oxidative metabolism in molluscs. Xenobiotica 19(10), 1041-1062.

Livingstone, D.R., Garcia Martinez, P., Michel, X., Narbonne, J.F., O'Hara, S., Ribera, D., Winston, G.W., 1990. Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, Mytilus edulis L., and other molluscs. Funct. Ecol. 4, 415-424.

Livingstone, D.R., Chipman, J.K., Lowe, D.M., Minier, C., Mitchelmore, C.L., Moore, M.N., Peters, L.D., Pipe, R.K., 2000. Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates: recent molecular, genotoxic, cellular and immunological studies on the common mussel (Mytilus edulis L.) and other mytilids. Int. J. Environ. Pollut. 13(1-6), 56-91.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Machala, M., Nezveda, K., Petcivalský, M., Jarošová, A.B., Piaèka, V., Svobodová, Z., 1997. Monooxygenase activities in carp as biochemical markers of pollution by polycyclic and polyhalogenated aromatic hydrocarbons: choice of substrates and effects of temperature, gender and capture stress. Aquat. Toxicol. 37, 113-123.

Masthanamma, P., Jayasundaramma, B., Ramamurthi, R., 1987. Metabolic response to altered pH of the ambient in the freshwater mussels Lamellidens marginalis (Lamark) and Perreysia rugosa (Gmelin). Adv. Aquat. Biol. Fish. 81-88.

Mattice, J.S., Dye, L.L., 1976. Thermal tolerance of the adult Asiatic clam. In: Esch, G.W., McFarlane, R.W. (Eds.), Thermal Ecology II. United States Energy Research and Development Administration Symposium Series, Washington, D.C., pp. 130-135.

McMahon, R.F., 1979. Response to temperature and hypoxia in the oxygen consumption of the introduced Asiatic freshwater clam Corbicula fluminea (Müller). Comp. Biochem. Physiol. 63A, 383-388.

Mora, P., Fournier, D., Narbonne, J.F., 1999a. Cholinesterases from the marine mussels Mytilus galloprovincialis Lmk. and M. edulis L. and from the freshwater bivalve Corbicula fluminea Müller. Comp. Biochem. Physiol. Part C 122, 353-361.

Mora P., Michel, X., Narbonne, J.F., 1999b. Cholinesterase activity as potential biomarker in two bivalves. Environ. Toxicol. Pharmacol. 7, 253-260.

Mouthon, J., 1981. Sur la présence en France et au Portugal de Corbicula (Bivalvia, Corbiculidae) originaire d'Asie. Basteria 45, 109-116.

Narbonne, J.F., Djomo, J.E., Ribera, D., Ferrier, V., Garrigues, P., 1999. Accumulation kinetics of polycyclic aromatic hydrocarbons adsorbed to sediment by the mollusk Corbicula fluminea. Ecotoxicol. Environ. Saf. 42, 1-8.

Neff, J.M., 1984. Bioaccumulation of organic micropollutants from sediments and suspended particulates by aquatic animals. Fresenius Z. Anal. Chem. 319, 132-136.

Oliveira-Filho, E.C., De-Carvalho, R.R., Paumgartten, F.J.R., 1999. The influence of environmental factors on the molluscicidal activity of Euphorbia milii latex. J. Environ. Sci. Health B34(2), 289-303.

Pannunzio, T.M., Storey, K.B., 1998. Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod Littorina littorea. J. Exp. Mar. Biol. Ecol. 221, 277-292.

Parant, M., 1998. Le métabolisme des xénobiotiques chez les bivalves. C. R. Acad. Sci. Paris, Sciences de la vie 321, 485-494.

Porte, C., Lemaire, P., Peters, L.D., Livingstone, D.R., 1995. Partial purification and properties of cytochrome P450 from digestive gland microsomes of the common mussel, Mytilus edulis L. Mar. Environ. Res. 39, 27-31.

Power, A., Sheehan, D., 1996. Seasonal variation in the antioxidant defence systems of gill and digestive gland of the blue mussel, Mytilus edulis. Comp. Biochem. Physiol. 114C(2), 99-103.

Regoli, F., 1998. Trace metals and antioxidant enzymes in gills and digestive gland of the Mediterranean mussel Mytilus Galloprovincialis. Arch. Environ. Contam. Toxicol. 34, 48-63.

Schubauer-Berigan, M.K., Dierkes, J.R., Monson, P.D., Ankley, G.T., 1993. pH-dependent toxicity of Cd, Cu, Ni, Pb and Zn to Ceriodaphnia dubia, Pimephales promelas, Hyalella azteca and Lumbriculus variegatus. Environ. Toxicol. Chem. 12, 1261-1266.

Sheehan, D., Power, A., 1999. Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. Comp. Biochem. Physiol. 123C, 193-199.

Sickel, J.B., 1986. Corbicula population mortalities: factors influencing population control. Am. Malacol. Bull., Special Edition 2, 89-94.

Stegeman, J.J., 1979. Temperature influence on basal activity and induction of mixed function oxygenase activity in Fundulus heteroclitus. J. Fish. Res. Board Can. 36, 1400-1404.

Viarengo, A., Canesi, L., Pertica, M., Livingstone, D.R., 1991. Seasonal variations in the antioxidant defence systems and lipid peroxidation of the digestive gland of mussels. Comp. Biochem. Physiol. 100C (1/2), 187-190.

Vidal, M-L., Narbonne, J-F., 2000. Characterization of glutathione S-transferase activity in the Asiatic clam Corbicula fluminea. Bull. Environ. Contam. Toxicol. 64, 455-462.

Vidal, M-L., Bassères, A., Narbonne, J-F., 2001. Potential biomarkers of trichloroethylene and toluene exposure in Corbicula fluminea. Environ. Toxicol. Pharmacol. 9, 87-97.

Way, C.M., Hornbach, D.J., Miller-Way, C.A., Payne, B.S., Miller, A.C., 1990. Dynamics of filter feeding in Corbicula fluminea (Bivalvia: Corbiculidae). Can. J. Zool. 68, 115-120.

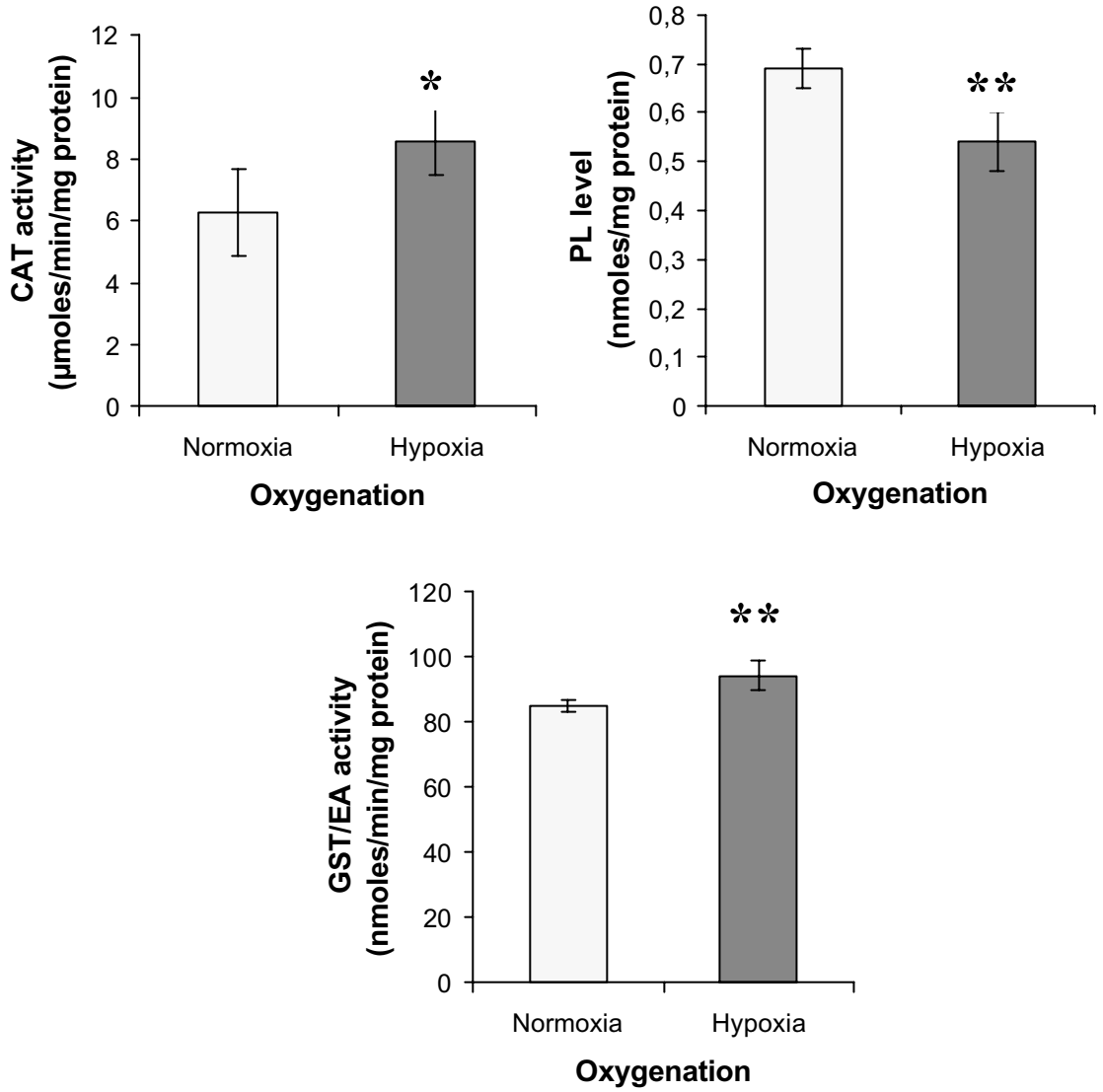


Figure 1. Biochemical parameters exhibiting significant responses compared to control (normoxia) after exposure of *Corbicula fluminea* to hypoxia: catalase (CAT) activity, level of peroxided lipids (PL) and glutathione *S*-transferase activity towards EA (GST/EA). Values are mean ± S.D. (n=5). Significant effect at p 0.05: * and p 0.01: **.

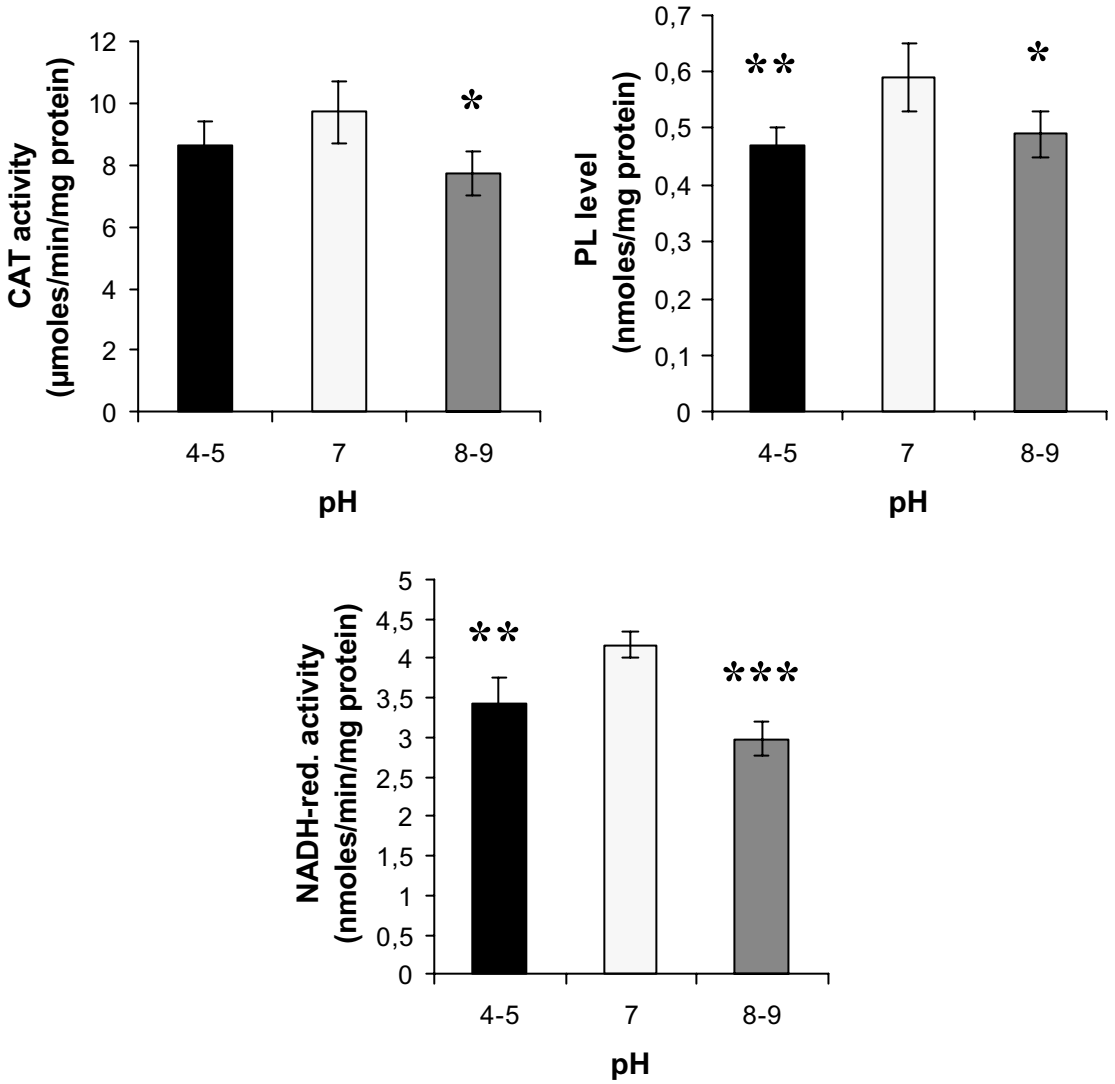


Figure 2. Biochemical parameters exhibiting significant responses compared to control (pH 7) after exposure of *Corbicula fluminea* to different pH conditions: catalase (CAT) activity, level of peroxided lipids (PL) and NADH-cytochrome *c* reductase (NADH-red.) activity. Values are mean ± S.D. (n=5). Significant effect at p 0.05: *, p 0.01: ** and p 0.001: ***.

Table 1. Responses of biochemical parameters to different experimental conditions of temperature, water-type and substrate. Values are mean ± S.D. (n=5).

Experimental conditions			Biochemical parameters						
Temperature	Water-type	Substrate	CAT [a]	PL [b]	GST/CDNB [c]	GST/EA [c]	NADPH-red. [c]	NADH-red. [c]	PChE [c]
10°C	Reverse-osmosis	-	9.6 ± 1.0	0.55 ± 0.05	701.1 ± 44.7	83.9 ± 3.1	1.67 ± 0.26	2.27 ± 0.16	3.33 ± 0.70
		Sand	7.8 ± 0.7	0.65 ± 0.06	684.1 ± 65.9	87.6 ± 5.6	1.52 ± 0.14	2.21 ± 0.15	4.20 ± 1.06
	Lake	-	7.7 ± 1.0	0.64 ± 0.04	648.5 ± 40.0	80.6 ± 5.8	2.08 ± 0.06	2.59 ± 0.21	3.65 ± 0.51
		Sand	6.8 ± 1.2	0.52 ± 0.04	651.3 ± 63.5	81.0 ± 6.7	1.56 ± 0.05	2.28 ± 0.35	3.20 ± 0.53
	Tap	-	7.5 ± 1.5	0.70 ± 0.10	680.8 ± 43.3	84.8 ± 6.1	1.87 ± 0.16	3.02 ± 0.32	4.24 ± 0.81
		Sand	8.5 ± 1.5	0.52 ± 0.03	736.8 ± 59.9	98.9 ± 4.5	1.97 ± 0.23	2.74 ± 0.14	4.19 ± 0.58
20°C	Reverse-osmosis	-	8.6 ± 1.3	0.53 ± 0.03	727.7 ± 36.0	87.6 ± 3.5	1.84 ± 0.16	2.57 ± 0.11	3.64 ± 0.48
		Sand	5.3 ± 0.1	0.51 ± 0.06	660.2 ± 41.4	94.1 ± 3.3	2.29 ± 0.37	2.32 ± 0.40	3.32 ± 0.25
	Lake	-	6.3 ± 1.4	0.69 ± 0.04	666.7 ± 39.3	84.7 ± 1.8	1.73 ± 0.41	2.29 ± 0.47	3.56 ± 0.89
		Sand	4.7 ± 0.8	0.67 ± 0.03	636.3 ± 30.6	86.5 ± 9.5	1.73 ± 0.23	2.22 ± 0.36	3.04 ± 0.56
	Tap	-	9.3 ± 1.2	0.48 ± 0.02	662.0 ± 33.8	89.7 ± 2.8	2.18 ± 0.20	2.89 ± 0.24	4.79 ± 0.89
		Sand	9.8 ± 2.2	0.58 ± 0.06	646.7 ± 66.5	80.5 ± 8.8	2.31 ± 0.26	2.78 ± 0.39	8.07 ± 0.49

[a, b, c] In μmoles/min/mg protein, nmoles/mg protein and nmoles/min/mg protein, respectively.

Table 2. Effects of abiotic factors (temperature, water-type, substrate and their interactions, oxygenation and pH) on biochemical parameter responses as given by ANOVA and MANOVA analyses. Significant effect or interaction at p 0.05: *, p 0.01: ** and p 0.001: ***; non-significant effect or interaction: ns.

Biochemical parameter	Abiotic factor effect								
	Temp.	Water	Subst.	Temp./Water	Temp./Subst.	Water/Subst.	Temp./Water/Subst.	Oxygen.	pH
CAT	ns	***	**	***	ns	**	ns	*	*
PL	ns	***	ns	***	**	*	***	**	**
GST/CDNB	ns	ns	ns	ns	ns	ns	ns	ns	ns
GST/EA	ns	*	ns	**	ns	ns	**	**	ns
NADPH-red.	**	**	ns	**	**	*	ns	ns	ns
NADH-red.	ns	***	*	ns	ns	ns	ns	ns	***
PChE	**	***	*	***	ns	***	***	ns	ns

Table 3. Index of variability (calculated as VI = maximal mean / minimal mean) of biochemical parameters of *Corbicula fluminea* exposed to different experimental conditions of temperature, water-type, substrate, oxygenation and pH.

Experimental conditions		Biochemical parameters [a]							**ΣVI** [g]
		CAT [b]	PL [c]	GST/CDNB [d]	GST/EA [d]	NADPH-red. [d]	NADH-red. [d]	PChE [d]	
Temperature [e]	*10°C*	8.0 ± 0.9	0.60 ± 0.07	683.8 ± 30.1	86.1 ± 6.2	1.78 ± 0.21	2.52 ± 0.29	3.80 ± 0.43	
	20°C	7.3 ± 2.0	0.58 ± 0.08	666.6 ± 29.2	87.2 ± 4.2	2.01 ± 0.25	2.51 ± 0.25	4.40 ± 1.73	
VI		**1.09**	**1.04**	**1.03**	**1.01**	**1.13**	**1.00**	**1.16**	***7.46***
Water-type [e]	*ROP*	7.8 ± 1.6	0.56 ± 0.05	693.3 ± 24.6	88.3 ± 3.7	1.83 ± 0.29	2.34 ± 0.14	3.62 ± 0.36	
	Lake	6.4 ± 1.1	0.63 ± 0.07	650.7 ± 10.8	83.2 ± 2.5	1.78 ± 0.19	2.35 ± 0.14	3.36 ± 0.25	
	Tap	8.8 ± 0.9	0.57 ± 0.08	681.6 ± 34.1	88.5 ± 6.8	2.08 ± 0.17	2.86 ± 0.11	5.32 ± 1.60	
VI		**1.38**	**1.13**	**1.07**	**1.06**	**1.17**	**1.22**	**1.58**	***8.61***
Substrate [e]	-	8.2 ± 1.1	0.60 ± 0.08	681.1 ± 26.5	85.2 ± 2.9	1.90 ± 0.18	2.61 ± 0.28	3.87 ± 0.50	
	Sand	7.2 ± 1.8	0.58 ± 0.06	669.2 ± 33.6	88.1 ± 6.6	1.90 ± 0.32	2.43 ± 0.24	4.34 ± 1.73	
VI		**1.14**	**1.03**	**1.02**	**1.03**	**1.00**	**1.07**	**1.12**	***7.41***
Oxygenation	*Normoxia*	6.3 ± 1.4	0.69 ± 0.04	666.7 ± 39.3	84.7 ± 1.8	1.73 ± 0.41	2.29 ± 0.47	3.56 ± 0.89	
	Hypoxia	8.6 ± 1.1	0.54 ± 0.06	652.2 ± 25.0	94.2 ± 4.6	1.82 ± 0.06	2.21 ± 0.35	3.74 ± 0.41	
VI		**1.37**	**1.28**	**1.02**	**1.11**	**1.05**	**1.04**	**1.05**	***7.92***
pH	*4-5*	8.6 ± 0.8	0.47 ± 0.03	737.6 ± 43.7	96.4 ± 7.4	2.91 ± 0.20	3.42 ± 0.32	2.31 ± 0.36	
	7	9.7 ± 1.0	0.59 ± 0.06	690.8 ± 29.8	95.6 ± 9.4	2.65 ± 0.17	4.17 ± 0.16	2.45 ± 0.34	
	8-9	7.7 ± 0.7	0.49 ± 0.04	676.8 ± 46.9	90.7 ± 2.8	2.74 ± 0.08	2.98 ± 0.21	2.11 ± 0.30	
VI		**1.26**	**1.26**	**1.09**	**1.06**	**1.10**	**1.40**	**1.16**	***8.33***
ΣVI [f]		***6.24***	***5.74***	***5.23***	***5.27***	***5.45***	***5.73***	***6.07***	

[a] Values are mean ± S.D (n=6, 4, 6, 5, 5 for temperature, water-type, substrate, oxygenation and pH, respectively).

[b, c, d] In µmoles/min/mg protein, nmoles/mg protein and nmoles/min/mg protein, respectively.

[e] Averages of all exposures for the considered abiotic factor.

[f, g] Sum of VI for each biochemical parameter and abiotic factor, respectively.

CHAPITRE III

Etude en laboratoire des effets du trichloroéthylène, du toluène, du chlorure de cadmium et d'une coupe de goudron de houille

&

Etude de terrain

Ce troisième chapitre est consacré en partie à l'étude en laboratoire des effets individuels de plusieurs contaminants modèles: le trichloroéthylène (TCE), le toluène (TOL), le cadmium (Cd, sous forme de $CdCl_2$) et une coupe de goudron de houille (GH, un mélange complexe d'hydrocarbures aromatiques polycycliques). Ces résultats sont présentés dans les **articles n°5** (TCE et TOL) et **n°6** (Cd et GH). Dans des conditions statiques, des *Corbicula fluminea* ont été exposées pendant cinq jours à quatre doses subléthales de TCE (de 1,56 à 100 mg/l), de TOL (de 7,5 à 60 mg/l), de Cd (0,154, 0,308, 0,615 et 1,230 mg/l) ou de GH (0,75, 1,5, 3 et 6 mg/l) *via* la colonne d'eau. Dans une approche multibiomarqueurs, les réponses de composants du métabolisme de (dé)toxication des phases I et II, de paramètres associés au stress oxydatif et de l'activité propionylcholinestérase ont été mesurées. A l'issue de l'exposition, des analyses chimiques de l'eau ont révélé une disparition partielle ou complète des polluants, représentative d'une contamination aiguë et ponctuelle. En combinant les résultats d'analyses de variance, de tests de Tukey et d'analyses discriminantes, les biomarqueurs les plus pertinents se sont avérés être les teneurs en cytochromes P450 et P418, les activités NADH-cytochrome *c* réductase et catalase, les teneurs en lipides peroxydés et peroxydables et le taux de peroxydation nette pour le TCE et le TOL; les teneurs en cytochromes P450 et P418, les activités NADPH-cytochrome *c* réductase et NADH-cytochrome *c* réductase, le taux de peroxydation nette pour le GH ainsi que l'activité catalase, l'activité glutathion *S*-transférase envers le 1-chloro-2,4-dinitrobenzène et le taux de peroxydation nette pour le Cd. Ces études ont souligné la persistance d'effets biochimiques malgré la disparition partielle ou complète des contaminants ainsi que l'importance d'une approche multibiomarqueurs dans les programmes de surveillance environnementale. Afin de mettre en valeur de tels aspects, une étude de terrain conduite sur un site industriel est présentée dans **l'article n°7**.

ARTICLE 5

Potential biomarkers of trichloroethylene and toluene exposure in *Corbicula fluminea*

Vidal, M-L., Bassères, A., Narbonne J-F.

Environmental Toxicology and Pharmacology, 2001, 9, 87-97.

ELSEVIER

Environmental Toxicology and Pharmacology 9 (2001) 87–97

ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY
ETAP

www.elsevier.com/locate/etap

Potential biomarkers of trichloroethylene and toluene exposure in *Corbicula fluminea*

Mary-Laure Vidal [a,*], Anne Bassères [b], Jean-François Narbonne [a]

[a] *Laboratoire de Physico-Toxicochimie des Systèmes Naturels (LPTC), UPRES-A 5472 CNRS, Département de Toxicologie Biochimique, Université Bordeaux 1, Avenue des Facultés, 33405 Talence, France*

[b] *ELF, Groupement de Recherches de Lacq, Service Environnement, BP 34, 64170 Lacq, France*

Received 31 May 2000; received in revised form 20 October 2000; accepted 3 November 2000

Abstract

Freshwater clams *Corbicula fluminea* were exposed in aquariums to four doses of trichloroethylene-TCE-(1.56 up to 100 mg/l) or toluene-TOL-(7.5 up to 60 mg/l) for 5 days. At the end of exposure, components of (de)toxification metabolism of phases I and II, parameters related to oxidative stress and propionylcholinesterase activity were assayed. Determination of TCE and TOL concentrations in water revealed an important evaporative loss during the experiment, characteristic of acute and occasional contaminations by such products occurring in the environment. Appropriate statistical methods such as ANOVA, Tukey test and discriminant analysis underlined the relevance of cytochromes *P*450 and *P*418, NADH-cytochrome *c* reductase, catalase, peroxided and peroxidizable lipids and net peroxidation as biomarkers of exposure to these solvents in *C. fluminea*. This experiment emphasised the importance of a multi-biomarker approach in environmental surveys and will be completed further by mesocosm studies.

Keywords: Biomarkers; *Corbicula fluminea*; Microcosms; Toluene; Trichloroethylene

1. Introduction

Organic solvents such as trichloroethylene (TCE) and toluene (TOL) are used widely for both industrial and domestic applications. Today, the major use of TCE, a chlorinated aliphatic hydrocarbon, is in metal cleaning or degreasing (International Agency for Research on Cancer — IARC, 1995). TOL, an aromatic solvent, is produced extensively and employed in a broad spectrum of applications, primarily as a solvent, as a component in gasoline and in the production of benzene (Fishbein, 1985). Both solvents have been detected mainly in air owing to their volatile properties but also at low levels in water supplies and frequently in groundwater. TCE and TOL concentrations of 0.1 up to 2.4 and of 0.8 μg/l, respectively, have been reported in the Rhine river in Germany (IARC, 1989, 1995). Higher levels were also reported, up to 10 000 μg/l of TCE in the groundwater of Japan near an electronics factory (IARC, 1995) or 42 up to 100 μg/l of TOL in well water in the vicinity of landfill sites in the US (IARC, 1989). TCE and TOL have become common environmental contaminants and, therefore, studies of their impact on freshwater aquatic ecosystems are requisite.

Filtering organisms as bivalves and especially mussels are able to bioconcentrate organic compounds in their tissues and so are used widely as sentinel organisms to assess the concentration of pollutants in aquatic ecosystems. Moreover, measuring responses of various biochemical parameters — or biomarkers — in these organisms provides an additional tool to evaluate effects of chronic and acute contaminations on the environment. Thus, the detection of sublethal and early effects can allow a preventive approach (Livingstone, 1993). As *Mytilus edulis* and *M. galloprovincialis* are used currently as sentinel organisms in seawater ecosystems (Solé et al., 1998; Narbonne et al., 1999a), *Corbicula fluminea* (Müller) could be their counterpart in freshwater ecosystems. This bivalve originating from

* Corresponding author. Tel.: +33-556-842456; fax: +-33-556-848719.

E-mail address: ml.vidal@lptc.u-bordeaux.fr (M.-L. Vidal).

1382-6689/01/$ - see front matter
PII: S1382-6689(00)00068-5

Asia has become a major component of many benthic communities in the US and, more recently, in Europe (Araujo et al., 1993). It is known to accumulate contaminants such as heavy metals (Baudrimont et al., 1997) or polycyclic aromatic hydrocarbons (Narbonne et al., 1999b). In order to validate this mollusc as a sentinel organism for the biomonitoring of freshwater ecosystems, it is important to study responses of several potential biomarkers after exposure to common environmental contaminants. In such a validation step, microcosm experiments in laboratory constitute prerequisite studies.

Thus, aims of this work were (i) to get preliminary information concerning toxic effects of TCE and TOL in *C. fluminea* for little is known about them in molluscs; (ii) to evaluate the potential utility of several biochemical parameters as biomarkers of exposure to TCE and TOL in this clam for further applications in environmental biomonitoring. In a static laboratory study, we exposed *C. fluminea* to four doses of either solvent during 5 days. We selected high concentrations with no renewal during the experiment to be representative of acute, occasional contaminations occurring in the vicinity of urban or industrial areas. At the end of exposure, biochemical parameters related to TCE or TOL toxicity mechanisms were measured. Two pathways are involved in TCE metabolism in mammals. The first and the prevailing pathway is the oxidative hepatic metabolism of TCE by cytochrome *P*450 enzymes leading to a proposed epoxide intermediate, which rearranges spontaneously to chloral. Chloral is then metabolised to trichloroethanol, trichloroethanol glucuronide and trichloroacetic acid (TCA). Some minor metabolites including dichloroacetic acid (DCA) are also produced (Goeptar et al., 1995). Metabolites such as TCA or DCA are known to induce oxidative stress in mammals (Larson and Bull, 1992). Induction of *P*450 level (Kawamoto et al., 1988) but also inactivation (Miller and Guengerich, 1983) have been reported in mammals. The second and the minor pathway involves conjugation of TCE with glutathione and further metabolism by the mercapturic acid pathway (Goeptar et al., 1995). TOL has a different structure to TCE, except in having an unsaturated carbon skeleton. As it is the case for TCE, TOL is also metabolised in mammals by *P*450 enzymes and could influence the relative amounts of these enzymes via induction or destruction (Nakajima and Wang, 1994). Its methyl group is oxidised by cytochrome *P*450 enzymes to benzylalcohol, which is then oxidised to benzaldehyde by alcohol dehydrogenase. Benzaldehyde was reported to be the metabolite responsible for the acceleration of the production of reactive oxygen species in rats (Mattia et al., 1991, 1993) and also for the destruction of some *P*450 enzymes via the formation of benzaldehyde-heme adducts, especially in the lung (Furman et al., 1998). Once synthesised, benzaldehyde is oxidised rapidly by aldehyde dehydrogenase to benzoic acid, the majority of which is conjugated with glycine and excreted in the urine as hippuric acid. To a minor extent, TOL is also converted by *P*450 enzymes to *o*- and *p*-cresol, which in turn are conjugated with sulphate of glucuronic acid and excreted in the urine (Mattia et al., 1993; Von Burg, 1993; Nakajima and Wang, 1994). TOL can also exert its toxicity through the inhibition of acetylcholinesterase activity (Korpela and Tähti, 1988). With respect to these toxic effects in mammals, the following biochemical parameters — already reported in *C. fluminea* or more generally in molluscs — were selected in our study, namely, components of phase I metabolism such as cytochromes *P*450, *P*418 and *b*5 (*P*450, *P*418 and *b*5, respectively), NADPH — cytochrome *P*450 reductase (NADPH-red) and NADH-cytochrome *b*5 reductase (NADH-red) activities, NADPH-independent ethoxyresorufin-*O*-deethylase (NADPH-ind. EROD) activity (Livingstone et al., 1989); components of phase II metabolism such as glutathione *S*-transferase activity using 1-chloro-2,4-dinitrobenzene (GST/CDNB) or ethacrynic acid (GST/EA) as a substrate (Fitzpatrick and Sheehan, 1993; Vidal and Narbonne, 2000); parameters related to oxidative stress such as catalase (CAT) activity, levels of peroxided and peroxidizable lipids (PL and PLI, respectively) and level of net peroxidation (NP) (Livingstone et al., 1992); propionylcholinesterase (PChE) activity (Mora et al., 1999a,b).

2. Materials and methods

2.1. Animal sampling and acclimatisation

One thousand adult *C. fluminea* were collected from the banks of the non-polluted freshwater Cazaux-Sanguinet lake (Aquitaine, France) in late September, 1998. They were at the beginning of the rest phase of their reproductive cycle. Thus, no embryo was detected in the gills and few oocytes were present in the gonadal tissue. No sexual differences were taken into account for Asiatic clams are hermaphroditic. Two plastic tanks each containing 500 *C. fluminea* in 50 l of lake water were used for the transport back to the laboratory and for an 8-day acclimatisation period. Two hundred litre of this water were sampled for the experiment. During transport and acclimatisation period, aeration was provided continuously by a diffuser connected to an air pump (Shego M2K3). The temperature was maintained at 20 ± 0.1°C in the laboratory. Clams were not fed to clear out gut content and were held under a natural light cycle.

2.2. Experimental design

The experiment was performed for 5 days in ten glass aquariums containing 10 l of lake water previously filtered through 50 μm and then 5 μm cotton cartridges using a Cole–Parmer Masterflex pump (model 7019-20, Cole–Parmer Instrument Co.). Filtration was performed to avoid adsorption of pollutants to particles and, thus, to privilege water-column contamination. The lake water can be considered of good quality for the Sanguinet lake is known to be a non polluted site (unpublished data and personal communication from the Centre National du Machinisme Agricole, du Génie Rural, des Eaux et des Forêts — CEMAGREF, France). In a preliminary study, sublethal doses of trichloroethylene (TCE) (SDS, Peypin, France) and toluene (TOL) (Carlo Erba, Rueil Malmaison, France) had been established for *C. fluminea*. According to these results, four doses were set for the experiment, 1.56, 6.25, 25 and 100 mg/l for TCE; 7.5, 15, 30 and 60 mg/l for TOL. As these products were hydrosoluble in the range of doses assayed, they were directly dissolved in the filtered lake water. Controls consisted in two aquariums each containing 10 l of filtered lake water. Aquariums were covered to limit water and/or product evaporation. Fifty *C. fluminea* were immersed in each aquarium. They were not fed and were held under a natural light cycle during the experiment. The temperature was maintained at 20 ± 0.1°C in the laboratory. Aeration was provided by intermittent air bubbling. A diffuser connected to an air pump (Shego M2K3) released air during 15 min every 12 h in each aquarium, included controls. We opted for this frequency to limit evaporative loss of solvents. Temperature, pH, conductivity and percentage of oxygen saturation were checked in each aquarium during the exposure (Universal Pocket Meter Multiline P4, WTW). No mortality occurred during the experiment, thus, no dead clam could have fouled the water.

2.3. Chemical analysis

At the beginning and the end of the exposure, samples of 250 ml of water (contaminated or not) were collected and stored in amber glass flasks filled to avoid air bubbles at 4°C. Chemical analysis were performed in the Laboratory of Control and Environment of Elf Aquitaine (Lacq, France). Measurements were carried out in triplicates. Water samples were injected directly without previous extraction. Determination of TCE concentrations was performed by gas chromatography coupled to mass spectrometry (GC–MS) with a Hewlett–Packard Model 5973 in selected ion monitoring (SIM) mode. Sample (0.5 μl) was injected on a dimethylpolysiloxane column (30 m, I.D. 0.32 mm, Hewlett–Packard). Ionisation was performed under electronic impact (70 eV). Temperatures of the injector, the source and the oven were 250, 150, and 90°C, respectively. Limits of detection and quantification were 0. 03 and 0. 1 mg/l, respectively. Determination of TOL concentrations was performed by high performance liquid chromatography (HPLC with a Hewlett–Packard Model 1090M. Twenty microlitres of sample was injected on a 250 × 4.6 mm C18 column (Chrompack) with acetonitrile–water (85/15%) as the mobile phase. Toluene was detected by way of a diode array detector at a wavelength of 210 nm. The flow rate was 1 ml/min. Limits of detection and quantification were 0.3 and 1 mg/l, respectively.

2.4. Preparation of subcellular fractions

At the end of the 5-day exposure, *C. fluminea* were collected and submitted to homogenisation steps at 4°C. After the shell and the crystalline style were removed, clams were dissected out to obtain entire soft bodies or visceral masses. For each experimental condition, five pools of five *C. fluminea* were prepared. Body samples were rinsed in 100 mM phosphate buffer (K_2HPO_4/KH_2PO_4), pH 7.4, dried on absorbent paper sheets, weighed and homogenised in the same phosphate buffer (1:4 weight:volume ratio) using an Ultra-Turrax Antrieb T25 (IKA Labortechnik). Entire soft bodies and visceral masses were homogenised at 13 500 and 8000 rd/min, respectively. Homogenates were then centrifuged at $9000 \times g$ for 30 min in a Sigma 3MK centrifuge. Submitochondrial fractions (S9) of soft entire bodies were then stored at −80°C. Those of visceral masses were centrifuged at $105\,000 \times g$ for 1 h in a Beckman LE-80 ultracentrifuge. Cytosolic fractions were then stored at −80°C. Microsomal pellets were resuspended in a 100 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 20% glycerol and stored at −80°C.

2.5. Biochemical assays

Measurements of enzymatic activities were carried out in duplicates or in triplicates for each pool at 25°C except for GST/CDNB, GST/EA, NADPH-red and NADH-red activities (30°C). All biochemical measurements were performed on a dual-beam temperature-controlled Kontron Uvikon 932 spectrophotometer except for NADPH-ind. EROD activity assay, which was carried out on a Shimadzu RF-540 spectrofluorophotometer. All assays were performed in S9 of soft entire bodies except for GST/CDNB and GST/EA activities (cytosol of visceral masses) as well as NADPH-red and NADH-red activities (microsomes of visceral masses). *P*450, *P*418 and *b*5 levels as well as NADPH-ind. EROD activity were measured in S9 of soft entire bodies because, in *C. fluminea*, variability is

less important in this fraction than in microsomes of visceral masses.

CAT activity was determined as described in Clairborne (1985). PL level was estimated according to Buege and Aust (1978) following the appearance of thiobarbituric acid reactive species (TBARs) at 532 nm. PLI level was determined likewise but after incubation of the samples 1 h at 37°C in a reaction mixture containing an iron (II)/dihydroxyfumaric acid as peroxidation initiator (Fraisse et al., 1993). NP was estimated as the difference between PLI level (stimulated peroxidation) and PL level (basal peroxidation). Cholinesterase activity was assayed using propionylthiocholine as a substrate as in Mora et al. (1999b) according to the method of Ellman et al. (1961). GST/CDNB and GST/EA activities were assayed as in Vidal and Narbonne (2000) by a method adapted from Habig et al. (1974). *P*450 and *P*418 levels were estimated according to a method adapted from Estabrook and Werringloer (1978). b_5 level was determined similarly as described in Estabrook and Werringloer (1978) but using sodium dithionite as a reducing agent. NADPH-red was assayed as described in Guengerich (1994). NADH-red was determined likewise, replacing NADPH by NADH. These two assays are indirect measurements of NADPH-cytochrome *P*450 reductase and NADH-cytochrome b_5 reductase activities, respectively. NADPH-ind. EROD activity was measured by a direct fluorimetric assay adapted from the method of Burke and Mayer (1974). No NADPH was added to the mixture to start the assay as NADPH was found to inhibit the reaction. Indeed, one-electron oxidation and the involvement of a peroxide is considered to be a possible mechanism of molluscan cytochrome *P*450 catalytic action. Such an NADPH-independent mechanism has already been reported for benzo(*a*)pyrene hydroxylase or ethoxycoumarine-*O*-deethylase activities in mussels (Livingstone et al., 1989). All biochemical parameter responses were expressed in relation to protein concentration measured according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

2.6. Statistical analysis

Statistica software (5.1 release, Statsoft) was used for all statistical evaluations. Data distributed normally were submitted to a Brown–Forsythe test for homogeneity of variances (across dose groups). Data fulfilling this requirement were then submitted to a univariate analysis of variance (ANOVA) with 'dose' (of TCE or TOL) as the independent variable and biochemical parameters as dependent variables. Whenever a significant ($P \leq 0.05$) dose effect was established on a biochemical parameter response, a Tukey HSD multiple comparison test was used to check significant ($P \leq 0.05$) differences between doses. Correlations between the dose and biochemical parameter responses were determined with the Pearson correlation coefficient (significant for $P \leq 0.05$). As additional studies, discriminant analysis (DA), often referred to as canonical variate analysis, were performed to try to discriminate doses (of TCE or TOL) from each other and to determine biochemical parameters allowing the best discrimination between doses. Unlike univariate analysis, multivariate analysis such as DA takes into account the whole biochemical parameter responses and, thus, provides an integrated approach. DA procedures and its applications in environmental biomonitoring have been described by Adams et al. (1994).

3. Results

3.1. Chemical analysis and parameters of water quality

Results of chemical analysis are presented in Table 1. As TCE and TOL are volatile products, an important loss was observed during the preparation of mixtures, due mainly to homogenisation (loss of 23 up to 44% for TCE and 6 up to 45% for TOL). For the following results, actual concentrations will be then considered, 1.2, 3.6, 14, 69.4 mg/l for TCE and 4.3, 8.2, 28.2 and 36.4 mg/l for TOL. After 5 days of exposure, the loss of product was considerable for both solvents, whatever the dose was.

During exposure, temperature of water was 20.7 ± 0.9°C and 20.7 ± 0.8°C for TCE and TOL, respectively, whereas pH was 7.19 ± 0.2 and 7.09 ± 0.3, respectively. Conductivity increased whereas percentage of oxygen saturation decreased according to the dose and time of exposure (control, from 193 up to 210 μS/cm and from 68 down to 48%, respectively; highest dose of TCE, from 193 up to 261 μS/cm and from 73.9 down to 31.4%, respectively; highest dose of TOL, from 194 up to 245 μS/cm and from 75.5 down to 14.3%, respectively).

3.2. Biochemical assays

Only biochemical parameters exhibiting significant responses compared with control for TCE-treated and TOL-treated *C. fluminea* will be considered. Results for CAT and NADH-red activities, PL, *P*450 and *P*418 levels for TCE-treated *C. fluminea* are given in Fig. 1. Results for CAT activity, PL, PLI, NP, *P*450 and *P*418 levels for TOL-treated *C. fluminea* are presented in Fig. 2. Degrees of significance of responses of exposed clams compared with those of control clams are given as results of a Tukey HSD multiple comparison test following an univariate ANOVA.

When *C. fluminea* were exposed to TCE, CAT activity increased significantly at the two lowest doses (+70 and +40% at 1.2 and 3.6 mg/l respectively, comparing exposed to control). This effect was no more observed at the two highest doses, as the level was similar sensibly to that of control. Another indicator of a change in the oxidative state, PL level, exhibited the same trend. It increased significantly of 29% at 1.2 mg/l and decreased slightly at the highest doses with no more significance. *P*450 and *P*418 levels as well as NADH-red activity were the only components of phase I metabolism exhibiting significant variations. *P*450 and *P*418 levels dramatically increased at 3.6 mg/l (+835 and +249%, respectively) and then decreased slightly at the two highest doses (with no more significance for *P*418 level). We observed a different trend for NADH-red activity as it decreased significantly below control at the two highest doses (−21 and −20% at 14 and 69.4 mg/l, respectively).

When *C. fluminea* were exposed to TOL, CAT activity and PL level exhibited a significant increase at the first dose (+26 and +33%, respectively) and then decreased slightly at the following doses, with no more significance. PLI and NP levels exhibited such an increase at 4.3 mg/l and in a greater extent at 8.2 mg/l (for NP level, +26 and +28%, respectively). No more significant variations were then observed at the two highest doses. *P*450 level increased according to the dose up to 28.2 mg/l (+1093%) and then decreased at 36.4 mg/l. *P*418 level followed the same trend with a significant increase at 28.2 mg/l (+240%). PChE activity exhibited a substantial decrease, though non-significant, at 4.3 mg/l and especially at 8.2 mg/l (−50%, data not shown). This effect was observed no more at the highest doses.

For both products, biochemical parameter responses were not correlated with the dose (data not shown). No dose–effect relationship could have then been established.

Synthesis of DA is given in Table 2. Biochemical parameters contributing significantly to discrimination between doses were practically the same for both solvents. *P*450 level was the parameter that contributed the most to discrimination, followed by two indicators of a modified oxidative state, namely PL level for TCE or NP level for TOL and CAT activity for both solvents. NADH-red activity was the last parameter mentioned for discrimination. The separation of groups relies on discriminating functions, which are linear combinations of the biochemical parameters. Thus, groups can be considered as entities with centroids (gravity centres) located in a space, which comprises as many dimensions as significant functions. The more the distance between two groups (the Mahalanobis distance) is high and significant, the more those entities are discriminated. These results are given in Table 3. For both solvents, the control group was well discriminated from the others. For TCE, all groups were discriminated from each other contrary to TOL, for which the first and the last dose groups were not discriminated.

Table 1
TCE and TOL concentrations in filtered lake water at the beginning (T0) and at the end (after 5 days, T5) of the exposure

Product	Theoretical concentration[a] at T0	Actual concentration[b] at T0	Loss of product during preparation[c]	Actual concentration[b] at T5	Loss of product after exposure[d]
TCE	0	<0.1[e]	–	<0.1[e]	–
	1.56	1.20 ± 0.08	23	0.10 ± 0.15	92
	6.25	3.58 ± 0.33	43	<0.1[e]	>97
	25	13.99 ± 1.82	44	0.22 ± 0.22	98
	100	69.44 ± 5.26	31	4.40 ± 0.04	94
TOL	0	<1[f]	–	<1[f]	–
	7.5	4.31 ± 0.11	43	<1[f]	>77
	15	8.19 ± 0.07	45	<1[f]	>88
	30	28.19 ± 0.82	6	<1[f]	>96
	60	36.43 ± 3.68	39	<1[f]	>97

[a] mg/l.
[b] mg/l. Values are mean ± S.D. ($n = 3$).
[c] Percent/theoretical concentration at T0.
[d] Percent/actual concentration at T0.
[e] Limit of TCE quantification.
[f] Limit of TOL quantification.

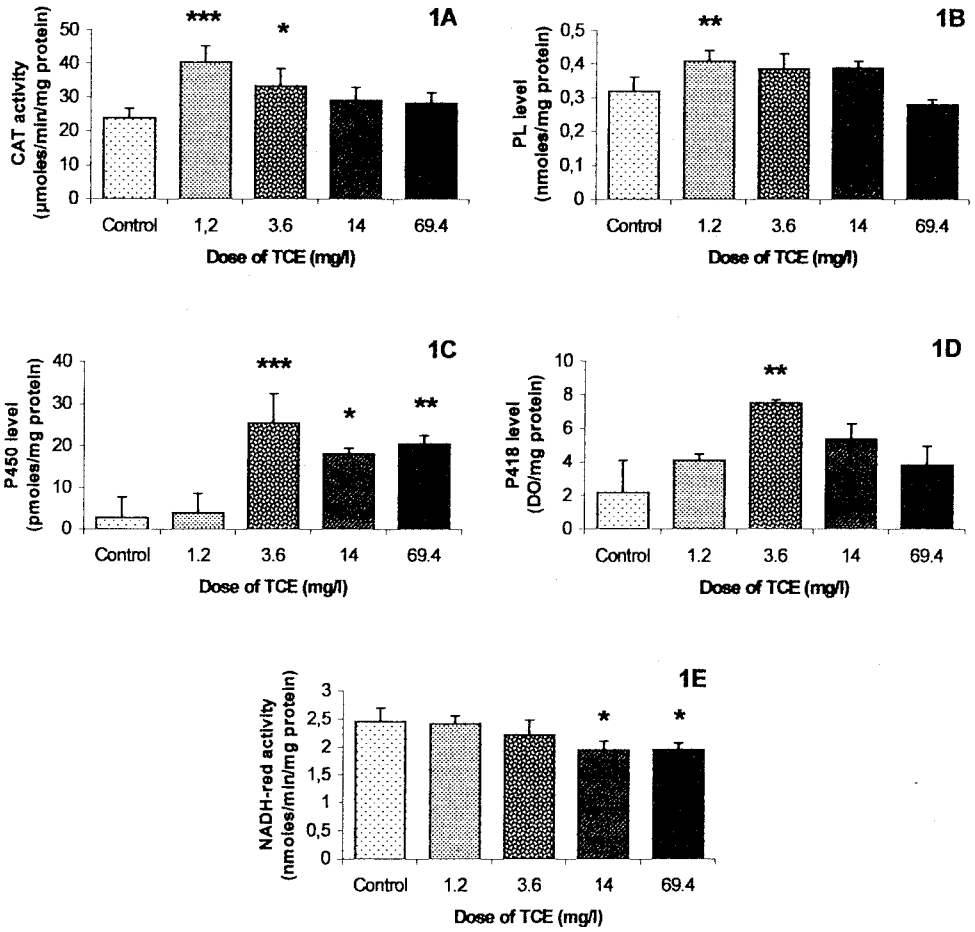


Fig. 1. Biochemical parameters exhibiting significant responses compared with control after exposure of *Corbicula fluminea* to different concentrations (measured ones) of TCE. 1A, CAT activity; 1B, PL level; 1C, *P*450 level; 1D, *P*418 level; 1E, NADH-red activity. Values are mean ± S.D. ($n = 5$). *, $P \leq 0.05$; **, $P \leq 0.01$ and ***, $P \leq 0.001$, comparing exposed to control.

4. Discussion

4.1. Chemical analysis

The loss of TCE and TOL during the preparation of the contaminated water and during the exposure was very important in spite of glass covers on aquariums and moderate, intermittent aeration to try to reduce evaporation. Because TCE and TOL are highly volatile solvents, they have been detected mainly at low levels in aquatic ecosystems (IARC, 1989, 1995). Nevertheless, in the case of a major or complete disappearance of such products in water after their release, residual biological effects may remain.

4.2. Biochemical parameters

Oxidative stress induced by TCE and/or its metabolites seemed to occur as we observed a significant increase of CAT activity at 1.2 and 3.6 mg/l paralleled by an increase of PL level. In mammals, TCE metabolites were reported to induce oxidative stress, including lipid peroxidation, excess free radical production and peroxisomal proliferation (Larson and Bull, 1992; Channel et al., 1998). Particularly, Goel et al. (1992) observed an induction of CAT activity, a marker enzyme of peroxisome, in mice liver after exposition to TCE. Oxyradical generation is an event also occurring in aquatic animals especially with exposure to xenobiotics (Winston, 1991). Invertebrates as mussels possess antioxidant systems, which have been well characterised (Ribera et al., 1989; Livingstone et al., 1992) and often considered as sensitive parameters that could be useful biomarkers for the evaluation of contaminated aquatic ecosystems (Livingstone et al., 1990; Cossu et al., 1997). Particularly, peroxisomal structures and related enzyme activities (as CAT activity) have been detected and studied in *M. galloprovincialis* (Cancio et al., 1998). Hence, their presence and induction by a xenobiotic such as TCE can be expected in *C. fluminea*. Likewise, as TCE metabolites more than TCE itself are able to induce oxidative stress, metabolism of TCE by *C. fluminea* could occur. The strong induction of *P*450 and *P*418 levels at 3.6 mg/l could support this hypothesis.

Such a *P*450 level induction in mammals was described already in literature. As an example, Kawamoto et al. (1988) observed a significant increase of *P*450 level in rat liver after a 5-day treatment. In *C. fluminea*, this event was less important at the highest doses (and no more significant for *P*418 level) and was paralleled by a recovery of indicators of oxidative stress, responses of which were no more significantly different from control. In mammals, it is assumed that metabolism of TCE proceeding through formation of a complex with oxygenated cytochrome *P*450 can lead to a suicidal heme destruction and, thus, an inactivation of cytochrome *P*450 and its related activities (Miller and Guengerich, 1983). Halmes et al. (1997) reported the appearance of a reactive metabolite of TCE bound covalently to a 50-KDa microsomal protein in mice. According to Pumford et al. (1997) it has now been demonstrated in rats that the major TCE-protein adduct was immunochemically reactive with cytochrome *P*4502E1, the major *P*450 enzyme implicated in TCE but also in TOL oxidative metabolism in rats (Nakajima et al., 1992). Thus, low TCE concentrations may lead to induction of cytochrome *P*450 while high concentrations may also cause induction of microsomal enzymes but in addition result in levels of reactive metabolites of TCE, which may inactivate cytochrome *P*450 (Kawamoto et al., 1988). Putative cytochrome *P*450 was partially purified in *M. edulis* by Porte et al. (1995) and at least five different digestive gland cytochrome *P*450 enzymes were recognised in this mollusc by polyclonal antibodies to *P*4501A, *P*4503A, *P*4504A, *P*4502B and *P*4502E from vertebrates (Peters et al., 1998). Then oxidative metabolism of TCE by *P*450 enzymes may take place in *C. fluminea*. Partial inactivation of that metabolism could lead to a diminution of metabolites inducing oxidative stress such as trichloroacetic acid and, thereby, limiting responses of CAT activity and PL level as we observed for *C. fluminea* at the highest doses.

As for TCE, events indicative of an oxidative stress occurred when *C. fluminea* were exposed to TOL, that is to say an increase of CAT activity and PL level at the lowest dose. Mattia et al. (1991) reported that benzaldehyde appeared to be the metabolite responsible for the

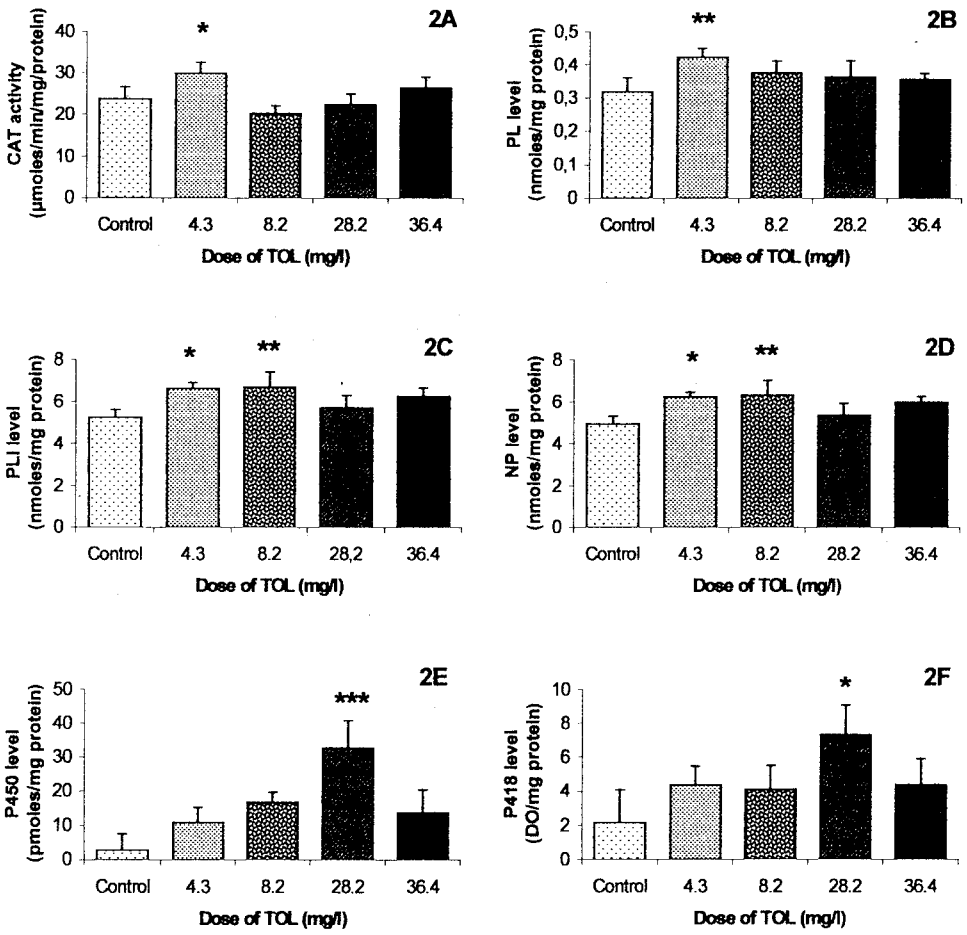


Fig. 2. Biochemical parameters exhibiting significant responses compared with control after exposure of *Corbicula fluminea* to different concentrations (measured ones) of TOL. 2A, CAT activity; 2B, PL level; 2C, PLI level; 2D, NP level; 2E, *P*450 level, 2F, *P*418 level. Values are mean ± S.D. ($n = 5$). *, $P \leq 0.05$, **, $P \leq 0.01$ and ***, $P \leq 0.001$, comparing exposed to control.

Table 2
Synthesis of discriminant analysis. Biochemical parameters contributing significantly to discrimination between doses of TCE or TOL, included control: *P*450 level, PL level, NP level, CAT activity, NADH-red activity[a]

Biochemical parameter	Wilks lambda	Partial lambda	*F* of exclusion (4.22)	*P*
TCE				
*P*450	0.042552	0.179304	25.17409	0.000000[c]
PL	0.029299	0.260409	15.62062	0.000003[c]
CAT	0.025502	0.299187	12.88316	0.000015[c]
NADH-red	0.016094	0.474086	6.10126	0.001845[b]
TOL				
*P*450	0.074071	0.176823	25.60458	0.000000[c]
NP	0.040181	0.325964	11.37301	0.000037[c]
CAT	0.030695	0.426692	7.38985	0.000624[c]
NADH-red	0.024632	0.531735	4.84349	0.005910[b]

[a] $n = 30$.
[b] Significant contribution to discrimination at $P \leq 0.01$.
[c] Significant contribution to discrimination at $P \leq 0.001$.

effect of TOL in accelerating the production of reactive oxygen species (ROS) within the nervous system of rats. Mattia et al. (1993) also observed a broad systemic elevation in the normal cellular rate of oxygen radical generation in rat liver. This ROS generation may be due to the release of superoxide anion generated during the catabolism of TOL by the mixed-function oxygenase (MWO) enzyme system and the oxidation of benzaldehyde. In *C. fluminea*, no more effects indicative of an oxidative stress were observed from 8.2 up to 36.4 mg/l. On the other hand, an increase of PLI and NP levels was observed but only at 8.2 mg/l. The decrease of PChE activity observed at 4.3 mg/l and especially at 8.2 mg/l, though non-significant, could be indicative of a membrane damage. Korpela and Tähti (1988) observed such an inhibition of acetylcholinesterase activity in rat erythrocytes and synaptosome membranes after exposure to TOL. Engelke et al. (1992) exposed that TOL, by inserting into the membrane, could disturb the lipid–protein interaction by two possible mechanisms, an increase of the membrane fluidity and/or TOL binding into hydrophobic enzyme pockets or concentration at the protein–lipid interface. Functions of integral membrane proteins such as *P*450 complex could also be affected. However, PChE inhibition was no more observed at the two highest doses. We observed a strong increase of *P*450 and *P*418 levels at 28.2 mg/l. Nakajima and Wang (1994) reported several *P*450 enzymes (such as CYP2E1, CYP2B1/2, CYP2C11, CYP2A1) involved in the metabolism of TOL in rat liver. TOL could influence the relative amounts of these enzymes via induction or destruction. This last event could occur via the formation of benzaldehyde-heme adducts (Furman et al., 1998). In *C. fluminea*, the absence of significant variation of *P*450 and *P*418 levels at the highest dose could be explained by a balance between induced and inhibited *P*450 enzymes.

No dose–effect relationship could have been established for TOL and TCE, whatever the biochemical parameter was. That could be explained by the fast decrease of solvent concentrations in water during exposure.

Besides biochemical effects, *C. fluminea* exhibited abnormal behaviour at the two highest doses at the beginning of the exposure such as largely parted valves, inflated foot and strongly extended siphons. These effects could be imputed to the toxic action of TCE and TOL. Indeed, both solvents are known to induce depression of central nervous system. TCE is characterised by its anaesthetic and analgesic properties (Waters et al., 1977). Narcotic properties of TOL have been also reported (Fishbein, 1985).

DA has already proven to be a very useful method for classifying the pollution status of different sites as reported by Adams et al. (1994, 1996), Ham et al. (1997), Van Der Oost et al. (1997). In a minor extent, it was applied also to emphasise differences between doses of contaminant in laboratory studies (Saint-Denis et al., 1999). DA was used in our study as an additional tool to discriminate doses (included control) from each other and also to show up biochemical parameters contributing significantly to this discrimination. DA performed in our study discarded automatically the irrelevant parameters, thus reducing the 'noise' and enhancing the discriminating potency between doses. For TCE, all doses were discriminated from each other considering responses of *P*450 and PL levels as well as CAT and NADH-red activities. Practically, the same parameters contributed to dose discrimination in the case of TOL, except that PL level was replaced by NP level and that the lowest dose was not discriminated from the highest one. Such parameters could be considered as relevant biomarkers of TCE or TOL exposure within our experimental conditions. *P*418 level for

Table 3
Distances between doses of TCE or TOL, included control, expressed as square of Mahalanobis distances between group centroids

	Group (dose of TCE[d])	1 (Control)	2 (1.2)	3 (3.6)	4 (14)	5 (69.4)
1	(Control)	–	47.69[c]	63.99[c]	35.88[c]	30.10[c]
2	(1.2)	–		41.27[c]	41.44[c]	79.90[c]
3	(3.6)	–	–	–	8.39[a]	35.47[c]
4	(14)	–	–	–	–	15.14[b]
5	(69.4)	–	–	–	–	–
	Group (dose of TOL[d])	1 (Control)	2 (4.3)	3 (8.2)	4 (28.2)	5 (36.4)
1	(Control)	–	27.03[c]	24.57[c]	55.29[c]	16.70[c]
2	(4.3)	–	–	22.93[c]	45.13[c]	5.57[ns]
3	(8.2)	–	–	–	29.74[c]	15.79[c]
4	(28.2)	–	–	–	–	23.65[c]
5	(36.4)	–	–	–	–	–

[a] Significant discrimination at $P \leq 0.05$.
[b] Significant discrimination at $P \leq 0.01$.
[c] Significant discrimination at $P \leq 0.001$. [ns]non-significant.
[d] mg/l (measured concentrations).

TCE, PL, PLI and *P*418 levels for TOL did not contribute significantly to the discrimination despite their significant responses compared with control, according to ANOVA results. Thus, they could be considered as less relevant biomarkers of exposure to TCE or TOL.

In conclusion, our study displayed some interesting knowledge on the biochemical effects of TCE and TOL in the mollusc *C. fluminea* and emphasised the importance of a multibiomarker approach in environmental studies. To be representative of an acute contamination, this experiment was carried out with high concentrations of TCE and TOL that were not maintained constant during exposure. The significant responses of some biochemical parameters observed after 5 days of exposure and despite the important loss of both solvents showed up the relevance of biological indicators in environmental biomonitoring. Indeed, the absence of detectable level of a chemical a few days after its release in water does not mean it has had (and it still has) no impact on the ecosystem. Contrary to the chemical approach, the biological one can allow the detection of such an impact as emphasised in our study, even after the complete disappearance of contaminants in water. Among the several biochemical parameters measured, components of phase I metabolism such as *P*450 level and NADH-red activity as well as parameters related to oxidative stress such as CAT activity, PL and NP levels could be considered as relevant biomarkers of TCE and TOL exposure in *C. fluminea*. Statistical methods such as DA allowed a more accurate selection among relevant biomarkers and proved to be interesting tools in environmental studies as underlined already by Adams et al. (1994). For further studies, it would be judicious to measure more parameters related to oxidative stress and phase I metabolism for most organic compounds exert their toxic effects on these targets. Nevertheless, our microcosm experiment was poorly representative of field conditions mainly because of the static exposure. Therefore, mesocosm experiments in which TCE or TOL would be injected in a flow-through system will be soon undertaken. They should allow a deeper understanding of TCE and TOL mechanisms of toxicity in *C. fluminea* as well as an advance in the validation of the use of MFO components and parameters related to oxidative stress as biomarkers of exposure to TCE and TOL.

Acknowledgements

This study was supported by a Ph.D. grant from the Environment Department of the Petroleum Society Elf Aquitaine. The authors are grateful to Muriel Audroin and Nadine Médevielle for their precious technical assistance and to the staff of the Laboratory of Control and Environment of Elf Aquitaine (Lacq, France) for chemical analysis.

References

Adams, S.M., Ham, K.D., Beauchamp, J.J., 1994. Application of canonical variate analysis in the evaluation and presentation of multivariate biological response data. Environ. Toxicol. Chem. 13 (10), 1673–1683.

Adams, S.M., Ham, K.D., Greeley, M.S., LeHew, R.F., Hinton, D.E., Saylor, C.F., 1996. Downstream gradients in bioindicator responses: point source contaminant effects on fish health. Can. J. Fish. Aquat. Sci. 53, 2177–2187.

Araujo, R., Moreno, D., Ramos, M.A., 1993. The Asiatic clam *Corbicula fluminea* (Müller, 1774) (Bivalvia: Corbiculidae) in Europe. Am. Malacol. Bull. 10, 39–49.

Baudrimont, M., Metivaud, J., Maury-Brachet, R., Ribeyre, F., Boudou, A., 1997. Bioaccumulation and metallothionein response

in the Asiatic clam (*Corbicula fluminea*) after experimental exposure to cadmium and inorganic mercury. Environ. Toxicol. Chem. 16, 2096–2105.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 50, 302–310.

Burke, M.D., Mayer, R.T., 1974. Ethoxyresorufin: direct fluorimetric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab. Dispos. 2 (6), 583–588.

Cancio, I., Orbea, A., Völkl, A., Fahimi, H.D., Cajaraville, M.P., 1998. Induction of peroxisomal oxidases in mussels: comparison of effects of lubricant oil and benzo(*a*)pyrene with two typical peroxisome proliferators on peroxisome structure and function in *Mytilus galloprovincialis*. Toxicol. Appl. Pharmacol. 149, 64–72.

Channel, S.R., Latendresse, J.R., Kidney, J.K., Grabau, J.H., Lane, J.W., SteelGoodwin, L., Gothaus, M.C., 1998. A subchronic exposure to trichloroethylene causes lipid peroxidation and hepatocellular proliferation in male B6C3F1 mouse liver. Toxicol. Sci. 43, 145–154.

Clairborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), Handbook of Methods for Oxygen Radical Research. CRC press, Boca Raton, FL, pp. 283–284.

Cossu, C., Doyotte, A., Jacquin, M.C., Babut, M., Exinger, A., Vasseur, P., 1997. Glutathione reductase, selenium-dependent glutathione peroxidase, glutathione levels, and lipid peroxidation in freshwater bivalves, *Unio tumidus*, as biomarkers of aquatic contamination in field studies. Ecotoxicol. Environ. Saf. 38, 122–131.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Engelke, M., Diehl, H., Tähti, H., 1992. Effects of toluene and *n*-hexane on rat synaptosomal membrane fluidity and integral enzyme activities. Pharmacol. Toxicol. 71, 343–347.

Estabrook, R.W., Werringloer, J., 1978. The measurement of difference spectra: application to the cytochromes of microsomes. Methods Enzymol. 52, 212–220.

Fishbein, L., 1985. An overview of environmental and toxicological aspects of aromatic hydrocarbons. II. Toluene Sci. Total Environ. 42, 267–288.

Fitzpatrick, P.J., Sheehan, D., 1993. Separation of multiple forms of glutathione *S*-transferase from the blue mussel *Mytilus edulis*. Xenobiotica 23, 851–861.

Fraisse, L., Verlhac, J.B., Roche, B., Rascle, M.C., Rabion, A., Seris, U., 1993. Long-chain-substituted uric acid and 5,6-diaminouracil derivatives as novel agents against free radical processes: synthesis and in vitro activity. J. Med. Chem. 36 (10), 1465–1473.

Furman, G.M., Silverman, D.M., Schatz, R.A., 1998. Inhibition of rat lung mixed function oxidase activity following repeated low-level toluene inhalation: possible role of toluene metabolites. J. Toxicol. Environ. Health Part A 54, 633–645.

Goel, S.K., Rao, G.S., Pandya, K.P., Shanker, R., 1992. Trichloroethylene toxicity in mice: a biochemical, hematological and pathological assessment. Indian J. Exp. Biol. 30, 402–406.

Goeptar, A.R., Commandeur, J.N.M., Van Ommen, B., Van Bladeren, P.J., Vermeulen, N.P.E., 1995. Metabolism and kinetics of trichloroethylene in relation to toxicity and carcinogenicity. Relevance of the mercapturic acid pathway. Chem. Res. Toxicol. 8, 3–21.

Guengerich, F.P., 1994. Analysis and characterization of enzymes. In: Hayes, A.W. (Ed.), Principles and Methods of Toxicology, Third ed. Raven Press, New York, pp. 1269–1270.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249 (22), 7130–7139.

Halmes, N.C., Samokyszyn, V.M., Pumford, N.R., 1997. Covalent binding and inhibition of cytochrome *P*4502E1 by trichloroethylene. Xenobiotica 27 (1), 101–110.

Ham, K.D., Adams, S.M., Peterson, M.J., 1997. Application of multiple bioindicators to differentiate spatial and temporal variability from the effects of contaminant exposure on fish. Ecotoxicol. Environ. Saf. 37, 53–61.

International Agency for Research on Cancer, 1989. Some organic solvents, resin monomers and related compounds, pigments and occupational exposures in paint manufacture and painting. IARC Monogr. Eval. Carcinog. Risks Hum. 47, 79–123.

International Agency for Research on Cancer, 1995. Dry cleaning, some chlorinated solvents and other industrial chemicals. IARC Monogr. Eval. Carcinog. Risks Hum. 63, 75–158.

Kawamoto, T., Hobara, T., Nakamura, K., Imamura, A., Ogino, K., Kobayashi, H., Iwamoto, S., Sakai, T., 1988. Induction of cytochrome *P*-450, cytochrome *b*-5, NADPH-cytochrome *c* reductase and change of cytochrome *P*-450 isozymes with long-term trichloroethylene treatment. Toxicology 53, 239–249.

Korpela, M., Tähti, H., 1988. The effect of in vitro and in vivo toluene exposure on rat erythrocyte and synaptosome membrane integral enzymes. Pharmacol. Toxicol. 63, 30–32.

Larson, J.L., Bull, R.J., 1992. Metabolism and lipoperoxidative activity of trichloroacetate and dichloroacetate in rats and mice. Toxicol. Appl. Pharmacol. 115 (2), 268–277.

Livingstone, D.R., 1993. Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. J. Chem. Technol. Biotechnol. 57, 195–211.

Livingstone, D.R., Kirchin, M.A., Wiseman, A., 1989. Cytochrome *P*-450 and oxidative metabolism in molluscs. Xenobiotica 19 (10), 1041–1062.

Livingstone, D.R., Garcia Martinez, P., Michel, X., Narbonne, J.F., O'Hara, S., Ribera, D., Winston, G.W., 1990. Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* L., and other molluscs. Funct. Ecol. 4, 415–424.

Livingstone, D.R., Lips, F., Garcia Martinez, P., Pipe, R.K., 1992. Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*. Mar. Biol. 112, 265–276.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mattia, C.J., LeBel, C.P., Bondy, S.C., 1991. Effects of toluene and its metabolites on cerebral reactive oxygen species generation. Biochem. Pharmacol. 42 (4), 879–882.

Mattia, C.J., Adams, J.D., Jr, Bondy, S.C., 1993. Free radical induction in the brain and liver by products of toluene catabolism. Biochem. Pharmacol 46 (1), 103–110.

Miller, R.E., Guengerich, F.P., 1983. Metabolism of trichloroethylene in isolated hepatocytes, microsomes, and reconstituted enzyme systems containing *P*-450. Cancer Res. 43 (3), 1145–1152.

Mora, P., Fournier, D., Narbonne, J.F., 1999a. Cholinesterases from the marine mussels *Mytilus galloprovincialis* Lmk. and *M edulis* L. and from the freshwater bivalve *Corbicula fluminea*. Müller. Comp. Biochem. Physiol. Part C 122, 353–361.

Mora, P., Michel, X., Narbonne, J.F., 1999b. Cholinesterase activity as potential biomarker in two bivalves. Environ. Toxicol. Pharmacol. 7, 253–260.

Nakajima, T., Wang, R.-S., 1994. Induction of cytochrome *P*450 by toluene. Int. J. Biochem. 26 (12), 1333–1340.

Nakajima, T., Wang, R.-S., Elovaara, E., Park, S.S., Gelboin, H.V., Vainio, H., 1992. A comparative study on the contribution of cytochrome *P*450 isozymes to metabolism of benzene, toluene and trichloroethylene in rat liver. Biochem. Pharmacol. 43 (2), 251–257.

Narbonne, J.F., Daubèze, M., Clérandeau, C., Garrigues, P., 1999a. Scale of classification based on biochemical markers in mussels: application to pollution monitoring in European coasts. Biomarkers 4 (6), 415–424.

Narbonne, J.F., Djomo, J.E., Ribera, D., Ferrier, V., Garrigues, P., 1999b. Accumulation kinetics of polycyclic aromatic hydrocarbons adsorbed to sediment by the mollusk *Corbicula fluminea*. Ecotoxicol. Environ. Saf. 42, 1–8.

Peters, L.D., Nasci, C., Livingstone, D.R., 1998. Immunochemical investigations of cytochrome *P*450 forms/epitopes (CYP1A, 2B, 2E, 3A and 4A) in digestive gland of *Mytilus sp.* Comp. Biochem. Physiol. Part C 121 (1–3), 361–369.

Porte, C., Lemaire, P., Peters, L.D., Livingstone, D.R., 1995. Partial purification and properties of cytochrome *P*450 from digestive gland microsomes of the common mussel, *Mytilus edulis* L. Mar. Environ. Res. 39, 27–31.

Pumford, N.R., Halmes, N.C., Hinson, J.A., 1997. Covalent binding of xenobiotics to specific proteins in the liver. Drug Metab. Rev. 29 (1/2), 39–57.

Ribera, D., Narbonne, J.F., Daubeze, M., Michel, X., 1989. Characterization, tissue distribution and sexual differences of some parameters related to lipid peroxidation in mussels. Mar. Environ. Res. 28, 279–283.

Saint-Denis, M., Narbonne, J.F., Arnaud, C., Thybaud, E., Ribera, D., 1999. Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil: effects of benzo(*a*)pyrene. Soil Biol. Biochem. 31, 1837–1846.

Solé, M., Peters, L.D., Magnusson, K., Sjölin, A., Granmo, Å., Livingstone, D.R., 1998. Responses of the cytochrome *P*450-dependent monooxygenase and other protective enzyme systems in digestive gland of transplanted common mussel (*Mytilus edulis* L.) to organic contaminants in the Skagerrak and Kattegat (North sea). Biomarkers 3 (1), 49–62.

Van Der Oost, R., Vindimian, E., Van Den Brink, P.J., Satumalay, K., Heida, H., Vermeulen, N.P.E., 1997. Biomonitoring aquatic pollution with feral eel (*Anguilla anguilla*). III. Statistical analyses of relationships between contaminant exposure and biomarkers. Aquat. Toxicol. 39, 45–75.

Vidal, M.L., Narbonne, J.F., 2000. Characterization of glutathione *S*-transferase activity in the Asiatic clam *Corbicula fluminea*. Bull. Environ. Contam. Toxicol. 64, 455–462.

Von Burg, R., 1993. Toxicology update. Toluene. J. Appl. Toxicol. 13 (6), 441–446.

Waters, E.M., Gerstner, H.B., Huff, J.E., 1977. Trichloroethylene. I. An overview. J. Toxicol. Environ. Health 2, 671–707.

Winston, G.W., 1991. Oxidants and antioxidants in aquatic animals. Comp. Biochem. Physiol. Part C 100 (1/2), 173–176.

ARTICLE 6

Effects of experimental exposure to cadmium and a coal tar fraction on (de)toxification metabolism and oxidative stress in the freshwater clam *Corbicula fluminea* (Müller)

Vidal, M-L., Bassères, A., Narbonne J-F.

Soumis à *Biomarkers*

Abstract

A laboratory study to assess biochemical effects of cadmium (Cd) and a complex mixture of polycyclic aromatic hydrocarbons (coal tar fraction: CT) in the clam *Corbicula fluminea* was conducted in view of future applications in fresh water biomonitoring programs. In static conditions, clams were exposed *via* water column to four unique sublethal doses of Cd (0.154, 0.308, 0.615 and 1.230 mg l^{-1}) or CT (0.75, 1.5, 3 and 6 mg l^{-1}) during five days. At the end of exposure, a partial or complete loss of pollutants was evidenced, representative of a punctual and acute contamination. In a multibiomarker approach, responses of: components of (de)toxification metabolism of phases I and II, parameters related to oxidative stress and propionylcholinesterase were assayed. Combining results of variance analysis, Tukey test and discriminant analysis, the most relevant biomarkers were levels of cytochromes P450 and P418, NADPH-cytochrome *c* (P450) reductase and NADH-cytochrome *c* (*b5*) reductase activities as well as net peroxidation level for CT; catalase activity, glutathione *S*-transferase activity towards 1-chloro-2,4-dinitrobenzene and net peroxidation level for Cd. This experiment underlined the persistence of biochemical effects despite partial or complete disappearance of Cd and CT and will be completed by mesocosm studies.

Introduction

Each day, a multitude of toxic compounds is released in the aquatic environment from point or diffuse sources. Among them, polycyclic aromatic hydrocarbons (PAHs) and cadmium (Cd), a non-essential heavy metal, are ubiquitous pollutants with both natural and anthropogenic origins, which are of long-standing environmental concern. Due to their capacity to accumulate high levels of organic and inorganic pollutants, molluscs and particularly marine mussels *Mytilus galloprovincialis* and *M. edulis* are suitable bioindicators of PAH- and heavy metal-contaminations (Taylor 1983, Neff 1984, Amodio-Cocchieri *et al.* 1993). Additionally, the measurement of biochemical and molecular markers in such sentinel organisms allows the detection of sublethal and early pollutant effects on aquatic ecosystems (Livingstone 1993). The need for such bioindicators in freshwater ecosystems has focused attention on the clam *Corbicula fluminea* (Müller). Originating from Asia, this opportunist species has progressively colonised USA and more recently Europe (Britton and Morton 1979, Araujo *et al.* 1993) to become a major component of benthic communities. Being both a filter- and a deposit-feeding species, *C. fluminea* is able to bioaccumulate PAHs (Narbonne *et al.* 1999) and heavy metals including Cd (Doherty *et al*. 1988, Inza *et al*. 1997, Labrot *et al.* 1998). Furthermore, responses of several biochemical parameters can be modulated by lead, uranium (Labrot *et al.* 1996), organophosphorus pesticides (Basack *et al.* 1998), toluene and trichloroethylene (Vidal *et al.* 2001). The present study belongs to a program of validation of *C. fluminea* as a sentinel organism in view of future field applications. Thus to expand our knowledge about potential biomarkers of the most common pollutants in this species, we have performed an experimental five-day exposure of these clams to four sublethal concentrations of Cd (as $CdCl_2$) or a coal tar fraction (CT) mainly composed of PAHs. High pollutant levels delivered as single doses at the beginning of the experiment were more representative of punctual, acute contaminations rather than chronic ones. We studied responses of a panel of biochemical parameters considered as general or specific biomarkers of pollution in most biomonitoring programs. Some PAHs such as benzo[*a*]pyrene (B[*a*]P) are of great environmental concern due to their activation to mutagenic and carcinogenic metabolites by the mixed-function oxygenase (MFO) system (Shaw and Connell 1994). Thus PAH-mediated induction of the cytochrome P450 isoform CYP1A is a well-known phenomenon in vertebrates, including fishes (Bucheli and Fent 1995). Some highly reactive metabolites can be inactivated *via* conjugation reactions catalysed by the glutathione *S*-transferases (GSTs), ubiquitous enzymes of phase II (Ketterer *et al.* 1988). Metabolism of PAHs can also generate

reactive oxygen species (ROS), e.g. *via* quinone compounds, leading to oxidative damages (Sjölin and Livingstone 1997). In mussels, principal MFO components as well as metabolism of B[*a*]P have been evidenced (Stegeman 1985, Livingstone *et al.* 1989, Michel *et al.* 1992). More recently, immunological and molecular studies have demonstrated the existence of several CYP-like proteins and genes in *Mytilus sp.* (Porte *et al.* 1995, Wootton *et al.* 1995, Wootton *et al.* 1996, Peters *et al.* 1998a). In particular, CYP1A-like protein was induced by organic pollutants such as PAHs and polychlorinated biphenyls (Solé *et al.* 1996, Peters *et al.* 1998b, Peters *et al.* 1999). Likewise, several GST isoenzymes have been purified and characterised in *M. edulis* (Fitzpatrick and Sheehan 1993) and more recently in *C. fluminea* (Vidal *et al.* unpublished results). However, modulation of molluscan GST activity by PAHs was not confirmed due to mitigated or contradictory results (Michel *et al.* 1993, Solé *et al.* 1996, Akcha *et al.* 2000). Mussels are also susceptible to undergo pollution-mediated oxidative stress (Livingstone *et al.* 1990). Molluscan antioxidant systems have been well characterised (Livingstone *et al.* 1992) and sometimes considered as responsive to PAH-exposure (Cajaraville *et al.* 1992, Eertman *et al.* 1995, Akcha *et al.* 2000). Several deleterious effects induced by heavy metals have been reported such as alterations of cell and lysosomal membranes particularly by the enhancement of lipid peroxidation, binding to proteins (including enzymes, metallothioneins) and low molecular weight compounds (e.g. GSH), modulation of protein synthesis and nuclear metabolism (Viarengo 1985). All these effects have been reported in invertebrates (Viarengo 1989) despite some controversial results concerning Cd-induced lipid peroxidation (Viarengo *et al.* 1990, Tejo Prakash and Jagannatha Rao 1995). In accordance with PAH- and Cd- toxicity mechanisms but also in a multibiomarker approach, we selected the following biochemical parameters: components of (de)toxification metabolism of phases I and II such as cytochromes P450, P418 and *b*5 (P450, P418 and b5, respectively), NADPH-cytochrome *c* (P450) reductase (NADPH-red.), NADH-cytochrome *c* (*b*5) reductase (NADH-red.), NADPH-independent ethoxyresorufin-*O*-deethylase (NADPH-ind. EROD) and GST using 1-chloro-2,4-dinitrobenzene (GST/CDNB) or ethacrynic acid (GST/EA) as a substrate; parameters related to oxidative stress such as the antioxidant enzyme catalase (CAT), peroxided and peroxidizable lipids (PL and PLI, respectively) and net peroxidation (NP). Additionally, we included cholinesterase (ChE), an enzyme involved in nervous functions. It has been purified in both marine mussels and *C. fluminea* by Mora *et al.* (1999a) and is usually considered as a specific biomarker of organophosphorus and carbamate pesticides (Basack *et al.* 1998, Mora *et al.* 1999b).

Nevertheless, ChE activity has been also reported to be modulated by PAHs and heavy metals (Regoli and Principato 1995, Labrot *et al.* 1996, Akcha *et al.* 2000).

Materials and methods

Chemicals

The coal tar fraction (CT) was provided by Chemco France (Poissy, France). It consisted of a complex mixture of PAHs, the most abundant being naphthalene, anthracene, phenanthrene, fluorene, fluoranthene and pyrene. $CdCl_2$, 2.5 H_2O was purchased from Interchim (Montluçon, France). Other chemicals were of the best technical grade available.

Animals

Sexually mature *C. fluminea* were collected from the banks of the freshwater Cazaux-Sanguinet lake (Aquitaine, France) in late September 1998, in their post-spawning period. No sexual differences were taken into account for Asiatic clams are hermaphroditic. The excellent water quality (personal communication from the Direction Départementale des Affaires Sanitaires et Sociales - DDASS, France) makes this lake a reference site in most environmental studies. Clams were transported back to the laboratory in plastic tanks filled with lake water and subsequently transferred into glass aquariums for an eight-day acclimatisation period. Aeration was provided continuously by air bubbling (Shego M2K3 air pump) and temperature was regulated at 20 ± 0.1°C. Clams were not fed during this period to clear out gut content and were submitted to a natural light cycle.

Experimental design

Each experimental unit consisted of a glass aquarium filled with 10 l of lake water previously filtered through 50 µm and then 5 µm cotton cartridges using a 7019-20 Cole-Parmer Masterflex pump to avoid adsorption of chemicals to particles and thus to privilege water-column contamination. Four sublethal doses of each product were tested: 0.75, 1.5, 3 and 6 mg l^{-1} of CT and 0.154, 0.308, 0.615 and 1.23 mg l^{-1} of Cd (0.31, 0.625, 1.25 and 2.5 mg l^{-1} of $CdCl_2$, 2.5 H_2O). As $CdCl_2$ was hydrosoluble in the range of doses assayed, it was mixed directly with filtered lake water. On the other hand, CT was dissolved previously in

dimethylsulfoxide (DMSO), final concentration of which was 0.5 g l^{-1}. Appropriate controls consisting of 10 l of filtered lake water (plus 0.5 g l^{-1} DMSO in the case of CT) were prepared. 50 *C. fluminea* were placed into each aquarium for a five-day exposure. They were not fed and were held under a natural light cycle, at 20 ± 0.1°C, during all the experiment. Aeration was provided by intermittent air bubbling *via* a diffuser connected to a Shego M2K3 air pump (15 min every 6 hr). Aquariums were covered to limit water and/or product evaporation. Water was not replaced and temperature, pH, conductivity and oxygen saturation were checked daily using a Universal Pocket Meter Multiline P4, WTW. Dead clams were removed to avoid water fouling (three for the first dose of Cd and one for the last dose of CT).

Chemical analysis

At the beginning and the end of the exposure, samples of 250 ml of water (contaminated or not) were collected and stored in amber glass flasks filled to avoid air bubbles, at 4°C. Chemical analyses were performed in the Laboratory of Control and Environment of TotalFinaElf (Lacq, France). Measurements were carried out in triplicates. Determination of CT concentrations was carried-out by high performance liquid chromatography (HPLC) with a Hewlett-Packard Model 1090M. 20 µl of sample were injected on a 250 × 4.6 mm C18 column (Chrompack) with acetonitrile / water (85% / 15%) as the mobile phase. Cd concentrations were measured by atomic absorption spectrophotometry according to the international standard method ISO (International Standardisation Organisation) 11885 (Organisation Internationale de Normalisation 1996).

Biochemical assays

At the end of the five-day exposure, *C. fluminea* were collected and dissected out to obtain entire soft bodies or visceral masses, after crystalline style was removed. The following homogenisation steps were then performed at 4°C. For each experimental condition, five pools of five *C. fluminea* were prepared. Body samples were rinsed in 100 mM phosphate buffer (K_2HPO_4/KH_2PO_4), pH 7.4, dried on absorbent paper towels, weighed and homogenised in the same phosphate buffer (1:4 weight:volume ratio) using an Ultra-Turrax Antrieb T25. Homogenates were centrifuged subsequently at 9000 g for 30 min in a Sigma 3MK centrifuge. Submitochondrial fractions (S9) of soft entire bodies were then stored at -80°C whereas those of visceral masses were centrifuged for additional 60 min at 105000 g in

a Beckman LE-80 ultracentrifuge. Cytosolic fractions were stored at -80°C. Microsomal pellets were resuspended in a 100 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 20% glycerol and stored at -80°C. All biochemical measurements were performed in duplicates or in triplicates for each pool on a dual-beam temperature-controlled Kontron Uvikon 932 spectrophotometer except for NADPH-ind. EROD activity assay, which was carried out on a Shimadzu RF-540 spectrofluorophotometer. Enzymatic activities were measured at 25°C in S9 of soft entire bodies except for GST/CDNB and GST/EA activities (30°C, cytosol of visceral masses) as well as NADPH-red. and NADH-red. activities (30°C, microsomes of visceral masses). P450, P418 and b_5 levels as well as NADPH-ind. EROD activity were determined in S9 of soft entire bodies for, in *C. fluminea*, variability is less important in this fraction than in microsomes of visceral masses. CAT activity was determined as described in Clairborne (1985). PL level was estimated according to Buege and Aust (1978) in S9 of soft entire bodies, following the appearance of thiobarbituric acid reactive species (TBARS) at 532 nm. PLI level was determined likewise but after incubation of the samples 1 hr at 37°C in a reaction mixture containing an iron (II)/dihydroxyfumaric acid as peroxidation initiator (Fraisse *et al.* 1993). NP level was estimated as the difference between PLI level (stimulated peroxidation) and PL level (basal peroxidation). P450, P418 and b5 levels were measured according to a method adapted from Estabrook and Werringloer (1978) using sodium dithionite as a reducing agent. NADPH-red. activity was assayed as described in Guengerich (1994) and NADH-red. activity was determined likewise, replacing NADPH by NADH. NADPH-ind. EROD activity was measured by a direct fluorimetric assay adapted from the method of Burke and Mayer (1974). No NADPH was added to the mixture to start the assay as NADPH was found to inhibit the reaction. Such a NADPH-independent mechanism has already been reported for benzo(*a*)pyrene hydroxylase or ethoxycoumarin-*O*-deethylase activities in mussels (Livingstone *et al.* 1989). GST/CDNB and GST/EA activities were assayed as in Vidal and Narbonne (2000) by a method adapted from Habig *et al.* (1974). ChE activity was assayed using propionylthiocholine as a substrate (PChE activity) as in Mora *et al.* (1999b) according to the method of Ellman *et al.* (1961). All biochemical parameter responses were expressed in relation to protein concentration measured according to the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Statistical analysis

All statistical evaluations were performed using Statistica software (5.1 release, Statsoft). Data fulfilling normal distribution and homogeneity of variances (across dose-groups) were submitted to a univariate analysis of variance (ANOVA) with 'dose' (of CT or Cd) as the independent variable and biochemical parameters as dependent variables. Whenever a significant ($p \leq 0.05$) dose-effect was established on a biochemical parameter response, a Tukey HSD multiple comparison test was used to check significant ($p \leq 0.05$) differences between doses. Non-parametric tests (Kruskall-Wallis ANOVA and Mann-Whitney test) were performed when data were not normally distributed or when they exhibited heterogeneous variances. Correlations among biochemical parameter responses and with dose were determined for each chemical with the Pearson correlation coefficient (significant for $p \leq 0.05$). Discriminant analyses (DA) were performed additionally, for they take into account the whole biochemical parameter responses and thus provide an integrated approach in environmental biomonitoring (Adams *et al.* 1994). Particularly, they informed us about the biochemical parameters contributing significantly to an eventual discrimination between doses of CT or Cd.

Results

Chemical analysis and water quality

Results of chemical analysis are presented in table 1. Despite actual CT concentrations at the beginning of exposure were slightly higher than the theoretical ones, the loss was practically complete after five days whatever the dose was (up to 100%). Initial Cd concentrations were slenderly inferior to the calculated ones. Contrary to CT, Cd was still present in relatively important amount at the end of the experiment, its disappearance being inversely proportional to the concentration (-67.8 and -23.8% for the lowest and the highest doses, respectively). For the following results, actual concentrations at the beginning of exposure will be considered: 1.17, 2.24, 3.58 and 7.84 mg l^{1} for CT and 0.118, 0.257, 0.543 and 1.141 mg l^{1} for Cd. During exposure, temperature was 19.7 ± 0.5°C and 20.3 ± 0.4°C whereas pH was 6.93 ± 0.2 and 7.29 ± 0.2 for CT- and Cd-contaminated water, respectively. Conductivity increased according to the exposure time for Cd-contaminated water (e.g. from 197 up to 214 µS cm^{-1}

for the highest Cd concentration) and also to the dose for CT-contaminated water (e.g. 211 and 257 µS cm^{-1} for 1.17 and 7.84 mg l^{-1} of CT, respectively, at the end of exposure). Oxygen saturation decreased along with time down to about 55% in water contaminated by either chemical.

Biochemical assays

Biochemical parameters displaying significant responses compared to control after exposure of *C. fluminea* to CT or Cd are depicted in figures 1 and 2, respectively. CAT activity and NP level were depressed significantly of 19% and induced of 20%, respectively, at 2.24 mg l^{-1} of CT. This effect was no more evidenced at higher concentrations, despite a significant decrease of 41% of PL level at 7.84 mg l^{-1}. That decrease concurred with an increase of 317 and 86% of P450 level and NADPH-red. activity, respectively. Such variations were not paralleled by those of P418 level and NADH-red. activity. P418 level increased generally when clams were exposed to CT, but only in a significant manner at 1.17 and 3.58 mg l^{-1} (+45 and 52%, respectively). Likewise, NADH-red. activity only showed a substantial increase at 1.17 and 2.24 mg l^{-1} (+32 and 41%, respectively).

CAT activity was correlated negatively with NP level and NADH-red. activity (r = - 0.4900 and - 0.3991, respectively). P450 level was correlated positively with P418 level and NADPH-ind. EROD activity, though this latter was not modulated significantly by CT exposure (r = 0.5788 and 0.5953, respectively). NAPDH-red. and NADH-red. activities were correlated negatively and positively with PL and NP levels, respectively (r = - 0.4860 and 0.5539, respectively). P450 level and NADPH-red. activity were correlated positively with the CT concentration (r = 0.9491 and 0.9548, respectively) whereas PL level was correlated negatively (r = - 0.9258). Though GST/EA activity increased accordingly with the CT dose (r = 0.9601), it was not sufficient to induce significant differences when comparing to control (data not shown).

When *C. fluminea* were exposed to Cd, CAT activity displayed a singular pattern, with a significant decrease of 39% at the lowest dose and a subsequent increase above control level at the highest doses (+31% at 0.543 and 1.141 mg l^{-1}). Coinciding with that induction, PLI and NP levels were both increased at the highest doses (+17 and 29% at 0.543 and 1.141 mg l^{-1}, respectively for PLI level and +29% at 1.141 mg l^{-1} for NP level). Responses of these two

parameters were correlated positively with the dose (r = 0.9312 and 0.9309 for PLI and NP levels, respectively). Though PL level did not vary significantly, it was also correlated positively with the dose (r = 0.8813). The P450-related NADPH-ind. EROD activity was strongly depressed at 0.118 mg l^{-1} and completely inhibited at the following Cd concentrations. A significant decrease of 29% of GST activity towards CDNB was only observed at the lowest dose. A similar but not substantial pattern was observed when using EA as a substrate.

A positive correlation was evidenced between CAT activity and PL, PLI and NP levels (r = 0.4800, 0.4855 and 0.4818, respectively). GST/CDNB and GST/EA activities were correlated positively (r = 0.6575). NADPH-ind. EROD activity was correlated inversely with PLI and NP levels (r = - 0.4219 and - 0.4362, respectively). Finally, PLI and NP levels were correlated positively (r = 0.9992).

Synthesis of DA is given in table 2. Biochemical parameters contributing significantly to discrimination between doses were mainly components of the MFO system and indicators of a modified oxidative state for CT and Cd, respectively. The separation of dose groups relies on discriminating functions, which are linear combinations of the biochemical parameters. The more the distance between two groups (the Mahalanobis distance) is high and significant, the more those groups are discriminated. These results are presented in table 3 for both chemicals [insert table 3 about here]. In the case of CT, the control group was well discriminated from the others, and particularly from that corresponding to the highest dose. More generally, all dose groups were discriminated from each other, except those related to the two lowest doses (1.17 and 2.24 mg l^{-1}). For Cd, the control group was also well discriminated from the others, and specially from the first-dose group. Only the second-dose group (0.257 mg l^{-1}) was not discriminated from the third and the fourth ones (0.543 and 1.141 mg l^{-1}, respectively).

Discussion

After five days of exposure, disappearance of CT was practically complete. Evaporation (despite covered aquariums and limited aeration), adsorption on aquarium walls and clamshells, photolytic degradation and clam bioaccumulation probably occurred. In a laboratory study, Narbonne *et al.* (1999) reported the accumulation by *C. fluminea* of

anthracene, phenanthrene, pyrene (major products of CT) and B[*a*]P from contaminated sediments. Kinetic data suggested that PAH uptake was related to desorption from sediment particles. Indeed, efficiency of PAH uptake from sediments is much lower than from water (Neff 1984). The loss of Cd was less important during exposure and probably due to adsorption on aquarium walls and clamshells, evaporation and bioaccumulation by *C. fluminea*, as for CT. This species has been reported to take up Cd readily, specially from the water column compared to sediment (Inza *et al.* 1997). Furthermore, the dominant species in fresh water displaying a pH around or below 8 is Cd^{2+}, which is considered to be a more bioavailable and toxic species than Cd chloro-complexes present in sea water (Jensen and Bro-Rasmussen 1992). Although disappearance of both CT and Cd during the experiment was relatively important, depending on the product, biological effects were still detectable, which is of great interest in monitoring acute and punctual pollution.

It is well known that metabolism of PAHs by the MFO system can lead to carcinogenicity *via* the formation of highly reactive intermediates (Shaw and Connell 1994). Among PAHs, B[*a*]P has been widely studied as a model inducer of MFO components. In mussels, its metabolism has been demonstrated both *in vitro* (Stegeman 1985, Michel *et al.* 1992) and *in vivo* (Michel *et al.* 1995) leading mainly to quinone compounds and to a lesser extent to diols and phenols. Quinones can suffer redox-cycling, thus giving rise to ROS. When the production of ROS is not sufficiently counterbalanced by the protective action of antioxidant enzymes/compounds, oxidative stress occurs leading to deleterious effects on proteins, lipids and nucleic acids. Redox-cycling of aromatic hydrocarbon quinones has been recently evidenced in *Mytilus edulis* (Sjölin and Livingstone 1997) and proposed as a potential mechanism of PAH-mediated toxicity in molluscs. Considering these assumptions, an increase in antioxidant defences could be anticipated after PAH exposure. Thus an increase of CAT activity, catalysing the transformation of the ROS hydrogen peroxide to molecular water has been reported in *M. galloprovincialis* after exposure to water accommodated fractions (WAF) of two crude oils and of a commercially lubricant oil (Cajaraville *et al.* 1992) or B[*a*]P (Akcha *et al.* 2000). Nevertheless, the contrary was observed in our case with a reduction of CAT activity at the CT dose of 2.24 mg l^{1}. Eertman *et al.* (1995) reported such a decrease after exposure of *M. edulis* to water contaminated with 6 µg l^{1} of B[*a*]P or fluoranthene. As this effect was not observed at lower concentrations, the authors advanced possible narcotic effects. However, in our case, CAT activity was decreased only at an intermediary, though important, dose and no more at higher concentrations. A similar diminution of CAT activity

was observed after a seven-day exposure of *M. galloprovincialis* to B[*a*]P or a lubricant oil WAF (Cancio *et al.* 1998). As this decrease suggests a lowered ability to protect cells against oxidative damage, we could have expected an enhancement of lipid peroxidation as previously noticed for *M. edulis* exposed to B[*a*]P (Livingstone *et al.* 1990) or collected in a PAH-contaminated site (Solé *et al.* 1996). An increase of NP level, representative of lipids remaining for peroxidation, as well as a reduction of PL level at the highest dose were however noticed. It could be explained by the enhancement of other antioxidant defences (e.g. glutathione peroxidase, NAD(P)H-dependent DT-diaphorase) not measured in that study. Beside potential inducers of oxidative stress, PAHs are known as inducers of cytochrome P4501A (CYP1A) in vertebrates and this feature has been successfully exploited in fishes to assess PAH-effects on aquatic ecosystems (Bucheli and Fent 1995). Less is known concerning the existence of such a P450 isoform in molluscs and its induction by PAHs. General components of the MFO system, i.e. cytochromes P450 (estimated by carbon monoxide difference spectra of dithionite-reduced samples) and *b5*, NADPH-cytochrome P450 reductase, NADH-cytochrome *b5* reductase and various P450-related activities, have been early evidenced in mussels (Livingstone *et al.* 1989). Subsequent immunological and molecular studies have revealed the existence of CYP1A-, CYP3A-, CYP2B-, CYP2E- and CYP4A-like proteins (Porte *et al.* 1995, Peters *et al.* 1998a) as well as CYP1A-, CYP3A-, CYP4A- and CYP11A- like genes (Wootton *et al.* 1995, Wootton *et al.* 1996) in *Mytilus sp.* More recently, the CYP4Y1 gene has been reported in *M. galloprovincialis* (Snyder 1998). Though such advanced studies have not been undertaken yet in *C. fluminea*, spectrophotometric measurements support the existence of the main components of the MFO system in this species and an increase of P450 level following trichloroethylene (chlorinated aliphatic hydrocarbon) or toluene (aromatic solvent) exposure has been evidenced previously (Vidal *et al.* 2001). The increase of P450 level (and to a lesser extent of P418 level) after exposure of *C. fluminea* to CT is in accordance with results from other laboratory and field studies in which mussels have been exposed to pure PAHs or complex PAH mixtures (Michel *et al.* 1993, Solé *et al.* 1994, Solé *et al.* 1996). More specifically, induction of P4501A-like protein by PAHs has been also reported (Solé *et al.* 1996, Peters *et al.* 1998b, Peters *et al.* 1999). An induction of NADPH-red. activity as observed in our case has been less evidenced in molluscs (Michel *et al.* 1993, Solé *et al.* 1994) and sometimes not detected at all (Livingstone *et al.* 1995, Solé *et al.* 1996). The strong correlation between P450 level or NADPH-red. activity and the CT dose makes these parameters good potential biomarkers of PAH-exposure in *C. fluminea*. Similar good correlations have been depicted previously in

Mytilus sp. (Solé *et al.* 1994, 1996). In vertebrates, including fishes, EROD activity is related to CYP1A. Its induction by PAHs is admitted and makes it a good biomarker of PAH-exposure in fishes (Bucheli and Fent 1995). In mussels, its existence has been rarely evidenced (Stegeman 1985). Thus its low level in *C. fluminea* is not surprising. Its independence to NADPH is a feature encountered with other P450-related activities from molluscs, possibly indicative of a P450-mediated one-electron oxidation mechanism (Livingstone *et al.* 1989, Michel *et al.* 1992, Dauberschmidt *et al.* 1997). Although NADPH-ind. EROD activity was correlated significantly with P450 level, it was not induced significantly by CT, therefore limiting its interest as a biomarker of PAH-exposure. The increase of NADH-red. activity could be related to its possible involvement in NADH-dependent redox-cycling of PAH-quinones (Sjölin and Livingstone, 1997). GSTs are phase II enzymes that can inactivate some PAH-derived reactive metabolites. However, we have observed a lack of modulation of GST activity, which is in accordance with other studies performed in molluscs (Livingstone *et al.* 1995, Solé *et al.* 1996). In some cases, an increase or a decrease, most of the time not correlated with the PAH concentration, has been observed in both fishes and mussels (Garrigues *et al.* 1990, Michel *et al.* 1993). In *C. fluminea*, GST activity towards EA, specific for Pi-class GSTs in vertebrates (Ketterer *et al.* 1988), increased with the dose but not in a significant manner. As this trend was not obvious using the non-specific substrate CDNB, a possible differential induction of GST isoenzymes could be suspected. As the main GSTs from *C. fluminea* have been recently characterised (Vidal *et al.* unpublished results), it would be pertinent to envisage a molecular approach to study effects of pollutants on GST isoenzymes' expression.

Harmful effects of metals are multiple, e.g. altering the permeability characteristics of the cell membranes specially by stimulating lipid peroxidation, reacting with various cytosolic (metallothioneins, proteins including enzymes, GSH, etc.) but also microsomal components (e.g. MFO system) as well as nucleic acids and proteins, modulating protein synthesis, altering physiology of lysosomes (Viarengo 1989). In our study CAT displayed opposite responses depending on the dose. At the lowest Cd-dose, CAT activity was inhibited but no concomitant increase of lipid peroxidation measured as TBARS was registered. Therefore the reduced intensity of CAT activity was still sufficient to protect cells from the deleterious effects of hydrogen peroxide and/or other protective antioxidant defences were possibly induced. At the two highest doses, induction of CAT activity was paralleled by an increase of peroxidizable lipids thus revealing an efficient protective action of that enzyme (and possibly

other antioxidant components) against an eventual Cd-induced oxidative stress. On this point, Cd is thought to induce oxidative stress in vertebrates, however mechanisms by which free radicals are produced are not well understood (Manca *et al.* 1991). Indeed Cd is a poor electron acceptor and donor and thus does not undergo redox-cycling reactions generating ROS as it is the case for copper or iron (Viarengo *et al.* 1990). In invertebrates, results are conflicting. The mussel *M. galloprovincialis* and the crab *Scylla serrata* exposed to Cd suffered no lipid peroxidation (Viarengo *et al.* 1990, Reddy 1997) whereas an increase of TBARS was reported in the marine bivalve *Perna viridis* (Tejo Prakash and Jagannatha Rao 1995). Such discrepancies could be due to different exposure duration and/or Cd-dose. Likewise, antioxidant defences including CAT activity were induced (Tejo Prakash and Jagannatha Rao 1995) or not affected (Reddy 1997) depending on the study. An inhibition of CAT activity was occasionally reported in vertebrates (fish: Roméo *et al.* 2000; rat: Sarkar *et al.* 1997). Inhibition of enzymatic activities can partly result from Cd-binding to sulfhydryl, hydroxyl, carboxyl, imidazole and amino residues of proteins as well as the -NH and C=O groups of the protein chain backbone (Viarengo *et al.* 1989). The microsomal localisation of MFO components combined with the high affinity of heavy metals for some protein residues make the MFO system a privileged target of Cd. Thus, inhibition of P450 and/or P450-related activities by heavy metals has sometimes been reported in fishes (Roméo *et al.* 1994) and mainly in rats (Rosenberg and Kappas 1991). In our case, inhibition of NADPH-ind. EROD activity from *C. fluminea*, not paralleled by a decrease of P450 level and an increase of PL level, could be a consequence of Cd-binding to some residues rather than an enhancement of lipid peroxidation of membranes leading to structural and functional alterations of related proteins. A slight but significant inhibition of GST activity was observed in *C. fluminea* but only at the lowest dose and with the non-specific substrate CDNB. Results concerning modulation of GST activity by heavy metals are greatly variable in invertebrates: induction in *P. viridis* exposed to lead, aluminium or Cd (Tejo Prakash and Jagannatha Rao 1995); induction or no change in *S. serrata* exposed to copper or Cd, respectively (Reddy 1997); inhibition in the Antarctic scallop *Adamussium colbecki* exposed to copper or mercury (Regoli *et al.* 1997). Therefore, GST activity does not appear as a reliable biomarker of Cd-exposure, at least in our experimental conditions. ChE activity is usually considered as a biomarker of organophosphate and carbamate exposure (Basack *et al.* 1998, Mora *et al.* 1999b) and in some cases of heavy metals (Regoli and Principato 1995, Labrot *et al.* 1996). However, PChE activity was not affected significantly by Cd-exposure in our experimental conditions. Thus modulation of ChE activity by heavy metals can not be generalised.

DA is a statistical method that has been applied successfully to the classification of the pollution status of different sites in field studies (Adams *et al.* 1994), but also to emphasise differences between doses of contaminants in laboratory experiments (Vidal *et al.* 2001). In our case, the control group was well discriminated from dose groups for both CT and Cd. The absence of discrimination between some dose groups (the first and the second ones for CT; the second and the third but also the fourth ones for Cd) suggests similar biochemical parameter responses when clams are exposed to these pollutant concentrations. Parameters contributing significantly to discrimination between doses were globally specific to each pollutant: MFO components for CT and parameters related to oxidative stress for Cd. However, some parameters displaying significant responses with respect to control, basing on Tukey test, were not considered as discriminating factors (CAT activity and PL level for CT, PLI level and NADPH-ind. EROD activity for Cd). On the contrary, parameters not affected significantly by either pollutant were considered as discriminating (PChE activity for CT, NADPH-red. and GST/EA activities for Cd). It could be explained by the fact that DA makes discrimination between all groups and not only between control and dose ones. Finally the most relevant biomarkers of CT or Cd exposure in *C. fluminea* can be considered as those discriminating dose groups but also displaying significant responses compared to control, i.e. NADPH-red. and NADH-red. activities, P450, P418 and NP levels for CT; CAT and GST/CDNB activities as well as NP level for Cd.

In conclusion, when exposing the freshwater clams *C. fluminea* to Cd or a complex mixture of PAHs, significant biochemical effects consistent with other studies were evidenced. Particularly, components of MFO system and parameters relative to oxidative stress proved to be relevant biomarkers of either pollutant exposure in our experimental conditions. These laboratory results contribute to consider *C. fluminea* as a sentinel organism, however additional studies, specially field ones, are needed. The rapid decrease of Cd and particularly CT concentrations in water was representative of an acute contamination. Nevertheless, certain biochemical responses were significant and sometimes dose-dependent five days after the initial contamination. Therefore, biomarkers are superior to chemical measurements in that they can reveal a pollution event even after partial or complete disappearance of contaminants. In this sense, a multibiomarker approach increases the probability of detecting past or current pollution. In order to validate these results, mesocosm studies are now required for a static laboratory study is not quite representative of realistic environmental conditions.

Aknowledgements

This study was supported by a PhD grant from the TotalFinaElf Company. The authors are grateful to Muriel Audroin and Nadine Médevielle for their precious technical assistance and to the Laboratory of Control and Environment of TotalFinaElf (Lacq, France) for chemical analyses.

References

Adams, S.M., Ham, K.D. and Beauchamp, J.J., 1994, Application of canonical variate analysis in the evaluation and presentation of multivariate biological response data. *Environmental Toxicology and Chemistry*, **13(10)**, 1673-1683.

Akcha, F., Izuel, C., Venier, P., Budzinski, H., Burgeot, T. and Narbonne, J-F., 2000, Enzymatic biomarker measurement and study of DNA adducts formation in benzo[a]pyrene-contaminated mussels, *Mytilus galloprovincialis*. *Aquatic Toxicology*, **49(4)**, 269-287.

Amodio-Cocchieri, R., Del Prete, U., Arnese, A., Giuliano, M. and Roncioni, A., 1993, Heavy metals and polycyclic aromatic hydrocarbons (PAH's) in marine organisms from the Ionian Sea (Italy). *Bulletin of Environmental Contamination and Toxicology*, **50**, 618-625.

Araujo, R., Moreno, D. and Ramos, M.A., 1993, The Asiatic clam *Corbicula fluminea* (Müller, 1774) (Bivalvia: Corbiculidae) in Europe. *American Malacological Bulletin*, **10**, 39-49.

Basack, S.B., Oneto, M.L., Fuchs, J.S., Wood, E.J. and Kesten, E.M., 1998, Esterases of *Corbicula fluminea* as biomarkers of exposure to organophosphorus pesticides. *Bulletin of Environmental Contamination and Toxicology*, **61**, 569-576.

Britton, J.C. and Morton, B., 1979, *Corbicula* in North America: the evidence reviewed and evaluated. *Proceedings of the First International Corbicula Symposium* (Fort Worth, Texas: Texas Christian University Research Foundation Publication), pp. 249-287.

Bucheli, T.D. and Fent, K., 1995, Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical Reviews in Environmental Science and Technology*, **25(3)**, 201-268.

Buege, J.A. and Aust, S.D., 1978, Microsomal lipid peroxidation. *Methods in Enzymology*, **50**, 302-310.

Burke, M.D. and Mayer, R.T., 1974, Ethoxyresorufin : direct fluorimetric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metabolism and Disposition*, **2(6)**, 583-588.

Cajaraville, M.P., Uranga, J.A. and Angulo, E., 1992, Comparative effects of the water accommodated fraction of three oils on mussels -3. Quantitative histochemistry of enzymes related to the detoxication metabolism. *Comparative Biochemistry and Physiology*, **103C(2)**, 369-377.

Cancio, I., Orbea, A., Völkl, A., Fahimi, H.D. and Cajaraville, M.P., 1998, Induction of peroxisomal oxidases in mussels: comparison of effects of lubricant oil and benzo(*a*)pyrene with two typical peroxisome proliferators on peroxisome structure and function in *Mytilus galloprovincialis*. *Toxicology and Applied Pharmacology*, **149**, 64-72.

Clairborne, A., 1985, Catalase activity. In *Handbook of Methods for Oxygen Radical Research*, edited by R.A. Greenwald (Boca Raton, Florida: CRC press), pp. 283-284.

Dauberschmidt, C., Dietrich, D.R. and Schlatter, C., 1997, Investigations on the biotransformation capacity of organophosphates in the mollusc *Dreissena polymorpha* P. *Aquatic Toxicology*, **37**, 283-294.

Doherty, F.G., Failla, M.L. and Cherry, D.S., 1988, Metallothionein-like heavy metal binding protein levels in Asiatic clams are dependent on the duration and mode of exposure to cadmium. *Water Research*, **22(7)**, 927-932.

Eertman, R.H.M., Groenink, C.L.F.M.G., Sandee, B. and Hummel, H., 1995, Response of the blue mussel *Mytilus edulis* L. following exposure to PAHs or contaminated sediment. *Marine Environmental Research*, **39**, 169-173.

Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M., 1961, A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, 7, 88-95.

Estabrook, R.W. and Werringloer, J., 1978, The measurement of difference spectra: application to the cytochromes of microsomes. *Methods in Enzymology*, 52, 212-220.

Fitzpatrick, P.J. and Sheehan, D., 1993, Separation of multiple forms of glutathione *S*-transferase from the blue mussel, *Mytilus edulis*. *Xenobiotica*, 23, 851-861.

Fraisse, L., Verlhac, J.B., Roche, B., Rascle, M.C., Rabion, A. and Seris, J.L., 1993, Long-chain-substituted uric acid and 5,6-diaminouracil derivatives as novel agents against free radical processes: synthesis and *in vitro* activity. *Journal of Medicinal Chemistry*, 36(10), 1465-1473.

Garrigues, P., Raoux, C., Lemaire, P., Mathieu, A., Ribera, D., Narbonne, J.F. and Lafaurie, M., 1990, *In situ* correlations between polycyclic aromatic hydrocarbons (PAH) and PAH metabolizing system activities in mussels and fish in the Mediterranean Sea: preliminary results. *International Journal of Environmental Analytical Chemistry*, **38**, 379-387.

Guengerich, F.P., 1994, Analysis and characterization of enzymes. In *Principles and Methods of Toxicology (third edition)*, edited by A.W. Hayes (New-York: Raven Press), pp. 1269-1270.

Habig, W.H., Pabst, M.J. and Jakoby, W.B., 1974, The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, **249(22)**, 7130-7139.

Inza, B., Ribeyre, F., Maury-Brachet, R. and Boudou, A, 1997, Tissue distribution of the inorganic mercury, methylmercury and cadmium in the Asiatic clam (*Corbicula fluminea*) in relation to the contamination levels of the water column and sediment. *Chemosphere*, **35(12)**, 2817-2836.

Jensen, A. and Bro-Rasmussen, F., 1992, Environmental cadmium in Europe. *Reviews of Environmental Contamination and Toxicology*, **125**, 101-181.

Ketterer, B., Meyer, D.J. and Clark, A.G., 1988, Soluble glutathione transferase isozymes. In *Glutathione Conjugation, Mechanisms and Biological Significance*, edited by B. Ketterer and H. Sies (London: Academic Press), pp. 73-135.

Labrot, F., Ribera, D., Saint-Denis, M. and Narbonne, J-F., 1996, *In vitro* and *in vivo* studies of potential biomarkers of lead and uranium contamination: lipid peroxidation, acetylcholinesterase, catalase and glutathione peroxidase activities in three non-mammalian species. *Biomarkers*, **1**, 21-28.

Labrot, F., Narbonne, J-F., Ville, P., Saint-Denis, M. and Ribera, D., 1998, Acute toxicity, toxicokinetics, and tissue target of lead and uranium in the clam *Corbicula fluminea* and the worm *Eisenia fetida*: comparison with the fish *Brachydanio rerio*. *Archives of Environmental Contamination and Toxicology*, **36(2)**, 167-178.

Livingstone, D.R., 1993, Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. *Journal of Chemical Technology and Biotechnology*, **57**, 195-211.

Livingstone, D.R., Kirchin, M.A. and Wiseman, A., 1989, Cytochrome P-450 and oxidative metabolism in molluscs. *Xenobiotica*, **19(10)**, 1041-1062.

Livingstone, D.R., Garcia Martinez, P., Michel, X., Narbonne, J.F., O'Hara, S., Ribera, D. and Winston, G.W., 1990, Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* L., and other molluscs. *Functional Ecology*, **4**, 415-424.

Livingstone, D.R., Lips, F., Garcia Martinez, P. and Pipe, R.K., 1992, Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*. *Marine Biology*, **112**, 265-276.

Livingstone, D.R., Lemaire, P., Matthews, A., Peters, L.D., Porte, C., Fitzpatrick, P.J., Förli, L., Nasci, C., Fossato, V., Wootton, N. and Goldfarb, P., 1995, Assessment of the impact of organic pollutants on Goby (*Zosterisessor ophiocephalus*) and mussel (*Mytilus galloprovincialis*) from the Venice Lagoon, Italy: biochemical studies. *Marine Environmental Research*, **39**, 235-240.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J., 1951, Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.

Manca, D., Ricard, A.C., Trottier, B. and Chevalier G., 1991, Studies on lipid peroxidation in rat tissues following administration of low and moderate doses of cadmium chloride. *Toxicology*, **67**, 303-323.

Michel, X.R., Cassand, P.M., Ribera, D.G. and Narbonne, J-F., 1992, Metabolism and mutagenic activation of benzo(a)pyrene by subcellular fractions from mussel (*Mytilus galloprovincialis*) digestive gland and sea bass (*Discenthrarcus labrax*) liver. *Comparative Biochemistry and Physiology*, **103C(1)**, 43-51.

Michel, X., Suteau, P., Robertson, L.W. and Narbonne, J-F., 1993, Effects of benzo(a)pyrene, 3,3',4,4'-tetrachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl on the xenobiotic-metabolizing enzymes in the mussel (*Mytilus galloprovincialis*). *Aquatic Toxicology*, **27**, 335-344.

Michel, X.R., Beasse, C. and Narbonne, J-F., 1995, *In vivo* metabolism of benzo(a)pyrene in the mussel *Mytilus galloprovincialis*. *Archives of Environmental Contamination and Toxicology*, **28**, 215-222.

Mora, P., Fournier, D. and Narbonne, J-F., 1999a, Cholinesterases from the marine mussels *Mytilus galloprovincialis* Lmk. and *M. edulis* L. and from the freshwater bivalve *Corbicula fluminea* Müller. *Comparative Biochemistry and Physiology*, **122C**, 353-361.

Mora P., Michel, X. and Narbonne, J-F., 1999b, Cholinesterase activity as potential biomarker in two bivalves. *Environmental Toxicology and Pharmacology*, **7**, 253-260.

Narbonne, J.F., Djomo, J.E., Ribera, D., Ferrier, V. and Garrigues, P., 1999, Accumulation kinetics of polycyclic aromatic hydrocarbons adsorbed to sediment by the mollusk *Corbicula fluminea*. *Ecotoxicology and Environmental Safety*, **42**, 1-8.

Neff, J.M., 1984, Bioaccumulation of organic micropollutants from sediments and suspended particulates by aquatic animals. *Fresenius' Zeitschrift für Analytische Chemie*, **319**, 132-136.

Organisation Internationale de Normalisation (editor) 1996, *Norme ISO 11885: Qualité de l'eau-Détermination de 33 éléments par spectroscopie d'émission atomique avec plasma couplé par induction* (Genève: ISO Publications).

Peters, L.D., Nasci, C. and Livingstone D.R., 1998a, Immunochemical investigations of cytochrome P450 forms/epitopes (CYP1A, 2B, 2E, 3A and 4A) in digestive gland of *Mytilus sp. Comparative Biochemistry and Physiology*, **121C**, 361-369.

Peters, L.D., Nasci, C. and Livingstone, D.R., 1998b, Variation in levels of cytochrome P4501A, 2B, 2E, 3A and 4A-immunopositive proteins in digestive gland of

indigenous and transplanted mussel *Mytilus galloprovincialis* in Venice Lagoon, Italy. *Marine Environmental Research*, **46(1-5)**, 295-299.

Peters, L.D., Shaw, J.P., Nott, M., O'Hara, S.C.M. and Livingstone, D.R., 1999, Development of cytochrome P450 as a biomarker of organic pollution in *Mytilus* sp.: field studies in United Kingdom ('Sea Empress' oil spill) and the Mediterranean Sea. *Biomarkers*, **4(6)**, 425-441.

Porte, C., Lemaire, P., Peters, L.D. and Livingstone, D.R., 1995, Partial purification and properties of cytochrome P450 from digestive gland microsomes of the common mussel, *Mytilus edulis* L. *Marine Environmental Research*, **39**, 27-31.

Reddy, P.S., 1997, Modulations of antioxidant enzymes in the gill and hepatopancreas of the edible crab *Scylla serrata* during exposure to cadmium and copper. *Fresenius Environmental Bulletin*, **6**, 589-597.

Regoli, F. and Principato, G., 1995, Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. *Aquatic Toxicology*, **31**, 143-164.

Regoli, F., Nigro, M., Bertoli, E., Principato, G. and Orlando, E., 1997, Defenses against oxidative stress in the Antarctic scallop *Adamussium colbecki* and effects of acute exposure to metals. *Hydrobiologia*, **355**, 139-144.

Roméo, M., Mathieu, A., Gnassia-Barelli, M., Romana, A. and Lafaurie, M., 1994, Heavy metal content and biotransformation enzymes in two fish species from the NW Mediterranean. *Marine Ecology Progress Series*, **107**, 15-22.

Roméo, M., Bennani, N., Gnassia-Barelli, M., Lafaurie, M. and Girard, J.P., 2000, Cadmium and copper display different responses towards oxidative stress in the kidney of the sea bass *Dicentrarchus labrax*. *Aquatic Toxicology*, **48**, 185-194.

Rosenberg, D.W. and Kappas, A., 1991, Induction of heme oxygenase in the small intestinal epithelium: a response to oral cadmium exposure. *Toxicology*, **67**, 199-210.

Sarkar, P., Yadav P. and Bhatnagar, D., 1997, Cadmium-induced lipid peroxidation and the antioxidant system in rat erythrocytes: the role of antioxidants. *Journal of Trace Elements in Medicine and Biology*, **11**, 8-13.

Shaw, G.R. and Connell, D.W., 1994, Prediction and monitoring of the carcinogenicity of polycyclic aromatic compounds (PACs). *Reviews of Environmental Contamination and Toxicology*, **135**, 1-62.

Sjölin, A.M. and Livingstone, D.R., 1997, Redox cycling of aromatic hydrocarbon quinones catalysed by digestive gland microsomes of the common mussel (*Mytilus edulis* L.). *Aquatic Toxicology*, **38**, 83-99.

Snyder, M.J., 1998, Cytochrome P450 enzymes belonging to the CYP4 family from marine invertebrates. *Biochemical and Biophysical Research Communications*, **249**, 187-190.

Solé, M., Porte, C. and Albaigés, J., 1994, Mixed-function oxygenase system components and antioxidant enzymes in different marine bivalves: its relation with contaminant body burdens. *Aquatic Toxicology*, **30**, 271-283.

Solé, M., Porte, C., Biosca, X., Mitchelmore, C.L., Chipman, J.K., Livingstone, D.R. and Albaigés, J., 1996, Effetcs of the "Aegean Sea" oil spill on biotransformation enzymes, oxidative stress and DNA-adducts in digestive gland of the mussel (*Mytilus edulis* L.). *Comparative Biochemistry and Physiology*, **113C(2)**, 257-265.

Stegeman, J.J., 1985, Benzo[a]pyrene oxidation and microsomal enzyme activity in the mussel (*Mytilus edulis*) and other bivalve mollusc species from the Western North Atlantic. *Marine Biology*, **89**, 21-30.

Taylor, D., 1983, The significance of the accumulation of cadmium by aquatic organisms. *Ecotoxicology and Environmental Safety*, **7**, 33-42.

Tejo Prakash, N. and Jagannatha Rao, K.S., 1995, Modulations in antioxidant enzymes in different tissues of marine bivalve *Perna viridis* during heavy metal exposure. *Molecular and Cellular Biochemistry*, **146**, 107-113.

Viarengo, A., 1985, Biochemical effects of trace metals. *Marine Pollution Bulletin*, **16(4)**, 153-158.

Viarengo, A., 1989, Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. *Reviews in Aquatic Sciences*, **1**, 295-316.

Viarengo, A., Canesi, L., Pertica, M., Poli, G., Moore, M.N. and Orunesu, M., 1990, Heavy metal effects on lipid peroxidation in the tissues of *Mytilus galloprovincialis* Lam. *Comparative Biochemistry and Physiology*, **97C(1)**, 37-42.

Vidal, M-L. and Narbonne, J-F., 2000, Characterization of glutathione *S*-transferase activity in the Asiatic clam *Corbicula fluminea*. *Bulletin of Environmental Contamination and Toxicology*, **64**, 455-462.

Vidal, M-L., Bassères, A. and Narbonne, J-F., 2001, Potential biomarkers of trichloroethylene and toluene exposure in *Corbicula fluminea*. *Environmental Toxicology and Pharmacology*, 9, 87-97.

Wootton, A.N., Herring, C., Spry, J.A., Wiseman, A., Livingstone, D.R. and Goldfarb, P.S., 1995, Evidence for the existence of cytochrome P450 gene families (CYP1A, 3A, 4A, 11A) and modulation of gene expression (CYP1A) in the mussel *Mytilus* spp. *Marine Environmental Research*, 39, 21-26.

Wootton, A.N., Goldfarb, P.S., Lemaire, P., O'Hara, S.C.M. and Livingstone, D.R., 1996, Characterization of the presence and seasonal variation of a CYP1A-like enzyme in digestive gland of the common mussel, *Mytilus edulis*. *Marine Environmental Research*, 42(1-4), 297-301.

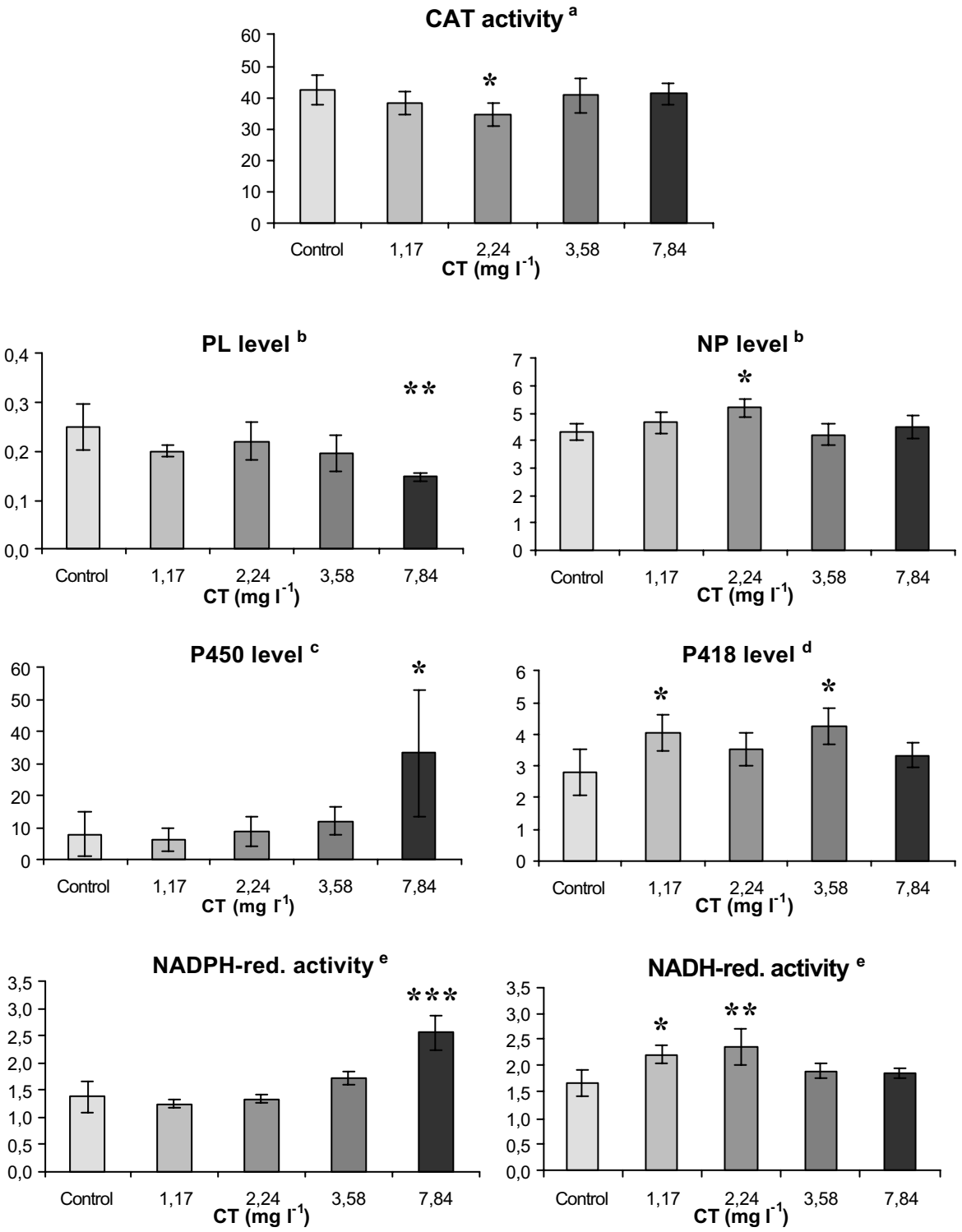


Figure 1. Biochemical parameters exhibiting significant responses compared to control after exposure of *Corbicula fluminea* to different concentrations (measured ones) of coal tar fraction (CT). Values are mean ± S.D. (n=5). [a, b, c, d, e] : in µmoles min^{-1} mg $protein^{-1}$, nmoles mg $protein^{-1}$, pmoles mg $protein^{-1}$, DO mg $protein^{-1}$ and nmoles min^{-1} mg $protein^{-1}$, respectively. *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.001$, comparing exposed to control.

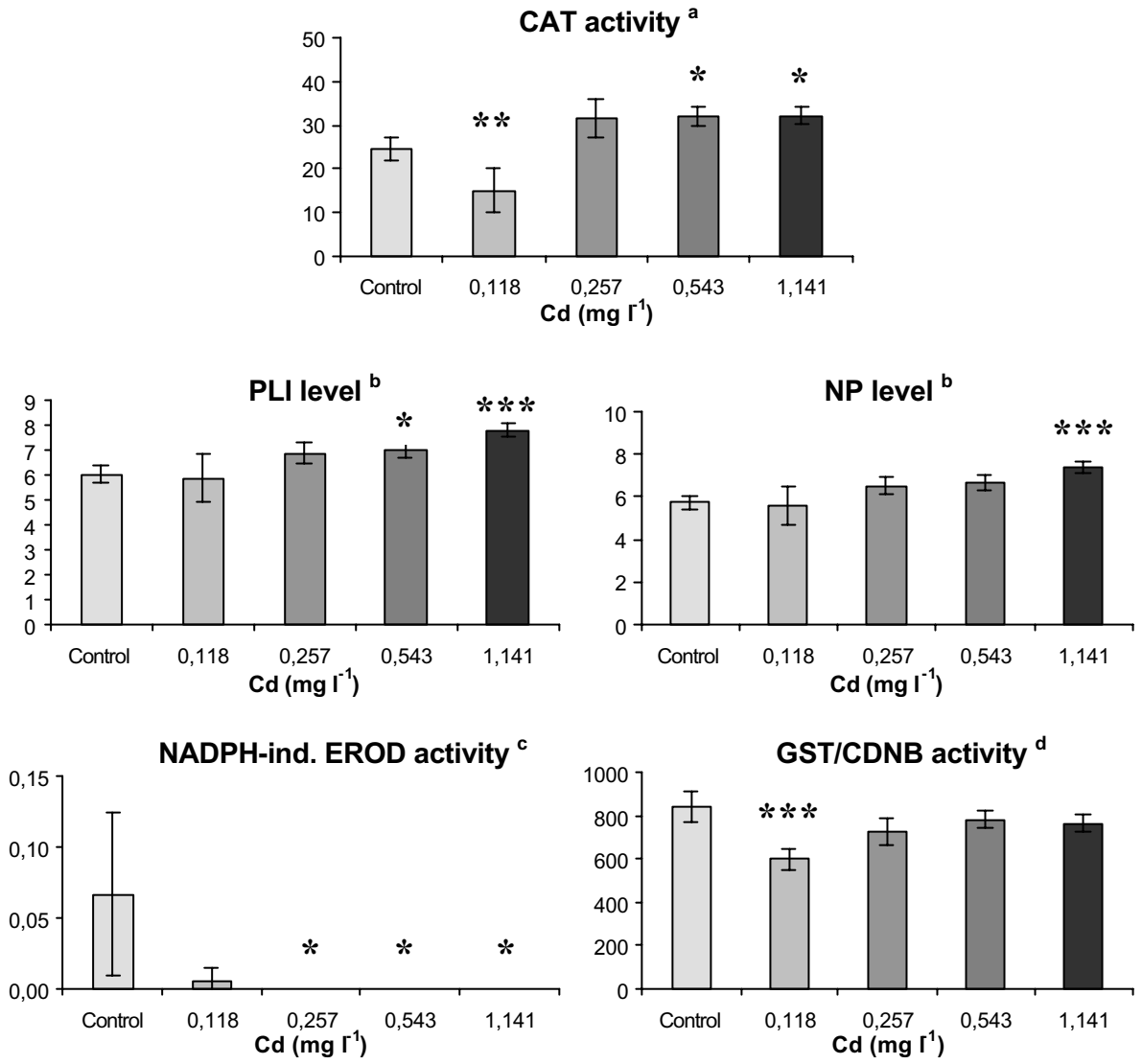


Figure 2. Biochemical parameters exhibiting significant responses compared to control after exposure of *Corbicula fluminea* to different concentrations (measured ones) of cadmium (Cd). Values are mean ± S.D. (n=5). [a, b, c, d] : in µmoles min^{-1} mg $protein^{-1}$, nmoles mg $protein^{-1}$, pmoles min^{-1} mg $protein^{-1}$, nmoles min^{-1} mg $protein^{-1}$, respectively. *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.001$, comparing exposed to control.

Table 1. Concentrations of coal tar fraction (CT) and cadmium (Cd) in filtered lake water at the beginning (D0) and at the end (after 5 days: D5) of the exposure.

Chemical	Theoretical concentration [a] at D0	Actual concentration [b] at D0	Actual concentration [b] at D5	Loss of chemical after exposure [c]
CT	0	nd	nd	-
	0.75	1.17 ± 0.13	0.000 ± 0.000	100.0
	1.5	2.24 ± 0.30	0.003 ± 0.005	99.9
	3	3.58 ± 1.02	0.043 ± 0.025	98.8
	6	7.84 ± 2.61	0.247 ± 0.047	96.8
Cd	0	nd	nd	-
	0.154	0.118	0.038	67.8
	0.308	0.257	0.140	45.5
	0.615	0.543	0.372	31.5
	1.230	1.141	0.869	23.8

[a] mg l^{-1}.

[b] mg l^{-1}. Values are mean ± S.D. (n=3) for CT.

[c] % / actual concentration at D0.

nd: not detected.

Table 2. Synthesis of discriminant analysis. Biochemical parameters contributing significantly to discrimination between doses of coal tar fraction (CT) or cadmium (Cd), included control: catalase (CAT) activity, level of net peroxidation (NP), levels of cytochromes P450 (P450) and P418 (P418), NADPH-cytochrome *c* reductase (NADPH-red.) and NADH-cytochrome *c* reductase (NADH-red.) activities, glutathione *S*-transferase activity towards 1-chloro-2,4-dinitrobenzene (GST/CDNB) or ethacrynic acid (GST/EA) and propionylcholinesterase (PChE) activity; n=35 (CT) or 30 (Cd).

Biochemical parameter	Wilks Lambda	Partial Lambda	F of exclusion	p [a]
	CT			
NADPH-red.	0.052469	0.131228	41.37708	***
P450	0.022478	0.306318	14.15366	***
NADH-red.	0.014143	0.486857	6.58745	***
P418	0.013423	0.512975	5.93383	**
PChE	0.010367	0.664173	3.16019	*
NP	0.010171	0.676961	2.98244	*
	Cd			
CAT	0.041215	0.208341	19.94911	***
GST/CDNB	0.040514	0.211946	19.52052	***
NP	0.025040	0.342921	10.05967	***
NADPH-red.	0.014952	0.574277	3.89192	*
GST/EA	0.014271	0.601719	3.47500	*

[a] Significant contribution to discrimination at *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

Table 3. Discriminant analysis: distances between doses of coal tar fraction (CT) or cadmium (Cd), included control, expressed as square of Mahalanobis distances between group centroids.

Group (dose of CT [d])		1 (Control)	2 (1.17)	3 (2.24)	4 (3.58)	5 (7.84)
1	(Control)	-	26.33 [c]	23.88 [c]	12.27 [b]	138.18 [c]
2	(1.17)	-	-	3.39 [ns]	35.60 [c]	234.35 [c]
3	(2.24)	-	-	-	33.43 [c]	204.89 [c]
4	(3.58)	-	-	-	-	101.53 [c]
5	(7.84)	-	-	-	-	-
Group (dose of Cd [d])		1 (Control)	2 (0.118)	3 (0.257)	4 (0.543)	5 (1.141)
1	(Control)	-	62.03 [c]	24.06 [c]	23.06 [c]	33.80 [c]
2	(0.118)	-	-	73.26 [c]	102.56 [c]	106.27 [c]
3	(0.257)	-	-	-	5.28 [ns]	7.94 [ns]
4	(0.543)	-	-	-	-	9.91 [a]
5	(1.141)	-	-	-	-	-

[a, b, c] Significant discrimination at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

[ns] Non-significant.

[d] mg l^{-1} (measured concentrations).

L'**article n°7** correspond en partie à la synthèse des résultats obtenus lors des contaminations avec le trichloroéthylène, le toluène, le cadmium et la coupe de goudron de houille présentés dans les deux articles précédents. Il met notamment l'accent sur l'importance d'une approche multibiomarqueurs. En particulier, une analyse discriminante a permis de mettre en évidence une discrimination des contaminants reposant sur l'ensemble des réponses des biomarqueurs. Ainsi un biomarqueur isolé est rarement spécifique d'un polluant donné. Par contre l'utilisation d'une palette de biomarqueurs permet l'obtention de profils de réponses spécifiques de chaque contaminant. Afin de valider un paramètre biochimique comme biomarqueur de pollution, des études de terrain s'imposent. Une telle étude conduite sur un site industriel est présentée dans l'article n°7. Des *Corbicula fluminea* ont été exposées pendant sept jours en cinq points d'une rivière situés en amont et en aval d'une sortie d'effluents industriels. A l'issue de cette période, les activités catalase, glutathion *S*-transférase (avec comme substrat le 1-chloro-2,4-dinitrobenzène) et propionylcholinestérase ainsi que les teneurs en lipides peroxydés et peroxydables ont été mesurées. Tous les paramètres biochimiques mesurés chez les animaux transplantés en aval du point de rejet ont présenté des réponses significatives par rapport à ceux mesurés chez les animaux transplantés en amont (site de référence). Grâce à une analyse discriminante, les cinq sites ont pu être distingués en termes d'intensité de pollution. Par ailleurs, plusieurs indicateurs concernant des niveaux d'organisation biologique supérieurs, tels que les biocénoses, ont été mesurés parallèlement par le Service Environnement du Groupement de Recherches de Lacq de TotalFinaElf. Ces résultats, présentés à la suite de l'article n°7, ont mis en évidence une bonne corrélation entre les réponses des biomarqueurs et celles des autres indicateurs biologiques, soulignant ainsi la signification écologique des biomarqueurs mais également leur sensibilité en terme de détection des effets biologiques. Afin de caractériser la pollution d'un point de vue qualitatif, des études complémentaires de laboratoire et de terrain permettant d'obtenir des profils typiques de réponses pour les principaux polluants présents dans les milieux dulçaquicoles seront nécessaires.

ARTICLE 7

Interest of a multibiomarker approach in the assessment of freshwater ecosystem quality: laboratory and field studies

Vidal, M-L., Bassères, A., Narbonne J-F.

***Water Science and Technology, 2001, 44 (2-3), 305-312*.**

ERRATUM

p. 230 (306), ligne 14: stabulation *au lieu* de stabilation.

p. 232 (308), ligne 36: P418 increased significantly at 1.17 mg/l (+45%) and 3.58 mg/l (+52%) *au lieu de* P418 increased significantly at 2.2 mg/l (+26%) and 7.8 mg/l (+19%).

p. 234 (310), tableau 5: CAT: - (CT) *au lieu de* CAT: ns (CT).

Interest of a multibiomarker approach in the assessment of freshwater ecosystem quality: laboratory and field studies

M.L. Vidal*, A. Bassères and J.F. Narbonne***

*Environmental and Toxicological Chemistry Laboratory, UPRES-A 5472 CNRS, Department of Biochemical Toxicology, University of Bordeaux 1, Avenue des Facultés, 33405 Talence, France

** ELF, Group of Research of Lacq, Department of Environment, BP 34, 64170 Lacq, France

Abstract The aims of this study were to validate several biochemical parameters as biomarkers of pollution in the freshwater bivalve *Corbicula fluminea* and to underline the interest of a multibiomarker approach in environmental biomonitoring. The study was divided into a laboratory exposure to 4 doses of trichloroethylene, toluene, cadmium chloride or a coal tar fraction for 5 days and a field exposure for one week in 5 sites surrounding an industrial effluent outlet. Whatever the product was, parameters that exhibited significant responses were mainly parameters related to oxidative stress and components of phase I metabolism. As a result of discriminant analysis, doses were clearly discriminated from the control and from each other. Likewise, products were discriminated from each other, based on results of the whole parameter responses obtained for the first dose. Concerning the field experiment, all biochemical parameters assayed exhibited significant responses for sites located downstream of the effluent outlet, compared to the upstream reference site. Through a discriminant analysis, sites could be distinguished from each other in terms of pollution intensity. In order to characterise pollution at a qualitative level, further laboratory and field studies are needed to obtain typical profiles for the main pollutants present in freshwater ecosystems.

Keywords Biomarkers; biomonitoring; caging; *Corbicula fluminea*; freshwater ecosystems; pollutants

Water Science and Technology **Vol 44 No 2-3 pp 305-312** © IWA Publishing 2001

Introduction

To assess the impact of pollutants on freshwater ecosystems, biomarkers make up a predictive method that concerns every molecular, biochemical, cellular, physiological or behavioural change occurring in an organism after an exposure to pollution. *Corbicula fluminea* is a freshwater bivalve originating from Asia that has become a major component of many benthic communities in Europe (Araujo *et al.*, 1993). As it is known to accumulate pollutants (Narbonne *et al.*, 1999) we study the possible use of this species as a sentinel organism of freshwater ecosystems. Thus, the aim of this study was to assess the potential use of several biochemical parameters as biomarkers in *C. fluminea* and to underline the interest of a multibiomarker approach in the assessment of freshwater ecosystem quality. Components of phase I and phase II (de)toxification metabolism, that is to say: cytochromes P450 (P450), P418 (P418), *b*5 (b5), NADPH and NADH-cytochrome *c* reductases (NADPH-red and NADH-red), NADPH-independent ethoxyresorufin *O*-deethylase (EROD), glutathione *S*-transferase using 1-chloro-2,4-dinitrobenzene or ethacrynic acid as a substrate (GST/CDNB and GST/EA), as well as parameters related to oxidative stress such as catalase (CAT), peroxided and peroxidizable lipids (PL and PLI), net peroxidation (NP) were measured. Propionylcholinesterase (PChE) was also considered. The first part of this study consisted of a laboratory experiment in which *C. fluminea* were exposed to 4 doses of 4 contaminants: trichloroethylene (TCE), toluene (TOL), cadmium chloride ($CdCl_2$) and a coal tar fraction (CT) with a high Polycyclic Aromatic Hydrocarbons content (PAHs). The second part of the study was a field experiment in which *C. fluminea* were transplanted by caging into 5 sites located around an industrial effluent outlet. After several

days of exposure, responses of biochemical parameters were studied and appropriate statistical analysis was performed, especially to discriminate doses, products (laboratory experiment) and sites (field experiment) from each other.

Methods

Chemicals

Trichloroethylene (TCE), toluene (TOL), cadmium chloride ($CdCl_2$) and the coal tar fraction (CT) were obtained from SDS (Peypin, France), Carlo Erba (Rueil Malmaison, France), Interchim (Montluçon, France) and Chemco (Poissy, France), respectively. Other chemicals were of the best technical grade available.

Animals

Adult *C. fluminea*, the anterior-posterior length of which ranged from 15 to 20 mm, were collected from the banks of the non-polluted freshwater Cazaux-Sanguinet lake (Aquitaine, France). They were placed in a plastic tank containing lake water for the transport back to the laboratory. They were maintained in stabilation for 8 days before the experiment in glass aquariums containing lake water at 20°C (laboratory experiment) or dechlorinated tap water at 18.5°C (field experiment). Aeration was provided by air bubbling. Clams were not fed during this period and were held under a natural light cycle. No sexual differences were taken into account for *C. fluminea* are hermaphroditic.

Laboratory experiment

Exposure studies were performed for 5 days in glass aquariums containing 10 l of lake water (contaminated or not) previously filtered through 50 µm and then 5 µm cotton cartridges (Cole-Parmer Masterflex® pump, model 7019-20, Cole-Parmer Instrument Co.). Ranges of doses assayed were of 1.56, 6.25, 25 and 100 mg/l for TCE; 7.5, 15, 30 and 60 mg/l for TOL; 0.31, 0.625, 1.25 and 2.5 mg/l for $CdCl_2$ (that is to say 0.154, 0.308, 0.615 and 1.23 mg/l expressed as Cd content) and 0.75, 1.5, 3 and 6 mg/l for CT. As $CdCl_2$, TCE and TOL were hydrosoluble in the range of doses assayed, they were directly mixed with filtered lake water. CT was previously mixed with dimethylsulfoxide (DMSO), the final concentration of which was 0.5 g/l. Appropriate controls consisting of filtered lake water (plus DMSO in the case of CT) were prepared. 50 *C. fluminea* were placed in each aquarium. They were not fed and were held under a natural light cycle at 20°C during the exposure. Aeration was provided by intermittent air bubbling (15 min every 6 hr for $CdCl_2$ and CT; 15 min every 12 hr for TCE and TOL).

Field experiment

60 *C. fluminea* were transplanted by caging into different points of a river in the vicinity of a factory. 5 sites were selected around the industrial effluent outlet. The first one was located just upstream of the effluent outlet and was considered as the reference site (UPS). The downstream sites were located 10 metres (DS1), one hundred metres (DS2), several hundred metres (DS3) and several kilometres (DS4) downstream of the effluent outlet. The exposure time was of 7 days.

Chemical analysis

Concentrations of TCE, TOL, $CdCl_2$ and CT were determined at the beginning (T0) and the end (T5) of the laboratory experiment. Analysis was performed by the Laboratory of Control and Environment of Elf Aquitaine (Lacq, France). Quantification of TCE, TOL, $CdCl_2$ (Cd content) and CT was performed by gas chromatography coupled to mass

spectrometry, High Performance Liquid Chromatography (HPLC), atomic absorption spectrophotometry and HPLC, respectively.

Preparation of subcellular fractions

All steps of homogenisation were achieved at 4°C. After the shell and the crystalline style were removed, *C. fluminea* were dissected-out to obtain entire soft bodies or visceral masses. For each experimental condition, 5 pools of 5 animals were prepared. Body samples were rinsed in 100 mM phosphate buffer, pH 7.4, dried on absorbent paper sheets, weighed and homogenised in the same phosphate buffer (1:4 weight:volume ratio) using an Ultra-Turrax® Antrieb T25 (IKA® Labortechnik). Homogenates were then centrifuged at 9,000 g for 30 min in a Sigma 3 MK centrifuge. In the case of soft entire bodies, supernatants consisting of the submitochondrial fractions (S9) were collected and stored at –80°C. For visceral masses, they were centrifuged at 105,000 g for 1 hr in a Beckman LE-80 ultracentrifuge. Supernatants (cytosolic fractions) were collected and stored at –80°C. Microsomal pellets were resuspended in a 100 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 20% glycerol, then collected and stored at –80°C.

Biochemical assays

Enzymatic activities were measured on a dual-beam temperature-controlled Kontron Uvikon 932 spectrophotometer except for EROD activity which was carried out on a Shimadzu RF-540 spectrofluorophotometer. Assays were run in triplicate for each pool. CAT, PChE, GST (CDNB/EA), EROD and NAD(P)H-red activities were assayed as described in Clairborne (1985), Ellman *et al.* (1961), Habig *et al.* (1974), Burke and Mayer – without NADPH – (1974) and Guengerich (1994), respectively. P450, P418 (denatured form of cytochrome P450) and b5 levels were measured as described in Estabrook and Werringloer (1978). PL and PLI levels were quantified as Thiobarbituric Acid Reactive Species (TBARs) as described in Buege and Aust (1978). NP level was calculated as the difference between PLI level and PL level. CAT, PChE, GST/CDNB (field experiment only), EROD activities and P450, P418, b5, PL and PLI levels were measured in S9 of entire soft bodies. GST (CDNB/EA) (laboratory experiment only) and NAD(P)H-red were assayed in cytosol and microsomes of visceral masses, respectively. Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Statistical analysis

Statistical analysis of data was performed with STATISTICA™ software (5.1 release, Statsoft®). Data fulfilling variance homogeneity conditions were submitted to a one-way analysis of variance (ANOVA) to estimate the effect of the contaminant dose or the site on biochemical parameter responses. Whenever a significant ($p \leq 0.05$) dose or site effect was observed on a biochemical parameter response, a Tukey HSD multiple comparison test was performed to check significant ($p \leq 0.05$) differences between doses or sites. Correlations between dose and biochemical parameter responses were determined with the Pearson correlation coefficient. For each contaminant, a discriminant analysis was carried out to distinguish doses from each other and to determine biochemical parameters allowing the best discrimination. Such an analysis was also performed to distinguish contaminants or sites from each other.

Results and discussion

Chemical analysis

Concerning TCE and TOL which are highly volatile solvents, the loss during preparation of contaminated water was very important (23 to 44% for TCE and 6 to 45% for TOL). Actual

concentrations at the beginning of the laboratory experiment (T0) were: 1.20, 3.58, 13.99 and 69.44 mg/l for TCE; 4.31, 8.19, 28.19 and 36.43 mg/l for TOL; 0.118, 0.257, 0.543 and 1.141 mg/l for $CdCl_2$ (quantified as Cd); 1.17, 2.24, 3.58 and 7.84 mg/l for CT. At the end of the experiment (T5), the loss was complete for TOL. Final concentrations were: 0.10, 0, 0.22 and 4.40 mg/l for TCE; 0.036, 0.140, 0.372 and 0.869 mg/l for $CdCl_2$ (quantified as Cd); 0, 0.0033, 0.043 and 0.247 mg/l for CT. For the following results, actual concentrations of products at T0 will be considered.

Laboratory experiment

Whatever the product was, biochemical parameters that exhibited significant responses compared to control were mainly parameters related to oxidative stress (CAT activity, PL, PLI and NP levels) and components of phase I metabolism (P450 and P418 levels, NAD(P)H-red and EROD activities). In the case of TCE, an increase of CAT activity (+70% and +40% at 1.2 mg/l and 3.6 mg/l respectively) and PL (+29% at 1.2 mg/l) could be indicative of an oxidative stress as it has been observed already for mice (Goel *et al.*, 1992). P418 and especially P450 exhibited significantly high responses at 3.6 mg/l (+249%) and from 3.6 mg/l up to 69.4 mg/l (from +835% to +649%) respectively. On the other hand, NADH-red activity significantly decreased by 21% and 20% at 14 mg/l and 69 mg/l, respectively. Regarding TOL, all parameters related to oxidative stress exhibited a significant increase at the two lowest doses (+26%, +33%, +26% and +26% for CAT activity, PL, PLI and NP levels respectively at 4.3 mg/l). Among components of phase I metabolism, P450 level and to a lesser extent P418 level, exhibited a significant response. Their increase (+1093% and +240% at 28 mg/l for P450 level and P418 level, respectively) was almost similar to that observed in the case of TCE. An increase of P450 level has already been described in mammals for both products (Kawamoto *et al.*, 1988; Nakajima and Wang, 1994). For $CdCl_2$, CAT activity exhibited a significant decrease at the lowest dose (–39%) followed by a significant increase at the two highest doses (+31%). PLI and NP responses were correlated with the dose ($r = 0.9312$ and $r = 0.9309$, respectively). They significantly increased at 1.1 mg/l of Cd (+29%). Indeed, cadmium is known to induce oxidative stress and to alter the antioxidant system (Sarkar *et al.*, 1998). EROD activity was annulled at the three highest doses. Such a dramatic decrease was previously observed in rats (Rosenberg and Kappas, 1991). GST/CDNB was significantly reduced but only at the lowest dose (–29%). Such an inhibition of GST activity by cadmium has been reported by Sidhu *et al.* (1997). For CT, NADPH-red activity as well as P450 and PL levels were correlated with the dose ($r = 0.9548$, $r = 0.9491$ and $r = -0.9258$ respectively). They were significant at the highest dose (+86%, +317% and –41% respectively compared to control). P418 level increased significantly at 2.2 mg/l (+26%) and 7.8 mg/l (+19%). Gilewicz *et al.* (1984) have already observed such an induction of P450 following exposure of *Mytilus galloprovincialis* to petroleum hydrocarbons. NADH-red activity and NP level also increased significantly but only at the lowest doses (+32% and +41% at 1.2 mg/l and 2.2 mg/l respectively for NADH-red activity; +20% at 2.2 mg/l for NP level).

Results of discriminant analysis are presented in Tables 1 and 2. For each product, control was discriminated from other doses. Generally, doses were discriminated from each other, except for the lowest dose and the highest one for TOL, the second dose and the third and the fourth ones for $CdCl_2$ and the two lowest doses for CT. For TCE and TOL, doses were discriminated thanks to parameters related to oxidative stress (CAT activity, PL and NP levels) and to components of phase I (P450 level and NADH-red activity). More biochemical parameters contributed to discrimination in the case of $CdCl_2$ and CT. However, some parameters exhibiting no significant response compared to control in the ANOVA analysis contributed to discrimination, especially PChE activity for CT. To try to

Table 1 Discriminant analysis: distances between doses of each product expressed as square of Mahalanobis distances between group centroids. Discriminated doses are notified * ($p \leq 0.05$), ** ($p \leq 0.01$) or *** ($p \leq 0.001$). Non-discriminated doses are notified ns

Products		Dose 1	Dose 2	Dose 3	Dose 4
TCE	Control	47.7 ***	63.9 ***	35.9 ***	30.1 ***
	Dose 1	-	41.3 ***	41.4 ***	79.9 ***
	Dose 2		-	8.4 *	35.5 ***
	Dose 3			-	15.1 **
TOL	Control	27.0 ***	24.6 ***	55.3 ***	16.7 ***
	Dose 1	-	22.9 ***	45.1 ***	5.6 ns
	Dose 2		-	29.7 ***	15.8 ***
	Dose 3			-	23.6 ***
$CdCl_2$	Control	62.0 ***	24.1 ***	23.1 ***	33.8 ***
	Dose 1	-	73.2 ***	102.6 ***	106.3 ***
	Dose 2		-	5.3 ns	7.9 ns
	Dose 3			-	9.9 *
CT	Control	26.3 ***	23.9 ***	12.3 **	138.2 ***
	Dose 1	-	3.4 ns	35.6 ***	234.3 ***
	Dose 2		-	33.4 ***	204.9 ***
	Dose 3			-	101.5 ***

Table 2 Discriminant analysis: biochemical parameters contributing significantly to discrimination between doses of each product. F of exclusion and corresponding degree of significance are in brackets (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$); n=30

Products	Biochemical parameters					
TCE	P450	PL	CAT	NADH-red		
	(25.2 ***)	(15.6 ***)	(12.9 ***)	(6.1 **)		
TOL	P450	NP	CAT	NADH-red		
	(25.6 ***)	(11.4 ***)	(7.4 ***)	(4.8 **)		
$CdCl_2$	CAT	GST/CDNB	NP	NADPH-red	GST/EA	
	(19.9 ***)	(19.5 ***)	(10.1 ***)	(3.9 *)	(3.5 *)	
CT	NADPH-red	P450	NADH-red	P418	PChE	NP
	(41.4 ***)	(14.1 ***)	(6.6 ***)	(5.9 **)	(3.2 *)	(2.9 *)

discriminate products from each other a discriminant analysis was performed based on the responses of biochemical parameters at the lowest dose. As presented in Table 3, products could be discriminated from each other. Biochemical parameters contributing to this discrimination are presented in Table 4. As observed previously for dose discrimination, parameters allowing a good discrimination between products were mainly parameters related to oxidative stress and components of phase I metabolism.

At the end of this laboratory study, typical profiles of biochemical parameter responses could be obtained for each product as described in Table 5. For the considered products, b5 level, GST/EA and PChE activities never exhibited a significant response compared to control.

Table 3 Discriminant analysis: distances between each product expressed as square of Mahalanobis distances between group centroids. Discriminated products are notified *** ($p \leq 0.001$)

Products	TCE	TOL	$CdCl_2$	CT
TCE	-	128.1 ***	184.2 ***	282.4 ***
TOL		-	348.2 ***	507.3 ***
$CdCl_2$			-	54.9 ***

Table 4 Discriminant analysis: biochemical parameters contributing significantly to discrimination between products. F of exclusion and corresponding degree of significance are in brackets (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$); n=20

Products	Biochemical parameters						
All	PL (27.4 ***)	CAT (19.6 ***)	NP (15.1 ***)	PChE (13.6 ***)	P450 (12.3 **)	NADH-red (11.6 **)	NADPH-red (4.6 *)

Table 5 Typical profiles of biochemical parameter responses obtained for each product (no consideration of dose). Significant increase or decrease of responses is notified + or –. Non-significant responses are notified ns

Biochemical parameters	Signification	Products $CdCl_2$	TCE	TOL	CT
CAT	Oxidative stress	–/+	+	+	ns
PL		ns	+	+	–
PLI		+	ns	+	ns
NP		+	ns	+	+
P450	Phase I metabolism	ns	+	+	+
P418		ns	+	+	+
b5		ns	ns	ns	ns
EROD		–	ns	ns	ns
NADPH-red		ns	ns	ns	+
NADH-red		ns	–	ns	+
GST/CDNB	Phase II metabolism	–	ns	ns	ns
GST/EA		ns	ns	ns	ns
PChE	Nervous system	ns	ns	ns	ns

Field experiment

For downstream sites, all biochemical parameters exhibited significant responses compared to those observed for the reference site. Results observed for CAT activity were contradictory. It increased significantly for DS1 (+43% compared to the reference site) and then began to decrease until it was significantly lower for DS3 and DS4 compared to the reference site (–71%). Both PL and PLI levels decreased for all downstream sites (–50% and –78% respectively for DS1). This tendency was slightly attenuated along with the pollution gradient for only PLI level. Concerning GST/CDNB activity, the highest increase was observed for the two sites nearest to the effluent outlet (+25% for DS1 and DS2). Then activity decreased along with distance and pollution gradient (+16% and +11% for DS3 and DS4, respectively). PChE activity was not significantly affected for the first downstream site (DS1) whereas it was for the next two (–24% and –23% for DS2 and DS3, respectively). This effect tended to attenuate along with the pollution gradient (no significant response was observed for DS4).

Results of discriminant analysis are presented in Tables 6 and 7. All downstream sites were discriminated from the reference site. DS1 and DS2 as well as DS3 and DS4 were not discriminated from each other. Biochemical parameters accounting for site discrimination were the only parameters related to oxidative stress.

Among downstream sites, we could distinguish two categories of sites as presented in Figure 1 (obtained from discriminant analysis): 1) near the effluent outlet, highly polluted sites (DS1 and DS2); 2) far from the effluent outlet, moderately polluted sites (DS3 and DS4). Thus, discriminant analysis based on biochemical parameter responses was suited to classifying the pollution status of different sites as previously described by Van der Oost *et al.* (1997). As the set of biochemical parameters was too restricted and because of the non-specificity of parameters related to oxidative stress, we were unable to express a hypothesis

Table 6 Discriminant analysis: distances between sites expressed as square of Mahalanobis distances between group centroids. Discriminated sites are notified *** ($p \leq 0.001$). Non-discriminated sites are notified ns

Sites	DS1	DS2	DS3	DS4
UPS	224.5 ***	216.5 ***	259.8 ***	244.7 ***
DS1	–	3.2 ns	156.3 ***	158.8 ***
DS2		–	123.9 ***	126.8 ***
DS3			–	0.4 ns

Table 7 Discriminant analysis: biochemical parameters contributing significantly to discrimination between sites. F of exclusion and corresponding degree of significance are in brackets (*: $p \leq 0.05$; ***: $p \leq 0.001$); n = 29

Sites	Biochemical parameters		
All	CAT (147.8 ***)	PLI (30.2 ***)	PL (3.1 *)

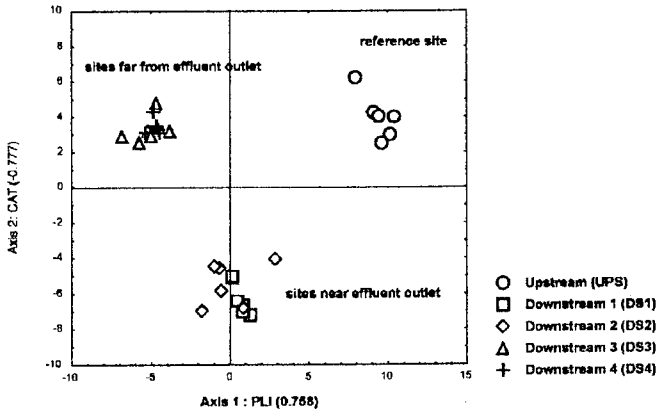


Figure 1 Representation of sites (field experiment) in the first factorial plane obtained from discriminant analysis. The biochemical parameter that is the highest correlated with each axis is specified (correlation coefficient bracketed)

concerning the nature of the effluent. Further studies to obtain typical profiles of more pollutants and the use of a larger set of biochemical parameters in field studies are needed.

Conclusions

In the laboratory experiment, most of the biochemical parameters studied could be considered as potential biomarkers in *C. fluminea*. Whereas product specificity of each parameter was limited, profiles including the whole parameter responses were typical of each product thus emphasising the interest of a multibiomarker approach. In the field experiment, biochemical parameters studied were of great interest for the detection of polluted effluents and it would now be interesting to include more than five parameters in further studies to obtain more accurate interpretations. A multibiomarker approach combined with appropriate statistics such as discriminant analysis could be a useful tool in the biomonitoring of freshwater ecosystem quality. In particular, sites could be distinguished in terms of pollution intensity. It would now be interesting to acquire data on other pollutants to generate typical profiles such as those obtained in the laboratory study and thus to be able to characterise pollution in field experiments.

References

Araujo, R., Moreno, D. and Ramos, M.A. (1993). The Asiatic clam *Corbicula fluminea* (Müller, 1774) (Bivalvia: Corbiculidae) in Europe. *American Malacol. Bull.*, **10**(1), 39–49.

Buege, J.A. and Aust, S.D. (1978). Microsomal lipid peroxidation. *Methods Enzymol.*, **50**, 302–310.

Burke, M.D. and Mayer, R.T. (1974). Ethoxyresorufin: direct fluorimetric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Dispos.*, **2**(6), 583–588.

Clairborne, A. (1985). Catalase activity. In: *Handbook of Methods for Oxygen Radical Research*, R.A. Greenwald (ed.), CRC Press, Boca Raton, pp. 283–284.

Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, **7**, 88–95.

Estabrook, R.W. and Werringloer, J. (1978). The measurement of difference spectra : application to the cytochromes of microsomes. *Methods Enzymol.*, **52**, 212–220.

Gilewicz, M., Guillaume, J.R., Carles, D., Leveau, M. and Bertrand, J.C. (1984). Effects of petroleum hydrocarbons on the cytochrome P450 content of the mollusc bivalve *Mytilus Galloprovincialis*. *Mar. Biol.*, **80**, 155–159.

Goel, S.K., Rao, G.S., Pandya, K.P. and Shanker, R. (1992). Trichloroethylene toxicity in mice: a biochemical, hematological and pathological assessment. *Indian J. Exp. Biol.*, **30**, 402–406.

Guengerich, F.P. (1994). Analysis and characterization of enzymes. In: *Principles and Methods of Toxicology*, A.W. Hayes (ed.), 3rd edn, Raven Press, New York, pp. 1269–1270.

Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974). The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.*, **249**(22), 7130–7139.

Kawamoto, T., Hobara, T., Nakamura, K., Imamura, A., Ogino, K., Kobayashi, H., Iwamoto, S. and Sakai, T. (1988). Induction of cytochrome *P*-450, cytochrome *b*-5, NADPH-cytochrome *c* reductase and change of cytochrome *P*-450 isozymes with long-term trichloroethylene treatment. *Toxicology*, **53**, 239–249.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

Nakajima, T. and Wang, R.S. (1994). Induction of cytochrome *P*450 by toluene. *Int. J. Biochem.*, **26**(12), 1333–1340.

Narbonne, J.F., Djomo, J.E., Ribera, D., Ferrier, V. and Garrigues, P. (1999). Accumulation kinetics of polycyclic aromatic hydrocarbons adsorbed to sediment by the mollusk *Corbicula fluminea*. *Ecotoxicol. Environ. Saf.*, **42**, 1–8.

Rosenberg, D.W. and Kappas, A. (1991). Induction of heme oxygenase in the small intestinal epithelium: a response to oral cadmium exposure. *Toxicology*, **67**, 199–210.

Sarkar, S., Yadav, P. and Bhatnagar, D. (1998). Lipid peroxidative damage on cadmium exposure and alterations in antioxidant system in rat erythrocytes: a study with relation to time. *BioMetals*, **11**(2), 153–157.

Sidhu, M., Prasad, R., Gill, K.D. and Nath, R. (1997). Alterations in isoforms of glutathione S-transferase in liver and kidney of cadmium exposed rhesus monkeys: purification and kinetic characterization. *Mol. Cell. Biochem.*, **166**, 55–63.

Van der Oost, R., Vindimian, E., Van den Brink, P.J., Satumalay, K., Heida, H. and Vermeulen, N.P.E. (1997). Biomonitoring aquatic pollution with feral eel (*Anguilla anguilla*). III. Statistical analyses of relationships between contaminant exposure and biomarkers. *Aquat. Toxicol.*, **39**, 45–75.

Lors de cette étude de terrain, des indicateurs concernant des niveaux d'organisation biologique supérieurs, tels que les biocénoses, ont été mesurés par le Service Environnement du Groupement de Recherches de Lacq de TotalFinaElf. Nous les présentons brièvement.

Deux catégories d'indicateurs biologiques ont été considérées (Bassères et Tramier, 2001):

- les indices biocénotiques :
    - l'indice biologique global (IBG) et l'indice biologique global normalisé (l'IBGN): ces indices reposent sur l'analyse qualitative et quantitative des populations d'invertébrés benthiques (macro-faune d'une taille supérieure à 500 µm). Ils sont déterminés à partir d'un échantillon représentatif prélevé dans la rivière à l'aide d'un surber (IBGN) ou recueilli *via* un piège artificiel (IBG).
    - l'indice de pollusensibilité spécifique (IPS) et l'indice de qualité biologique des sédiments (IOBS): ces indices reposent respectivement sur l'analyse des populations de diatomées (algues de 10 à 500 µm) et d'oligochètes.

- les indices microbiologiques:
    - les dénombrements bactériens: la flore totale présente dans l'eau (FTE) et les sédiments (FTS), la flore spécifique des hydrocarbures présente dans l'eau (FHE) et les sédiments (FHS), la flore totale (bactéries actives et dormantes) présente dans l'eau et les sédiments déterminée par épifluorescence (EPIE et EPIS, respectivement), l'estimation de l'activité microbiologique par dosage de l'adénosine-tri-phosphate (ATP).
    - le test Microtox: ce test de toxicité aiguë est basé sur l'inhibition de la croissance et donc de la luminescence de la bactérie marine *Photobacterium phosphoreum*. Il permet la détermination de la concentration en effluent entraînant une inhibition de croissance de 50% (CI 50). La toxicité est exprimée en unité de toxicité ou équitox (100/CI 50). Elle a été déterminée pour l'eau (MICROE) et les sédiments (MICROS).

Les résultats obtenus pour chacun de ces indices, ainsi que pour les biomarqueurs, sont présentés dans le tableau 6 et la figure 14.

Tableau 6. Réponses des indicateurs biologiques et des biomarqueurs obtenues lors de l'étude de terrain.

Biomarqueur / Indicateur biologique [a]	**Site**		
	Amont	*Aval proche* [b]	*Aval lointain* [c]
CAT (µmoles/min/mg protéine)	44,2	55,8	12,9
LP (nmoles/mg protéine)	0,444	0,261	0,237
LPI (nmoles/mg protéine)	0,582	0,145	0,165
GST/CDNB (nmoles/min/mg protéine)	335,8	420	371,7
PChE (nmoles/min/mg protéine)	5,14	3,89	4,37
IBGN (note/20)	11	1	10
IBG (note/20)	11	2	11
IPS (note/20)	19,1	16,54	17,6
IOBS (note/20)	8,4	0	2,4
FTE (nombre de bactéries/ml)	12000	12000	3750
FTS (nombre de bactéries/ml)	10500	2150000	46500
FHE (nombre de bactéries/ml)	7500	47500	21000
FHS (nombre de bactéries/ml)	37500	21000000	1150000
EPIE (nombre de bactéries/ml)	938000	207000	1500000
EPIS (nombre de bactéries/ml)	98300000	36600000	79300000
ATP (quantité d'ATP en pg)	497	1112	1040
MICROE (équitox)	38,54	5491	5,07
MICROS (équitox)	1,3	5,9	1,3

[a] CAT, LP, LPI, GST/CDNB, PChE: activité catalase, teneur en lipides peroxydés, teneur en lipides peroxydables, activité glutathion *S*-transférase envers le 1-chloro-2,4-dinitrobenzène, propionylcholinestérase.

[b] A 100 m du point de rejet.

[c] A 6 km du point de rejet.

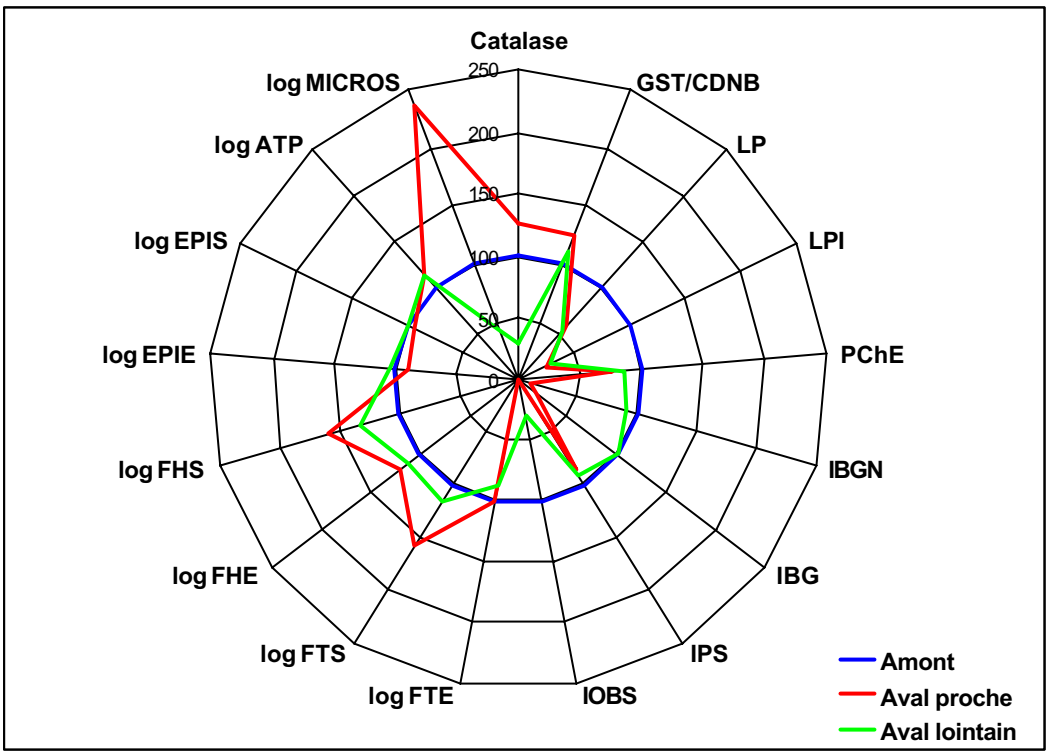


Figure 14. Réponses des indicateurs biologiques et des biomarqueurs obtenues lors de l'étude de terrain: représentation «radar». Le test MICROE n'est pas mentionné en raison des valeurs trop importantes obtenues à l'aval proche.

D'une manière générale, les indicateurs biologiques et en particulier l'IBG(N), l'IOBS, le FTS, le FHS, le MICROE et le MICROS ont présenté de fortes variations à l'aval proche, témoignant ainsi de l'impact important de l'effluent industriel sur l'écosystème. Comme nous l'avons souligné dans l'article n° 7, les différents biomarqueurs ont également présenté des variations significatives à l'aval proche. A l'aval lointain, la plupart des indicateurs biologiques ont pratiquement recouvré leur valeur initiale et ce, au contraire des biomarqueurs. A l'issue de cette étude de terrain, il est apparu qu'une bonne corrélation existait entre les réponses des indicateurs biologiques et celles des biomarqueurs à l'aval proche, soulignant ainsi la signification écologique de ces derniers. De plus, l'utilisation des biomarqueurs a mis en évidence la persistance des effets de l'effluent -bien qu'atténués- à l'aval lointain. Bien que d'autres études de terrain soient nécessaires, il apparaît que la sensibilité des biomarqueurs ainsi que leur caractère prédictif font de ces derniers des outils de choix dans l'évaluation de la qualité des écosytèmes dulçaquicoles.

CONCLUSIONS GENERALES & PERSPECTIVES

Le but de notre travail était de valider le mollusque bivalve *Corbicula fluminea* comme organisme sentinelle des milieux dulçaquicoles et ce, en privilégiant une approche multibiomarqueurs.

Des études préliminaires de terrain effectuées dans notre laboratoire (non présentées dans ce manuscrit) ont permis de mettre en évidence plusieurs paramètres biochimiques susceptibles d'être utilisés comme biomarqueurs de pollution chez cette espèce, c'est à dire présentant des réponses significatives après exposition à un ou plusieurs contaminants. Ces paramètres sont essentiellement des indicateurs de stress oxydatif et des composants du métabolisme de (dé)toxication des phases I et II. Des études moléculaires permettant de connaître la nature des biomarqueurs potentiels étudiés ainsi que la signification de leurs réponses sont indispensables dans une telle démarche de validation. Elles ont été réalisées ou sont en cours de réalisation chez la moule marine. En ce qui concerne plus particulièrement *C. fluminea*, les cholinestérases présentes chez ce mollusque ont été purifiées et caractérisées dans notre laboratoire (Mora, 1999a; 1999b). Dans le cadre de notre travail nous avons choisi d'étudier plus particulièrement les glutathion *S*-transférases (GSTs) en raison de leur rôle fondamental dans les réactions de (dé)toxication des xénobiotiques.

Nous avons dans un premier temps étudié l'activité GST envers quatre substrats: le 1-chloro-2,4-dinitrobenzène (CDNB), substrat non spécifique métabolisé par la majeure partie des classes de GSTs; l'acide éthacrynique (AE), spécifique des GSTs de classe Pi; le 3,4-dichloro-1-nitrobenzène (DCNB), spécifique des GSTs de classe Mu et le 1,2-époxy-3-(*p*-nitrophénoxy)propane (EPNP), spécifique des GSTs de classe Thêta. Seuls le CDNB et l'AE nous ont permis d'obtenir des activités GSTs significatives et suffisamment fiables pour pouvoir être mesurées lors d'études environnementales. Les conditions de dosage des activités envers ces deux substrats ont été optimisées et les paramètres cinétiques apparents (K_m et V_{max}) déterminés pour chacun d'entre eux. L'existence de plusieurs isoenzymes a été pressentie lors de cette première étude. Elle a ensuite été confirmée lors de la purification des GSTs d'extraits cytosoliques de masse viscérale et de branchies. Nous avons ainsi purifié et caractérisé pour la première fois chez *C. fluminea* trois isoenzymes cytosoliques de GST présentes dans la masse viscérale (GSTc1, GSTc2 et GSTc3). Nous avons obtenu par HPLC en phase inverse des profils de sous-unités caractéristiques de la masse viscérale et des branchies. Toutes ces sous-unités sont apparentées à la classe Pi. Certaines sous-unités de masse viscérale sont également apparentées à la classe Mu. Des études complémentaires sont

nécessaires, notamment la détermination de leur structure primaire, pour mieux les caractériser et confirmer leur appartenance à une classe donnée. Il serait également intéressant d'identifier les isoenzymes présentes dans les branchies mais aussi dans d'autres organes. Nous avons montré que les activités GSTs envers le CDNB et l'AE présentent de faibles variations saisonnières et sont peu affectées par les facteurs abiotiques, ce qui leur confère des avantages par rapport à d'autres paramètres biochimiques tels que les composants du métabolisme de phase I et les indicateurs de stress oxydatif. Cependant, comme elles présentent peu ou pas de variations significatives après exposition des animaux en laboratoire à plusieurs familles de contaminants, il est possible qu'une simple mesure d'activité enzymatique ne soit pas suffisante pour mettre en évidence la sur- ou la sous-expression d'une isoenzyme ou d'une sous-unité particulière de GST. C'est pourquoi il est désormais essentiel d'étudier l'expression des isoenzymes et/ou des sous-unités de GST que nous avons purifiées et caractérisées, après exposition des *C. fluminea* à différents contaminants en laboratoire et sur le terrain, ceci afin de valider ou d'invalider définitivement les GSTs comme biomarqueurs de pollution chez cet organisme.

La variabilité saisonnière des biomarqueurs de pollution ainsi que leur sensibilité à différents facteurs abiotiques peuvent masquer ou fausser les effets des contaminants. Il est donc nécessaire de les connaître pour pouvoir en tenir compte dans les études environnementales. Parmi les paramètres biochimiques que nous avons étudiés, les activités catalase, propionylcholinestérase et NADH-cytochrome *c* réductase ont présenté une forte variabilité saisonnière liée au cycle de reproduction et/ou à la température de l'eau. Ce sont ces mêmes paramètres qui ont été le plus affectés par les facteurs abiotiques tels que la température, le pH, l'oxygénation, le type d'eau et la présence ou l'absence de sable. Les activités GSTs et NADPH-cytochome *c* réductase ont quant à elles présenté peu ou pas de variations. Cependant, comme la plupart des paramètres sensibles aux facteurs biotiques et abiotiques ont présenté des variations significatives après exposition des *C. fluminea* à différents contaminants, il serait inopportun de les éliminer lors d'une approche multibiomarqueurs. Par contre, il est absolument indispensable de contrôler tous ces facteurs lors des études de laboratoire et d'en tenir compte lors des études de terrain en choisissant un site de référence approprié. En particulier, l'homogénéité génétique des organismes sentinelles, que ce soit intra- ou inter-population(s), est primordiale en particulier lors des études de terrain conduites sur différents sites. En effet, des réponses plus élevées ou plus faibles observées chez certains animaux pourraient être attribuées, à tort, à une pollution alors qu'elles correspondent

simplement à des niveaux de base différents. D'une manière analogue, il est primordial de connaître les caractéristiques physico-chimiques du ou des milieux étudié(s), le cycle de reproduction et l'état général des animaux. Ainsi il est possible que les animaux soient plus sensibles et meurent plus facilement en période de reproduction. De plus, l'encagement d'animaux gravides dans des rivières naturellement exemptes de *C. fluminea* constitue un risque écologique majeur puisque cette espèce n'est pas autochtone. Afin d'éviter une pollution d'ordre « biologique », deux solutions se présentent: éviter l'encagement en période de reproduction ou utiliser des cages à mailles très serrées. Concernant l'état général des animaux, l'incorporation systématique de paramètres tels que le poids frais, le poids sec, l'index de condition et les teneurs en protéines, lipides et glucides permettrait une meilleure évaluation de l'état physiologique des animaux exposés. Ces paramètres ont déjà prouvé leur efficacité lors d'études environnementales antérieures (Cantelmo-Cristini *et al*., 1985; Doherty, 1990; Sujatha *et al*., 1995; Black et Belin, 1998).

L'étude des effets de plusieurs facteurs abiotiques nous a permis de souligner la nécessité de se conformer à certaines règles, en particulier lors des études de laboratoire: acclimater les animaux à la température expérimentale, en particulier lorsque les animaux sont prélevés l'été et l'hiver; pratiquer la stabulation et l'expérimentation dans l'eau du lieu de collecte; bien oxygéner l'eau; contrôler les paramètres physico-chimiques; conduire les expériences en présence de sédiment. Il est évident que certaines de ces recommandations ne peuvent pas s'appliquer dans le cas où l'on souhaite étudier les effets d'une eau prélevée sur un site pollué ou bien les effets d'une contamination *via* la colonne d'eau et non *via* le sédiment. Il est alors nécessaire de tenir compte des effets « parasites » susceptibles d'interférer avec les effets des contaminants.

Des études supplémentaires permettant d'évaluer les effets d'un contaminant donné en fonction de différentes conditions de température, de pH ou d'oxygénation permettraient d'appréhender plus précisément l'impact de tels facteurs (a)biotiques.

Après exposition des *C. fluminea* au trichloroéthylène, au toluène, au chlorure de cadmium et à une coupe de goudron de houille, tous les paramètres biochimiques, excepté l'activité propionylcholinestérase et l'activité GST envers l'AE, ont présenté des réponses significatives et peuvent donc être considérés comme biomarqueurs potentiels de pollution chez *C. fluminea*. Parmi ces paramètres, certains ont présenté des variations significatives pour la

plupart des produits, tels que l'activité catalase, les indicateurs de stress oxydatif et les teneurs en cytochromes P450 et P418. D'autres n'ont présenté des réponses significatives que pour un seul contaminant comme par exemple l'activité éthoxyrésorufine-*O*-déséthylase NADPH-indépendante et l'activité GST envers le CDNB. Cependant, la notion de spécificité d'un paramètre biochimique pour un contaminant (ou une famille de contaminants) donné n'est que relative. A titre d'exemple, l'activité éthoxyrésorufine-*O*-déséthylase NADPH-indépendante n'a pas présenté de variations significatives après exposition à un mélange d'hydrocarbures aromatiques polycycliques mais a été inhibée après exposition à du chlorure de cadmium. Par contre, une approche multibiomarqueurs nous a permis d'obtenir des profils de réponses spécifiques pour chacun des contaminants étudiés. De plus, ces réponses ont été obtenues après une exposition représentative d'une contamination ponctuelle et transitoire (disparition de la plupart des contaminants après cinq jours d'exposition) ce qui démontre la persistance des effets biologiques -et donc l'intérêt des biomarqueurs dans les études environnementales- après une disparition totale des contaminants.

Il convient néanmoins de préciser que peu de corrélations dose-réponse ont été obtenues. Pour certains contaminants et certains paramètres biochimiques (ex. indicateurs de stress oxydatif pour le trichloroéthylène et le toluène), les réponses significatives ont été obtenues pour les doses les plus faibles. La disparition rapide des produits dans les milieux d'exposition et/ou la fermeture des valves des animaux aux plus fortes concentrations pourraient expliquer ce phénomène.

L'étude de terrain que nous avons effectué sur un site industriel s'est avérée concluante. Les réponses combinées de plusieurs paramètres biochimiques ont permis la détection des sites contaminés et leur classification en fonction de leur degré de pollution. La mesure en parallèle d'indices se situant à des niveaux supérieurs d'organisation biologique (biocénoses, dénombrements bactériens, tests Microtox) a mis en évidence la signification écologique et la sensibilité des biomarqueurs. L'ensemble de ces résultats de terrain a souligné l'importance d'une approche multibiomarqueurs, soit dans ce contexte une approche combinant des marqueurs liés à différents niveaux d'organisation biologiques (individus, populations, communautés). Dans les études de terrain futures, il serait intéressant d'étudier une plus large palette de paramètres biochimiques ainsi que des indices d'état généraux.

L'utilisation combinée de plusieurs outils statistiques s'est avérée d'un intérêt majeur lors de l'interprétation des résultats. En particulier, l'analyse discriminante a permis de différencier successivement des saisons, des doses de contaminants, des contaminants et des sites contaminés et ce, en considérant les réponses obtenues pour l'ensemble des paramètres biochimiques. L'analyse discriminante possède également un caractère prédictif qu'il serait intéressant d'exploiter dans le futur. En multipliant les études de contamination, une fonction discriminante (correspondant à un profil de réponses) pourrait être établie pour chaque contaminant et permettre ainsi d'émettre des hypothèses quant à la nature des contaminants présents dans le milieu.

A l'issue de ce travail et au vu des résultats obtenus, *C. fluminea* pourrait constituer un organisme sentinelle satisfaisant dans le cadre d'une approche multibiomarqueurs. Cependant, des études complémentaires s'imposent afin de valider les réponses obtenues lors des études de laboratoire. Dans ce but, des études de contamination chronique en mésocosmes sont actuellement conduites au sein du groupe TotalFinaElf.

BIBLIOGRAPHIE

Aceto, A., Di Ilio, C., Bucciarelli, T., Pantani, C., Dell'Agata, M., Pannunzio, G., Federici, G., 1991. Characterization of glutathione transferase from *Gammarus italicus*. *Comparative Biochemistry and Physiology*, **99B(3)**, 523-527.

Adams, S.M., Ham, K.D. and Beauchamp, J.J., 1994. Application of canonical variate analysis in the evaluation and presentation of multivariate biological response data. *Environmental Toxicology and Chemistry*, **13(10)**, 1673-1683.

Adams, S.M., Ham, K.D., Greeley, M.S., LeHew, R.F., Hinton, D.E., Saylor, C.F., 1996. Downstream gradients in bioindicator responses: point source contaminant effects on fish health. *Canadian Journal of Fisheries and Aquatic Sciences*, **53**, 2177-2187.

Agence de l'Eau Adour-Garonne, 1996. *Cahier géographique du bassin Adour-Garonne: Dordogne* (Toulouse: Publications de l'Agence de l'Eau Adour-Garonne), 41 p.

Ajele, J.O., Afolayan, A., 1992. Purification and characterisation of glutathione transferase from the giant African snail, *Archachatina marginata. Comparative Biochemistry and Physiology*, **103B(1)**, 47-55.

Akcha, F., Izuel, C., Venier, P., Budzinski, H., Burgeot, T., Narbonne, J-F., 2000. Enzymatic biomarker measurement and study of DNA adducts formation in benzo[a]pyrene-contaminated mussels, *Mytilus galloprovincialis*. *Aquatic Toxicology*, **49(4)**, 269-287.

Amiard, J-C., Caquet, T., Lagadic, L., 1998. Les biomarqueurs parmi les méthodes d'évaluation de la qualité de l'environnement. In *Utilisation de biomarqueurs pour la surveillance de la qualité de l'environnement*, edited by par L. Lagadic, T. Caquet, J.C. Amiard and F. Ramade (Paris: Lavoisier, Technique et Documentation), pp. XXI-XXXI.

Amodio-Cocchieri, R., Del Prete, U., Arnese, A., Giuliano, M., Roncioni, A., 1993. Heavy metals and polycyclic aromatic hydrocarbons (PAH's) in marine organisms from the Ionian Sea (Italy). *Bulletin of Environmental Contamination and Toxicology*, **50**, 618-625.

Andrews, P., Thyssen, J., Lorke, D., 1982. The biology and toxicology of molluscicide, Bayluscide. *Pharmacology and Therapeutics,* **19(2)**, 245-295.

Angelucci, S., Sacchetta, P., Moio, P., Melino, S., Petruzzelli, R., Gervasi, P., Di Ilio, C., 2000. Purification and characterization of glutathione transferases from the sea bass (*Dicentrarchus labrax*) liver. *Archives of Biochemistry and Biophysics*, **373(2)**, 435-441.

Araujo, R., Moreno, D., Ramos, M.A., 1993. The Asiatic clam *Corbicula fluminea* (Müller, 1774) (Bivalvia: Corbiculidae) in Europe. *American Malacological Bulletin*, **10**, 39-49.

Armstrong, R.N., 1991. Glutathione S-transferases: reaction mechanism, structure, and function. *Chemical Research in Toxicology*, **4(2)**, 131-140.

Aruoma, O.I., 1998. Free radicals, oxidative stress, and antioxidants in human health and disease. *JAOCS*, **75(2)**, 199-212.

Association Française de Normalisation -AFNOR-, 1984. *Norme T 90-117: Dosage de la chlorophylle a et des phéopigments par spectrométrie d'absorption moléculaire* (Paris: Publications de l'AFNOR), 7 p.

Bachinski, N., Koziol, C., Batel, R., Labura, Z., Schröder, H.C., Müller, W.E.G., 1997. Immediate and early response of the marine sponge *Suberites domuncula* to heat stress: reduction of trehalose and glutathione concentrations and glutathione S-transferase activity. *Journal of Experimental Marine Biology and Ecology*, **210**, 129-141.

Balabaskaran S.C.S., Chew S., Segaran M., 1986. Studies on glutathione *S*-transferase in molluscs. *Comparative Biochemistry and Physiology*, **85B**, 183-192.

Baldwin, W.S., LeBlanc, G.A., 1996. Expression and induction of an immunochemically related class of glutathione S-transferases in *Daphnia magna*. *Comparative Biochemistry and Physiology*, **113B(2)**, 261-267.

Basack, S.B., Oneto, M.L., Verrengia Guerrero, N.R., Kesten, E.M., 1997. Accumulation and elimination of pentachlorophenol in the freshwater bivalve *Corbicula fluminea*. *Bulletin of Environmental Contamination and Toxicology*, **58**, 497-503.

Basack, S.B., Oneto, M.L., Fuchs, J.S., Wood, E.J. and Kesten, E.M., 1998. Esterases of *Corbicula fluminea* as biomarkers of exposure to organophosphorus pesticides. *Bulletin of Environmental Contamination and Toxicology*, **61**, 569-576.

Bassères, A., Tramier, B., 2001. Characterisation of the impact of aqueous industrial waste in mesocosms: biological indicators and pilot streams. *Water Science and Technology*, **44 (2-3)**, 135-143.

Baudrimont, M., Lemaire-Gony, S., Ribeyre, F., Métivaud, J., Boudou, A., 1997a. Seasonal variations of metallothionein concentrations in the Asiatic clam (*Corbicula fluminea*). *Comparative Biochemistry and Physiology*, **118C(3)**, 361-367.

Baudrimont, M., Metivaud, J., Maury-Brachet, R., Ribeyre, F., Boudou, A., 1997b. Bioaccumulation and metallothionein response in the Asiatic clam (*Corbicula fluminea*) after experimental exposure to cadmium and inorganic mercury. *Environmental Toxicology and Chemistry*, **16**, 2096-2105.

Beckett, G.J., Hayes, J.D., 1993. Glutathione *S*-transferases: biomedical applications. *Advances in Clinical Chemistry*, **30**, 281-380.

Belanger, S.E., 1991. The effect of dissolved oxygen, sediment, and sewage treatment plant discharges upon growth, survival and density of Asiatic clams. *Hydrobiologia*, **218**, 113-126.

Belanger, S.E., Farris, J.L., Cherry, D.S., Cairns, J.Jr., 1985. Sediment preference of the freshwater Asiatic clam, *Corbicula fluminea*. *The Nautilus*, **99(2-3)**, 66-73.

Beuffe, H., 1995. *Bilan trophique des plans d'eau landais et suivi des effets des aménagements sur le bassin versant du lac de Parentis-Biscarosse* (Bordeaux: Publications du Centre National du Machinisme Agricole, du Génie Rural, des Eaux et des Forêts -CEMAGREF-), 43 p.

Beukema, J.J., De Bruin, W., 1977. Seasonal changes in dry weight and chemical composition of the soft parts of the tellinid bivalve *Macoma balthica* in the Dutch Wadden sea. *Netherlands Journal of Sea Research*, **11(1)**, 42-55.

Beyer, J., Sandvik, M., Hylland, K., Fjeld, E., Egaas, E., Aas, E., Skåre, J.U., Goksøyr, A., 1996. Contaminant accumulation and biomarker responses in flounder (*Platichthys flesus* L.) and Atlantic cod (*Gadus morhua* L.) exposed by caging to polluted sediments in Sørfjorden, Norway. *Aquatic Toxicology*, **36**, 75-98.

Black, M.C., Belin, J.I., 1998. Evaluating sublethal indicators of stress in Asiatic clams (*Corbicula fluminea*) caged in urban stream. *Environmental Toxicology and Risk Assessment*, **7**, 76-91.

Blanchette, B.N., Singh, B.R., 1999. Purification and characterization of the glutathione-S-transferases from the northern quahog *Mercinaria mercinaria*. *Marine Biotechnology*, **1**, 74-80.

Blanco, P., Machado, A., Satrustegui, J., 1987. Variations due to hyperoxia and ageing in the activities of glutathione *S*-transferase and NADPH-cytochrome *c* reductase. *Mechanisms of Ageing and Development*, **39**, 11-19.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, **37(8)**, 911-917.

Board, P.G., Baker, R.T., Chelvanayagam, G., Jermiin, L.S., 1997. Zeta, a novel class of glutathione transferases in a range of species from plants to humans. *Biochemical Journal*, **328**, 929-935.

Board, P.G., Coggan, M., Chelvanayagam, G., Easteal, S., Jermün, L.S., Schulte, G.K., Danley, D.E., Hoth, L.R., Griffor, M.C., Kamath, A.V., Rosner, M.H., Chrunyk, B.A.,

Perregaux, D.E., Gabel, C.A., Geoghegan, K.F., Pandit, J., 2000. Identification, characterization, and crystal structure of the Omega class glutathione transferases. *Journal of Biological Chemistry*, **275(32)**, 24798-24806.

Bocquené, G., Roig, A., Fournier, D., 1997. Cholinesterases from the common oyster (*Crassostrea gigas*). Evidence for the presence of a soluble acetylcholinesterase insensitive to organophosphate and carbamate inhibitors. *FEBS Letters*, **407(3)**, 261-266.

Bolton, R.M., Ahokas, J.T., 1997. Purification and characterization of hepatic glutathione transferases from an insectivorous marsupial, the brown antechinus (*Antechinus stuartii)*. *Xenobiotica*, **27(6)**, 573-586.

Borgeraas, J., Nilsen, K., Stenersen, J., 1996. Methods for purification of glutathione transferases in the earthworm genus *Eisenia*, and their characterization. *Comparative Biochemistry and Physiology*, **114C(2)**, 129-140.

Boryslawskyj, M., Garrood, A.C., Pearson, J.T., Woodhead, D., 1988. Elevation of glutathione-*S*-transferase activity as a stress response to organochlorine compounds, in the freshwater mussel, *Sphaerium corneum*. *Marine Environmental Research*, **24**, 101-104.

Britton, J.C., Morton, B., 1979. *Corbicula* in north America: the evidence reviewed and evaluated. In *Proceedings of the First International Corbicula Symposium*, edited by J.C. Britton, J.S. Mattice, C.E. Murphy and L.W. Newland (Fort Worth, Texas: Texas Christian University Research Foundation Publication), pp. 249-287.

Britton, J.C., Morton, B., 1982. A dissection guide, field and laboratory manual for the introduced bivalve *Corbicula fluminea*. *Malacological Review,* **supplement 3**, 1-82.

Brown, D.J., Clark, G.C., Van Beneden, R.J., 1998. A new cytochrome P450 (CYP30) family identified in the clam, *Mercenaria mercenaria*. *Comparative Biochemistry and Physiology*, **121C**, 351-360.

Bucheli, T.D., Fent, K., 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical Reviews in Environmental Science and Technology*, **25(3)**, 201-268.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. *Methods in Enzymology*, **50**, 302-310.

Burke, M.D., Mayer, R.T., 1974. Ethoxyresorufin: direct fluorimetric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metabolism and Disposition*, **2(6)**, 583-588.

Buttner, J.K., Heidinger, R.C., 1981. Rate of filtration in the Asiatic clam, *Corbicula fluminea*. *Transactions of the Illinois State Academy of Sciences*, **74(3/4)**, 13-17.

Cairns, J.Jr., Bidwell, J.R., 1996. Discontinuities in technological and natural systems caused by exotic species. *Biodiversity and Conservation*, **5**, 1085-1094.

Cajaraville, M.P., Uranga, J.A., Angulo, E., 1992. Comparative effects of the water accommodated fraction of three oils on mussels -3. Quantitative histochemistry of enzymes related to the detoxication metabolism. *Comparative Biochemistry and Physiology*, **103C(2)**, 369-377.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *Science of the Total Environment*, **247**, 295-311.

Cancio, I., Orbea, A., Völkl, A., Fahimi, H.D., Cajaraville, M.P., 1998. Induction of peroxisomal oxidases in mussels: comparison of effects of lubricant oil and benzo(*a*)pyrene with two typical peroxisome proliferators on peroxisome structure and function in *Mytilus galloprovincialis*. *Toxicology and Applied Pharmacology*, **149**, 64-72.

Cancio, I., Ibabe, A., Cajaraville, M.P., 1999. Seasonal variation of peroxisomal enzyme activities and peroxisomal structure in mussels *Mytilus galloprovincialis* and its

relationship with the lipid content. *Comparative Biochemistry and Physiology*, **123C**, 135-144.

Canesi, L., Viarengo, A., Leonzio, C., Filippelli, M., Gallo, G., 1999. Heavy metals and glutathione metabolism in mussel tissues. *Aquatic Toxicology*, **46**, 67-76.

Cantelmo-Cristini, A., Hospod, F.E., Lazell, R.J., 1985. An *in situ* study on the adenylate energy charge of *Corbicula fluminea* in a freshwater system. In *Marine Pollution and Physiology: Recent Advances*, edited by A. Calabrese, F.P. Thurberg, F.J. Vernberg and W. Vernberg (Columbia: University South Carolina Press), pp. 45-62.

Channel, S.R., Latendresse, J.R., Kidney, J.K., Grabau, J.H., Lane, J.W., Steel-Goodwin, L., Gothaus, M.C., 1998. A subchronic exposure to trichloroethylene causes lipid peroxidation and hepatocellular proliferation in male B6C3F1 mouse liver. *Toxicological Sciences*, **43**, 145-154.

Cheung, C.C.C., Zheng, G.J., Li, A.M.Y., Richardson, B.J., Lam, P.K.S., 2001. Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*. *Aquatic Toxicology*, **52(3-4)**, 189-203.

Clairborne, A, 1985. Catalase activity. In *Handbook of Methods for Oxygen Radical Research*, edited by R.A. Greenwald (Boca Raton, Florida: CRC press), pp. 283-284.

Clark A.G., 1989. The comparative enzymology of the glutathione *S*-transferases from non-vertebrate organisms. *Comparative Biochemistry and Physiology*, **92B**, 419-446.

Cohen, R.R.H., Dresler, P.V., Phillips, E.J.P., Cory, R.L., 1984. The effect of the Asiatic clam, *Corbicula fluminea*, on phytoplankton of the Potomac River, Maryland. *Limnology and Oceanography,* **29(1)**, 170-180.

Cossu, C., Doyotte, A., Jacquin, M.C., Babut, M., Exinger, A., Vasseur, P., 1997. Glutathione reductase, selenium-dependent glutathione peroxidase, glutathione levels, and lipid peroxidation in freshwater bivalves, *Unio tumidus*, as biomarkers of aquatic contamination in field studies. *Ecotoxicology and Environmental Safety*, **38**, 122-131.

Counts, C.L., 1981. *Corbicula fluminea* (Bivalvia: Sphaeriacea) in British Columbia. *The Nautilus*, **95(1)**, 12-13.

Dauberschmidt, C., Dietrich, D.R., Schlatter, C., 1997. Investigations on the biotransformation capacity of organophosphates in the mollusc *Dreissena polymorpha* P. *Aquatic Toxicology*, **37**, 283-294.

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.

Deslous-Paoli, J-M., Zanette, Y., Heral, M., Massé, H., Garnier, J., 1982. Amélioration de la forme et de la qualité de l'huître *Crassostrea gigas* Thunberg dans les claires de Marennes-Oléron. *Revue des Travaux de l'Institut des Pêches Maritimes*, **45(3)**, 181-194.

Dierickx P.J., 1984. Glutathione *S*-transferase in aquatic macro-invertebrates and its interaction with different organic micropollutants. *Science of the Total Environment*, **40**, 93-102.

Doherty, F.G., Failla, M.L., Cherry, D.S., 1988. Metallothionein-like heavy metal binding protein levels in Asiatic clams are dependent on the duration and mode of exposure to cadmium. *Water Research*, **22(7)**, 927-932.

Doherty, F.G., 1990. The Asiatic clam, *Corbicula* spp., as a biological monitor in freshwater environments. *Environmental Monitoring and Assessment*, **15**, 143-181.

Dominey, R.J., Nimmo, I.A., Cronshaw, A.D., Hayes, J.D., 1991. The major glutathione S-transferase in salmonid fish livers is homologous to the mammalian pi-class GST. *Comparative Biochemistry and Physiology*, **100B**, 93-98.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, **28(3)**, 350-356.

Dubois, C., 1995. Biologie et démo-écologie d'une espèce invasive, *Corbicula fluminea* (Mollusca: Bivalvia) originaire d'Asie: étude *in situ* (Canal Latéral à la Garonne, France) et en canal expérimental. *Thèse (Université Paul Sabatier de Toulouse)*, 169 p.

Eertman, R.H.M., Groenink, C.L.F.M.G., Sandee, B., Hummel, H., 1995. Response of the blue mussel *Mytilus edulis* L. following exposure to PAHs or contaminated sediment. *Marine Environmental Research*, **39**, 169-173.

Egaas, E., Sandvik, M., Fjeld, E., Källqvist, T., Goksøyr, A., Svensen, A., 1999. Some effects of the fungicide propiconazole on cytochrome P450 and glutathione *S*-transferase in brown trout (*Salmo trutta*). *Comparative Biochemistry and Physiology*, **122C**, 337-344.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, **7**, 88-95.

Engelke, M., Diehl, H., Tähti, H., 1992. Effects of toluene and n-hexane on rat synaptosomal membrane fluidity and integral enzyme activities. *Pharmacology and Toxicology*, **71**, 343-347.

Escartín, E., Porte, C., 1997. The use of cholinesterase and carboxylesterase activities from *Mytilus galloprovincialis* in pollution monitoring. *Environmental Toxicology and Chemistry*, **16(10)**, 2090-2095.

Estabrook, R.W., Werringloer, J., 1978. The measurement of difference spectra: application to the cytochromes of microsomes. *Methods in Enzymology*, **52**, 212-220.

Fishbein, L., 1985. An overview of environmental and toxicological aspects of aromatic hydrocarbons. II. Toluene. *Science of the Total Environment*, **42**, 267-288.

Fisher, S.W., Hwang, H., Atanasoff, M., Landrum, P.F., 1999. Lethal body residues for pentachlorophenol in zebra mussels (*Dreissena polymorpha*) under varying conditions of temperature and pH. *Ecotoxicology and Environmental Safety*, **43**, 274-283.

Fitzpatrick, P.J., Sheehan, D., 1993. Separation of multiple forms of glutathione *S*-transferase from the blue mussel, *Mytilus edulis*. *Xenobiotica*, **23**, 851-861.

Fitzpatrick, P.J., Krag, T.O.B., Højrup, P., Sheehan, D., 1995a. Characterization of a glutathione *S*-transferase and a related glutathione-binding protein from gill of the blue mussel, *Mytilus edulis*. *Biochemical Journal*, **305**, 145-150.

Fitzpatrick, P.J., Sheehan, D., Livingstone, D.R., 1995b. Studies on isoenzymes of glutathione S-transferase in the digestive gland of *Mytilus galloprovincialis* with exposure to pollution. *Marine Environmental Research*, **39**, 241-244.

Fitzpatrick, P.J., O'Halloran, J., Sheehan, D., Walsh, A.R., 1997. Assessment of glutathione S-transferase and related proteins in the gill and digestive gland of *Mytilus edulis* (L.), as potential organic pollution biomarkers. *Biomarkers*, **2**, 51-56.

Foe, C., Knight, A., 1985. The effect of phytoplankton and suspended sediment on the growth of *Corbicula fluminea* (Bivalvia). *Hydrobiologia*, **127**, 105-115.

Foe, C., Knight, A., 1986. A thermal energy budget for juvenile *Corbicula fluminea*. *American Malacological Bulletin*, **Special Edition 2**, 143-150.

Foley, V., Sheehan, D., 1998. Glutathione S-transferases of the yeast *Yarrowia lipolytica* have unusually large molecular mass. *Biochemical Journal*, **333**, 839-845.

Fontan, B., Meny, J., 1996. *Note sur l'invasion de* Corbicula fluminea *dans le réseau hydrographique de la région Aquitaine et précision sur son spectre écologique* (Aquitaine: Publications de la Préfecture de la région Aquitaine, de la Direction Régionale de l'Environnement Aquitaine et du Service de l'Eau et des Milieux Aquatiques), 16 p.

Fraisse, L., Verlhac, J.B., Roche, B., Rascle, M.C., Rabion, A., Seris, J.L., 1993. Long-chain-substituted uric acid and 5,6-diaminouracil derivatives as novel agents against free radical processes: synthesis and *in vitro* activity. *Journal of Medicinal Chemistry*, **36(10)**, 1465-1473.

Furman, G.M., Silverman, D.M., Schatz, R.A., 1998. Inhibition of rat lung mixed-function oxidase activity following repeated low-level toluene inhalation: possible role of toluene metabolites. *Journal of Toxicology and Environmental Health*, **54A**, 633-645.

Gamble, S.C., Goldfarb, P.S., Porte, C., Livingstone, D.R., 1995. Glutathione peroxidase and other antioxidant enzyme function in marine invertebrates (*Mytilus edulis*, *Pecten maximus*, *Carcinus maenas* and *Asterias rubens*). *Marine Environmental Research*, **39**, 191-195.

Garrigues, P., Raoux, C., Lemaire, P., Mathieu, A., Ribera, D., Narbonne, J.F., Lafaurie, M., 1990. *In situ* correlations between polycyclic aromatic hydrocarbons (PAH) and PAH metabolizing system activities in mussels and fish in the Mediterranean Sea: preliminary results. *International Journal of Environmental Analytical Chemistry*, **38**, 379-387.

George, S.G., Young, P., 1988. Purification and properties of plaice liver cytosolic glutathione-*S*-transferases. *Marine Environmental Research*, **24**, 93-96.

Gilewicz M., Guillaume J.R., Carles D., Leveau M., Bertrand J.C., 1984. Effects of petroleum hydrocarbons on the cytochrome P450 content of the mollusc bivalve *Mytilus Galloprovincialis*. *Marine Biology*, **80**, 155-159.

Girardi, H., 1989-1990. Deux bivalves d'eau douce récents pour la faune française (Mollusca, Bivalvia). *Bulletin de la Société d'Etudes des Sciences Naurelles du Vaucluse*, 87-93.

Goel S.K., Rao G.S., Pandya K.P., Shanker R., 1992. Trichloroethylene toxicity in mice: a biochemical, hematological and pathological assessment. *Indian Journal of Experimental Biology*, **30**, 402-406.

Goeptar, A.R., Commandeur, J.N.M., Van Ommen, B., Van Bladeren P.J., Vermeulen, N.P.E., 1995. Metabolism and kinetics of trichloroethylene in relation to toxicity and carcinogenicity. Relevance of the mercapturic acid pathway. *Chemical Research in Toxicology*, **8**, 3-21.

Graney, R.L.Jr., Cherry, D.S., Cairns, J.Jr., 1984. The influence of substrate, pH, diet and temperature upon cadmium accumulation in the Asiatic clam (*Corbicula fluminea*) in laboratory artificial streams. *Water Research*, **18(7)**, 833-842.

Gruet, Y., 1992. Un nouveau mollusque bivalve pour notre région: *Corbicula* sp. (Heterodonta sphaeriacea). *Bulletin de la Société des Sciences Naturelles de l'Ouest de la France*, **14(2)**, 37-43.

Guengerich, F.P., 1994. Analysis and characterization of enzymes. In *Principles and Methods of Toxicology (third edition)*, edited by A.W. Hayes (New-York: Raven Press), pp. 1269-1270.

Gutteridge, J.M.C., 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clinical Chemistry*, **41(12)**, 1819-1828.

Haber, F., Weiss, J., 1934. The catalytic decomposition of hydrogen peroxide by iron salts. *Proceedings of the Royal Society of London*, **147A**, 332-351.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, **249(22)**, 7130-7139.

Hahn, M.E., 1998. The aryl hydrocarbon receptor: a comparative perspective. *Comparative Biochemistry and Physiology*, **121C(1-3)**, 23-53.

Halkier, B.A., 1996. Catalytic reactivities and structure/function relationships of cytochrome P450 enzymes. *Phytochemistry*, **43(1)**, 1-21.

Halliwell, B., 1978. Biochemical mechanisms accounting for the toxic action of oxygen on living organisms: the key role of superoxide dismutase. *Cell Biology International Reports*, **2**, 113-128.

Halliwell, B., Cross, C.E., 1994. Oxygen-derived species: their relation to human disease and environmental stress. *Environmental Health Perspectives*, **102(10)**, 5-12.

Halmes, N.C., Samokyszyn, V.M., Pumford, N.R., 1997. Covalent binding and inhibition of cytochrome P4502E1 by trichloroethylene. *Xenobiotica*, **27(1)**, 101-110.

Ham, K.D., Adams, S.M., Peterson, M.J., 1997. Application of multiple bioindicators to differentiate spatial and temporal variability from the effects of contaminant exposure on fish. *Ecotoxicology and Environmental Safety*, **37**, 53-61.

Hayes, J.D., Mantle, T.J., 1986. Anomalous electrophoretic behaviour of the glutathione *S*-transferase Ya and Yk subunits isolated from man and rodents. *Biochemical Journal*, **237**, 731-740.

Heinonen, J., Kukkonen, J., Penttinen, O-P., Holopainen, I.J., 1997. Effects of hypoxia on valve-closure time and bioaccumulation of 2,4,5-trichlorophenol by the freshwater clam *Sphaerium corneum* [L.]. *Ecotoxicology and Environmental Safety*, **36**, 49-56.

Hillis, D.M., Patton, J.C., 1982. Morphological and electrophoretic evidence for two species of *Corbicula* (Bivalvia: Corbiculidae) in North America. *The American Midland Naturalist*, **108(1)**, 74-80.

Hinson, J.A., Kadlubar, F.F., 1988. Glutathione and glutathione transferases in the detoxification of drug and carcinogen metabolites. In *Glutathione Conjugation, Mechanisms and Biological Significance*, edited by B. Ketterer and H. Sies (London: Academic Press), pp. 235-280.

Hong, S-H., Park, H-J., Kong, K-H., 1999. Purification and biochemical properties of glutathione *S*-transferase from *Oryza sativa. Comparative Biochemistry and Physiology*, **122B**, 21-27.

Howlett, D., Baker, R., 1999. *Corbicula fluminea* (Müller): new to UK. *Journal of Conchology*, **36(6)**, 83.

International Agency for Research on Cancer (IARC), 1989. Some organic solvents, resin monomers and related compounds, pigments and occupational exposures in paint manufacture and painting. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, **47**, 79-123.

International Agency for Research on Cancer (IARC), 1995. Dry cleaning, some chlorinated solvents and other industrial chemicals. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, **63**, 75-158.

Inza, B., Ribeyre, F., Maury-Brachet, R., Boudou, A., 1997. Tissue distribution of the inorganic mercury, methylmercury and cadmium in the Asiatic clam (*Corbicula fluminea*) in relation to the contamination levels of the water column and sediment. *Chemosphere*, **35(12)**, 2817-2836.

Isom, B.G., 1986. Historical review of Asiatic clam (*Corbicula*) invasion and biofouling of waters and industries in the Americas. *American Malacological Bulletin*, **Special Edition 2**, 1-5.

Janero, D.R., 1990. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biology and Medicine*, **9**, 515-540.

Jensen, A., Bro-Rasmussen, F., 1992. Environmental cadmium in Europe. *Reviews of Environmental Contamination and Toxicology*, **125**, 101-181.

Jimenez, B.D., Burtis, L.S., 1989. Influence of environmental variables on the hepatic mixed-function oxidase system in bluegill sunfish, *Lepomis macrochirus*. *Comparative Biochemistry and Physiology*, **93C(1)**, 11-21.

Johnson, P.D., McMahon, R.F., 1998. Effects of temperature and chronic hypoxia on survivorship of the zebra mussel (*Dreissena polymorpha*) and Asian clam (*Corbicula fluminea*). *Canadian Journal of Fisheries and Aquatic Sciences*, **55**, 1564-1572.

Kamisaka, K., Habig, W.H., Ketley, J.N., Arias, M., Jakoby, W.B., 1975. Multiple forms of human glutathione S-transferase and their affinity for bilirubin. *European Journal of Biochemistry*, **60(1)**, 153-161.

Kappus, H., 1991. Lipid peroxidation: mechanism and biological relevance. In *Free Radicals and Food Additives*, edited by O.I. Aruoma and B. Halliwell (London: Taylor and Francis), pp. 59-75.

Kaur, H., Perkins, M.J., 1991. The free-radical chemistry of food additives. In *Free Radicals and Food Additives*, edited by O.I. Aruoma and B. Halliwell (London: Taylor and Francis), pp. 17-35.

Kawamoto T., Hobara T., Nakamura K., Imamura A., Ogino K., Kobayashi H., Iwamoto S., Sakai T., 1988. Induction of cytochrome *P*-450, cytochrome *b*-5, NADPH-cytochrome *c* reductase and change of cytochrome *P*-450 isozymes with long-term trichloroethylene treatment. *Toxicology*, **53**, 239-249.

Keeran, W.S., Lee, R.F., 1987. The purification and characterization of glutathione *S*-transferase from the hepatopancreas of the blue crab, *Callinectes sapidus*. *Archives of Biochemistry and Biophysics*, **255(2)**, 233-243.

Ketterer, B., Meyer, D.J., Clark, A.G., 1988. Soluble glutathione transferase isozymes. In *Glutathione Conjugation, Mechanisms and Biological Significance*, edited by B. Ketterer and H. Sies (London: Academic Press), pp. 73-135.

Ketterer, B., Meyer, D.J., 1989. Glutathione transferases: a possible role in the detoxication and repair of DNA and lipid hydroperoxides. *Mutation Research*, **214**, 33-40.

Kirchin, M.A., Wiseman, A., Livingstone, D.R., 1992. Seasonal and sex variation in the mixed-function oxygenase system of digestive gland microsomes of the common mussel, *Mytilus edulis* L. *Compartive Biochemistry and Physiology*, **101C**, 81-91.

Klaunig, J.E., Xu, Y., Isenberg, J.S., Bachowski, S., Kolaja, K.L., Jiang, J., Stevenson, D.E., Walborg, E.F.Jr., 1998. The role of oxidative stress in chemical carcinogenesis. *Environmental Health Perspectives*, **106(1)**, 289-295.

Klotz, A.V., Stegeman, J.J., Woodin, B.R., Snowberger, E.A., Thomas, P.E., Walsh, C., 1986. Cytochrome P-450 isozymes from the marine teleost *Stenotomus chrysops*: their roles in steroid hydroxylation and the influence of cytochrome b_5. *Archives of Biochemistry and Biophysics*, **249**, 326-338.

Korpela, M., Tähti, H., 1988. The effect of *in vitro* and *in vivo* toluene exposure on rat erythrocyte and synaptosome membrane integral enzymes. *Pharmacology and Toxicology*, **63**, 30-32.

Kraemer, L.R., 1979. *Corbicula* (Bivalvia: Sphaeriacea) *vs.* indigenous mussels (Bivalvia: Unionacea) in U.S. rivers: a hard case for interspecific competition ? *American Zoology*, **19**, 1085-1096.

Kraemer, L.R., Galloway, M.L., 1986. Larval development of *Corbicula fluminea* (Müller) (Bivalvia: Corbiculacea): an appraisal of its heterochrony. *American Malacological Bulletin*, **4(1)**, 61-79.

Kraemer, L.R., Swanson, C., Galloway, M., Kraemer, 1986. Biological basis of behavior in *Corbicula fluminea*, II. Functional morphology of reproduction and development and review of evidence for self-fertilization. *American Malacological Bulletin*, **Special Edition 2**, 193-201.

Labrot, F., Ribera, D., Saint-Denis, M., Narbonne, J-F., 1996. *In vitro* and *in vivo* studies of potential biomarkers of lead and uranium contamination: lipid peroxidation, acetyl-

cholinesterase, catalase and glutathione peroxidase activities in three non-mammalian species. *Biomarkers*, **1**, 21-28.

Labrot, F., Narbonne, J-F., Ville, P., Saint-Denis, M., Ribera, D., 1998. Acute toxicity, toxicokinetics, and tissue target of lead and uranium in the clam *Corbicula fluminea* and the worm *Eisenia fetida*: comparison with the fish *Brachydanio rerio*. *Archives of Environmental Contamination and Toxicology*, **36(2)**, 167-178.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Lagadic, L., Caquet, T., 1996. Marqueurs biologiques de pollution: des outils au service de l'écotoxicologie. *Phytoma - La Défense des Végétaux*, **480**, 10-13.

Larson, J.L., Bull, R.J., 1992. Metabolism and lipoperoxidative activity of trichloroacetate and dichloroacetate in rats and mice. *Toxicology and Applied Pharmacology*, **115(2)**, 268-277.

Lauritsen, D.D., 1986. Filter-feeding in *Corbicula fluminea* and its effect on seston removal. *Journal of the North American Benthological Society*, **5(3)**, 165-172.

LeBlanc, G.A., Cochrane, B.J., 1987. Identification of multiple glutathione *S*-transferases from *Daphnia magna*. *Comparative Biochemistry and Physiology*, **88B(1)**, 39-45.

Lee, R.F., 1981. Mixed function oxygenases (MFO) in marine invertebrates. *Marine Biology Letters*, **2**, 87-105.

Lee, R.F., 1988. Glutathione S-transferase in marine invertebrates from Langesundfjord. *Marine Ecology Progress Series*, **46**, 33-36.

Lee R.F., Sheehan W.S., Pickwell G.V., 1988. Marine invertebrate glutathione-*S*-transferases : purification, characterization and induction. *Marine Environmental Research*, **24**, 97-100.

Leff, L.G., Burch, J.L., McArthur, J.V., 1990. Spatial distribution, seston removal, and potential competitive interactions of the bivalves *Corbicula fluminea* and *Elliptio complanata*, in a coastal plain stream. *Freshwater Biology*, **24**, 409-416.

Lemaire, P., Livingstone, D.R., 1993. Pro-oxidant/antioxidant processes and organic xenobiotic interactions in marine organisms, in particular the flounder *Platichthys flesus* and the mussel *Mytilus edulis*. *Trends in Comparative Biochemistry and Physiology*, 1, 1119-1149.

Lemaire, P., Förlin, L., Livingstone, D.R., 1996. Responses of hepatic biotransformation and antioxidant enzymes to CYP1A-inducers (3-methylcholanthrene, â-naphtoflavone) in sea bass (*Dicentrarchus labrax*), dab (*Limanda limanda*) and rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology*, **36**, 141-160.

Lemaire-Gony, S., Boudou, A., 1997. Mantle and gill fine structure in the freshwater Asiatic clam, *Corbicula fluminea* (Müller). *Annales de Limnologie*, **33(3)**, 163-178.

Lenártová, V., Holovská, K., Pedrajas, J-R., Martínez-Lara, E., Peinado, J., López-Barea, J., Rosival, I., Košúth, P., 1997. Antioxidant and detoxifying fish enzymes as biomarkers of river pollution. *Biomarkers*, **2**, 247-252.

Levine, S.L., Oris, J.T., Wissing, T.E., 1995. Influence of environmental factors on the physiological condition and hepatic ethoxyresorufin *O*-deethylase (EROD) activity of gizzard shad (*Dorosoma cepedianum*). *Environmental Toxicology and Chemistry*, **14(1)**, 123-128.

Lindström-Seppä, P., Koivusaari, U., Hänninen, O., 1983. Metabolism of foreign compounds in freshwater crayfish (*Astacus astacus* L.) tissues. *Aquatic Toxicology*, **3**, 35-46.

Livingstone D.R., 1991. Organic xenobiotic metabolism in marine invertebrates. In *Advances in Comparative and Environmental Physiology*, edited by R. Gilles (Berlin: Springer-Verlag), p 45.

Livingstone, D.R., 1993. Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. *Journal of Chemical Technology and Biotechnology*, **57**, 195-211.

Livingstone, D.R., Farrar, S.V., 1984. Tissue and subcellular distribution of enzyme activities of mixed-function oxygenase and benzo[a]pyrene metabolism in the common mussel *Mytilus edulis* L. *Science of the Total Environment*, **39**, 209-235.

Livingstone, D.R., Kirchin, M.A., Wiseman, A., 1989. Cytochrome P-450 and oxidative metabolism in molluscs. *Xenobiotica*, **19(10)**, 1041-1062.

Livingstone, D.R., Garcia Martinez, P., Michel, X., Narbonne, J.F., O'Hara, S., Ribera, D., Winston, G.W., 1990. Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* L., and other molluscs. *Functional Ecology*, **4**, 415-424.

Livingstone, D.R., Lips, F., Garcia Martinez, P., Pipe, R.K., 1992. Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*. *Marine Biology*, **112**, 265-276.

Livingstone, D.R., Lemaire, P., Matthews, A., Peters, L.D., Porte, C., Fitzpatrick, P.J., Förli, L., Nasci, C., Fossato, V., Wootton, N., Goldfarb, P., 1995. Assessment of the impact of organic pollutants on Goby (*Zosterisessor ophiocephalus*) and mussel (*Mytilus galloprovincialis*) from the Venice Lagoon, Italy: biochemical studies. *Marine Environmental Research*, **39**, 235-240.

Livingstone, D.R., Nasci, C., Solé, M., Da Ros, L., O'Hara, S.C.M., Peters, L.D., Fossato, V., Wootton, A.N., Goldfarb, P.S., 1997. Apparent induction of a cytochrome P450 with immunochemical similarities to CYP1A in digestive gland of the common mussel (*Mytilus galloprovincialis* L.) with exposure to 2,2',3,4,4',5'-hexachlorobiphenyl and Arochlor 1254. *Aquatic Toxicology*, **38**, 205-224.

Livingstone, D.R., Chipman, J.K., Lowe, D.M., Minier, C., Mitchelmore, C.L., Moore, M.N., Peters, L.D., Pipe, R.K., 2000. Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates: recent molecular, genotoxic, cellular and immunological studies on the common mussel (*Mytilus edulis* L.) and other mytilids. *International Journal of Environment and Pollution*, **13(1-6)**, 56-91.

Lotti, M., 1995. Cholinesterase inhibition: complexities in interpretation. *Clinical Chemistry*, **41(12)**, 1814-1818.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.

Machala, M., Nezveda, K., Petøivalský, M., Jarošová, A.B., Piaèka, V., Svobodová, Z., 1997. Monooxygenase activities in carp as biochemical markers of pollution by polycyclic and polyhalogenated aromatic hydrocarbons: choice of substrates and effects of temperature, gender and capture stress. *Aquatic Toxicology*, **37**, 113-123.

Manca, D., Ricard, A.C., Trottier, B., Chevalier G., 1991. Studies on lipid peroxidation in rat tissues following administration of low and moderate doses of cadmium chloride. *Toxicology*, **67**, 303-323.

Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M.K., Warholm, M., Jornvall, H., 1985. Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. *Proceedings of the National Academy of Sciences of the United States of America*, **82(21)**, 7202-7206.

Mansuy, D., 1998. The great diversity of reactions catalyzed by cytochromes P450. *Comparative Biochemistry and Physiology*, **121C**, 5-14.

Mansuy, D., Battioni, P., Battioni, J-P., 1989. Chemical model systems for drug-metabolizing cytochrome-P-450-dependent monooxygenases. *European Journal of Biochemistry*, **184**, 267-285.

Martínez-Lara, E., Toribio, F., López-Barea, J., Bárcena, J.A., 1996. Glutathione-*S*-transferase isoenzyme patterns in the gilthead seabream (*Sparus aurata*) exposed to environmental contaminants. *Comparative Biochemistry and Physiology*, **113C(2)**, 215-220.

Martínez-Lara, E., George, S.G., López-Barea, J., Bárcena, J.A., 1997. Purification and characterization of multiple glutathione transferase isoenzymes from grey mullet liver. *Cellular and Molecular Life Sciences*, **53**, 759-768.

Massoulié, J., Bon, S., 1993. L'acétylcholinestérase: une structure originale pour une fonction vitale. *Annales de l'Institut Pasteur / Actualités*, **4(1)**, 35-49.

Massoulié, J., Pezzementi, L., Bon, S., Krejci, E., Vallette, F-M., 1993. Molecular and cellular biology of cholinesterases. *Progress in Neurobiology*, **41**, 31-91.

Massoulié, J., Anselmet, A., Bon, S., Krejci, E., Legay, C., Morel, N., Simon, S., 1999. The polymorphism of acetylcholinesterase: post-translational processing, quaternary associations and localization. *Chemico-Biological Interactions*, **119/120**, 29-42.

Masthanamma, P., Jayasundaramma, B., Ramamurthi, R., 1987. Metabolic response to altered pH of the ambient in the freshwater mussels *Lamellidens marginalis* (Lamark) and *Perreysia rugosa* (Gmelin). *Advances in Aquatic Biology and Fisheries*, 81-88.

Mattia, C.J., LeBel, C.P., Bondy, S.C., 1991. Effects of toluene and its metabolites on cerebral reactive oxygen species generation. *Biochemical Pharmacology*, **42(4)**, 879-882.

Mattia, C.J., Adams, J.D.Jr., Bondy, S.C., 1993. Free radical induction in the brain and liver by products of toluene catabolism. *Biochemical Pharmacology*, **46(1)**, 103-110.

Mattice, J.S., Dye, L.L., 1976. Thermal tolerance of the adult Asiatic clam. In *Thermal Ecology II*, edited by G.W. Esch and R.W. McFarlane (Washington, D.C.: United States Energy Research and Development Administration Symposium Series), pp. 130-135.

McLellan, T., 1982. Electrophoresis buffers for polyacrylamide gels at various pH. *Analytical Biochemistry*, **126(1)**, 94-99.

McLeod, M.J., 1986. Electrophoretic variation in North American *Corbicula*. *American Malacological Bulletin*, **Special edition 2**, 125-132.

McMahon, R.F., 1979. Response to temperature and hypoxia in the oxygen consumption of the introduced Asiatic freshwater clam *Corbicula fluminea* (Müller). *Comparative Biochemistry and Physiology*, **63A**, 383-388.

Meyer, D.J., Thomas, M., 1995. Characterization of rat spleen prostaglandin H D-isomerase as a sigma-class GSH transferase. *Biochemical Journal*, **311**, 739–742.

Meyer, D.J., Coles, B., Pemble, S.E., Gilmore, K.S., Fraser, G.M., Ketterer, B., 1991. Theta, a new class of glutathione transferases purified from rat and man. *Biochemical Journal*, **274**, 409-414.

Michel, X.R., Cassand, P.M., Ribera, D.G., Narbonne, J-F., 1992. Metabolism and mutagenic activation of benzo(a)pyrene by subcellular fractions from mussel (*Mytilus galloprovincialis*) digestive gland and sea bass (*Discenthrarcus labrax*) liver. *Comparative Biochemistry and Physiology*, **103C(1)**, 43-51.

Michel, X., Suteau, P., Robertson, L.W., Narbonne, J-F., 1993. Effects of benzo(a)pyrene, 3,3',4,4'-tetrachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl on the xenobiotic-metabolizing enzymes in the mussel (*Mytilus galloprovincialis*). *Aquatic Toxicology*, **27**, 335-344.

Michel, X.R., Beasse, C., Narbonne, J-F., 1995. *In vivo* metabolism of benzo(a)pyrene in the mussel *Mytilus galloprovincialis*. *Archives of Environmental Contamination and Toxicology*, **28**, 215-222.

Miller, R.E., Guengerich, F.P., 1983. Metabolism of trichloroethylene in isolated hepatocytes, microsomes, and reconstituted enzyme systems containing P-450. *Cancer Research*, **43(3)**, 1145-1152.

Mora, P., Fournier, D., Narbonne, J-F., 1999a. Cholinesterases from the marine mussels *Mytilus galloprovincialis* Lmk. and *M. edulis* L. and from the freshwater bivalve *Corbicula fluminea* Müller. *Comparative Biochemistry and Physiology*, **122C**, 353-361.

Mora P., Michel, X., Narbonne, J-F., 1999b. Cholinesterase activity as potential biomarker in two bivalves. *Environmental Toxicology and Pharmacology*, **7**, 253-260.

Morgenstern, R., DePierre, J.W., 1983. Microsomal glutathione transferase. Purification in unactivated form and further characterization of the activation process, substrate specificity and amino acid composition. *European Journal of Biochemistry*, **134**, 591-597.

Mouthon, J., 1981. Sur la présence en France et au Portugal de *Corbicula* (Bivalvia, Corbiculidae) originaire d'Asie. *Basteria*, **45**, 109-116.

Murray, M., Reidy, G.F., 1990. Selectivity in the inhibition of mammalian cytochromes P-450 by chemical agents. *Pharmacological Reviews*, **42(2)**, 85-101.

Nakajima T., Wang R.S.; 1994. Induction of cytochrome *P*450 by toluene. *International Journal of Biochemistry*, **26**(12), 1333-1340.

Nakajima, T., Wang, R.S., Elovaara, E., Park, S.S., Gelboin, H.V., Vainio, H., 1992. A comparative study on the contribution of cytochrome P450 isozymes to metabolism of benzene, toluene and trichloroethylene in rat liver. *Biochemical Pharmacology*, **43(2)**, 251-257.

Narbonne, J.F., Djomo, J.E., Ribera, D., Ferrier, V., Garrigues, P., 1999a. Accumulation kinetics of polycyclic aromatic hydrocarbons adsorbed to sediment by the mollusk *Corbicula fluminea*. *Ecotoxicology and Environmental Safety*, **42**, 1-8.

Narbonne, J.F., Daubèze, M., Clérandeau, C., Garrigues, P., 1999b. Scale of classification based on biochemical markers in mussels: application to pollution monitoring in European coasts. *Biomarkers*, **4(6)**, 415-424.

Nebert, D.W., Gonzalez, F.J., 1987. P450 genes: structure, evolution, and regulation. *Annual Review of Biochemistry*, **56**, 945-993.

Neff, J.M., 1984. Bioaccumulation of organic micropollutants from sediments and suspended particulates by aquatic animals. *Fresenius' Zeitschrift für Analytische Chemie*, **319**, 132-136.

Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., Nebert D.W., 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, **6**, 1-42.

Oliveira-Filho, E.C., De-Carvalho, R.R., Paumgartten, F.J.R., 1999. The influence of environmental factors on the molluscicidal activity of *Euphorbia milii* latex. *Journal of Environmental Science and Health*, **34B(2)**, 289-303.

Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *Journal of Biological Chemistry*, **239**, 2370-2378.

Organisation Internationale de Normalisation, 1996. *Norme ISO 11885: Qualité de l'eau - Détermination de 33 éléments par spectroscopie d'émission atomique avec plasma couplé par induction* (Genève: ISO Publications), 13 p.

Ostlund Farrants, A-K., Meyer, D., Coles, B., Southan, C., Aitken, A., Johnson, P.J., Ketterer, B., 1987. The separation of glutathione transferase subunits by using reverse-phase high-pressure liquid chromatography. *Biochemical Journal*, **245**, 423-428.

Pannunzio, T.M., Storey, K.B., 1998. Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod *Littorina littorea*. *Journal of Experimental Marine Biology and Ecology*, **221**, 277-292.

Parant, M., 1998. Le métabolisme des xénobiotiques chez les bivalves. *Comptes Rendus de l'Académie des Sciences de Paris, Sciences de la vie*, **321**, 485-494.

Pascal, S., Debrauwer, L., Ferte, M-P., Anglade, P., Rouimi, P., Scalla, R., 1998. Analysis and characterization of glutathione *S*-transferase subunits from wheat (*Triticum aestivum* L.). *Plant Science*, **134**, 217-226.

Pasteur, N., Pasteur, G., Bonhomme, F., Catalan, J., Britton-Davidian, J., 1987. *Manuel technique de génétique par électrophorèse des protéines* (Paris: Lavoisier, Technique et Documentation), 217 p.

Paulson K.E., 1989. Xenobiotic regulation of glutathione *S*-transferase Ya gene expression. *Molecular Toxicology*, **2**, 215-235.

Pelmont, J., 1995. *Enzymes, catalyseurs du monde vivant* (Grenoble: Presses Universitaires de Grenoble), 1039 p.

Pemble, S.E., Wardle, A.F., Taylor, J.B., 1996. Glutathione S-transferase class Kappa: characterization by the cloning of rat mitochondrial GST and identification of a human homologue. *Biochemical Journal*, **319**, 749-754.

Pérez-López, M., Anglade, P., Bec-Ferté, M.P., Debrauwer, L., Perdu, E., Cravedi, J.P., Rouimi, P., 2000. Characterization of hepatic and extrahepatic glutathione S-transferases in rainbow trout (*Oncorhynchus mykiss*) and their induction by 3,3',4,4'-tetrachlorobiphenyl. *Fish Physiology and Biochemistry*, **22**, 21-32.

Peters, L.D., Nasci, C., Livingstone D.R., 1998a. Immunochemical investigations of cytochrome P450 forms/epitopes (CYP1A, 2B, 2E, 3A and 4A) in digestive gland of *Mytilus sp. Comparative Biochemistry and Physiology*, **121C**, 361-369.

Peters, L.D., Nasci, C., Livingstone, D.R., 1998b. Variation in levels of cytochrome P4501A, 2B, 2E, 3A and 4A-immunopositive proteins in digestive gland of indigenous and transplanted mussel *Mytilus galloprovincialis* in Venice Lagoon, Italy. *Marine Environmental Research*, **46(1-5)**, 295-299.

Peters, L.D., Shaw, J.P., Nott, M., O'Hara, S.C.M., Livingstone, D.R., 1999. Development of cytochrome P450 as a biomarker of organic pollution in *Mytilus* sp.: field studies in United Kingdom ('Sea Empress' oil spill) and the Mediterranean Sea. *Biomarkers*, **4(6)**, 425-441.

Ploemen J.H.T.M., Van Ommen B., Bogaards J.J.P., Van Bladeren P.J., 1993. Ethacrynic acid and its glutathione conjugate as inhibitors of glutathione *S*-transferases. *Xenobiotica*, **23**, 913-923.

Porte, C., Lemaire, P., Peters, L.D., Livingstone, D.R., 1995. Partial purification and properties of cytochrome P450 from digestive gland microsomes of the common mussel, *Mytilus edulis* L. *Marine Environmental Research*, **39**, 27-31.

Power, A., Sheehan, D., 1996. Seasonal variation in the antioxidant defence systems of gill and digestive gland of the blue mussel, *Mytilus edulis*. *Comparative Biochemistry and Physiology*, **114C(2)**, 99-103.

Power, A., McCarthy, R., Raggett, E., Sheehan, D., 1996. Ligand-binding properties of the glutathione-binding protein of the mussel, *Mytilus edulis*. *Comparative Biochemistry and Physiology*, **115B(4)**, 439-443.

Prapanthadara, L-A., Koottathep, S., Promtet, N., Hemingway, J., Ketterman, A.J., 1996. Purification and characterization of a major glutathione *S*-transferase from the mosquito *Anopheles dirus* (species B). *Insect Biochemistry and Molecular Biology*, **26(3)**, 277-285.

Pumford, N.R., Halmes, N.C., Hinson, J.A., 1997. Covalent binding of xenobiotics to specific proteins in the liver. *Drug Metabolism Reviews*, **29 (1/2)**, 39-57.

Raoux, C., Lemaire, P., Mathieu, A., Garrigues, P., Lafaurie, M., 1989. Bioprotective system activities in *Serranus scriba* from the Mediterranean Sea: relation between pollutant content and enzymatic activities. *Océanis*, **15(4)**, 623-627.

Reddy, P.S., 1997. Modulations of antioxidant enzymes in the gill and hepatopancreas of the edible crab *Scylla serrata* during exposure to cadmium and copper. *Fresenius Environmental Bulletin*, **6**, 589-597.

Regoli, F., 1998. Trace metals and antioxidant enzymes in gills and digestive gland of the Mediterranean mussel *Mytilus Galloprovincialis*. *Archives of Environmental Contamination and Toxicology*, **34**, 48-63.

Regoli, F., Principato, G., 1995. Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. *Aquatic Toxicology*, **31**, 143-164.

Regoli, F., Nigro, M., Bertoli, E., Principato, G., Orlando, E., 1997. Defenses against oxidative stress in the Antarctic scallop *Adamussium colbecki* and effects of acute exposure to metals. *Hydrobiologia*, **355**, 139-144.

Réseau de Bassin Adour-Garonne, 1997. *Atlas et Données sur l'eau* (Toulouse: Publications de l'Agence de l'Eau Adour-Garonne), 149 p.

Ribera, D., Narbonne, J-F., Daubeze, M., Michel, X., 1989. Characterisation, tissue distribution and sexual differences of some parameters related to lipid peroxidation in mussels. *Marine Environmental Research*, **28**, 279-283.

Ricci, G., Lo Bello, M., Caccuri, A.M., Galiazzo, F., Federici, G., 1984. Detection of glutathione transferase activity on polyacrylamide gels. *Analytical Biochemistry*, **143**, 226-230.

Robinson, J.V., Wellborn, G.A., 1988. Ecological resistance to the invasion of a freshwater clam, *Corbicula fluminea*: fish predation effects. *Oecologia*, **77**, 445-452.

Roméo, M., Mathieu, A., Gnassia-Barelli, M., Romana, A., Lafaurie, M., 1994. Heavy metal content and biotransformation enzymes in two fish species from the NW Mediterranean. *Marine Ecology Progress Series*, **107**, 15-22.

Roméo, M., Bennani, N., Gnassia-Barelli, M., Lafaurie, M., Girard, J.P., 2000. Cadmium and copper display different responses towards oxidative stress in the kidney of the sea bass *Dicentrarchus labrax*. *Aquatic Toxicology*, **48**, 185-194.

Rosenberg, D.W., Kappas, A., 1991. Induction of heme oxygenase in the small intestinal epithelium: a response to oral cadmium exposure. *Toxicology*, **67**, 199-210.

Rouimi, P., Debrauwer, L., Tulliez, J., 1995. Electrospray ionization-mass spectrometry as a tool for characterization of glutathione *S*-transferase isozymes. *Analytical Biochemistry*, **229**, 304-312.

Rouimi, P., Anglade, P., Debrauwer, L., Tulliez; J., 1996. Characterization of pig liver glutathione S-transferases using HPLC-electrospray-ionization mass spectrometry. *Biochemical Journal*, **317**, 879-884.

Saint-Denis M., Narbonne, J.F., Arnaud, C., Thybaud, E., Ribera, D., 1999. Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil: effects of benzo(a)pyrene. *Soil Biology and Biochemistry*, **31**, 1837-1846.

Sarkar, P., Yadav P., Bhatnagar, D., 1997. Cadmium-induced lipid peroxidation and the antioxidant system in rat erythrocytes: the role of antioxidants. *Journal of Trace Elements in Medicine and Biology*, **11**, 8-13.

Sarkar S., Yadav P., Bhatnagar D., 1998. Lipid peroxidative damage on cadmium exposure and alterations in antioxidant system in rat erythrocytes: a study with relation to time. *BioMetals*, **11**(2), 153-157.

Schaffner, W., Weissmann, C., 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Analytical Biochemistry*, **56**, 502-514.

Schlenk, D., 1999. Necessity of defining biomarkers for use in ecological risk assessments. *Marine Pollution Bulletin*, **39(1-12)**, 48-53.

Schrenk C., Pflugmacher S., Brüggemann R., Sandermann H. Jr., Steinberg C.E.W., Kettrup A., 1998. Glutathione *S*-transferase activity in aquatic macrophytes with emphasis on habitat dependence. *Ecotoxicology and Environmental Safety*, **40**, 226-233.

Schubauer-Berigan, M.K., Dierkes, J.R., Monson, P.D., Ankley, G.T., 1993. pH-dependent toxicity of Cd, Cu, Ni, Pb and Zn to *Ceriodaphnia dubia*, *Pimephales promelas*, *Hyalella azteca* and *Lumbriculus variegatus*. *Environmental Toxicology and Chemistry*, **12**, 1261-1266.

Seed, J., Brown, R.P., Olin, S.S., Foran, J.A., 1995. Chemical mixtures: current risk assessment methodologies and future directions. *Regulatory Toxicology and Pharmacology*, **22**, 76-94.

Shaw, G.R., Connell, D.W., 1994. Prediction and monitoring of the carcinogenicity of polycyclic aromatic compounds (PACs). *Reviews of Environmental Contamination and Toxicology*, **135**, 1-62.

Sheehan, D., Power, A., 1999. Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. *Comparative Biochemistry and Physiology*, **123C**, 193-199.

Sickel, J.B., 1986. *Corbicula* population mortalities: factors influencing population control. *American Malacological Bulletin*, **Special Edition 2**, 89-94.

Sidhu M., Prasad R., Gill K.D., Nath R., 1997. Alterations in isoforms of glutathione S-transferase in liver and kidney of cadmium exposed rhesus monkeys: purification and kinetic characterization. *Molecular and Cellular Biochemistry*, **166**, 55-63.

Sies, H., Cadenas, E., 1983. Biological basis of detoxication of oxygen free radicals. In *Biological Basis of Detoxication*, edited by J. Caldwell and W.B. Jakoby (London: Academic Press), pp. 181-211.

Sjölin, A.M., Livingstone, D.R., 1997. Redox cycling of aromatic hydrocarbon quinones catalysed by digestive gland microsomes of the common mussel (*Mytilus edulis* L.). *Aquatic Toxicology*, **38**, 83-99.

Snyder, M.J., 1998. Cytochrome P450 enzymes belonging to the CYP4 family from marine invertebrates. *Biochemical and Biophysical Research Communications*, **249**, 187-190.

Snyder, M.J., 2000. Cytochrome P450 enzymes in aquatic invertebrates: recent advances and future directions. *Aquatic Toxicology*, 48, 529-547.

Solé, M., Porte, C., Albaigés, J., 1994. Mixed-function oxygenase system components and antioxidant enzymes in different marine bivalves: its relation with contaminant body burdens. *Aquatic Toxicology*, **30**, 271-283.

Solé, M., Porte, C., Biosca, X., Mitchelmore, C.L., Chipman, J.K., Livingstone, D.R., Albaigés, J., 1996. Effetcs of the "Aegean Sea" oil spill on biotransformation enzymes, oxidative stress and DNA-adducts in digestive gland of the mussel (*Mytilus edulis* L.). *Comparative Biochemistry and Physiology*, **113C(2)**, 257-265.

Solé, M., Peters, L.D., Magnusson, K., Sjölin A., Granmo, Å., Livingstone, D.R., 1998. Responses of the cytochrome P450-dependent monooxygenase and other protective enzyme systems in digestive gland of transplanted common mussel (*Mytilus edulis* L.) to organic contaminants in the Skagerrak and Kattegat (North sea). *Biomarkers*, **3(1)**, 49-62.

Stegeman, J.J., 1979. Temperature influence on basal activity and induction of mixed function oxygenase activity in *Fundulus heteroclitus*. *Journal of Fisheries Research from Board of Canada*, **36**, 1400-1404.

Stegeman, J.J., 1985. Benzo[a]pyrene oxidation and microsomal enzyme activity in the mussel (*Mytilus edulis*) and other bivalve mollusc species from the Western North Atlantic. *Marine Biology*, **89**, 21-30.

Stenersen J., Guthenberg C., Mannervik B., 1979. Glutathione *S*-transferases in earthworms (Lumbricidae). *Biochemical Journal*, **181**, 47-50.

Stenersen, J., Kobro, S., Bjerke, M., Arend, U., 1987. Glutathione transferases in aquatic and terrestrial animals from nine phyla. *Comparative Biochemistry and Physiology*, **86C(1)**, 73-82.

Sujatha, C.H., Nair, S.M., Chacko, J., 1995. Tissue lipid levels of the clam, *Villorita cyprenoides* var. *cochinensis*, following exposure to endosulfan, malathion, and methyl parathion. *Environmental Toxicology and Water Quality*, **10**, 231-235.

Suteau, P., Daubeze, M., Migaud, M.L., Narbonne, J.F., 1988. PAH-metabolizing enzymes in whole mussels as biochemical tests for chemical pollution monitoring. *Marine Ecology Progress Series*, **46**, 45-49.

Talesa, V., Grauso, M., Giovannini, E., Rosi, G., Toutant, J-P., 1995. Acetylcholinesterase in tentacles of *Octopus vulgaris* (cephalopoda). Histochemical localization and characterization of a specific high salt-soluble and heparin-soluble fraction of globular forms. *Neurochemistry International*, **27(2)**, 201-211.

Talesa, V., Romani, R., Calvitti, M., Rosi, G., Giovannini, E., 1998. Acetylcholinesterase at high catalytic efficiency and substrate specificity in the optic lobe of *Eledone moschata* (Cephalopoda: Octopoda): biochemical characterization and histochemical localization. *Neurochemistry International*, **33(2)**, 131-141.

Talesa, V., Romani, R., Antognelli, C., Giovannini, E., Rosi, G., 2001. Soluble and membrane-bound acetylcholinesterases in *Mytilus galloprovincialis* (Pelecypoda: Filibranchia) from the northern Adriatic sea. *Chemico-Biological Interactions*, **134(2)**, 151-166.

Tan Tiu, A., Prezant, R.S., 1992. The role of environment in shell growth dynamics of the Asian clam *Corbicula fluminea* (Mollusca: Bivalvia). *Malacological Review*, **25**, 109-117.

Tang, S-S., Lin, C-C., Chang, G-G., 1994. Isolation and characterization of octopus hepatopancreatic glutathione S-transferase. Comparison of digestive gland enzyme with lens S-crystallin. *Journal of Protein Chemistry*, **13(7)**, 609-618.

Taylor, D., 1983. The significance of the accumulation of cadmium by aquatic organisms. *Ecotoxicology and Environmental Safety*, **7**, 33-42.

Tejo Prakash, N., Jagannatha Rao, K.S., 1995. Modulations in antioxidant enzymes in different tissues of marine bivalve *Perna viridis* during heavy metal exposure. *Molecular and Cellular Biochemistry*, **146**, 107-113.

Timbrell, J.A., Draper, R., Waterfield, C.J., 1994. Biomarkers in toxicology: new uses for some old molecules? *Toxicology and Ecotoxicology News*, **1(1)**, 4-14.

Trundle, D., Marcial, G., 1988. Detection of cholinesterase inhibition. The significance of cholinesterase measurements. *Annals of Clinical and Laboratory Science*, **18(5)**, 345-352.

Van Bladeren, P.J., 1988. Formation of toxic metabolites from drugs and other xenobiotics by glutathione conjugation. *Trends in Pharmacological Sciences*, **9**, 295-299.

Van der Oost R., Vindimian E., Van den Brink P.J., Satumalay K., Heida H., Vermeulen N.P.E., 1997. Biomonitoring aquatic pollution with feral eel (*Anguilla anguilla*). III. Statistical analyses of relationships between contaminant exposure and biomarkers. *Aquatic Toxicology*, **39**, 45-75.

Viarengo, A., 1985. Biochemical effects of trace metals. *Marine Pollution Bulletin*, **16(4)**, 153-158.

Viarengo, A., 1989. Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. *Reviews in Aquatic Sciences*, **1**, 295-316.

Viarengo, A., Canesi, L., 1991. Mussels as biological indicators of pollution. *Aquaculture*, **94**, 225-243.

Viarengo, A., Canesi, L., Pertica, M., Poli, G., Moore, M.N., Orunesu, M., 1990. Heavy metal effects on lipid peroxidation in the tissues of *Mytilus galloprovincialis* Lam. *Comparative Biochemistry and Physiology*, **97C(1)**, 37-42.

Viarengo, A., Canesi, L., Pertica, M., Livingstone, D.R., 1991. Seasonal variations in the antioxidant defence systems and lipid peroxidation of the digestive gland of mussels. *Comparative Biochemistry and Physiology*, **100C (1/2)**, 187-190.

Von Burg, R., 1993. Toxicology update. Toluene. *Journal of Applied Toxicology*, **13(6)**, 441-446.

Vos, R.M.E., Van Bladeren, P.J., 1990. Glutathione *S*-transferases in relation to their role in the biotransformation of xenobiotics. *Chemico-Biological Interactions*, **75**, 241-265.

Walling, C., 1975. Fenton's reagent revisited. *Accounts of Chemical Research*, **8**, 139-142.

Waters, E.M., Gerstner, H.B., Huff, J.E., 1977. Trichloroethylene. I. An overview. *Journal of Toxicology and Environmental Health*, **2**, 671-707.

Way, C.M., Hornbach, D.J., Miller-Way, C.A., Payne, B.S., Miller, A.C., 1990. Dynamics of filter feeding in *Corbicula fluminea* (Bivalvia: Corbiculidae). *Canadian Journal of Zoology*, **68**, 115-120.

Wilce, M.C.J., Parker, M.W., 1994. Structure and function of glutathione *S*-transferases. *Biochimica et Biophysica Acta*, **1205**, 1-18.

Willett, K.L., Wilson, C., Thomsen, J., Porter, W., 1999. Evidence for and against the presence of polynuclear aromatic hydrocarbon and 2,3,7,8-tetrachloro-p-dioxin binding proteins in the marine mussels, *Bathymodiolus* and *Modiolus modiolus*. Aquatic Toxicology, **48**, 51-64.

Williams, C.J., McMahon, R.F., 1989. Annual variation of tissue biomass and carbon and nitrogen content in the freshwater bivalve *Corbicula fluminea* relative to downstream dispersal. *Canadian Journal of Zoology*, **67**, 82-90.

Winston, G.W., 1991. Oxidants and antioxidants in aquatic animals. *Comparative Biochemistry and Physiology*, **100C (1/2)**, 173-176.

Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology*, **19**, 137-161.

Wootton, A.N., Herring, C., Spry, J.A., Wiseman, A., Livingstone, D.R., Goldfarb, P.S., 1995. Evidence for the existence of cytochrome P450 gene families (CYP1A, 3A, 4A, 11A) and modulation of gene expression (CYP1A) in the mussel *Mytilus* spp. *Marine Environmental Research*, **39**, 21-26.

Wootton, A.N., Goldfarb, P.S., Lemaire, P., O'Hara, S.C.M., Livingstone, D.R., 1996. Characterization of the presence and seasonal variation of a CYP1A-like enzyme in digestive gland of the common mussel, *Mytilus edulis*. *Marine Environmental Research*, **42(1-4)**, 297-301.

ANNEXES

ANNEXE 1:

Qualité des eaux du lac de Cazaux-Sanguinet et de la rivière Dronne

Le lac de Cazaux-Sanguinet et la rivière Dronne sont respectivement situés dans le département des Landes et de la Gironde (région Aquitaine, Sud-Ouest de la France), à une distance d'environ 150 km l'un de l'autre (figure 14). Les sites de prélèvement des *Corbicula fluminea* dans le lac et la rivière sont présentés sur les figures 15 et 16. Les résultats des analyses physico-chimiques de l'eau présentés à la suite sont issus de la Direction Départementale des Affaires Sanitaires et Sociales (DDASS) pour le lac et de l'Agence de l'Eau Adour-Garonne pour la rivière. La grille de détermination de la qualité des eaux utilisée par cette dernière est également présentée.

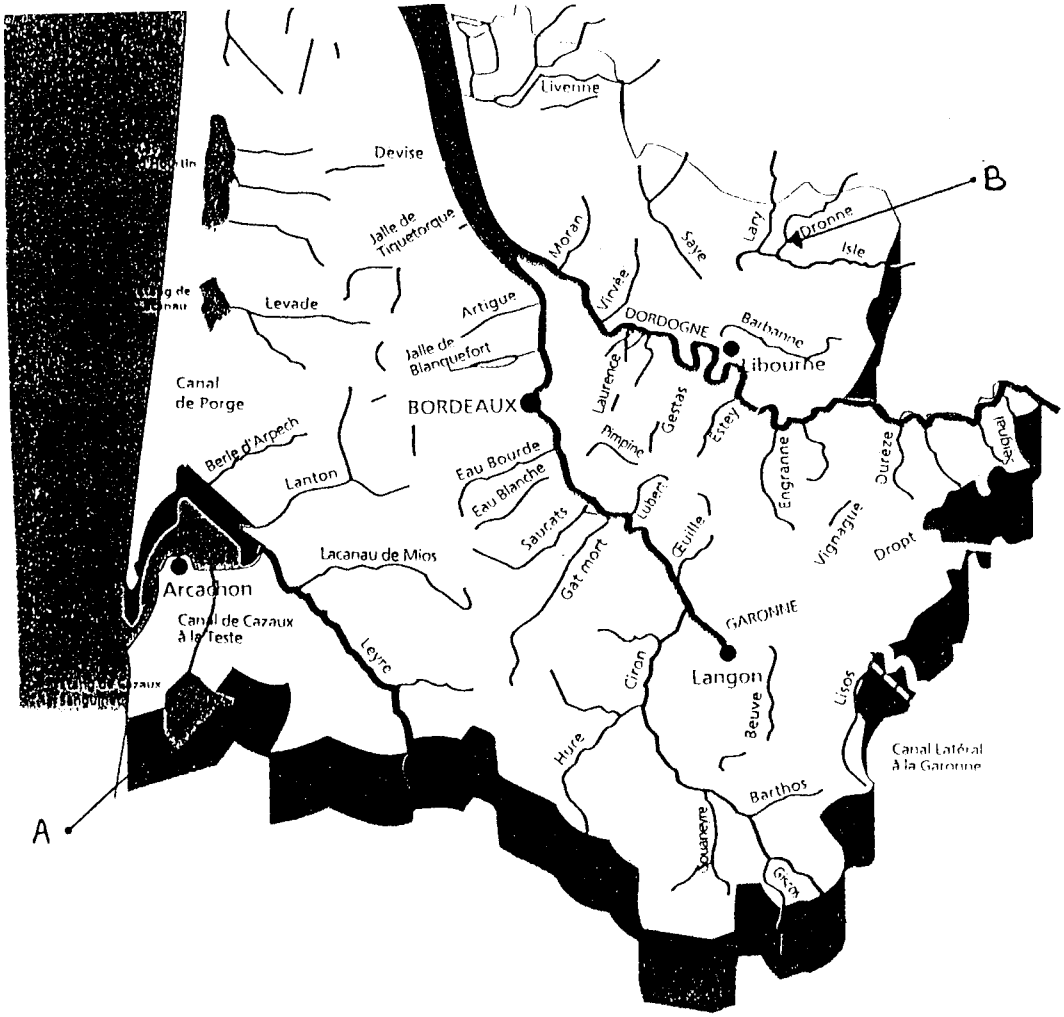


Figure 15. Sites de prélèvement des *Corbicula fluminea*: (A) lac de Cazaux-Sanguinet, (B) rivière Dronne.

Figure 16. Site de prélèvement des *Corbicula fluminea* sur les berges du lac de Cazaux-Sanguinet (département des Landes).

Figure 17. Site de prélèvement des *Corbicula fluminea* sur les berges de la rivière Dronne (département de la Gironde).

MINISTERE DU TRAVAIL
ET DES AFFAIRES SOCIALES
DIRECTION DEPARTEMENTALE DES
AFFAIRES SANITAIRES ET SOCIALES
DE LA GIRONDE

REPUBLIQUE FRANCAISE

Bordeaux le: 02/07/1997

Code du prélèvement	00032202
Prélevé le	Lundi 28 Avril 1997
Par	MME LEROI , DDASS

Unité de gestion	DISTRICT ARCACHON
Installation	CAP LAC CAZAUX
Point de surveillance	LA TESTE
0000001328	EXHAURE
	ROBINET
Type de visite	RS

MONSIEUR LE DIRECTEUR DDASS
Terrasse du Maréchal KOENIG

33062 BORDEAUX

	Unités	Résultats	Normes
Mesures terrains			
. Température de l'Eau	°C	15,00	25
. Couleur (0=r.a.s.,sinon =1,cf comm.	qualit.	0	
. Odeur (0=r.a.s., sinon=1 cf comm.)	qualit.	0	
. Saveur (0=r.a.s., sinon=1 cf comm.)	qualit.	0	
Mesures laboratoires 00032149 97/E/12042 LABORATOIRE DE L'INSTITUT EUROPEEN DE L'ENVIRONNEMENT, BORDEAUX			
PARAMETRES MICROBIOLOGIQUES			
. ColiformesThermotolérants/100ml(MS)	n/100ml	6	20000
. Streptocoques Fécaux/ 100ml(MS)	n/100ml	3	10000
CARACTERISTIQUES ORGANOLEPTIQUES			
. Turbidite Néphélométrique	NTU	22,0	
. Coloration	mg/l Pt	5	
EQUILIBRE CALCO-CARBONIQUE			
. pH à 20°C	unitépH	6,50	
. Hydrogénocarbonates	mg/l	12,20	
MINERALISATION			
. Conductivité à 20°C	µS/cm	215	
. Calcium	mg/l	5,95	
. Magnésium	mg/l	4,10	
. Potassium	mg/l	2,75	
. Sodium	mg/l	22,25	
. Sulfates	mg/l	13,15	250
. Chlorures	mg/l	42,25	200
. Silicates (en SiO2)	mg/lSiO	2,30	
FER ET MANGANESE			
. Fer Total	µg/l	1390	2000
. Manganèse total	µg/l	32	
PARAMETRE AZOTES ET PHOSPHORES			
. Azote Kjeldhal (en N)	mg/l	0,40	
. Ammonium (en NH4)	mg/l	0,07	1,5
. Nitrites (en NO2)	mg/l	<0,01	
. Nitrates (en NO3)	mg/l	0,15	50
. Orthophosphates (enPO4)	mg/l	<0,05	
OXYGENE ET MATIERES ORGANIQUES			
. Oxygène Dissous	mg/l	8,05	
. Carbone Organique Total	mg/l C	3,95	
. DBO5	mg/l O2	1	
. DCO	mg/l O2	18	273
. Matières En Suspension	mg/l	3	

MINISTERE DU TRAVAIL
ET DES AFFAIRES SOCIALES
DIRECTION DEPARTEMENTALE DES
AFFAIRES SANITAIRES ET SOCIALES
DE LA GIRONDE

REPUBLIQUE FRANCAISE

Bordeaux le: 02/07/1997

Code du prélèvement	00032202
Prélevé le	Lundi 28 Avril 1997
Par	MME LEROI , DDASS
Unité de gestion	DISTRICT ARCACHON
Installation	CAP LAC CAZAUX
Point de surveillance	LA TESTE
0000001328	EXHAURE
	ROBINET
Type de visite	RS

MONSIEUR LE DIRECTEUR DDASS
Terrasse du Maréchal KOENIG

33062 BORDEAUX

	Unités	Résultats	Normes
. Hydrogène sulfuré	mg/l	<0,005	
OLIGO-ELEMENTS ET MICROPOLLUANTS M.			
. . Aluminium Total	mg/l	0,009	
. . Baryum	mg/l	0,019	1
. . Cadmium	µg/l	<0,1	5
. . Cuivre	mg/l	<0,001	
. . Fluorures	µg/l	60	
. . Plomb	µg/l	<1	50
. . Zinc	mg/l	0,006	5
SOUS-PRODUIT DE DESINFECTION			
. . Chloroforme	µg/l	<0,5	
. . Dichloromonobromomethane	µg/l	<0,5	
. . Monochlorodibromomethane	µg/l	<1	
. . Bromoforme	µg/l	<2	
COMPOSES ORGANOHALOGENES VOLATILES			
. . 1,1,1-Trichloroéthane	µg/l	<0,5	
. . 1,1,2,2-Tétrachloroéthane	µg/l	<2	
. . 1,1,2,2-Tétrachloroéthylène	µg/l	<0,5	
. . 1,1,2-Trichloroéthane	µg/l	<0,5	
. . 1,1-Dichloroéthane	µg/l	<10	
. . 1,1-Dichloroéthylène	µg/l	<10	
. . 1,2-Dichloroéthane	µg/l	<10	
. . 1,2-Dichloroéthylène cis	µg/l	<10	
. . Dichlorométhane	µg/l	<10	
. . Tétrachlorure de Carbone	µg/l	<0,1	
. . Trichloroéthylène	µg/l	<0,5	
. . Trichlorofluorométhane	µg/l	<0,5	
. . Trichlorotrifluoroéthane	µg/l	<0,5	
HYDROCARB. POLYCYCLIQUES AROMATIQU			
. . Hydrocarb. Polycycl.Arom.(6subst.)	µg/l	<0,200	0,2
. . Benzo(1,12)Perylène	µg/l	<0,050	
. . Fluoranthène	µg/l	<0,010	
. . Benzo(3,4)Fluoranthène	µg/l	<0,010	
. . Benzo(11,12)Fluoranthène	µg/l	<0,010	
. . Benzo(a)Pyrène	µg/l	<0,010	
. . Indéno(1,2,3-Cd)Pyrène	µg/l	<0,050	
PESTICIDES ORGANOCHLORES			
. . Aldrine	µg/l	<0,01	
. . DDD-2-4'	µg/l	<0,03	
. . DDD-4-4'	µg/l	<0,03	
. . DDE-2,4'	µg/l	<0,03	
. . DDE-4,4'	µg/l	<0,03	
. . DDT-4,4'	µg/l	<0,03	274
. . Dieldrine	µg/l	<0,01	

MINISTERE DU TRAVAIL
ET DES AFFAIRES SOCIALES
DIRECTION DEPARTEMENTALE DES
AFFAIRES SANITAIRES ET SOCIALES
DE LA GIRONDE

REPUBLIQUE FRANCAISE

Bordeaux le: 02/07/1997

Code du prélèvement	00032202
Prélevé le	Lundi 28 Avril 1997
Par	MME LEROI , DDASS

Unité de gestion	DISTRICT ARCACHON
Installation	CAP LAC CAZAUX
Point de surveillance	LA TESTE
0000001328	EXHAURE
	ROBINET
Type de visite	RS

MONSIEUR LE DIRECTEUR DDASS
Terrasse du Maréchal KOENIG

33062 BORDEAUX

	Unités	Résultats	Normes
. . HCH Alpha	µg/l	<0,03	
. . HCH Béta	µg/l	<0,03	
. . Heptachlore	µg/l	<0,03	
. . Heptachlore Epoxide	µg/l	<0,03	
. . Hexachlorobenzène	µg/l	<0,005	
PESTICIDES TRIAZINES			
. . Atrazine	µg/l	0,05	
. . Simazine	µg/l	<0,05	
PLASTIFIANTS			
. . PolychloroBipheniles(PCB)	µg/l	<0,1	

Commentaires

Eau brute conforme aux normes en vigueur pour les paramètres mesurés.

Pour le Directeur,

Terrasse du Maréchal Koenig - B.P .922 - 33062 BORDEAUX CEDEX - Tél. 05.56.90.88.88 - Télécopie 05.56.96.29.31

LE 20/10/98

FICHIER DE LA QUALITE DES EAUX SUPERFICIELLES

PRESENTATION STATISTIQUE DES RESULTATS

BASSIN : ADOUR GARONNE
COURS D'EAU : DRONNE
DEPARTEMENT : 33 GIRONDE

LOCALISATION: PONT DE COUTRAS DE LA D10 AVAL DISTILLERIES

POINT NO: 030-000
PERIODE : DU 26/02/96 AU 23/04/98

PARAMETRE ET UNITE			NB.	CONCENTRATION MAXIMALE DANS N% DES ANALYSES							MOY.GEN.
				MINIMUM	10%	25%	50%	75%	90 %	MAXIMUM	
Débit		m3/s	22	2,200	3,360	4,490	9,080	14,100	17,500	237,000	20,738
Température Eau		Degré C	25	7,1	8,8	10,5	13,6	18,2	20,0	26,2	14,8
pH			25	7,2	7,3	7,7	8,1	8,1	8,2	8,3	7,9
Conduct.à 20 °C		µS/cm	25	212	337	370	400	420	435	448	394
M.E.S.		mg/L	25			3	5	10	13	86	10
DBO5		mg/L	23	0,4	0,5	0,8	1,2	1,4	1,6	2,3	1,2
DCO		mg/L	10	4	4	4	8	9	13	18	8
Carbone Organique		mg/L	23	2,4	2,5	2,6	3,2	3,8	4,4	6,5	3,5
Oxygène Dissous		mg/L	25	6,1	6,2	7,9	8,8	10,3	11,0	11,8	9,1
Taux Saturation en O2		%	25	66	71	77	89	94	97	104	87
Calcium	Ca++	mg/L	5	64	NON CALC.	NON CALC.	NON CALC.	NON CALC.	NON CALC.	81	74
Magnesium	Mg++	mg/L	5	3,8	NON CALC.	NON CALC.	NON CALC.	NON CALC.	NON CALC.	6,4	4,9
Sodium	Na+	mg/L	5	8,0	NON CALC.	NON CALC.	NON CALC.	NON CALC.	NON CALC.	8,7	8,3
Potassium	K+	mg/L	5	1,8	NON CALC.	NON CALC.	NON CALC.	NON CALC.	NON CALC.	2,8	2,3
Ammoniaque	NH4+	mg/L	25	0,04	0,04	0,14	0,16	0,28	0,34	0,60	0,21
Nitrites	NO2-	mg/L	23	0,02	0,02	0,03	0,05	0,13	0,19	0,25	0,09
Nitrates	NO3-	mg/L	25	3,15	4,00	5,60	10,00	13,70	14,80	17,20	10,27
Azote Kjeldahl		mg/L	23	0,2	0,3	0,4	0,6	0,8	0,9	1,2	0,6
Bicarbonates	HCO3-	mg/L	23	115	195	222	244	250	256	272	235
Chlorures	Cl-	mg/L	23	12,9	13,5	14,0	14,8	15,2	16,5	19,3	15,0
Sulfates	SO4--	mg/L	23	7,3	10,0	11,0	12,4	13,2	13,5	16,1	12,2
Phosphates	PO4---	mg/L	25	0,03	< 0,05	0,06	0,12	0,16	0,20	0,69	< 0,16
Phosphore total		mg/L P	23	0,030	0,030	0,050	0,070	0,090	0,140	0,200	0,087
Cadmium	Cd	mg/L	10	0	0	0	0	0	0,0010	0,0010	0,0002
Chrome Total	Cr	mg/L	10	0	0	0	0,0010	0,0010	0,0020	0,0100	0,0016
Cuivre	Cu	mg/L	10	0,0020	0,0020	0,0020	0,0030	0,0070	0,0080	0,0530	0,0092
Plomb	Pb	mg/L	10	0	0	0,0010	0,0010	0,0020	0,0020	0,0020	0,0014
Zinc	Zn	mg/L	10	0,0010	0,0010	0,0050	0,0140	0,0170	0,0290	0,0400	0,0155

Les grilles de détermination de la qualité des eaux

Grille des paramètres généraux utilisés pour évaluer la qualité des eaux

Code	Groupes de paramètres		1A Excellente	1B Bonne	2 Passable	Mauvaise	
	Conductivité	µ S/cm	≤ 400	400 à 750	750 à 1500	1500 à 3000	> 3000
	Chlorures	mg/l	≤100	100 à 200	200 à 400	400 à 1000	> 1000
	Température	°C	≤ 20	20 à 22	22 à 25	25 à 30	> 30
	pH		6,5 à 8,5	-	6 à 6,5 ou 8,5 à 9	5,5 à 6 ou 9 à 9,5	< 5,5 ou > 9,5
MES	Mat. en Suspension	mg/l	≤ 30	-	-	30 à 70	> 70
OX Matières organiques (oxydables)	O2 Dissous	mg/l	> 7	5 à 7	3 à 5	≤ 3	-
	% Saturation	%	> 90	70 à 90	50 à 70	≤ 50	-
	DBO5	mg/l	≤ 3	3 à 5	5 à 10	10 à 25	> 25
	DCO	mg/l	≤ 20	20 à 25	25 à 40	40 à 80	> 80
A Ammoniaque	NH4	mg/l	≤ 0,1	0,1 à 0,5	0,5 à 2	2 à 8	> 8
	N K	mg/l	≤ 1	1 à 2	2 à 3	> 3	-
N Nitrates	NO3	mg/l	< 5	5 à 25	25 à 50	50 à 100	> 100
P Matières phosphorées	PO4	mg/l	< 0,2	0,2 à 0,5	0,5 à 1	1 à 5	> 5
	Phosphore Total	mg/l P	< 0,1	0,1 à 0,25	0,25 à 0,5	0,5 à 2,5	> 2,5

Grilles des paramètres complémentaires donnés à titre indicatif

Code	Paramètre		Pollution normale	Pollution modérée	Pollution notable	Pollution importante	
T Matières toxiques	Fer (Fe)	mg/l	≤ 0,5	0,5 à 1	1 à 1,5	> 1,5	-
	Manganèse (Mn)	mg/l	≤ 0,1	0,1 à 0,25	0,25 à 0,5	> 0,5	-
	Cuivre (Cu)	mg/l	≤ 0,02	0,02 à 0,05	0,05 à 1	> 1	-
	Zinc (Zn)	mg/l	≤ 0,5	0,5 à 1	1 à 5	> 5	-
	Arsenic (As)	mg/l	≤ 0,01	-	0,01 à 0,05	> 0,05	-
	Cadmium (Cd)	mg/l	≤ 0,001	-	-	> 0,001	-
	Chrome (Cr)	mg/l	≤ 0,05	-	-	> 0,05	-
	Cyanure (CN)	mg/l	≤ 0,05	-	-	> 0,05	-
	Plomb (Pb)	mg/l	≤ 0,05	-	-	> 0,05	-
	Sélénium (Se)	mg/l	≤ 0,01	-	-	> 0,01	-
	Mercure (Hg)	mg/l	≤ 0,0005	-	-	> 0,0005	-
	Fluor (F)	mg/l	≤ 0,7	0,7 à 1,7	-	> 1,7	-
	Phénols	mg/l	0	0 à 0,01	0,01 à 0,05	0,05 à 0,5	> 0,5
	Détergents	mg/l	≤ 0,2	-	0,2 à 0,5	> 0,5	-

Code	Paramètre	Excellente	Bonne	Passable	Médiocre	
I Indice biologique	IBG	20 à 17	16 à 13	12 à 9	8 à 5	< 5

Code		4 à 9 prélèvements Plus de 10 prélèvements			
B Bactério	Coliformes totaux	80 % < 500 95 % < 10 000	95 % < 10 000	5 à 33 % < 10 000	Plus de 33 % > 10 000
	Coliformes fécaux	80 % < 100 95 % < 2 000	95 % < 2 000	5 à 33 % < 2 000	Plus de 33 % > 2 000
	Streptocoques fécaux	95 % < 100			

ANNEXE 2:

Etude de génétique biochimique sur les populations de *Corbicula fluminea* peuplant le lac de Cazaux-Sanguinet et la rivière Dronne

Les populations de *Corbicula fluminea* peuplant le lac de Cazaux-Sanguinet (département des Landes) et la rivière Dronne (département de la Gironde) présentent des différences phénotypiques importantes. Les coquilles de *C. fluminea* du lac sont de couleur brune et possèdent une longueur antério-postérieure maximale d'environ 30 mm alors que les coquilles des animaux de la rivière sont de couleur jaune-olivâtre et possèdent une longueur antério-postérieure maximale d'environ 20-25 mm (figure 17).

Figure 18. *Corbicula fluminea* provenant du lac de Cazaux-Sanguinet et de la rivière Dronne.

Pour vérifier l'existence d'éventuelles différences génotypiques entre les individus des deux populations mais également entre les individus de chacune des populations, une étude de génétique biochimique a été conduite. Cette étude repose sur l'analyse d'une série de systèmes enzymatiques dont l'activité est révélée après une migration électrophorétique sur gel d'amidon. Des différences inter-individuelles au niveau de la distance de migration de la ou des protéine(s) appartenant à un système enzymatique donné peuvent traduire des différences au niveau génétique.

Pour ce faire, 44 animaux sexuellement matures ont été prélevés à la fin du mois d'octobre 1999 dans le lac de Cazaux-Sanguinet et la rivière Dronne. Ils ont été maintenus en stabulation en aquarium pendant trois jours, dans l'eau du lac ou de la rivière thermostatée à

20 ± 0.1°C et constamment aérée par un diffuseur connecté à une pompe à air (Shego M2K3). Après dissection, les tissus mous de chaque animal ont été pesés et homogénéisés dans un volume égal de tampon Tris-HCl 10 mM, pH 6,8, EDTA 1 mM, NADP 1% à l'aide d'un Ultra-Turrax Antrieb T25, puis centrifugés à 20000 g pendant 30 min dans une ultracentrifugeuse Beckman LE-80. Les surnageants ont ensuite été stockés à -80°C. Des électrophorèses sur gel d'amidon à 12% (dans du tampon Tris (21 mM) - citrate (5 mM), pH 8) ont été effectuées pour chacun des 44 individus de chaque population. La migration, d'une durée totale de 4 à 6 h, a été réalisée à 40A puis à 80A dans du tampon Tris (0,62 M) - citrate (0,14 M), pH 8. Dix systèmes enzymatiques ont ensuite été étudiés à l'aide de solutions de révélation appropriées (tableau 6) (Pasteur *et al.*, 1987).

Au sein de chaque population, les différents systèmes enzymatiques étudiés se sont avérés monomorphes. Par contre les estérases ont présenté un polymorphisme entre les deux populations. Ces résultats préliminaires ne signifient pas obligatoirement que nous nous trouvons en présence de deux espèces différentes. Cependant, de telles différences doivent être prises en compte lors d'études environnementales impliquant ces deux populations.

Tableau 7. Systèmes enzymatiques étudiés et solutions de révélation correspondantes.

Système enzymatique	Particularités	Solution de révélation [a]
Aspartate-amino-transférase (AAT) E.C. 2.6.1.1.	Dimère (en général 2 loci)	Tris-HCl 0,2 M, pH 8 Acide L-aspartique Acide cétoglutarique Pyridoxal-5-phosphate Fast blue BB
Estérase (EST) E.C. 3.1.1.1.	Monomère ou dimère (nombreux loci)	KH_2PO_4/Na_2HPO_4 0,1 M, pH 6,5 á-naphthyl-acétate 2% â-naphthyl-acétate 2% Fast blue BB
Glucose-6-phosphate- déshydrogénase (G6PD) E.C. 1.1.1.49.	Dimère	Tris-HCl 0,2 M, pH 8 Glucose-6-phosphate $MgCl_2$ 0,5 M, NADP 1% NBT 1%, MTT 1%, PMS 1%
Glucose-phosphate- isomérase (GPI) E.C. 5.3.1.9.	Dimère	Tris-HCl 0,2 M, pH 8 $MgCl_2$ 0,5 M Fructose-6-phosphate G6PD, NAD 1%, NADP 1% MTT 1%, PMS 1%, agarose 1,5%
Hexokinase (HK) E.C. 2.7.1.1.	Monomère	Tris-HCl 0,2 M, pH 8 á -D^+-glucose ATP, NAD 1%, NADP 1% $MgCl_2$ 0,5 M, G6PD NBT 1%, PMS 1%, agarose 1,5%
Leucine-amino-peptidase (LAP)	Monomère (nombreux loci)	Tris-maléate 0,2 M, pH5,5 L-leucyl-â-naphthyl-amide $MgCl_2$ 0,5 M Black K Salt
Malate-déshydrogénase (MDH) E.C. 1.1.1.37.	Dimère (2 loci)	Tris-HCl 0,2 M, pH 8 Acide malique 2 M $MgCl_2$ 0,5 M, NAD 1% NBT 1%, MTT 1%, PMS 1%

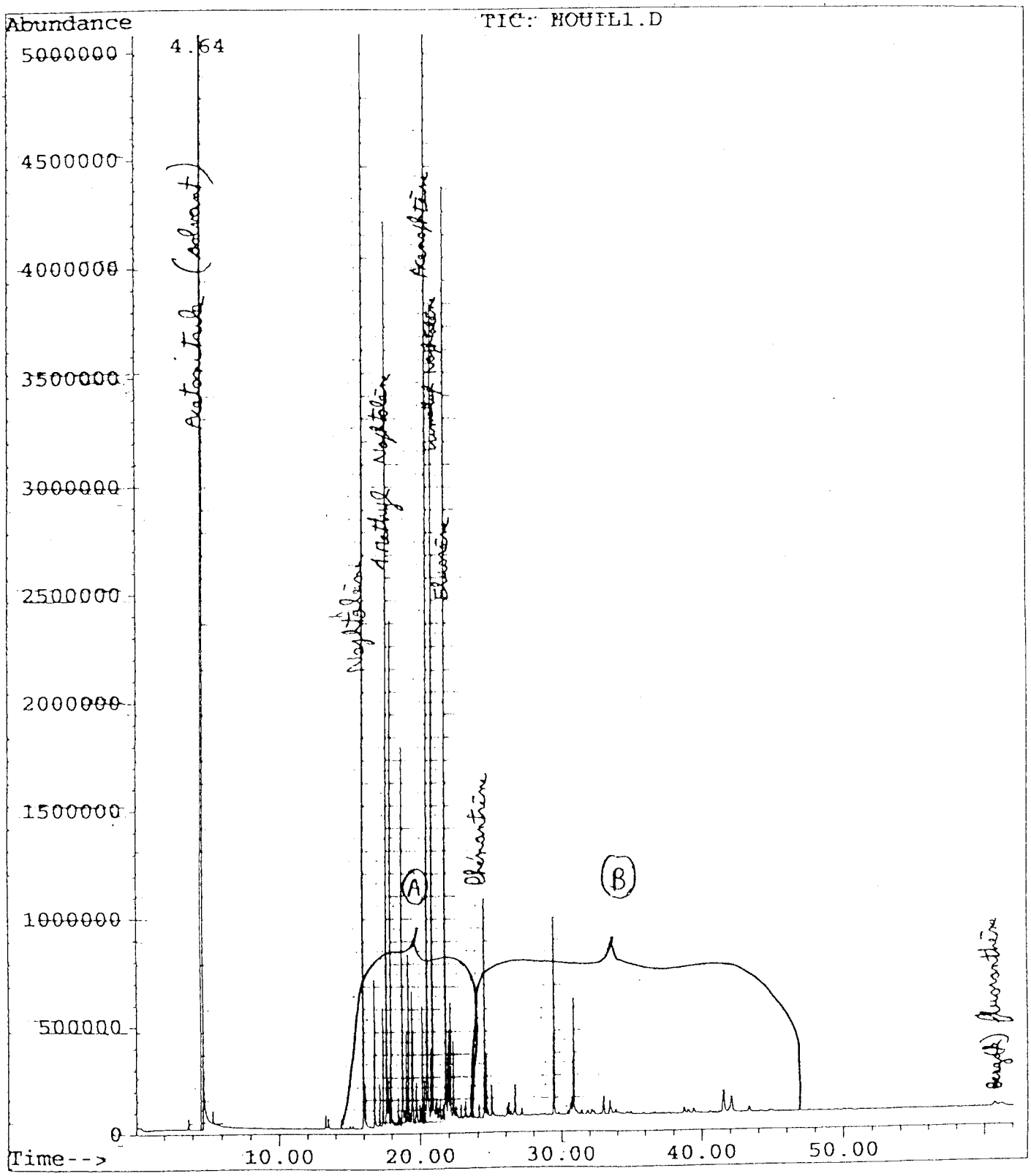
Tableau 7 (suite).

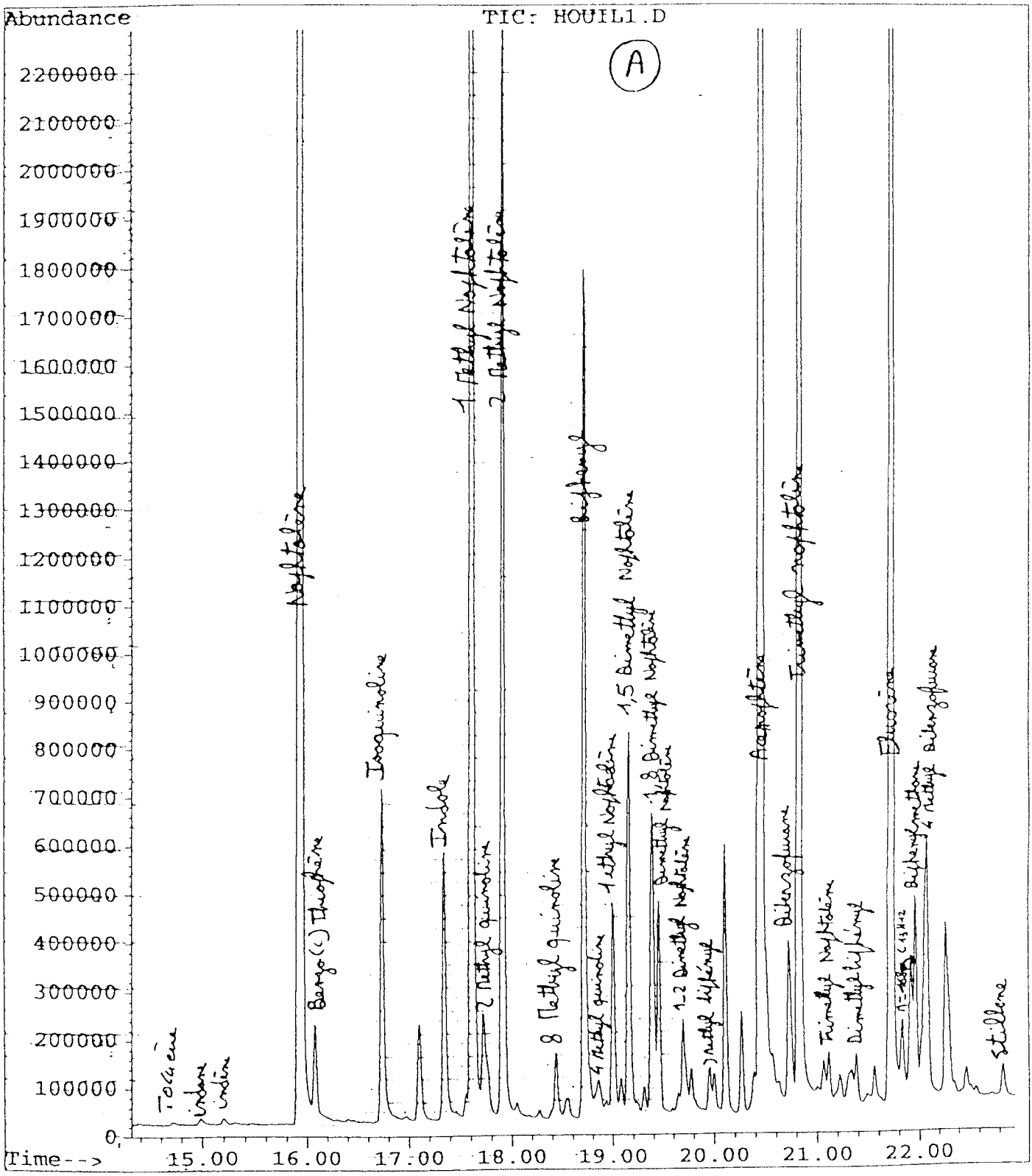
Système enzymatique	Particularités	Solution de révélation
Malico-enzyme (ME) E.C. 1.1.1.40.	Dimère (2 loci)	Tris-HCl 0,2 M, pH 8 Acide malique 2 M $MgCl_2$ 0,5 M, NADP 1% NBT 1%, MTT 1%, PMS 1%
Phosphoglucomutase (PGM) E.C. 2.7.5.1.	Monomère	Tris-HCl 0,2 M, pH 8 $MgCl_2$ 0,5 M Glucose-1-phosphate NAD 1%, NADP 1%, G6PD NBT 1%, MTT 1%, PMS 1% Agarose 1,5 %
Phosphogluconate- déshydrogénase (PGD) E.C. 1.1.1.43.	Dimère	Tris-HCl 0,2 M, pH 8 Acide 6-phosphogluconique $MgCl_2$ 0,5 M, NADP 1% NBT 1%, MTT 1%, PMS 1%

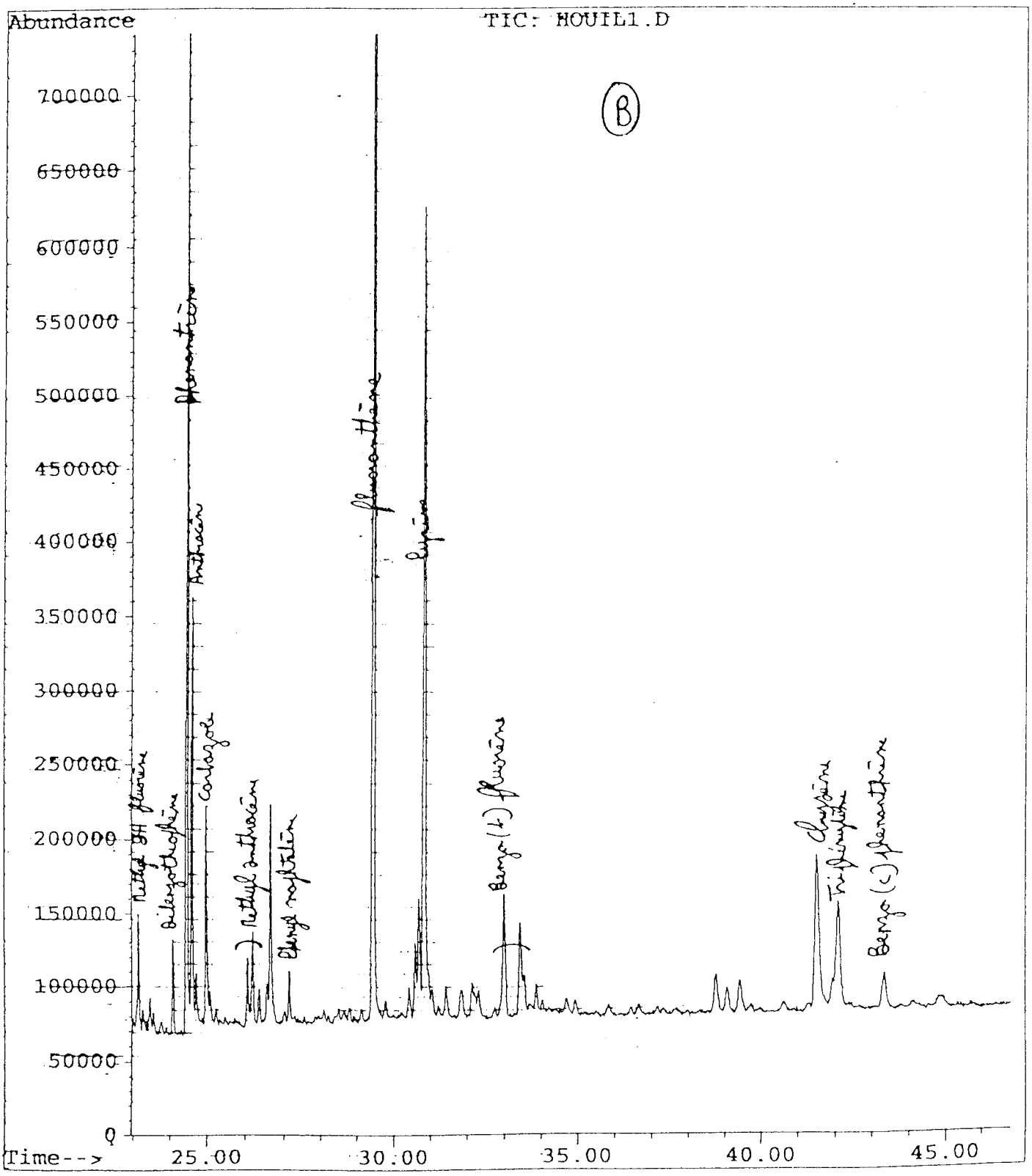
[a] MTT, NBT, PMS: Thiazolyl Blue, Nitro Blue Tetrazolium et Phénazine Méthosulfate, respectivement.

ANNEXE 3:

Caractérisation de la coupe de goudron de houille par chromatographie en phase gazeuse couplée à la spectrométrie de masse (Laboratoire Contrôle et Environnement, Groupement de Recherches de Lacq, Groupe TotalFinaElf)




Abundance
TIC: HOUIL1.D
A
2200000
2100000
2000000
1900000
1800000
1700000
1600000
1500000
1400000
1300000
1200000
1100000
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
Time-->
15.00
16.00
17.00
18.00
19.00
20.00
21.00
22.00
Toluène
Indan
Indène
Naphtalène
Benzo(c) Thiophène
Isoquinoline
Indole
1 Méthyl Naphtalène
2 Méthyl Naphtalène
7 Méthyl quinoline
8 Méthyl quinoline
Biphényle
4 Méthyl quinoline
1 éthyl Naphtalène
1,5 Diméthyl Naphtalène
1,8 Diméthyl Naphtalène
1,2 Diméthyl Naphtalène
Méthyl biphényl
Acénaphtène
Dibenzofurane
Triméthyl naphtalène
Triméthyl Naphtalène
Diméthylbiphényl
Fluorène
Biphénylméthane
4 méthyl Dibenzofurane
Stilbène


Abundance
TIC: HOUIL1.D
B
700000
650000
600000
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
Time-->
25.00
30.00
35.00
40.00
45.00
Fluoranthène
Pyrène
Benzo(c)phénanthrène

Résumé: Notre travail s'inscrit dans une démarche de validation du mollusque bivalve *Corbicula fluminea* comme organisme sentinelle des milieux dulçaquicoles. Nous avons étudié les réponses de plusieurs biomarqueurs potentiels de pollution (composants du métabolisme de (dé)toxication des phases I et II, indicateurs de stress oxydatif, activité propionylcholinestérase) après exposition à des conditions naturelles ou contrôlées. Au préalable, nous avons optimisé les conditions de dosage de l'activité enzymatique des glutathion *S*-transférases (GSTs, enzymes de phase II) envers plusieurs substrats, puis purifié et caractérisé trois isoenzymes cytosoliques de la masse viscérale. La variabilité saisonnière des biomarqueurs de pollution ainsi que leur sensibilité à différents facteurs abiotiques peuvent masquer ou fausser les effets des contaminants. Afin de les connaître nous avons étudié les variations saisonnières des différents paramètres et les effets en laboratoire de la température, du pH, de l'oxygénation, du type d'eau et du sédiment sur leurs réponses. Dans les deux cas, les activités catalase, propionylcholinestérase et NADH-cytochrome *c* réductase ont présenté une forte variabilité, au contraire des activités GSTs et NADPH-cytochome *c* réductase. Après exposition en laboratoire à plusieurs contaminants (trichloroéthylène, toluène, chlorure de cadmium, coupe de goudron de houille), la plupart des paramètres biochimiques ont présenté des variations significatives. L'approche multibiomarqueurs nous a permis d'obtenir des profils de réponses spécifiques pour chacun des contaminants étudiés. De plus, une étude de terrain sur un site industriel a démontré son efficacité pour évaluer la contamination des écosystèmes aquatiques. Au vu de l'ensemble des résultats obtenus, *C. fluminea* pourrait constituer un organisme sentinelle satisfaisant. Des études complémentaires en mésocosmes sont désormais nécessaires afin de confirmer ces résultats.

Study of biochemical markers of pollution in the freshwater bivalve mollusc *Corbicula fluminea* (Müller). Purification and characterisation of glutathione *S*-transferases.

Abstract: Our work belongs to a program of validation of the mollusc bivalve *Corbicula fluminea* as a sentinel organism of freshwater ecosystems. We studied responses of several potential pollution biomarkers (components of (de)toxification metabolism of phases I and II, indicators of oxidative stress, propionylcholinesterase activity) after exposure to natural or controlled conditions. At first, we have optimised assay conditions of enzymatic activity of glutathione *S*-transferases (GSTs, enzymes of phase II) towards several substrates and then purified and characterised three cytosolic isoenzymes from visceral mass. Seasonal variability of pollution biomarkers as well as their sensibility to different abiotic factors can occult or distort effects of contaminants. To know them, we studied seasonal variations of the different parameters and laboratory effects of temperature, pH, oxygenation, water-type and sediment on their responses. In both cases, catalase, propionylcholinesterase and NADH-cytochrome *c* reductase activities displayed high variability, contrary to GST and NADPH-cytochrome *c* reductase activities. After a laboratory exposure to several contaminants (trichloroethylene, toluene, cadmium chloride and a coal tar fraction), most of biochemical parameters exhibited significant variations. The multibiomarker approach made possible the obtention of response profiles specific to each contaminant. Moreover, a field study around an industrial site attested its efficacy to assess contamination of aquatic ecosystems. Considering our whole results, *C. fluminea* could be an acceptable sentinel organism. Henceforth, complementary studies in mesocosms are needed to confirm these results.

ECOTOXICOLOGIE

Mots-clés: biomarqueurs, bivalve, chlorure de cadmium, *Corbicula fluminea*, (dé)toxication, eau douce, environnement, glutathion *S*-transférases, hydrocarbures aromatiques polycycliques, purification, lac, mollusque, pollution, rivière, toluène, trichloroéthylène

Laboratoire de Physico-Toxico-Chimie des Systèmes Naturels (LPTC), UPRES-A 5472 CNRS, Département de Toxicologie Biochimique, Université Bordeaux 1, Avenue des Facultés, 33405 Talence, France.