UNIVERSIDADE DO ALGARVE

UNIVERSITY OF NICE-SOPHIA ANTIPOLIS

STUDY OF THE EFFECTS OF CONTAMINANTS IN A SENTINEL SPECIES, THE CLAM *RUDITAPES DECUSSATUS*, THROUGH PROTEIN EXPRESSION ANALYSIS: APPLICATION TO MARINE ENVIRONMENT MONITORING

Suze Chainho Chora

(Dissertation presented at the University of Algarve to obtain the degree of Doctor in Philosophy in Environmental Sciences and Technologies, Area of Aquatic Environment)

PhD Thesis Jury:

President – Doctor João Pinto Guerreiro, University of Algarve, Portugal
Doctor Amparo Torreblanca Tamarit, University of Valência, Spain
Doctor Vítor Manuel de Oliveira e Vasconcelos, University of Porto, Faculty of Sciences, Portugal
Doctor David Sheehan, University College Cork, Ireland
Doctor Maria João Bebianno, University of Algarve, Portugal
Doctor Michèle Roméo, University of Nice-Sophia Antipolis, France

Faro

2009

UNIVERSIDADE DO ALGARVE

UNIVERSITY OF NICE-SOPHIA ANTIPOLIS

STUDY OF THE EFFECTS OF CONTAMINANTS IN A SENTINEL SPECIES, THE CLAM *RUDITAPES DECUSSATUS*, THROUGH PROTEIN EXPRESSION ANALYSIS: APPLICATION TO MARINE ENVIRONMENT MONITORING

Suze Chainho Chora

(Dissertation presented at the University of Algarve to obtain the degree of Doctor in Philosophy in Environmental Sciences and Technologies, Area of Aquatic Environment)

Faro

2009

UNIVERSIDADE DO ALGARVE

UNIVERSITY OF NICE-SOPHIA ANTIPOLIS

ESTUDO DO EFEITO DE CONTAMINANTES NA ESPÉCIE SENTINELA, A AMÊIJOA *RUDITAPES DECUSSATUS*, POR INTERMÉDIO DA ANÁLISE DA EXPRESSÃO PROTEICA: APLICAÇÃO À MONITORIZAÇÃO DO MEIO MARINHO

ÉTUDE DES EFFETS DES CONTAMINANTS CHEZ L'ESPÈCE SENTINELLE, LA PALOURDE *RUDITAPES DECUSSATUS*, PAR L'ANALYSE DE L'EXPRESSION PROTÉIQUE: APPLICATION À LA SURVEILLANCE DU MILIEU MARIN

STUDY OF THE EFFECTS OF CONTAMINANTS IN A SENTINEL SPECIES, THE CLAM *RUDITAPES DECUSSATUS*, THROUGH PROTEIN EXPRESSION ANALYSIS: APPLICATION TO MARINE ENVIRONMENT MONITORING

Suze Chainho Chora

Supervisors:

Professor Doutora Maria João Bebianno, University of Algarve, Portugal
Doctor Michèle Roméo, University of Nice-Sophia Antipolis, France

Faro

2009

This thesis has been accomplished in the Ecotoxicology and Environmental Chemistry group from the Faculty of Sciences and Technology (F.C.T) of the Algarve University and in the University of Nice-Sophia Antipolis, laboratory EA 4228 ECOMERS, thanks to a PhD fellowship SFRH/BD/19477/2004 from the Portuguese Fundação para a Ciência e Tecnologia (FCT) of the Ministry of Science and Technology of Portugal.

Resumo Geral

As regiões costeiras, que englobam alguns dos ecossistemas mais produtivos do meio marinho e que são vitais à manutenção do equilíbrio da região litoral, são também lugares preferenciais para a instalação de várias estruturas humanas, tais como portos, centros urbanos, indústrias e actividades agrícolas. Como consequência, numerosos e variados poluentes são libertados nestas áreas, infligindo vários impactos negativos. Estes poluentes, que podem existir em solução ou em suspensão na água ou ainda associados a sedimentos, são classificados em poluentes: orgânicos, metais ou organo-metálicos. A presença destes contaminantes e, consequentemente, a sua persistência e biodisponibilidade, dependem de vários factores tais como as suas propriedades químicas e factores bióticos e abióticos. Tendo em conta este cenário, várias medidas foram aplicadas com o intuito de monitorizar a poluição do meio marinho que pode ter sérias repercussões não só ao nível dos organismos marinhos como também nas populações humanas que os consomem. Durante a década de 70, esta monitorização baseava-se na detecção de compostos químicos e na quantificação de moléculas potencialmente perigosas presentes na água, nos sedimentos e nos organismos marinhos. Contudo, tais análises não fornecem qualquer informação relativa ao efeito produzido pelos poluentes nos organismos marinhos. Por esta razão, passados trinta anos, surgiu uma nova abordagem no âmbito da ecotoxicologia, baseada em medidas bioquímicas, fisiológicas e comportamentais, denominada biomarcadores. Estes biomarcadores constituem um sistema de alarme precoce para situações de poluição podendo estar associados à exposição ou a efeitos tóxicos de um ou vários poluentes. Existem diferentes tipos de biomarcadores consoante a especificidade da sua resposta aos diferentes tipos de contaminantes. Todavia, estes biomarcadores, conhecidos por "biomarcadores clássicos", não fornecem informações acerca dos processos celulares a que os organismos recorrem para reagirem e se adaptarem às situações de exposição a compostos nocivos. Adicionalmente para a maioria de poluentes faltam biomarcadores robustos, específicos e sensíveis. Com o intuito de ultrapassar estas lacunas, passados 10 anos surgiram novas técnicas, denominadas "ómicas", que permitem um análise holística do efeito dos poluentes. Estas técnicas englobam: a genómica (o estudo da expressão génica), a transcriptómica (o estudo da expressão do ARNm), a metabolómica (o estudo dos metabolitos) e a proteómica (o estudo da expressão proteica). Contudo, estas técnicas inicialmente eram essencialmente aplicadas a espécies geneticamente bem caracterizadas, tais como microorganimos, roedores e o Homem. A análise proteómica, em particular, permite efectuar a análise quantitativa da expressão do proteoma definido como o

complemento proteico total expresso por um genoma num dado momento em resposta a uma determinada condição. A proteómica tem sido aplicada com sucesso em biomedicina com o intuito de definir assinaturas de expressão proteica específicas de células ou tecidos associadas a doenças ou administração de fármacos, fornecendo novos biomarcadores de patologias humanas. Recentemente começou também a ser aplicada no âmbito da ecotoxicologia na avaliação da poluição ambiental, propondo novos biomarcadores de poluição. A análise proteómica permite estabelecer assinaturas de expressão proteica de células ou tecidos específicas de stresses particulares e a identificação de novos biomarcadores em organismos, estudados como modelos na área da ecotoxicologia. Contudo, são escassos os estudos onde esta abordagem é aplicada a bivalves, consideradas espécies bioindicadoras importantes devido à sua abundância, fácil colheita, dimensões aceitáveis, natureza séssil, repartição geográfica abrangente, considerável tolerância ao stress e capacidade para bioacumulação de poluentes. Estes organismos desenvolveram uma série de estratégias de acumulação, regulação e imobilização de poluentes sendo comummente utilizados em programas de monitorização de poluição marinha. O bivalve marinho *Ruditapes decussatus* (L.), também conhecida por amêijoa mediterrânica, preenche a maior parte dos requisitos anteriormente mencionados sendo considerada uma espécie bioindicadora, permitindo uma avaliação temporal e espacial da contaminação ambiental, tanto a curto como a longo prazo. Este bivalve está presente na Europa e países do mar Mediterrânico, sobretudo nas zonas estuarinas e lagunares. Em Portugal, particularmente no Sul do país (lagoa da Ria Formosa), a produção desta amêijoa é intensa, constituindo um importante recurso económico para a região, representando cerca de 80 % das amêijoas produzidas em Portugal. O cádmio (Cd) e o nonilfenol (NP) são dois poluentes presentes na Ria Formosa. O primeiro é um metal não essencial e a sua entrada nos ambientes marinhos advém de uma grande variedade de fontes antropogénicas (e.g. fertilizantes fosfatados, indústrias de exploração de metais; desgaste de pneus) e algumas naturais (e.g. erosão de rochas e actividade vulcânica). O NP, por seu turno, resulta da degradação dos etoxilatos de nonilfenol (NPE) que são amplamente utilizados entrando na composição de vários detergentes, lubrificantes, etc. Este composto é extremamente resistente à degradação e, consequentemente, bastante persistente nos ambientes marinhos. Ambos os contaminantes são bioacumulados por organismos marinhos, sendo extremamente tóxicos para os mesmos: são disruptores endócrinos e causam stress oxidativo. O stress oxidativo é originado nas células quando os sistemas de defesas antioxidantes (nomeadamente as enzimas antioxidantes tais como a superóxido dismutase, a catalase, a glutationa peroxidase, a

glutationa, o ascorbato, etc) deixam de ter capacidade para neutralizar as denominadas "espécie reactivas de oxigénio". Estes radicais livres de oxigénio têm origem em fontes endógenas ao organismo (e.g. enzimas da Phase I e ciclo redox) e exógenas (e.g. vários poluentes marinhos). Como são extremamente reactivos e não selectivos, estes radicais interagem com várias moléculas e, consequentemente, causam efeitos nocivos para os organismos, tais como: peroxidação lipídica, danos ao nível do ADN (e.g. modificações das bases, "strand-breaks"), apoptose celular e alterações e modificações das proteínas. Ao nível das proteínas os principais danos causados pelos radicais de oxigénio descritos são: a oxidação directa dos grupos hidroxilo, a formação de aldeído/cetonas (carbonilação), a oxidação dos resíduos S (e.g. cisteína), a racemização, a ubiquitinação e efeitos no padrão das ligações disulfido (e.g. glutationilação e modificações no grupo tiol). Estas modificações induzem alterações funcionais, nomeadamente ao nível do metabolismo celular sendo bastante nocivas para os organismos. A carbonilação é uma modificação pós-transducional e irreversível que se traduz na adição de grupos carbonilo C=O às proteínas. A ubiquitinação é igualmente uma modificação pós-transducional, que consiste na fixação covalente de uma ou mais ubiquitina (proteína) a uma ou várias lisinas. Esta modificação pode ser reversível e marca as proteínas para serem degradadas via o proteassoma, também conhecido pelo sistema ubiquitina-proteassoma. Recentemente, a análise da glutationilação, do conteúdo de tióis, e da carbonilação e ubiquitinação de proteínas têm sido aplicados com êxito como biomarcadores de stress oxidativo em algumas espécies bioindicadoras de bivalves. Neste contexto, o objectivo principal da presente tese foi analisar as modificações de expressão proteica induzidas na amêijoa *R. decussatus* devido à exposição a dois poluentes químicos, Cd (metal) e NP (um composto orgânico). Seleccionou-se a abordagem proteómica "bottom-up" com separação de proteínas efectuada por intermédio da electroforese bidimensional em condições desnaturantes (2DE SDS-PAGE). A 2DE SDS-PAGE é uma técnica correntemente utilizada na análise proteómica para a separação de proteínas, separando-as inicialmente em função do respectivo ponto isoeléctrico (focalização isoelétrica) e de seguida pelo peso molecular (electroforese propriamente dita). Contudo, os protocolos disponíveis para a separação 2DE SDS-PAGE, elaborados para aplicação em mamíferos, não se mostraram adequados aos tecidos dos bivalves, devido ao elevado teor em água do mar e de lípidos. Os sais interferem com o processo de electroforese enquanto que a associação de proteínas a lípidos reduz a solubilidade destas e altera o ponto isoelétrico e a massa molecular das mesmas. Face a isto, a primeira etapa desta tese incidiu sobre a optimização de um protocolo adequado a este tipo de organismos

marinhos. O protocolo resultante permite obter géis reprodutíveis, mesmo quando gerados em diferentes laboratórios o que possibilita uma boa análise de imagem. Deve realçar-se ainda que este protocolo foi aplicado com sucesso a outras espécies de moluscos marinhos. De seguida procedeu-se ao ensaio de exposição durante o qual as amêijoas foram expostas 21 dias a concentrações sub-letais de Cd (40 $\mu g.l^{-1}$) e de NP (100 $\mu g.l^{-1}$). De salientar que os dados disponíveis, referentes a ensaios prévios de exposição similares ao anteriormente descrito, demonstraram ocorrer uma acumulação significativa de cádmio nas brânquias (órgão filtrador com uma grande área de contacto com o meio externo) e na glândula digestiva (principal órgão de armazenamento e desintoxicação) da espécie em estudo. O conteúdo de NP bioacumulado foi calculado por cromatografia gasosa (GC) acoplada a espectrometria de massa, equipada com quadrupolo simples. Esta técnica revelou a ocorrência de uma acumulação significativa de NP nos dois tecidos estudados comparativamente ao controlo (43.52 ± 0.53 $\mu g.g^{-1}$ ps nas brânquias e 55.29 ± 0.60 $\mu g.g^{-1}$ ps na glândula digestiva). Os perfis de expressão proteica (PEPs) obtidos em géis com 10% de poliacrilamida das brânquias e da glândula digestivas das amêijoas expostas foram comparados com os dos animais controlo e analisados por intermédio do software PDQuest (V8.0, Bio-Rad). Esta análise revelou que a exposição ao Cd e ao NP induziu profundas modificações nos proteomas de ambos os tecidos, nomeadamente uma expressiva redução do número total de proteínas. Estas diferenças dependem do tipo de tecido e são consequência das funções desempenhadas por cada um deles na acumulação dos dois poluentes. A exposição ao Cd induziu um maior número de novas proteínas, nomeadamente ao nível da glândula digestiva, o que pode ser justificado pelo facto deste órgão constituir o principal local de armazenamento de Cd nas amêijoas. Este estudo permitiu definir assinaturas de expressão proteica (PESs) específicas da exposição aos dois poluentes e da exposição a cada um dos poluentes em separado. Nestas PESs foi visível a tendência para a sobre-expressão de proteínas após exposição aos poluentes. Adicionalmente, procedeu-se à identificação de algumas das proteínas cuja expressão foi drasticamente alterada através de sequenciação por LC-MS/MS (cromatrografia liquida acoplada a massas). Esta identificação foi dificultada por existirem poucas sequências de proteínas de moluscos presentes nas bases de dados utilizados para a identificação. Contudo, permitiu verificar que a exposição a Cd e NP alteram a expressão de proteínas envolvidas: na estrutura do citoesqueleto, na manutenção das células e resposta ao stress, transporte celular e transdução de sinal. O maior número de identificações de proteínas relacionadas com o citoesqueleto justifica-se pela sua maior abundância e prevalência nas bases de dados. No

entanto, deve realçar-se a identificação de 4 proteínas que não estão no grupo de péptidos vulgarmente e repetidamente identificados neste tipo de estudos: a aldeído desidrogenase (ALDH), a acil-CoA desidrogenase da cadeia média (MCAD), a serum albumina precursor e a proteína "histona-binding" (RBBP7). Deve salientar-se ainda que a identificação das proteínas ALDH, MCAD e RBBP7 foi confirmada por sequenciação *de novo*. As proteínas "RabGDP inhibitor dissociation" e "histona-binding" (RBBP7) são sugeridas como possíveis novos biomarcadores de exposição ao Cd e ao NP, sendo sobre-expressadas após exposição ao Cd e inibidas pelo NP. Os péptidos «calreticulin precursor» (CRP55) e serum albumina precursor por seu lado são propostas para eventuais biomarcadores de exposição ao NP, sendo sub-expressas por este. Adicionalmente, a subunidade beta da ATP sintase e a acil-CoA desidrogenase da cadeia média (MCAD) são indicados como potenciais novos biomarcadores para exposição ao Cd: a primeira é sub-expressa enquanto que a segunda é sobre-expressa. Contudo é necessária a sua validação para poderem ser utilizados como biomarcadores de exposição a Cd e NP nesta espécie. Procedeu-se também ao estudo do stress oxidativo causado pelos mesmos poluentes recorrendo à aplicação da técnica denominada "proteómica redox" para analisar as proteínas carboniladas e ubiquitinadas nas brânquias e glândula digestiva de *R. decussatus* após a exposição a Cd e NP. Neste estudo recorreu-se à utilização de amêijoas provenientes do ensaio de exposição anteriormente descrito e à separação de proteínas por 2DE SDS-PAGE seguida de imunodetecção por western-blotting em presença de anti-corpos específicos. Os padrões das proteínas carboniladas e ubiquitinadas foram analisados recorrendo ao software de análise de imagem PDQuest (V 8.0, Bio-Rad). Em ambos os tecidos a exposição ao Cd e NP originou diferentes perfis para a carbonilação e ubiquitinação de proteínas. As diferenças detectadas foram específicas de cada um dos tecidos e de cada um dos poluentes. Estes resultados revelam que estas duas modificações são processos independentes entre si e específicos do Cd e do NP possuindo grande potencial para constituírem bons marcadores de stress oxidativo nesta espécie. Os resultados obtidos na presente tese permitiram definir assinaturas de expressão proteica e identificar algumas proteínas envolvidas na resposta da espécie bioindicadora *R. decussatus* à exposição ao Cd e NP que poderão constituir novos biomarcadores. No seu conjunto, os dados apresentados validam o potencial da análise proteómica no âmbito da ecotoxicologia, constituindo uma ferramenta que poderá ser utilizada no futuro na avaliação de risco da contaminação química do ambiente marinho.

Palavras-chave: ecossistemas marinhos, *Ruditapes decussatus*, poluição, cádmio, nonilfenol, biomarcadores, proteómica, bivalves, ROS.

Résumé

Les régions côtières, qui abritent des écosystèmes parmi les plus productifs du milieu marin et qui contribuent au maintien des équilibres littoraux, sont aussi le lieu préférentiel d'installation de ports, de villes et d'activités industrielles. De nombreux polluants y sont rejetés et ont par conséquent un impact significatif. Ces polluants existent dans l'eau en solution, suspension ou associés à des particules sédimentaires et sont classés en: organiques, métaux et organo-métalliques. Le devenir de ces contaminants, et donc leur persistance et leur biodisponibilité, dépend de leurs propriétés chimiques, de facteurs environnementaux biotiques et abiotiques. Des mesures ont été prises en compte pour surveiller la pollution du milieu marin qui peut avoir des répercussions sur les organismes et sur l'homme, consommateurs des produits de la mer. Dans les années 70, cette surveillance reposait sur la détection chimique et la quantification de molécules potentiellement dangereuses dans l'eau et sédiments et dans les organismes. Cependant, ces mesures n'évaluent pas les effets des polluants sur les organismes. Depuis une trentaine d'années ne nouvelle approche écotoxicologique a été développée, fondée sur des mesures biochimiques, physiologiques et comportementales, appelées biomarqueurs, qui peuvent être reliées à une exposition ou à des effets toxiques d'un ou de plusieurs polluants, et qui constituent un système d'alarme précoce de la pollution. Cependant, ces biomarqueurs «classiques» n'ont pas la capacité de déterminer les processus cellulaires mis en place par les organismes afin de s'adapter à des conditions d'exposition. Depuis une dizaine d'années, de nouvelles techniques, appelées «omiques»: génomique (expression des gènes), transcriptomique (expression de l'ARNm), métabolomique (métabolites) et protéomique (expression des protéines) ont été utilisées en écotoxicologie. La protéomique, en particulier, permet d'analyser quantitativement l'expression du protéome (complément protéique total exprimé par un génome, à un moment donné et en réponse à des conditions spécifiques). L'électrophorèse bidimensionelle en condition dénaturantes (2DE SDS-PAGE) est une technique couramment utilisée en protéomique pour séparer les protéines selon leur point isoélectrique et leur poids moléculaire. Cette méthode permet l'établissement des signatures de l'expression de protéines de cellules ou de tissus à des stress particuliers et l'identification de nouveaux biomarqueurs chez des organismes, étudiés comme modèles en écotoxicologie. Cependant, il n'y a pas beaucoup d'études qui appliquent la protéomique à l'étude des bivalves qui constituent des espèces bioindicatrices importantes du fait de leur abondance, leur caractère sessile, leur large répartition géographique, leur tolérance aux stress et leur capacité de bioacumulation des polluants. Le

bivalve marin *Ruditapes decussatus* (L.), ou palourde méditerranéenne, est une espèce sentinelle de la contamination chimique de l'environnement, présente en Europe et en Méditerranée, surtout dans les zones estuariennes et lagunaires. Au Portugal, notamment au Sud (lagune Ria Formosa), ce bivalve représente un enjeu économique important. L'objectif principal de la thèse a été d'analyser chez *R. decussatus* les modifications d'expression protéique induites par l'exposition à deux polluants chimiques, le cadmium (métal) et le nonylphénol (détergent). Ces contaminants ont été choisis car ils sont présents dans la lagune Ria Formosa où ils sont accumulés par la palourde.

Dans ce cadre l'approche protéomique «bottom-up» a été choisie. Comme les protocoles standard d'électrophorèse à deux dimensions (2DE) disponibles, élaborés pour les mammifères, ne sont pas adaptés aux tissus des bivalves, un protocole a dû être optimisé et testé chez les bivalves marins. Les résultats montrent une bonne reproductibilité des gels d'électrophorèse. Des palourdes ont alors été exposées pendant 21 jours, au laboratoire, au Cd (40 $\mu g.l^{-1}$) et au nonylphénol NP (100 $\mu g.l^{-1}$). Dans ces conditions de contamination, des données précédentes avaient montré, chez *R. decussatus*, une accumulation significative du cadmium dans les branchies, organe de filtration et les glandes digestives, organe de stockage et de détoxification. Dans le cas de l'exposition au NP, la bioaccumulation (mesurée par chromatographie en phase gazeuse, CG) est significative dans les deux tissus (43.52 ± 0.53 $\mu g.g^{-1}$ ps pour les branchies et 55.29 ± 0.60 $\mu g.g^{-1}$ ps pour la glande digestive) par rapport aux témoins. Les profils d'expressions des protéines obtenus dans les branchies et les glandes digestives des palourdes contaminées ont été comparés avec les profils des animaux témoins et analysés par le logiciel PDQuest (V8.0, Bio-Rad). L'exposition au Cd et au NP a induit des modifications profondes dans les protéomes des branchies et des glandes digestives, notamment une grande réduction du nombre des protéines. Cette étude a fourni des « patterns » des signatures protéiques et des protéines spécifiques de l'exposition au Cd et NP, qui ont démontré que ces polluants modifient l'expression des protéines impliquées dans la structure, réponse au stress, métabolisme, le transport et la transduction du signal dans la palourde (protéines ont été identifiées par LC-MS/MS). Quatre protéines (aldéhyde deshydrogénase (ALDH), acyl-CoA déshydrogénase à chaîne moyenne (MCAD), sérum albumine précurseur et la protéine «histone-binding» (RBBP7)) ne sont pas incluses dans le groupe des protéines couramment identifiées. L'aldéhyde deshydrogénase, la MCAD et la RBBP7 ont été identifiées de manière indubitable par le séquençage *de novo*. Les protéines RabGDP et «histone-binding» (RBBP7) pourraient être des possibles nouveaux biomarqueurs possibles de l'exposition au Cd et NP (augmentant

après l'exposition au Cd et disparaissant après l'exposition au NP) ; les protéines «calreticulin precursor» (CRP55) et sérum albumine précurseur sont présentées comme des éventuels biomarqueurs de l'exposition au NP (diminuant après l'exposition). Les protéines «ATP synthase sous-unité béta» et l'acyl-CoA déshydrogénase à chaîne moyenne (MCAD) pourraient être proposées comme des biomarqueurs spécifiques de l'exposition au Cd: l'ATP synthase sous-unité beta et l'acyl-CoA déshydrogénase diminue et la MCAD augmente après l'exposition. Dans les mêmes conditions de contamination, pour les mêmes contaminants, l'étude du stress oxydant a été abordée par la mesure de protéines carbonylées et ubiquitinées analysées par la proteomique «redox». La carbonylation, addition d'un groupe carbonyle, C=O aux protéines, est une modification post-traductionnelle, oxydative et irréversible des protéines. L'ubiquitination, fixation covalente d'une ou de plusieurs protéines d'ubiquitine sur une ou plusieurs lysines des protéines, est aussi une modification post-traductionnelle, réversible, permettant la dégradation des protéines par le protéasome (système ubiquitine-protéasome). L'ubiquitination et la carbonylation des protéines ont été analysées par le logiciel d'analyse d'image PDQuest (V8.0, Bio-Rad) après avoir réalisé des gels bidimensionnels par SDS-PAGE couplés à une immunodétection par Western Blot en présence d'anticorps spécifiques. La contamination au Cd et au NP conduit à des profils différents pour l'ubiquitination et la carbonylation des protéines dans les branchies et les glandes digestives. Ces résultats montrent que l'ubiquitination et la carbonylation des protéines sont spécifiques de chaque polluant et mettent en jeu des mécanismes indépendants. Cependant, ces deux mécanismes pourraient être considérés des bons marqueurs du stress oxydant chez *R. decussatus*. Les résultats obtenus ont permis d'identifier des signatures protéiques et quelques protéines impliquées dans la réponse aux polluants marins et de mieux comprendre les mécanismes mis en œuvre par la palourde en réponse à une exposition au Cd et au NP. L'ensemble de ces données confirment l'intérêt de l'analyse protéomique en écotoxicologie et constituent un outil qui pourra être utilisé à terme dans le cadre de l'étude du risque chimique dans l'environnement marin.

Mots clés: écosystèmes marins, *Ruditapes decussatus,* pollution, cadmium, nonylphénol, biomarqueur, protéomique, bivalves, ROS.

General Abstract

Marine ecosystems not only comprise and sustain habitats vital for many organisms, but also draw human activities. Consequently, these habitats are threatened by continuous influx of pollutants. Due to the high ecological and economic importance of marine ecosystems, the impact of pollutants on marine communities and human health is a continuous concern. Thus, there has been a growing awareness of the need to develop new methodologies to detect and assess the adverse effects of pollutants in organisms. The integrated use of chemical analysis and organism's biological parameters levels in response to pollutants exposure (biomarkers) is a common procedure for detecting the impact of pollutants in aquatic environments. Nonetheless, the conventional biomarkers do not provide specific information on the mechanisms of pollutants toxicity. This gap can be bridged by the application of multi-endpoint approaches such as proteomics that have good capacity to find new sensitive biomarkers. The proteome analysis has become a promising tool in ecotoxicology for the assessment of pollutant effects. Proteomics has the capacity to detect and quantify changes in protein expression between different tissues or conditions, allowing the follow up of the organism responses to different pollutant concentrations over time, helping to understand the kinetics of specific pollutant effects. Differential expression profiles of proteins can be compared among chemicals, different concentrations or complex mixtures in different natural environments. Nonetheless, few proteomic studies involved bivalves, considered good biological indicators. The metal cadmium (Cd) and the organic compound nonylphenol (NP), easily accumulated by the clam *Ruditapes decussatus*, are two contaminants found in marine ecosystems that affect marine bivalves causing endocrine disruption and increased reactive oxygen species (ROS) production.

Therefore, the aim of the present thesis was to study the impact of Cd and NP exposure in the sentinel specie *R. decussatus* using a proteomic approach. In order to accomplish this, the classical proteomic bottom-up analysis was applied. A 2DE SDS-PAGE protocol suitable for bivalve organisms was optimized, obtaining gels well resolved and providing good reproducibility (Chapter 2). Afterwards, clams were exposed to Cd (40 µg.l^{-1}, 21 days) and to NP (100 µg.l^{-1}, 21 days). The protein expression profiles (PEP) in gill and digestive gland of *R. decussatus*, exposed to both pollutants, were compared between them and to unexposed ones, using PDQuest software (V 8.0, Bio-Rad) (Chapter 3). The results of this study showed protein expression signatures (PESs) and single protein markers (identified by liquid chromatography tandem mass spectrometry (LC-MS/MS) that could characterize Cd

and NP exposure, as well as the NP content accumulated in clam's tissues after NP exposure. Additionally, protein expression signatures specific and common to both pollutants were also obtained (Chapter 3). Subsequently, the effects of Cd and NP exposure in proteins ubiquitination and carbonylation were assessed through a proteomic approach (redox proteomics), (Chapter 4).

Cadmium presents a higher tendency toward up-regulation while NP tends to suppress proteins. They also confirmed the NP accumulation in clam tissues (43.52 ± 0.53 $\mu g.g^{-1}$ *dw* in gill and 55.29 ± 0.60 $\mu g.g^{-1}$ *dw* in digestive gland). The protein expression signatures characterized could are proposed as biomarkers for the studied pollutants. Both contaminants interfere in cytoskeletal structure/function, cell maintenance and stress response, metabolism and signal transduction. Four of the identified proteins are not included in the "hit parade" of repeatedly, identified differentially expressed proteins (aldehyde dehydrogenase (ALDH), medium-chain acyl-CoA dehydrogenase (MCAD), serum albumin precursor, and histone-binding protein (RBBP7)) whereas three of them were identified by *de novo* sequencing (MCAD, ALDH and RBBP7). RabGDP dissociation inhibitor alpha and RBBP7 are indicated as potential biomarkers for both contaminants, being up-regulated after Cd exposure and suppressed in clams exposed to NP; calreticulin precursor (CRP55) and serum albumin precursor are recommended as possible biomarker specific for NP exposure, being down-regulated, whereas ATP synthase subunit beta is suggested as biomarker for Cd exposure, being down-regulated as well as the MCAD, that is induced. The results also confirmed that Cd and NP generate ROS in *R. decussatus*. The distinct patterns obtained for ubiquitination and carbonylation after exposure to these pollutants have capacity as markers of oxidative stress inflicted by Cd and NP in this species. The present thesis reinforce the importance of proteomics in ecotoxicology, mainly concerning bivalves, not only for finding new biomarkers but also for establishing patterns (PESs) specific and common to Cd and NP exposure.

Keywords: marine ecosystems, *Ruditapes decussatus*, pollution, cadmium, nonylphenol, biomarkers, proteomics, bivalves, ROS.

Acknowledgements/Agradecimentos

Apresente tese permitiu-me adquirir conhecimentos em técnicas laboratoriais e participar em diversas conferências e congressos científicos, contribuindo em muito para a minha formação científica e pessoal. Quero por isso agradecer àqueles que me proporcionaram esta oportunidade e que me deram o apoio para concluir esta tese.

I would like to thanks to Professor Dr. Maria João Bebianno and Dr. Michèle Roméo for offering me the opportunity to do this thesis and for introducing me to the world of ecotoxicology, mainly proteomics. For their trust and for all the support during this PhD;

Special thanks to Dr. Mireille Starita-Geribaldi for her valuable help and comments in the field of proteomics;

Thanks a million to David Sheehan for offering me the opportunity to learn some new techniques. Thanks to all my laboratory mates in Cork (Brian, Ryan and Sara);

Aos meus colegas de laboratório da F.C.T (Rui, Cristina, Bli, Ângela, Xana, Luísa, Luís, Vera e Alexandra) pelo apoio e amizade;

Um especial obrigado à Catarina Almeida, Catarina Pereira, Maria-Rey e Tânia Gomes pelo carinho e ajuda;

To my laboratory mates in Nice, specially to Ginette, Laure and Adrien. Thanks for all the help and friendship;

To all the friends I met in Nice, especially to Marie-Lu, Maria-Francesca, Laure, Aline, Angie, Miguel and Julien;

To all my dearest and closest friends, thank you for all your support, faith and unconditional friendship;

Finalmente, muito obrigada aos meus pais e irmã e, especialmente, à minha avó, à memória da qual dedico esta tese. MUITO OBRIGADA.

Index

Index

Figure Index

Table Index

Acronym List

Acronym List

2DE - two dimensional electrophoresis

AChE - acetylcholinesterase

ADP - adenosine Diphosphate

ALDH - aldehyde dehydrogenase

APEOs - alkylphenol ethoxylates

ATP - adenosine Triphosphate

BSA - bovine serum albumin

CAT- catalase

CHAPS - 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate;

CRP55 - Calreticulin precursor

Cu/ZnSOD – superoxide dismutase (Cu-Zn)

DDE - p,p'-dichlorodiphenyldichloroethylene

DMSO - dimethyl sulfoxide

DTT – dithiothreitol

EDTA - ethylenediamine tetraacetic acid

ESI-MS/MS - electrospray ionization tandem mass spectrometry;

HSP70II - heat shock 70 kDa protein

IEF - isoelectric focusing

IPG - immobilized pH gradient

LC - liquid chromatography

LC-MS/MS - liquid chromatography tandem mass spectrometry

NL - non linear

NP - nonylphenol

NPEs - nonylphenol ethoxylates

OS - oxidative stress

PAGE - polyacrylamide gel electrophoresis

PEPs - protein expression profiles

PES - protein expression signature;

PMSF - phenylmethylsulfonyl fluoride

RBBP7 - histone-binding protein

ROS - reactive oxygen species

SDS - sodium dodecyl sulfate

UPP - ubiquitin-proteasome pathway

Vtg - vitellogenin

Chapter 1.

General Introduction

1.1. Pollution in aquatic environment

The continuous increase of human population leads to an intensification of anthropogenic activities and consequent environment quality deterioration. Marine environments are threatened by continuous influx of pollutants arising from human activities (Ali and Sreekrishnan, 2001; Allen and Moore, 2004; Chouksey *et al.*, 2004), particularly in anthropogenic areas, like coastal zones, estuaries and lagoons. These environments constitute the ultimate sink for many contaminants originated from direct discharges (e.g. municipal, industrial and domestic effluents), diffuse sources (e.g. agriculture and urban runoff) or from atmospheric input (Stegeman and Hahn, 1994; Zorita *et al.*, 2007). Due to the high ecological and economic importance of these ecosystems, the impact of contaminants on marine communities is a growing concern in the conservation of marine life. The way contaminants interact with environmental and biological systems depends on the chemical properties of the contaminants (e.g. water solubility, hydrophobicity, lipophilicity and persistence), abiotic factors (e.g. salinity, temperature, concentration of other chemicals and redox potential), biotic factors (e.g. the organism's mode of feeding, trophic position, lipid concentration and metabolism), and bioavailability (Morrison *et al.*, 1996; Gobas *et al.*, 1999; Lee *et al.*, 2000; Shin and Lam, 2001). According to these, final exposure and effect estimation will be subject to uncertainty due to intrinsic variability/complexity of both environmental and biological systems (Figure 1.1). The persistence is determined by the chemical structure and by the environment that the chemical faces. Bioavailability is defined as the fraction of the total amount of the chemical present in soil/sediment and water (water column and interstitial) that can be taken up during the organism's lifetime (Belfroid et al., 1996). In faunal suspension-feeders, such as bivalves, contaminants (e.g. metal and organic

pollutants) are accumulated in their tissues up to a level that exceeds environmental concentrations. Following bioaccumulation, organisms have the capacity to eliminate chemicals. Several chemicals may be excreted in their original form, but others need metabolic transformation (biotransformation) before elimination can occur (Ahokas *et al.*, 1994). During biotransformation a more hydro-soluble metabolite is generated and is excreted more easily than the original compound (Vermeulen, 1996).

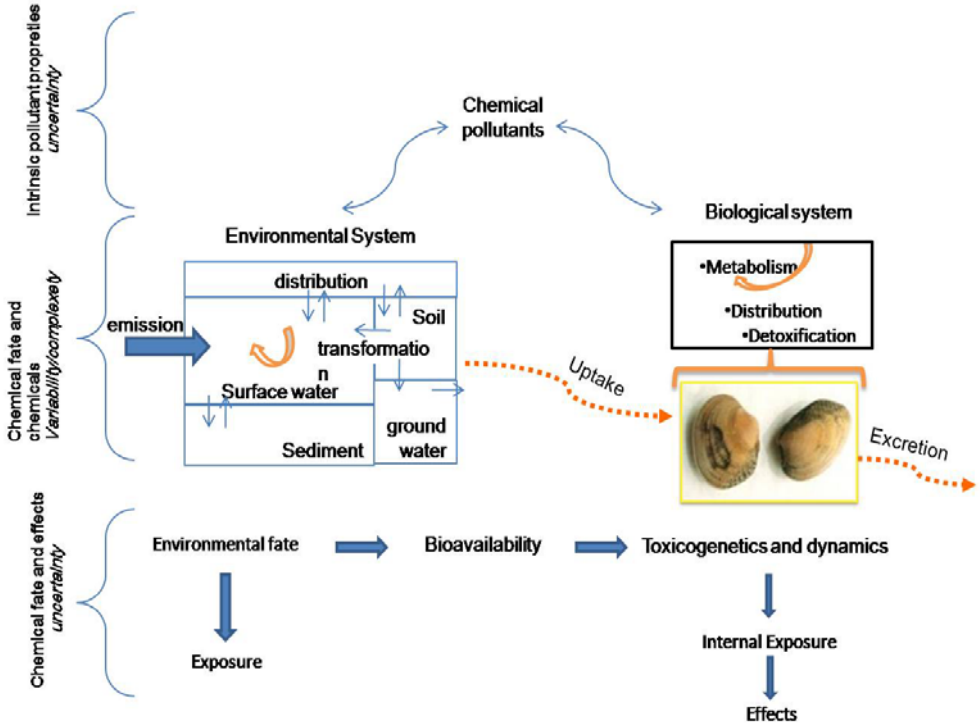

Figure 1.1 - Pollutant input, distribution and fate in the environmental and biological systems (adapted from Schwarzenbach *et al.*, 2006).

Pollutants can be classified into three categories: inorganic, organic and organometallic. Inorganic pollutants are metals that can be divided in essential (e.g. copper, zinc) and non essential (e.g. cadmium, mercury). Many marine bivalves are able to accumulate rather high concentrations of metals, including cadmium and retain it during a relatively long time (Simkiss *et al.*, 1982). Cadmium (Cd) is a non essential metal, widely distributed in the marine environment being toxic to biological systems, bivalves

included. Small quantities of this metal enter naturally in the marine environment by leaching/erosion of rocks, volcanic activity and forest fires (Satarug *et al.*, 2000). However, anthropogenic emissions exceed those from natural sources. Therefore, contamination of the marine environment with Cd results from zinc and lead extraction, agricultural use of phosphate fertilizers, and manufacturing industries and their products that use this metal, such as steel, plastics, paints, Ni-Cd batteries, cadmium pigments, cadmium stabilisers, cadmium coatings, cadmium alloys and cadmium electronic compounds (Foy *et al.*, 1978; WHO, 1993; Waalkes and Misra, 1996). In marine ecosystems Cd exists almost entirely in the dissolved form generally as chloride ($CdCl^+$, $CdCl_2$, $CdCl_3^-$) (Clark, 2001). This metal is very mobile in the aquatic environment having great persistence (t 1/2 of 10-30 years), is toxic at low concentrations and can be bioaccumulated. The health effects of Cd exposure are of great concern. In marine organisms, and especially bivalves, Cd is taken up directly from water and through food and its concentration in tissues increase with the time of exposure (Bebianno and Serafim, 1998). Cadmium can modify several metabolic processes, mainly energy metabolism, membrane transport and protein synthesis and may inflict DNA damage, directly or indirectly, through interference in genetic control and repair mechanisms (Yamada *et al.*, 1993; Hartwig, 1994; Hassoun and Stohs, 1996; Beyersmann and Hechtenberg, 1997; Dixon and Prusky, 2002). Although a non-redox metal unable to take part in Fenton-type reactions, Cd can originate reactive oxygen species (ROS) (Zhong *et al.*, 1990; Manca *et al.*, 1994; Kumar *et al.*, 1996; Stohs *et al.*, 2001; Watanabe *et al.*, 2003). These effects combined with the capacity to trigger protooncogenes, to interfere with cellular signalling, and to suppress apoptotic response, justify the carcinogenic properties attributed to Cd (Shimada *et al.*, 1998; Dixon and Prusky, 2002; Filipic *et al.*, 2006). Concerning aquatic organisms, Cd can cause many

deleterious effects: in limpets it reduces the ability to utilise glucose; in copepods causes decrease in reproduction rates and population numbers; in several fish species inflicts changes in immune function; in juvenile fish and invertebrates is responsible for depressed growth (Bryan and Langston, 1992; Thuvander, 1989). In bivalves Cd effects are well documented. In *Mizuhopecten yessoensis* Cd inhibits ADP-stimulated respiration reducing mitochondrial efficiency and coupling and stimulating proton leak inhibition (Sokolova, 2004); in *Mytilus galloprovincialis* alters membrane permeability and inhibits the oxidative phosphorylation and protein synthesis (Viarengo *et al.*, 1989); in *M. edulis* causes ROS formation (McDonagh and Sheehan, 2006) in *Crassostrea gigas* inflicts embryotoxicity (Damiens *et al.*, 2006); in *Mya arenaria* modifies the chemical composition of the gonads and vitellin (Gagné *et al.*, 2002), and reduces glycogen storage, essential for reproduction (Gauthier-Clerc *et al.*, 2002). In *Ruditapes decussatus* Cd toxicity, kinetics of bioaccumulation and elimination and biomarker responses to its exposure, are well reported (Serafim and Bebianno, 2007b). In this clam, Cd exposure decreases the condition index, causes depressed growth and stimulates metallothioneins synthesis (Langston and Bebianno, 1998; Serafim and Bebianno 2007b) lipid peroxidation (Geret *et al.*, 2002) and is also involved in endocrine disruption (Smaoui-Damak *et al.*, 2006; Ketata *et al.*, 2007 b).

Organic pollutants range from the simple methane molecule to long-chained, multi-ringed, halogenated structures (e.g. polycyclic aromatic hydrocarbons (PAHs), nonylphenols (NPs), octylphenols (OPs), phthalates pentachlorophenol, hexabromobiphenyl, polychlorinated biphenyls (PCBs), organomercury, organotin and organolead compounds) (UNEP, 2003). Like metals, these pollutants are very abundant and diverse and vary in persistence and effects on aquatic organism. The Stockholm

Convention on Persistent Organic Pollutants identified an initial twelve chemicals or chemical groups for priority action, known as "dirty dozen", composed by: aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex, toxaphene, PCBs, polychlorinated dioxins and polychlorinated furans (Fu *et al*., 2003).

Nonylphenol (NP) is a by-product of nonylphenol ethoxylates (NPEs) for oil soluble detergents and for emulsifiers that can be additionally refined to produce anionic detergents, lubricants, emulsifiers, oil additives, antioxidants in rubber and plastics, etc. Nonylphenol polyethoxylates constitute 80% of alkylphenol polyethoxylates that are released during microbial breakdown in sewage treatment plants and in the aquatic environment (Andrew *et al.*, 2008). Land application of NP-containing biosolids can also introduce large quantities of this contaminant into the environment (Xia *et al.*, 2003). This compound was detected in several fresh and marine waters from concentrations below detection limit to significantly high concentrations (95 $\mu g.l^{-1}$) (Dachs *et al.*, 1990). NP, a lipophilic chemical, is considered a critical compound due to its resistance towards biodegradation, toxicity and ability to bioaccumulate in aquatic organisms, marine bivalves included (Ekelund *et al.*, 1990; Staples *et al.*, 2004; Lietti *et al.*, 2007; Ricciardi *et al.*, 2008). This compound causes several deleterious effects in marine organisms: induces changes in population growth rates (Matozzo *et al.*, 2003; Mäenpää *et al.*, 2006), in *Tapes philipinarum* it affects the re-burrowing capacity and inhibits the antioxidant enzymes (Matozzo *et al.*, 2003) and reduces the stability of lysosomal membranes of *Mytilus galloprovincialis* (Canesi *et al.*, 2004, 2007). Furthermore, NP induce DNA adduct formation, and/or mutations or genomic rearrangements (*Balanus amphitrite*, Atiezar *et al.*, 2002). In addition, NP is an endocrine disruptor, having the capacity to mimic the action of endogenous estrogens

by binding to estrogens receptors, and leading to the disruption of several reproduction processes (Butwell *et al.*, 2002; Vazquez-Duhalt *et al.*, 2005). This capacity have been reported in several fish species (Arukwe *et al.*,1997; Madsen *et al.*, 1997; Christiansen *et al.*, 1998 a,b,c; Korsgaard and Pedersen, 1998) and molluscs (*Dreissena polymorpha* Quinn *et al.*, 2006; *Elliptio complanata*, Gagné and Blaise, 2003; *Tapes philippinarum*, Matozzo and Marin, 2005; *Saccostrea glomerata*, Andrew *et al.*, 2008; *Cerastoderma glaucum*, Marin *et al.*, 2008; *Mytilus galloprovincialis*, Ricciardi *et al.*, 2008). Taking into account the deleterious effects caused by Cd and NP mentioned, it could be hypothesized that both pollutants could cause alterations in protein expression profiles of proteins involved with cytoskeletal structure maintenance, cell maintenance and stress response, metabolism and in signal transducer transcription regulation.

During the last decades of the 20th century, modern societies became more and more aware of the potential long-term negative effects of contaminants and their by-products and of their risk for aquatic and terrestrial ecosystems and human health. Until the 80's, marine pollution assessment was based only on chemical analysis. However, in the last decades, international organisations and environmental agencies realised that marine pollution assessment simply cannot be based on chemical analysis of environmental samples (e.g. water and sediments), since they provide information on the incidence of potentially toxic compounds in the environment, but fail to estimate synergistic, additive or antagonistic effects, that provide a measure of their potential deleterious biological effects. Consequently, there was a growing awareness of the need for new methodologies to detect and assess the effects of contaminants in organisms. In this context, a new approach was developed based on the interaction between a pollutant and an organism response, called "biomonitoring". In order to monitor marine pollution, several biomonitoring programs, based on measurements of pollutants in marine

organisms, bivalves included, were developed ('Mussel Watch', NAS, 1980; Phillips, 1986). The use of living organisms for monitoring marine pollution has been increasingly recognized due to their biotic, spatial and temporal significance. Since bivalves, due to their filter-feeding habits, can accumulate pollutants in their tissues up to a concentration of 1,000 to 10,000 times that of surrounding waters, they have a lot of attributes as bioindicators. Several bivalve species are widely used as sentinel organisms for the monitoring of marine pollution, providing information on the spreading and levels of pollutants in the aquatic environments (Goldberg *et al.*, 1978; Simkiss *et al.*, 1982; Bebianno *et al.*, 2004; Ruokojärvi *et al.*, 2004, Porte *et al.*, 2006; Schiedek *et al.*, 2006). These attributes are:

- a correlation can be established between the pollutant content in the organism and the average concentration in the environment;

- bivalves are cosmopolitan, allowing comparing data from markedly different areas;

- bivalves are relatively tolerant to several pollutants and can survive in habitats contaminated within a considerable range of pollution;

- since bivalves are sedentary, they can better represent the study area than mobile species;

- bivalves are frequently abundant in quite stable populations, allowing repeatedly sampling throughout the study area;

- bivalves have generally a reasonable size, providing sufficient quantity of tissue for analysis;

- bivalves are easy to sample and robust enough to survive in laboratory, - bivalves tolerate a wide range of environmental variables (e.g. salinity, temperature), and are robust enough to be used in transplant experiments;

The integrated use of chemical analysis and organism's biological parameters levels in response to pollutants is a common procedure for detecting the impact of pollutants in aquatic environments. In this context, a wide range of biomarkers, have been proposed (Stagg, 1998; UNEP/RAMOGE, 1999). Biomarker is commonly defined as "a biochemical, cellular, physiological or behavioural modification measured in tissue- or body fluid samples, or at the organism level, that attests exposure to and/or effects of one or more pollutants" (McCarthy and Shugart, 1990; Peakall, 1994; Cajaraville *et al.*, 2000; Bebianno *et al.*, 2004) (Figure 1.2). They constitute an early warning system of chemical stress in organisms, allowing the initiation of remediation strategies before irreversible environmental damage occurs (Cajaraville *et al.*, 2000; Bebianno *et al.*, 2004; Monserrat *et al.*, 2007; Zorita *et al.*, 2007). Biomarkers of population, community and ecosystem levels have greater toxicological relevance; however provide a slower response (Peakall, 1994; Lopez-Barea, 1995; Timbrell, 2001).

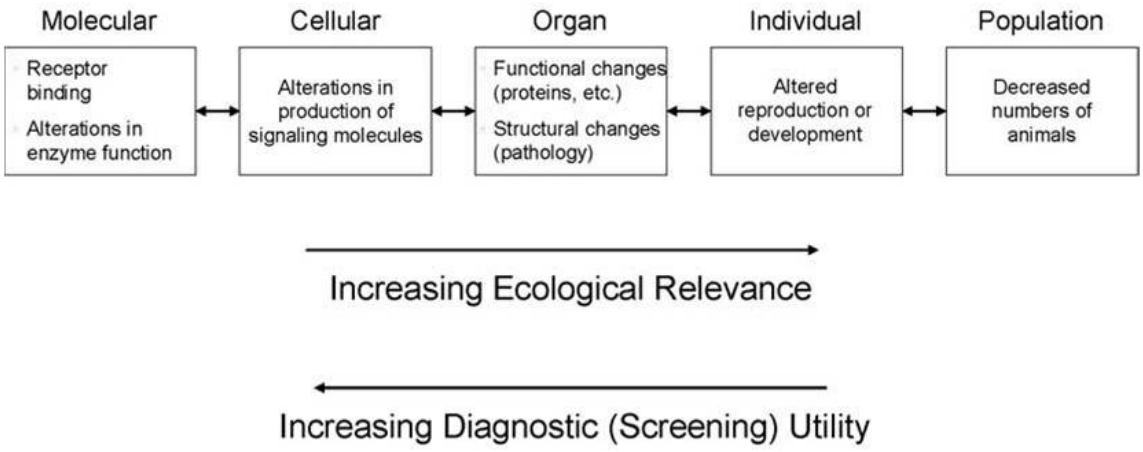

Figure 1.2 - Scheme ilustrating the sequential order of responses to pollutant stress within a biological system (Van der Oost *et al.*, 2003).

Besides the early warning capacity, biomarkers should respond to the following criteria (Van der Oost *et al.*, 2003):

- be reliable, easy to measure and cheap;
- be sensitive to pollutant exposure/effect;
- their baseline data must be well established allowing to differentiate natural variability from pollutant induced alterations;
- the impact of confounding factors should be well established;
- the intrinsic mechanism of the interactions between biomarker response and pollutant exposure (dosage and time) must be established;
- the toxicological significance of the biomarker should be established.

They are classified as biomarkers of exposure, effect and susceptibility (NRC, 1987; WHO, 1993). Exposure biomarkers should confirm and assess that the organism was

exposed to a pollutant or to its metabolites, measuring their interaction with some specific molecule or cell and establishing an association between external exposure and internal concentration. Nevertheless, they do not provide information about the level of pollutant that induced alterations or of its consequences in the organisms. Effect biomarkers assess the magnitude of the organism's response to a pollutant. Biomarkers of susceptibility express the inherent or acquired capacity of an organism to face the exposure to a specific pollutant. They help to understand the variations in the magnitude of organism response to pollutant exposure, including genetic factors and changes in receptors which alter the susceptibility of an organism to a specific exposure (Van der Oost *et al.*, 2003).

Nowadays, biomarkers are routinely used in biomonitoring programs (Prichard *et al.*, 1997; Moore *et al.*, 2004; Hyne and Maher, 2003; Wang *et al.*, 2005; Bonacci *et al.*, 2007; De la Torre *et al.*, 2007; Kopecka and Pempkowiak, 2008). Biomarkers have also been included in the Joint Monitoring Programme (OSPAR convention) of which Portugal and France are members (Cajaraville *et al.*, 2000). This approach should be multi-parametric, employing different and complementary biomarkers (Narbonne *et al.*, 1989), providing information about the effects of different pollutants, associated with other chemical and biological measurements.

1.1.1 Detoxification of pollutants

Essential and non essential metals

In order to resist to metal toxicity, aquatic organisms developed several mechanisms for their accumulation, regulation and mobilization (Viarengo and Nott, 1993; Geret *et al.*, 2002). These mechanisms comprise soluble metallothionein-like protein (MTLP) such

as metallothioneins (MT) and some antioxidant enzymes, or metal-depositing membrane compartments such as lysossomes, granules, vesicles and spheroblasts (Langston and Bebianno, 1998; Chelomin *et al.*, 2005). Heat-shock proteins, along with MTs, act by providing high-affinity sinks for nonessential metals, such as Cd, reducing their interactions with macromolecules (Tedengren *et al.*, 1999; Shi *et al.*, 2003; Cheung *et al.*, 2007), whereas lysosomal size and number, and antioxidant enzyme activities increase in bivalves in reaction to exposure to metals (Cajaraville *et al.*, 1989; Marigómez *et al.*, 1989; Regoli *et al.*, 1998; Cajaraville *et al.*, 2000).

Organic pollutants

Some organic contaminants are biotransformed by several reactions in order to promote their excretion (Commandeur et al., 1995). This process involves three phases: Phase I, Phase II and Phase III (Figure 1.3) (Van der Oost *et al.*, 2003). The first phase involves oxidation, reduction or hydrolysis (Goeptar *et al.*, 1995), Phase II enzymes catalyze synthetic conjugation reactions of phase I products with an endogenous ligand, while Phase III type enzymes (e.g. peptidases, hydrolases and blyase) catalyze the catabolism of conjugated metabolites to form easily excretable products (Commandeur *et al.*, 1995). During **Phase I,** the cytochrome P450-dependent monooxygenase system (MFO) is responsible for most chemical oxidation. Its principal components are: the cytochrome P450 (CYP P450), the flavoprotein NADPH cytochrome P450 reductase (P450 RED) and the cytochrome b_5 (cyt *b5*) (Stegeman and Hahn, 1994). In bivalves, these isoenzymes activities are mainly detected in the endoplasmatic reticulum and digestive gland (Livingstone and Ferrar, 1984; Porte *et al.*, 1995).

The second phase (**Phase II**) includes enzymes (e.g. glutathione transferase (GSH), aldehyde dehydrogenase (ALDH), and glucuronic acid (GA), UDPGT) that catalyze the conjugation of the contaminant, or its metabolites, with an endogenous polar group (sugars and amino acids) (Lech and Vodicnik, 1985). The addition of more polar groups will transform the contaminant into a more water-soluble molecule, promoting its excretion (Commandeur et al., 1995; Mulder et al., 1990). In this context, Phase II enzymes have a crucial role in homeostasis as well as in the detoxification and clearance of contaminants. Nevertheless, minor alterations in Phase II enzymes activity could be adverse to an organism (Stegeman et al., 1992; Lindström-Seppa and Oikari, 1991; Van der Oost *et al.*, 1996). The glutathione S-transferases are dimeric, multifunctional, primarily soluble enzymes that play an important role in intracellular transport (heme, billirubin and bile acids) and in the biosynthesis of leukotrienes and prostaglandins. Another important function is the defense against oxidative damage and peroxidative products of DNA and lipids (George, 1994). GSTs catalyze the conjunction reactions of electrophilic compounds with the reduced form of glutathione (GSH) and its induction is mediated by Ah receptor (Hoarau *et al.*, 2002).

$$GSH + R\text{-}X \leftrightarrow GS\text{-}R + HX$$

These enzymes are classified into four classes (a, m, p and q) based on species-specificity, immunological cross-reactivity and protein sequence data (George, 1994). Concerning marine bivalves, Fitzpatrick and Sheehan (1993) have reported five GSTs isoforms with high activity in *Mytilus edulis* gill.

In marine bivalves sulphatation is another possible pathway, being the preferred route for only a few compounds (George, 1994). Consists in the conjugation with sulphate ($PAPSO_3^-$):

$R\text{-}OH + PAPSO_3^- \leftrightarrow R\text{-}O\text{-}SO_3^- + PAP$

Sulphatation was assessed in the digestive gland of *Ruditapes philippinarum* (Kobayashi, 1985). Sulfo-transferases are phase II enzymes, however, this pathway is only effective at very low substrate concentrations (George, 1994).

The **Phase III** type enzymes (e.g. peptidases, hydrolases and β-lyase) and other detoxification systems (P-glycoproteins and and the multixenobiotic resistance mechanism) are responsible for the elimination of Phase I and II products. Therefore, these enzymes and systems are involved in membrane transport, such as ATP pumps GS-X dependent (Ishikawa 1992), multi-specific organic anion transporters (Heijin *et al*., 1992), dinitrophenol S-GSH transporters, P-glycoproteins (P-gp) (Smital, 2003) and the multixenobiotic resistance mechanism (MXR) (Achard *et al*., 2004).

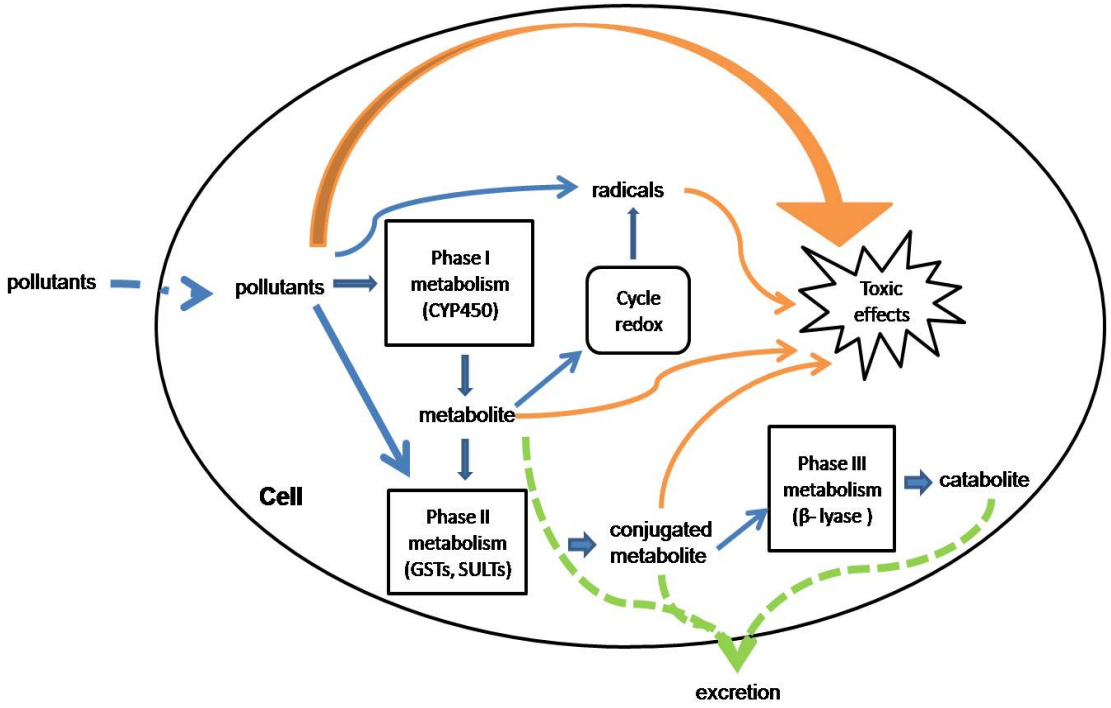

Figure 1.3 - Scheme illustrating common organic pollutant metabolic pathways (adapted from Van der Oost *et al*., 2003).

1.2. Oxidative stress

Oxidative stress (OS) is produced in cells by reactive oxygen species (ROS), "oxygen-derived species" or oxyradicals, which are important harmful factors. Since they are free unstable radicals having one or more impaired electrons, to reach stability they obtain electrons from other molecules (Manduzio *et al.*, 2005). Therefore, ROS are potent oxidants that participate in reactions with other important cellular compounds (nucleic acids, lipids, proteins, polysaccharides), possibly leading to several deleterious effects (Di Giulio *et al*., 1989; Halliwell and Gutteridge, 1999; Winzer 2001). Their levels depend on physiological and biochemical disturbances occurring during adaptation to fluctuations in the environment (Livingstone *et al.*, 1990; Sheehan and Power, 1999; Filho *et al.*, 2001; Livingstone, 2001). OS occurs when the generation of ROS in a

system exceeds the system's ability to neutralize and eliminate them. Therefore, the imbalance between oxidants and antioxidants in favor of the oxidants occurs when:

- cellular antioxidant defenses are overcome;
- cellular antioxidant defenses are inhibited, blocking the oxy-radical elimination.

Table 1.1 summarizes the reactice oxygen species.

Table 1.1 - Reactive oxygen species (Livingstone, 2001).

Radicals	Non-radicals
Superoxide: $O_2^{-\bullet}$	Hydrogen peroxide: H_2O_2
Hydroxyl: $OH^{\bullet}$	Singlet oxygen: $1O_2$
Peroxyl: $ROO^{\bullet}$	Hypochlorid acid (HOCl)
Alcoxyl: $RO^{\bullet}$	Ozone: O_3
Hydroperoxyl: $HO_2^{\bullet}$	Peroxynitrite: $OONO^-$
Nitric oxide $NO^{\bullet}$	

The main purpose of the endogenous production of ROS is the regulation of several molecules such as the mechanisms of signal transduction like protein kinase C (Dalton *et al.*, 1999). The mitochondria constitute the main site of ROS production since they consume over 90% of the cellular oxygen (Lenaz, 1998; Staniek and Nohl, 1999). Nevertheless, other sources of endogenous cellular oxyradicals have been identified such as the Phase I enzymes (cytochrome P450 monooxygenases) and redox cycles (Staniek and Nohl, 2000, Manduzio *et al.*, 2005). Several exogenous compounds can also stimulate ROS production. Some of them (e.g. nitroaromatic compounds, quinines and derived from the bypiridium) act by catalysing the microsomal transfer of electrons from the NAD(P)H towards oxygen (Lemaire *et al.*,1994; Peters *et al.*, 1996; Lemaire and Livingstone, 1997; Livingstone *et al.*, 2000). Some metals catalyse the production

of hydroxyl radicals via Haber-Weiss or Fenton reactions (Kadiiska *et al.*, 1993; Bremmer, 1998; Kehrer, 2000). Although Cd is a non-redox metal, which does not participate in Fenton reactions, it enhances ROS, mainly by inhibiting the enzymatic defence system (Stohs, 2001).

ROS generally are non-specific concerning biochemical targets causing several acute modifications and damages: lipid peroxidation (LPO), protein inactivation/modifications, DNA damage and, ultimately, cell death (Halliwell and Gutteridge 1999; Kehrer, 2000).

1.2.1. Lipid peroxidation

Lipids play important structural and functional roles in cell membranes. Therefore, the disruption of their functions can potentially lead to cell death. Changes in the cell membrane structure activate lipoxygenases (LOX), which transform polyunsaturated fatty acids (PUFAs) to lipid hydroperoxide molecules (LOOHs). When cells are intensely damaged, this physiological process switches to the non-enzymatic lipid peroxidation (LPO). Lipid peroxidation generates lipid peroxides (LOO^{-}) and a complex mixture of lipid degradation products (acetone, malonedialdehyde MDA and other aldehydes), which are very toxic for the cells, due to their high affinity for thiol and amino groups of peptides, enzymes, and nucleic acids (Viarengo, 1989; Knight and Voorhees, 1990).

1.2.2. Protein oxidation

The protein oxidation results from the direct interaction between ROS or indirect interaction with alcoxyl ($RO^{\bullet}$) or peroxyl ($ROO^{\bullet}$) radicals generated during LPO. The main protein redox lesions are: direct oxidation of hydroxyl radicals, formation of aldehyde/ketones (carbonylation), oxidation of S-containing residues (such as methionine and cysteine), racemization, ubiquitination and effects on disulphide patterns, like glutathionylation, and on changes on protein thiol status (Biswas *et al.*, 2006; McDonagh and Sheehan, 2006, 2007). These structural modifications induce functional alterations, mainly in the cellular metabolism. **Ubiquitin** is a highly–conserved protein (20 kDa) that targets the protein-transport machinery to transport the abnormal cytosolic and nuclear proteins to the proteasome for degradation via the ubiquitin-proteasome pathway (UPP) (Marques *et al.*, 2004). Damaged proteins are removed from cells by proteolysis, mainly *via* UPP. Therefore, this process is essential for normal cell growth and viability (Sherman and Goldberg, 2001). Ubiquitination is a covalent attachment between ubiquitin and signally proteins through an isopeptide bond (Figure 1.4). Alterations in ubiquitination are known to be involved in many human diseases such as neurodegenerative diseases and cancer (Friguet, 2006; Layfield *et al.*, 2001).

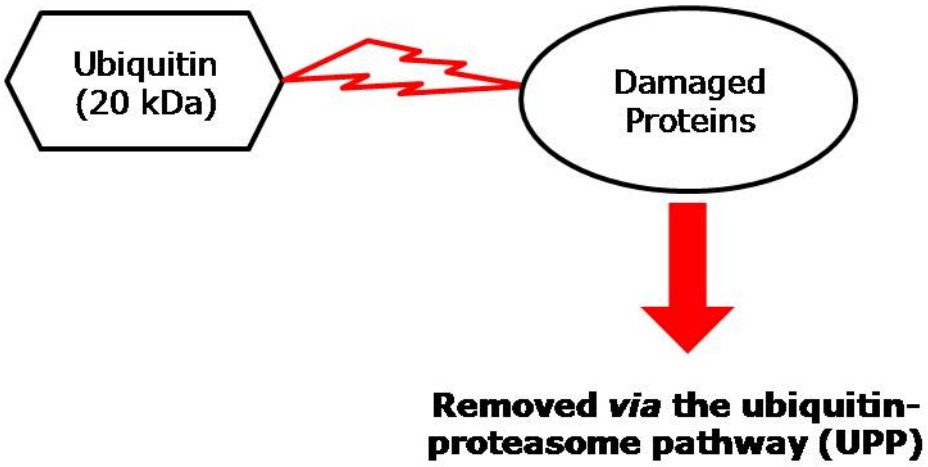

Figure 1.4 - Scheme ilustrating ubiquitination of proteins.

Carbonylation is an irreversible modification of amino acid side-chains into aldehyde or ketone groups, which lead to protein aggregation, inactivation or degradation (Levine *et al.*, 1990; Costa *et al.*, 2002). Protein carbonylation is involved in Alzheimer's disease pathology (Askenov *et al.*, 2001). The use of protein carbonyl groups as biomarker of oxidative stress provides some advantage when compared with the measurement of other oxidation products, since they have a relative early formation and are very stable (Davies and Goldberg, 1987; Augustin *et al.*, 1997).

In bivalves, glutathionylation, protein thiol status, carbonylation and/or ubiquitination of proteins have been recently successfully applied as biomarkers of oxidative stress inflicted by several environmental stressors (Table 1.2).

Table 1.2 - Glutathionylation, protein thiol status, carbonylation and/or ubiquitination of proteins measured in bivalves.

Organism	Environmental stressor	Measure	Authors
Mytilus trossolus	Exxon Valdez oil spill	Ubiquitination	Downs *et al*., 2002
Mytilus edulis	Copper	Carbonylation	Kirchin *et al.*, 1992
	Polluted sites and H_2O_2	Carbonylation; glutathionylation	McDonagh *et al*., 2005
	H_2O_2; $CdCl_2$; menadione	Ubiquitination; carbonylation	McDonagh and Sheehan, 2006
	Menadione	Ubiquitination; carbonylation; glutathionylation; protein thiol status	McDonagh and Sheehan, 2007
	Gold nanoparticles	Ubiquitination; carbonylation	Tedesco *et al.*, 2008
	PAHs and lindane	Carbonylation	Kaloyianni *et al*., 2009
Ruditapes decussatus	DDE	Carbonylation	Dowling *et al*., 2006

1.2.3. DNA damage

ROS can interact with nucleic acids causing: single and double strand-breaks, base modifications (e.g. deoxyguanosine oxidation into 8-hydroxy-2'-deoxyguanosine) and DNA-protein crosslinks (Meneghini, 1988; Spencer *et al.*, 1996). The DNA damage inflicted by ROS is mainly caused by the hydroxyl radical ($OH^{\bullet}$). Nevertheless, the superoxide radical and peroxynitrite could also cause some deleterious effects.

1.2.4. Antioxidant defence systems

Aerobic and aero-tolerant anaerobic organisms have antioxidant defence systems to limit the potential deleterious effects of ROS. These systems allow adaptation to natural and anthropogenic stressors that induces ROS formation in these organisms and include two kinds of antioxidant systems exist: antioxidant enzymes and molecules without enzymatic activity (Manduzio *et al.*, 2005).

Antioxidant enzymes

The antioxidant enzymes include: superoxide dismutase (SOD), catalase (CAT), glutathione-dependent peroxidase (GPx) and glutathione reductase (GRED). Therefore, their catalytic activity is often measured as a biomarker of OS (Van der Oost et al., 2003). The **superoxide dismutases** (SOD; EC 1.15.1.1) are a group of metalloenzymes that catalyse the conversion of reactive superoxide anions ($O2^{-\bullet}$) in hydrogen peroxide (H_2O_2) (Stegeman et al., 1992), which is then detoxified by catalase (CAT) and glutathione dependent peroxidases (GPXs):

$$2\ O_2^{-\bullet} + 2\ H+ \leftrightarrow H_2O_2 + O2$$

Four classes of SOD were identified according to the metal cofactor: Cu/Zn-SOD (32 kDa), Mn-SOD (86 kDa), Fe-SOD (41 kDa) and Ni-SOD (13.4 kDa). Nonetheless, despite of the metal cofactor, all forms of SOD share the same mechanism and speed in dismutating $O_2^{-\bullet}$ (Ahmad, 1995). When facing excessive oxidative stress, such as by pro-oxidants, the eukaryotic Cu/Zn-SOD is usually quickly induced whereas Mn-SOD or Fe-SOD usually are not. Cu/Zn-SOD is located in the cytosol and nucleus, whereas Mn-SOD and Fe-SOD are located in the mithocondria.

The **catalase** (CAT; EC 1.11.1.6) enzymes are a heme-dependent superfamily that follows SOD activity in radical detoxification from the cellular environment. They catalyze the removal of H_2O_2 which is metabolized to H_2O and O_2 (Di Giulio *et al.*, 1995):

$$2\ H_2O_2 \leftrightarrow 2\ H_2O + O_2$$

Since CAT is only activated at high H_2O_2 concentrations (Halliwell and Gutteridge, 1984), they play a minor role at H_2O_2 low levels. Nevertheless, they are crucial when H_2O_2 production is enhanced by oxidative stress (Ahmad, 1995). They are mainly located in peroxisomes. This location is considered strategic since the peroxisomes possess many of the cellular enzymes that originate H_2O_2, like glycolate oxidase and flavoprotein dehydrogenases by a direct two-electron reduction of O_2, and without the intermediation of $O2^{-\bullet}$ radical (Khessiba *et al.*, 2005).

The **glutathione peroxidases** (GPx; EC 1.11.1.9) are very important in defence against oxidative damage since they catalyse: i) the reduction of organic hydroperoxides (ROOH) to their correspondent alcohols (ROH) and water ii) the hydrogen peroxide reduction to water by reduced glutathione (GSH) producing glutathione disulphide (GSSG) (Günzler and Flohé, 1985):

$$ROOH + 2\ GSH \rightarrow ROH + GSSG + H_2O;$$

$$H_2O_2 + 2\ GSH \rightarrow 2\ H_2O + GSSG$$

These enzymes are localised in the cytoplasm and the mitochondrial matrix of the cells.

Other Antioxidant defence systems

Organisms have also no enzymatic compounds that act as antioxidant defences against ROS such as water-soluble reductants, non enzymatic, such as glutathione, uric acid, ascorbate (vitamin C), metallothioneins and lipid-soluble radical scavengers, such as α-tocopherol (vitamin E), β-carotene (vitamin A) and various xanthophylls (Remacle *et al.*, 1992).

Generally, **glutathione** is present in cells in the reduced form, GSH. Glutathione-reductase (EC 1.8.1.7) is the enzyme responsible for GSH formation: it reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH. Reduced glutathione (GSH) plays an important role in detoxification mechanism of contaminants.

Similarly to GSH, **ascorbate** (vitamin C) in chloroplasts can scavenge oxyradicals such as $OH^{\bullet}$, and can also serve as a cofactor for ascorbate peroxidase (AsPx) (Halliwell and Gutteridge, 1999). Its auto-oxidations, particularly in the presence of transitions metals, (e.g. Fe(III) and Cu(II)) give rise to oxyradicals (Cohen *et al.*, 1981). Additionally **α-tocopherol (vitamin E), β-carotene (vitamin A)**, located in cell membrane, can stop the chain reactions, mainly the lipoperoxidation by reacting with lipid radicals (Kruk, 1998). **Metallothioneins** (MTs) are also known to play a protective role against oxidative damage by binding and sequestering transitions metals or scavenging oxyradicals (Viarengo *et al.*, 1999; 2000; Cavaletto *et al.*, 2002).

1.3. "Omics" approach

The utility of classic biomarkers has been biased not only by the requirement of a deep knowledge of their mechanism of toxicity but also because of the lack of robust, sensitive and specific biomarkers for the great majority of pollutants. Therefore, new technologies, called "omics", are considered promising since they provide a deeper understanding of the mechanisms for regulating molecular homeostasis and responding to environmental stressors (Snape *et al.*, 2004; Miracle and Ankley, 2005; Samuelsson *et al.*, 2006). The current nomenclature of "omics" includes: genomics (for DNA variants); transcriptomics (for mRNA); proteomics (for proteins); and metabolomics (for intermediate products of metabolism). The power of "omics" technologies is that they are complementary and synergistic, offering a holistic and dynamic picture of biological systems. Nevertheless, very few field studies used combined "omic" approaches and, in general, individually "omic" techniques have been applied to genetically well-characterized organisms such as rodents, humans and microorganisms and to a less extent to marine species.

1.3.1. Genomics

Genomics, the study of the expression of genes and their function, aims to understand the structure of the genome, including the mapping of genes and the sequencing of the DNA. This tool allows analysing the molecular mechanisms and the interplay of genetic and environmental factors, providing a dynamic picture of the way organisms cope with environmental changes (Gracey *et al.*, 2001; Snape *et al.*, 2004). Consequently, efforts were made to establish a common gene database for chemical effects in model organisms (e.g. www.genomesonline.org). However, for non model species, such as bivalves, few genomic studies exist, mainly due to the small number of gene clones available for these organisms (Gueguen *et al.*, 2003; Dondero *et al.*, 2006; Venier *et al.*,

2006). The recent development of vectors for constructing libraries of large pieces of DNA (in the order of millions of base pairs) has facilitated genomic analysis of non-model species. However, among bivalves, only genes of the commercial oysters (*Crassostrea gigas*) exist in BAC (Bacterial Artificial Chromosome Resource Network's) library. Nowadays, genomics is based mainly in the application of DNA arrays (microarrays, oligonucleotide microarrays, macroarrays) which allow the expression of hundreds to thousands of genes to be monitored simultaneously in a single experiment (Gracey *et al.*, 2001; Brown *et al.*, 2006; Dondero *et al.*, 2006, Taris *et al.*, 2008). This approach allows to establish differential gene expression in stressor-treated versus control cells and tissue populations (Nuwaysir *et al.*, 1999).

1.3.2. Transcriptomics

Transcriptomics is the study of the set of all messenger RNA (mRNA) molecules (transcripts), obtained in one/several cells or even a whole organism; that can fluctuate face to an external environmental conditions, such as chemical stressors (Williams *et al.*, 2006, 2008). Biological pathways assessed by transcriptomics allow a good insight into mechanisms of toxicity (Pennie *et al.*, 2004). Generally, this approach can be based on the application of micro-arrays and differential expression cloning techniques such as Suppression Subtractive Hybridization (SSH) (Tanguy *et al.*, 2008). This approach was applied in fish following exposure to several classes of pollutants: the carp *Cyprinus carpio* (Moens *et al.*, 2006) and the rainbow trout, *Oncorhynchus mykiss* (Koskinen *et al.*, 2004; Hook *et al.*, 2006; Tilton *et al.*, 2006; Finne *et al.*, 2007). In bivalves this methodology was recently applied for assessing pollutants effects in molluscs: *Unio tumidus* (Doyen *et al.*, 2006), *Corbicula fluminea* (Bigot *et al.*, 2009), *Argopecten purpuratus* (Zapata *et al.*, 2009). Nevertheless, the existence of

dynamically, fluctuating transcripts and post-transcriptional regulation and modification could be considered a handicap (Pennie *et al.*, 2004).

1.3.3. Metabolomics

Metabolomics refers to the global study of all metabolites in a sample (Trethewey *et al.*, 1999; Fiehn *et al.*, 2000; Trethewey, 2004). This tool provides information to identify cellular processes switched-off by environmental changes. The development of new mass detectors and techniques like liquid chromatorgraphy/Mass spectrometry (LC-MS-MS), gas chromatography/mass spectrometry (GC–MS) and capillary electrophoresis/mass spectrometry (CE–MS), supports this approach, allowing measuring the whole metabolome, rather than only target compounds. Metabolomics, initially restricted to drug discovery and disease diagnosis and treatment (Horning and Horning, 1971; Matsumoto and Kuhara, 1996; Frank and Hargreaves, 2003), is now starting to be applied to the study pollutants effects in organisms (Samuelsson *et al.*, 2006).

1.3.4. Proteomics

Proteomics is the study of the "proteome", a catalogue of all proteins defined as the protein complement of a genome (Wilkins *et al.*, 1996). However, due to splicing and both co- and post-translational modifications, there is a high potential for molecular variation between genes and corresponding proteins. Consequently, proteomes are more complex than their genomes (Humphrey-Smith *et al.*, 1997; Rabilloud, 2000). Furthermore, gene activity and relative abundance of proteins are not always directly related. Indeed, while the genome activity is relatively stable, the proteome is very dynamic, since the protein content of cells varies in response to changes of the

environment, physiological state of the cell, stress, drug administration, health and disease (Rabilloud, 2000; Liebler, 2002). Additionally, different cell types within an organism have different proteomes, whereas the genome remains relatively unchanged (Rabilloud, 2000). Proteomics tools have been successfully applied in biomedicine to define protein expression signatures (PESs) of a specific cell or tissue that associate protein expression and post-translational modifications with disease state or drug administration, providing new biomarkers of human diseases (Wulfkuhle *et al.*, 2001; Starita-Geribaldi *et al.*, 2003; Wittke *et al.*, 2004). More recently, this approach was applied to environmental pollution assessment, in the determination of new sensitive biomarkers for environmental pollution (Alban *et al.*, 2003; Knigge *et al.*, 2004). The recent advances in genome sequencing and in analytical and bioinformatic methods allowed the evolution of proteomics to occur (Dowsey *et al.*, 2003; Patterson and Aebersold, 2003; Gorg *et al.*, 2004). There are two approaches for proteomic analysis: the bottom-up (A) and the top-down (B) analysis (Figure 1.5). In bottom-up proteomics, the proteins are digested, and partial sequence of each element in the ordered gene library is compared to sequence information obtained from the genome or transcriptome. The bottom-up approach is particularly suited for protein identification, despite the fact that only some of the detected peptides originate useful fragmentation sequences. Additionally, bottom-up analysis commonly provides incomplete protein sequence coverage and the loss of information on post-translational modifications (PTMs), or degradation, due to proteolytic digestion. Both types of analyses are complementary and depend on bioinformatics tools to cross-check experimentally produced mass spectrometric data with protein and genome sequence databases (Chait, 2006).

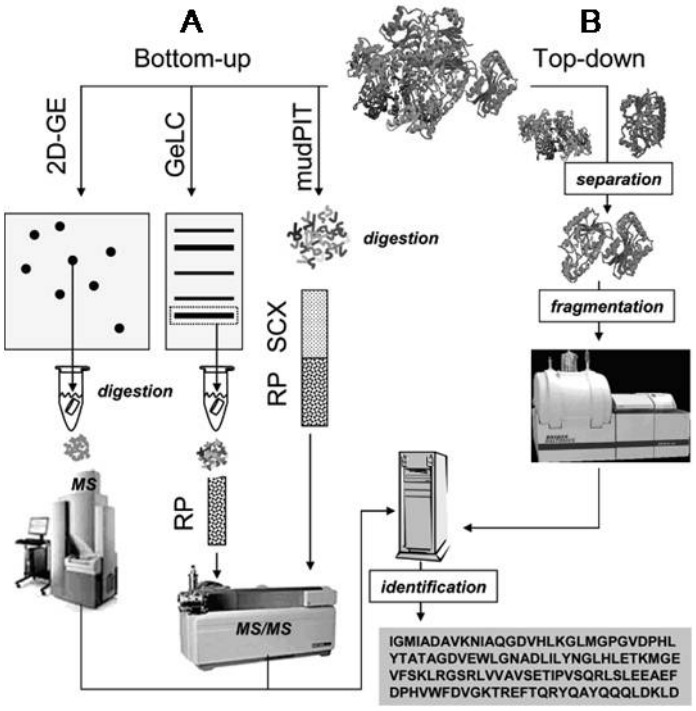

Figure 1.5 - Strategies for protein identification using mass spectrometry-based proteomics. A: bottom-up; B: top-down. GeLC: gradient elution linear chromatography; mudPIT: multidimensional protein identification technology; RP: reversed-phase; SCX: strong cation-exchange (adapted from Nesatyy and Suter, 2007).

In the top-down analysis polypeptide identity is obtained from determining the sequence through mass spectrometer analysis and by comparison with nucleotide sequences (Kettman *et al.*, 2001). Therefore, it allows the deduction of the primary structure of the protein, showing modifications such as PTMs and site-specific mutations (Chait, 2006). In this procedure, the transcriptome is obtained by array analysis using information obtained from the genome or transcriptome of the common gene products (Kettman *et al.*, 2001). This thesis will focus in the classical bottom-up analysis that can be subdivided in several phases: protein separation, visualisation, digestion, and identification based on available sequence information and gene ontology (Rabilloud, 2000).

Proteins separation

The proteins separation methods commonly used are: *i)* methods based on HPLC (high performance liquid chromatography) like mudPIT (multidimensional protein identification technology) and GeLC (*ii)* classic two-dimensional electrophoresis, particularly the 2DE SDS-PAGE. HPLC can perform peptide and protein separation by: *i)* reverse phase high performance liquid chromatography and *ii)* gel filtration and ion exchange chromatography. In the two-dimensional electrophoresis, proteins are separated, first by their isoelectric point followed by separation by their molecular weight. MudPIT, or shot-gun proteomics, is a technique for the separation and identification of complex protein and peptide mixtures. This technique involves digestion of the entire protein lysate and its subsequent analysis and identification without prior fractionation, often necessary in 2-DE gel methodology. Therefore, the separation can be interfaced directly with the ion source of a mass spectrometer (Washburn *et al.*, 2001). MudPIT has the advantage to detect and identify lower abundance proteins, frequently missed by gel based techniques. In GeLC technique samples are separated using 1-D SDS-PAGE. After separation the gel lane is divided into similar sized segments and proteins in each segment are reduced and alkylated, following gel digestion performed using a site-specific protease (e.g. trypsin). A LC-MS/MS experiment is then performed to generate peptide sequence information and identify the proteins in each segment (Pflieger et al., 2000; Schirle et al., 2003). Due to their good analytical potential, the HPLC techniques are more used, however, they are much more expensive than the traditional two-dimensional electrophoresis (Wang and Hanash, 2003; Gorg *et al.*, 2004). Furthermore, two-dimensional electrophoresis is well-

established and relatively easy to execute. In this context, the 2DE SDS-PAGE protein separation methodology was the technique used in this thesis.

2DE SDS-PAGE

This technique allows the separation of several thousands of proteins from a sample on a 2D polyacrylamide slab gel, comprises two steps: the isoelectric focusing (IEF) and the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Gorg *et al.*, 2004). In the isoelectric focusing an electric field is applied to a tube gel containing the protein sample and carrier ampholytes, separating proteins according to their molecular charge (isoelectric point, p*I*) (Figure 1.6 A).

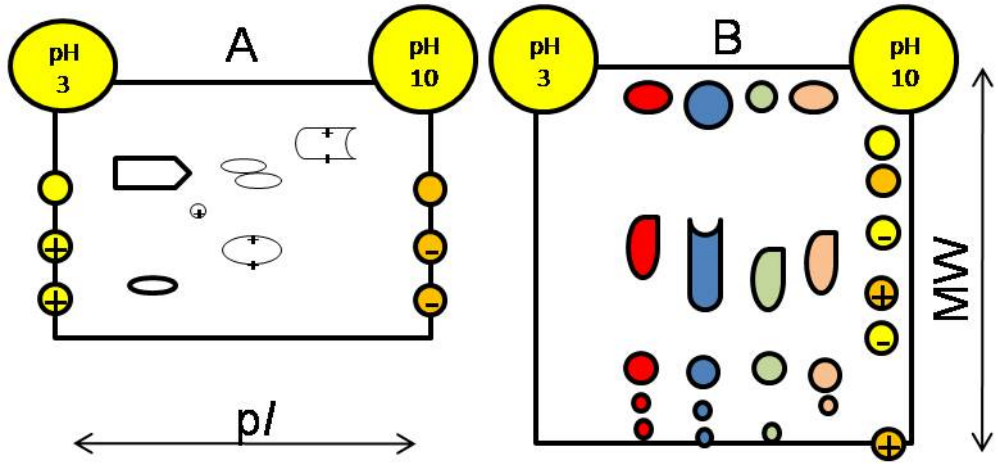

Figure 1.6 - Scheme illustrating 2DE SDS-PAGE: A – isoelectric focusing; B – SDS-PAGE.

Following this step, the focused proteins are transferred to a polyacrylamide gel and then separated by their molecular mass (M*r*) through a conventional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), originating a two-dimensional map (Figure 1.6 B) (Rabilloud, 2000).

Protein visualisation

The proteins in the two-dimensional map can be visualized and quantified by radiolabeling or by using one of several protein dyes and stains, such as Coomassie blue (CB), silver staining, or fluorescent dyes (e.g. Sypro Ruby and fluorencents Cys dyes). Traditionally, proteins visualisation is made either by silver or CB staining. However, silver staining is more sensitive. Nevertheless, since both methods can originate slight variations of spot pattern and the staining intensity, multiple gel replicates are required to warrant reliable data on the expression of each protein. These problems were overwhelmed by the introduction of fluorescent dyes. Their principal advantage is their 3-4 orders of magnitude linear response range that exceeds the 1 order of magnitude obtained with silver or CB staining. It allows a more efficient, sensitive, and reliable way of protein visualisation and quantification with a small number of replicates gels (Unlu *et al.*, 1997). The Sypro Ruby has a wider dynamic range than silver staining and the same sensitivity (Nesatyy and Suter, 2007; Lopez *et al.*, 2000; Lanne and Panfilov, 2005; Mezhoud *et al.*, 2008). The differential gel electrophoresis (DIGE) uses mass- and charge-matched fluorescent cyanide dyes to label protein samples prior to 2-D GE, allowing both the control and the treated samples to be run in the same gel, reducing the number of replicate gels (Unlu *et al.*, 1997).

Image analysis

There are several image analysis softwares (e.g. PDQuest, Group Warp Strategy of Delta 2D, Image Master 2D Platinum, Proteinweaver) for 2DE gel analysis. The principal function of software-assisted 2DE gel analysis is protein spot detection and quantitation, gel matching and identification of any differences in protein expression

between sets of samples, allowing the determination of differentially expressed protein spots. The successful quantification of protein expression levels relies on the algorithms for spot matching, normalization and background subtraction provided by the 2DE analysis software.

Following image analysis proteins spots of interest can be cut out from the gel and further identified by mass spectrometry based techniques.

Proteins identification

The identification of proteins relies on the comparison of experimental mass spectra against theoretical spectra computed from protein sequences stored in databases. The recent development of mass spectrometry like high sensitivity and automation of protein identification and of some post-translational modifications (PTMs) significantly increased the number of large-scale proteomics projects. The peptide mass fingerprinting (PMF) technique matches molecular weights of the peptide ions produced during digestion by a specific proteolytic enzyme against peptide ions produced *in silico* through digesting the protein using the same protease (James *et al.*, 1993; Pappin *et al.*, 1993). However, PMF identification relies on observing a considerable number of peptides, usually more than 5, from the same protein at high mass accuracy. Additionally, this technique is not suitable when dealing with complex mixtures of proteins and keratin contamination often renders difficult protein identification (Nesatyy and Suter, 2007). Protein identification by tandem mass spectra, often abbreviated as Tandem MS or MS/MS is based on the same principle of protein digestion as PMF and compares experimental and theoretical peptide sequence data. Programs (e.g. SEQUEST, PepIdent, TagIdent, Profound, MASCOT) match experimental data against

in silico produced fragmentation patterns from the protein sequences available in the databases (Mann and Wilm, 1994; Wilm *et al.*, 1996). According to the way the sample is placed into the tandem mass spectrometer, ESI (electrospray ion), FAB (fast atom bombardment) and LSI (liquid secondary ion) could precede the tandem MS. Nowadays, the tandem MS-based protein identification is usually chosen, due to is capacity to deal with complex mixtures and potential in obtaining good information on sequence, probable PTMs and mutations (Nesatyy and Suter, 2007). Nevertheless, this technique presents some problems:

- It cannot be used for the identification of proteins from unknown genomes;
- Often, after database searching, good quality spectra could remain unexplained, due to splicing, PTMs and mutation absence from databases or sequence errors;

A new approach, the *de novo* sequencing, is a viable option to solve some of these problems. In this process partial or complete peptide sequences are directly derived from tandem MS/MS signals by *de novo* sequencing of *i)* underivative peptides (using PEAKS studio software) or *ii)* derivative peptides (using N-terminal sulfonation of peptides with 4-sulfophenyl isothiocyanate). This approach does not need a prior knowledge of the protein sequence, and is suitable for organisms with unknown genomes (Marekov and Steinert, 2003; Yergey *et al.*, 2002).

Western blot analysis associated with 2DE SDS-PAGE separations can also be useful to identify specific proteins. In this approach, application of specific antibodies allows the detection of the protein of interest in mixtures of several proteins. This methodology has been successfully applied to assess ROS in marine bivalves exposed to several environmental stressors. This approach, also known as redox proteomics, was mainly

focused in glutathionylation, disulfide bond formation, carbonylation and ubiquitination of proteins (Table 1.3).

Table 1.3 – Redox proteomics in marine bivalve species.

Environmental stressor	Measures	Authors
	Mytilus edulis	
Polluted site; H_2O_2	Glutathionylation and carbonylation	McDonagh *et al.*, 2005
H_2O_2; $CdCl_2$; menadione	Ubiquitination and carbonylation	McDonagh and Sheehan, 2006
H_2O_2	Glutathionylation, carbonylation and disulfide bond formation	McDonagh *et al.*, 2006
Copper, 95 octane petrol	Carbonylation and disulfide bond formation	Prevodnik *et al.*, 2007
Menadione	Carbonylation and disulfide bond formation	McDonagh and Sheehan, 2008
Gold nanoparticles	Ubiquitination and carbonylation	Tedesco *et al*., 2008
	Ruditapes decussatus	
DDE	carbonylation	Dowling *et al.*, 2006

Recently, the use of protein arrays, mainly microchip, associated with protein identification techniques has also proved to be very promising technique for a more accurately protein identification (LaBaer *et al.*, 2005; Armenta, 2009).

1.4. Proteomics applied in ecotoxicology

Proteomics can be a very useful tool on the study of how organisms are affected by chemicals released into the environment by human activities. Protein expression is expected to be specific to the type and degree of stress and consequently, has the capacity to distinguish exposure and/or effect to pollutants (Shepard and Bradley,

2000). Differential expression of proteins can be compared among chemicals, concentrations or complex mixtures in different natural environments. The up-or down-regulated proteins can be combined within protein expression signatures (PESs) that are specific to the condition and the level of environmental stress (Shepard and Bradley, 2000). The PESs by themselves constitute a biomarker, in the assessment of the presence of certain pollutants, thus protein identification is not indispensable for adverse environmental conditions diagnosis (Shepard and Bradley, 2000, Petricoin, 2002). Nevertheless, in order to know the protein role and results consistency uphold, identification must be carried out, helping in understanding the pollutant effects in organisms

In this context, field and laboratory studies using proteomics have been applied in bivalves, showing different PEPs and identifying proteins altered by several stress exposures (Table 1.4). The relatively low number of identified proteins results from the limited presence of proteins from molluscs and other invertebrate species. Additionally, the existing proteins are generally limited to cytoskeletal proteins in current databases (López et al., 2001, Chu et al., 2000).

Table 1.4 - Summary of bivalve studies involving proteomics analysis. Id. method: identification methodology: SELDI-TOF-MS; MS/MS; MALDI-TOF; ESI-MS-MS; Immuno = immunolocalization.

Environmental stressor	Concentration	Time of exposure	Id. method	Identified proteins	References
			Mytilus edulis		
Cu and Cu sulfate	Cu 0, 20,40, 60, and 80 $\mu g.l^{-1}$Cu sulfate	24h			Shepard and Bradley, 2000
Copper, Aroclor 1248	Copper (70 $\mu g.l^{-1}$), Aroclor 1248 (1 $\mu g.l^{-1}$)	7 days			Shepard *et al,*. 2000
Exposure to PAHs (acenaphtene, acenaphthylene, anthracene, benz(a)anthracene, benzo(a)pyrene, benzo-(b)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)perylene, chrysene, dibenzo(a,h)anthracene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, pyrene) and metals (Cu, Zn, Pb and Cd)		Field, island of Karmøy, Norway	SELDI-TOF-MS		Knigge *et al.,* 2004
PCB and PAH	1.5×10^{-3} $\mu g.l^{-1}$PCB, 2×10^{-3} $\mu g.l^{-1}$ PCB, 347×10^{-3} $\mu g.l^{-1}$ PAH, 112×10^{-3} $\mu g.l^{-1}$ PAH	6 days	Immuno	Hsp 58, Hsp 62, Hsp 65, Hsp 70, Hsp 71, Hsp73, Hsp 82, Hsp 90	Olsson *et al.,* 2004

North Sea oil; alkylphenols and extra PAHs	0.5 x 10^3 µg.l^{-1} North Sea oil; 0.1 x 10^3 µg.l^{-1} alkylphenols +1 x 10^3 µg.l^{-1} extra PAHs	21 days	MS/MS MALDI	Heat shock protein 70 (*Dicentrarchus labrax*) Glutamyl tRNA synthetase (*Rhodobacter capsulatus*) Actin (*Fugu rubripes*) Replication factor c (*Mus musculus*) Probable arginase (*Arabidopsis thaliana*) Cyclin D1 (*Gallus gallus*) Fructose-biphosphate aldolase (*Caenorhabditis elegans*) Aminopeptidase (*Homo sapiens*) Cyclin D1 (*Gallus gallus*) Probable protease inhibitor (*Homo sapiens*) Tropomyosin (*Theragra chalcogramma*) Glutathione S-transferase (*Rattus norvegicus*) Thioredoxine peroxydase (*Homo sapiens*) Actin (*Debaryomyces hansenii*) ATP synthase b chain (*Rickettsia prowazekii*) Mitochondrial porin (*Bos taurus*) Malate dehydrogenase (*Tetratrichimonas gallinarum*) Heavy metal binding protein (*M. edulis*) Heavy metal binding protein (*M. edulis*)	Manduzzio *et al., 2005*
DAP, PBDE and BPA	38.3 x 10^6 µg.l^{-1} DAP, 0.23 x 10^6 µg.l^{-1} PBDE-47, 59.4 x 10^6 µg.l^{-1} BPA	3 weeks	MALDI-TOF ESI-MS/MS	Hydroxyacid oxidase 1 Glutathione S-transferase Aldehyde dehydrogenase 4A1 Carbonic anhydrase Mn-superoxide dismutase ATP synthase beta subunit Peroxin 10 Cytochrome P450 2A6 Enoyl-CoA hydratase Phospholipase A_2 Cytochrome C oxidase subunit II ß-tubulin alcohol dehydrogenase Catalase	*Apraiz et al., 2006*
Crude oil, APEOs and PAHs	0.5 x 10^3 µg.l^{-1} crude oil; 0.5 x 10^3 µg.l^{-1} crudeoil +	3 weeks	MALDI-TOF	Actin (*Placopecten magellanicus)* Major vault protein fragment (*M. edulis*)	Jonsson *et al*,. 2006

	164 x 10^3 µg.l^{-1}, APEOs + 36 x 10^3 µg.l^{-1} PAHs				
H_2O_2	1mM H_2O_2	24h	Immuno	Actin, carbonylated and glutathionated proteins	McDonagh *et al.*, 2005
H_2O_2, $CdCl_2$ and menadione	H_2O_2, $CdCl_2$ and menadione	24h	Immuno	Ubiquitinated and glutathionated proteins	McDonagh and Sheehan, 2006a
H_2O_2	1 mM H_2O_2	24h	Immuno	Disulphide bridges, carbonylated and glutathionated proteins	McDonagh *et al.*, 2006a
menadione	menadione	24h	Immuno	Actin and disulphide bridges	McDonagh and Sheehan, 2008
crude oil, crude oil+PAHs+APs	crude oil, crude oil+PAHs+APs	21 days :	SELDI		Monsinjon *et al.*, 2006
		Gothenburg harbor, Sweden	ESI-MS/MS	Cathepsin B like cysteine proteinase Putatitve AMP binding protein Acyl_CoA dehydrogenase Glutathione transferase Phosphoglycerate kinase Pex1 HSC70 HSC71 Fascin-like protein Aldehyde dehydrogenase 1A2	*Amelina et al.* ,2007
GNP, menadione, GNP+menadione	GNP, menadione ; GNP+menadione	24h	Immuno		Tedesco *et al,.* 2008
			Mytilus galloprovincialis		
		Bay of Biscay, Spain	ESI-MS/MS Immuno	EH, PLP, SOX, AOX, PH, SOD	Mi *et al.,* 2005
			Mytilus sp		

				heat shock protein 70, calreticulin albumin precursor-1-ho, transporter ATP-binding protein-ho ATP-synthase A chain	Lopez *et al*., 2002
			Crassostrea virginica		
Cd	0.44 µM Cd	96 h	MS	CvMT-I/II family	Jenny *et al*.,2004
			Chamaelea gallina		
Aroclor, Cu(II), TBT and As(III)	Aroclor 1254 (10,100,1000) mg/L; Cu(II) (0.1, 1, 5 x 10^3 µg.l^{-1}); TBT (0.3, 1, 3 x 10^6 µg.l^{-1}); As(III) (0.1, 1,10 x 10^3 µg.l^{-1})	7 days	MS	Tropomyosin (*Haliotis diversicolor)* Light chain of myosin (*Mercenaria mercenaria)* Actin (*Crassostrea gigas)* *Act87E* gene product (*Drosophila melanogaster)*	Rodríguez-Ortega *et al.,* 2003
identify possible CYP1A immunopositive proteins			Immuno	tropomyosin	Grøsvik *et al.,* 2006
			Ruditapes decussatus		
4,4_DDE, methoxychlor, imidazole	4,4_DDE, methoxychlor, imidazole	48 h	Immuno	GST	Hoarau *et al.,* 2004
DDE	40 x 10^6 µg.l^{-1} DDE	48 h	immuno	HSP60 HSP70 HSP90	Dowling *et al,.* 2006

	Scrobicularia plana			
	Guadalquivir Estuary, Spain	MALDI-TOF MS/MS.	HPRT G3PDH Hypoxanthine phosphoribosyltransferase Homeotic protein Hox B7 Acetoin[2,6-dichlorophenolindophenol] oxidoreductase Cytochrome-c peroxidase precursor Protein-tyrosine kinase, receptor precursor RNA-directed DNA polymerase Coat protein – apple chlorotic leaf spot virus DNA-directed RNA polymerase, beta'-2 chain Heat-labile enterotoxin chain B precursor Transcription factor isl-1 DNA-directed RNA polymerase beta chain Thymosin beta-4 Superoxide dismutase (Cu-Zn) Heat shock protein 21 precursor Coat protein – apple chlorotic leaf spot virus Ribosomal protein XL1a Cell surface antigen 4F2 heavy chain Hemoglobin alpha-D chain Profilin II – *Acanthamoeba castellanii* Imidazoleglycerol-phosphate dehydratase yeast Phosphotransferase system enzyme II, glucose-specific Coat protein – alfalfa mosaic virus Tropomyosin alpha chain Beta-casein	Romero-Ruiz *et al.*, 2006

1.5. Selected species

The clam *Ruditapes decussatus* (Linnaeus, 1758) belongs to the Veneridae family, order Veneroida (Figure 1.7). Similar to the asiatic species *Ruditapes philippinarum* (Adams and Reeve, 1850), *R. decussatus* has less angulated shell both posteriorly and anteriorly and the unfused siphons. This bivalve is widely distributed in European (from the United Kingdom of Mauritania) and Mediterranean coastal waters and due to its economic importance is heavily harvested in many countries, particularly in Portugal where its production has a relevant economic impact, mainly in the Ria Formosa lagoon (southern coast of Portugal) (Bebianno *et al.*, 2004).

Figure 1.7 - *Ruditapes decussatus* (Linnaeus, 1758).

R. decussatus is a syphonate suspended feeder that lives burrowed in sand and muddy-sand sediments in the inter-tidal level of bays, estuaries and coastal lagoons. *R. decussatus* is used for human consumption, and, since is rich in polyunsaturated fatty acids, particularly in eicosapentanoic acid, is investigated for human health purposes in the prevention of cardio-vascular diseases (Beninger and Stephan, 1985). This clam is considered gonochoristic, however, the occasional occurrence of individual hermaphrodites was reported (Delgado and Pérez Camacho, 2002). Usually the sexual cycle begins in March with external fecundation. However two periods of reproduction

occur one in June-July and another in September-October. Similar to other bivalves, *R. decussatus* are often used as sentinel organism for the monitoring of marine pollution due to its capacity to bioaccumulate contaminants, sessile nature, capacity to tolerate a wide range of environmental conditions, filter-feeding habits and widespread distribution (Bebianno and Serafim, 2003; Bebianno *et al.*, 2004). Like many other bivalves, in order to cope with changes in environmental conditions, these clams have developed subcellular systems for accumulation, regulation and immobilization of pollutants, mainly metals, which could be used as biomarkers. Therefore many biomarkers have been assessed in this clam: metallothioneins (MT), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx) (total and selenium-dependent), lipid peroxidation, glutathione S-transferase (GST) and acetylcholinesterase (AChE) (Geret *et al.*, 2002; Hoarau, 2002; Bebianno and Serafim, 2003; Hamza-Chaffai *et al.*, 2003; Simes *et al.*, 2003; Bebianno *et al.*, 2004; Dellali *et al.*, 2004; Geret and Bebianno, 2004; Smaoui-Damak *et al.,* 2006; Barreira *et al.*, 2007; Ketata *et al.*, 2007, a,b; Serafim and Bebianno, a,b 2007, 2009; Barreira and Bebianno, 2009). Nonetheless, there are few proteomic studies using this species. These include only protein carbonylation and HSP response due to p,p'-dichlorodiphenyldichloroethylene (DDE) exposure (Dowling *et al.*, 2006).

1.6. Objectives of the thesis

The aim of the present thesis was to assess the effects of cadmium (Cd) and nonylphenol (NP) in the sentinel specie *Ruditapes decussatus* using a proteomic approach, in order to find new biomarkers in the field of ecotoxicology.

In order to accomplish this objective, the thesis was structured into several chapters as follows:

The first chapter of this dissertation (**Chapter 1**), a general introduction, reviews the widespread distribution of persistent pollutants in aquatic environments, including the characterization of Cd, a non essential metal, and the organic pollutant NP, and the available techniques in marine pollution assessment. This introduction also describes the process of reactive oxygen species production, their main effects and the antioxidant defence systems in organisms. Furthermore, the importance of "omics" technologies in ecotoxicology, in particular the proteomic approach and redox proteomics are also reviewed. Additionally, a description of the selected species, *R. decussatus* was also included.

Bivalves are important because of their use as bioindicators in biomonitoring studies. However, the application of standard two-dimensional electrophoresis (2DE SDS-PAGE) protocols developed for mammalian tissues was proved not to be straight forward when used in bivalves, mainly due to the excess of salt water present and the lipid content of some of particular tissues. Thus, in **Chapter 2,** a description of the analytical procedure to develop a 2DE SDS-PAGE protocol suitable for bivalve species is described, in particularly for the tissues of the clam *R. decussatus*. The final 2DE protocol optimized for bivalve species is also detailed.

In order to understand the effects of model pollutants in protein expression of *R. decussatus* several laboratory experiments were conducted. Thus, in **Chapter 3** the protein expression profiles (PEP) in the tissues (gill and digestive gland) of *R. decussatus*, exposed to Cd (40 $\mu g.l^{-1}$, 21 days), were compared to unexposed ones. Single protein markers that could characterize Cd exposure were also identified. This chapter also describes the content of NP accumulated in the same clam tissues after NP exposure (100 $\mu g.l^{-1}$, 21 days), the subsequent alterations in PEP and potential single

markers for NP effects. At the end of this Chapter, a comparison between PEPs obtained for both tissues was made following exposure to Cd and NP and protein expression signatures (PESs), common and specific were defined. In **Chapter 4** laboratory studies were performed to study the potential of ubiquitination and carbonylation of proteins to assess oxidative stress (OS) in *R. decussatus* through a redox proteomic approach after exposure to both pollutants (Cd and NP). Thus, this chapter describes the effects of cadmium exposure (40 $\mu g.l^{-1}$, 21 days) on ubiquitination and carbonylation of proteins in the clam *R. decussatus*. Similarly, the effects of nonylphenol (100 $\mu g.l^{-1}$, 21 days) exposure on ubiquitination and carbonylation of proteins in the clam *R. decussatus* were also studied.

Finally, a general discussion (**Chapter 5**) is presented, comparing the effects Cd and Np in protein expression of *R. decussatus* tissues and the advantages of the use of a proteomic approach.

The final conclusions and future works are summarized in the last chapter (**Chapter 6**).

1.7. References

Achard, M., Baudrimont, M., Boudou, A., Bourdineaud, J.P., 2004. Induction of a multixenobiotic resistance protein (MXR) in the Asiatic clam *Corbicula fluminea* after heavy metals exposure. *Aquatic Toxicology* **67**, 347–357.

Ahmad, S., 1995. *Oxidative Stress and Antioxidant Defenses in Biology*. Chapman & Hall. New York. pp. 457.

Ahokas, J.T., Holdway, D.A., Brennan, S.E., Goudey, R.W., Bibrowska, H.B., 1994. MFO activity in carp (*Cyprinus carpio*) exposed to treated pulp and paper mill effluent in lake Coleman, Victoria, Australia, in relation to AOX, EOX and muscle PCDD/PCDF. *Environmental Toxicology and Chemistry* **13**, 41-50.

Alban, A., David, S.O., Bjorkesten, L., Andersson, C., Sloge, E., Lewis, S., Currie, I., 2003. A novel experimental design for comparative two-dimensional gel analysis: two-

dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics* **3**, 36-44, 2003.

Ali, M. and Sreekrishnan, T.R., 2001. Aquatic toxicity from pulp and paper mill effluents: a review. *Advances in Environmental Research* **2**, 175–96.

Allen, J.I. and M. N. Moore, 2004. Environmental prognostics: Is the current use of biomarkers appropriate for environmental risk evaluation? *Marine Environmental Research* **58**, 227-232.

Amelina, H., Apraiz, I., Sun, W., Cristobal, S., 2007. Proteomics-based method for the assessment of marine pollution using liquid chromatography coupled with two-dimensional electrophoresis. *Journal of Proteomic Research* **6**, 2094-2104.

Andrew, M. N., Dunstan, R. H., O'Connor, W. A., Van Zwieten, L., Nixona, B.,, MacFarlanea, G.R., 2008. Effects of 4- nonylphenol and 17_ ethynylestradiolexposure in the Sydney rock oyster, *Saccostrea glomerata*: Vitellogenin induction and gonadal development. *Aquatic Toxicology* **88**, 39-47.

Apraiz, I., Mi, J., Cristobal, S., 2006. Identification of proteomic signatures of exposure to marine pollutants in mussels (*Mytilus edulis*). *Molecular and Cellular Proteomics* **5**, 1274-1285.

Armenta, J.M., 2009. Microfluidic chips for protein differential expression profiling. *Electrophoresis* **30**, 1145-1156.

Arukwe, A., Knudsen, F. R. and A. Goksoyr, 1997. Fish zona radiata (eggshell) protein: A sensitive biomarker for environmental estrogens. *Environmental Health Perspectives* **105,** 418-422.

Ashfield, L. A., Pottinger, T. G., J. P. Sumpter, 1998. Exposure of female juvenile rainbow trout to alkylphenolic compounds results in modifications to growth and ovosomatic index. *Environmental Toxicology Chemistry* **17:** 679-686.

Askenov, M., Askenova, M., Butterfield, D., Geddes, J., Markesbery, W., 2001. Protein oxidation in the brain in Alzheimer's disease. *Neuroscience* **103**, 373–383.

Atiezar, F.A., Billinghust, Z., Depledge, M.H., 2002. 4-n-nonylphenol and 17- β estradiol may induce common DNA effects in developing barnacle larvae. *Environmental Pollution* **120,** 735-738.

Augustin, W.,Wiswedel, I., Noack, H., Reinheckel, T., Reichelt, O., 1997. Role of endogenous and exogenous antioxidants in the defence against functional damage and lipid peroxidation in rat liver mitochondria. *Molecular and cellular biochemistry* **174**, 199–205.

Barreira, L., Mudge, S.M.; Bebianno, M.J., 2007. Oxidative stress in the clam *Ruditapes decussatus* (Linnaeus, 1758) in relation to polycyclic aromatic hydrocarbon body burden. *Environmental Toxicology* **22**, 203-21.

Barreira, L. and Bebianno, M.J., 2009. Polycyclic aromatic hydrocarbons concentrations and biomarker responses in the clam *Ruditapes decussatus* transplanted in the Ria Formosa lagoon. *Ecotoxicology and Environmental Safety* **72(7)**, 1849-60.

Bebianno, M.J. and Serafim, M.A., 1998. Comparison of metallothionein induction in response to cadmium in the gills of the bivalve molluscs *Mytilus galloprovincialis* and *Ruditapes decussatus*. *The Science of the Total Environment* **214(1-3)**, 123-131.

Bebianno, M.J. and Serafim, A., 2003. Variation of metal and metallothionein concentrations in a natural population of *Ruditapes decussatus*. *Archives of Environmental Contamination and Toxicology* **44**, 53-66.

Bebianno, M.J., Geret, F., Hoarau, P., Serafim, M.A., Coelho, M.R., Gnassia-Barelli, M., Roméo, M., 2004. Biomarkers in *Ruditapes decussatus*: a potential bioindicator species. *Biomarkers* **9**, 305-330.

Belfroid, A.C., Sijm, D.T.H.M., Van Gestel, C.A.M., 1996. Bioavailability and toxicokinetics of hydrophobic aromatic compounds in benthic and terrestrial invertebrates. *Environmental Reviews* **4**, 276-299.

Beninger, P.G. and Stephan, G., 1985. Seasonal variations in a fatty acids of the triacylglycerols and phospholipids of two populations of adult clam (*Tapes decussatus* L. and *Tapes philippinarum*) reared in a common habitat. *Comparative Biochemistry and Physiology* **81B**, 591-601.

Beyersmann, D. and Hechtenberg, S., 1997. Cadmium, gene regulation and cellular signaling in mammalian cells. *Toxicology and Applied Pharmacology* **144**: 247-261.

Bigot, A., Doyen, P., Vasseur, P., Rodius, F., 2009. Metallothionein coding sequence identification and seasonal mRNA expression of detoxification genes in the bivalve *Corbicula fluminea. Ecotoxicology and Environmental Safety* **72**, 382-387.

Biswas, S., Chida, A.S., Rahman, I., 2006. Redox modifications of protein-thiols: emerging roles in cell signaling. *Biochemical pharmacology* **71**,551-564.

Bonacci, S., Iacocca, A., Fossi, S., Lancini, L., Caruso, T., Corsi, I., 2007. Biomonitoring aquatic environmental quality in a marine protected area: a biomarker approach. *AMBIO* **36**, 308–15.

Bremmer, I., 1998. Manifestations of copper excess. *The American Journal of Clinical Nutrition* **67**, 1069-1073.

Brown, M., Davies, I.M., Moffat, C.F., Craft, J.A., 2006. Application of SSH and a macroarray to investigate altered gene expression in Mytilus edulis in response to exposure to benzo[a]pyrene. *Marine Environmental Research* **62**, S128–S135.

Bryan, G.W. and Langston, W.J., 1992. Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. *Environmental Pollution* **76,** 89-131

Butwell, A. J., Hetheridge, M., James, H. A., Johnson, A. C., Young, W. F., (eds.) 2002. *Endocrine disrupting chemicals in wastewater: A review of occurrence and removal*, UK Water Industry Research Limited, London.

Cajaraville, M.P., Marigómez, J.A., Angulo, E., 1989. A stereological survey of lysosomal structure alterations in *Littorina littorea* exposed to 1-naphthol. *Comparative biochemistry and physiology* **93C**, 231-237.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C. and Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *The Science of the Total Environment* **247(2-3)**, 295-311.

Canesi, L., Lorusso, L. C., Ciacci, C., Betti, M., Gallo, G., 2004. Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways. *General and Comparative Endocrinology* **138**, 58-69.

Canesi, L., Lorusso, L. C., Ciacci, C., Betti, M., Rocchi, M., Pojana, G., Marcomini, A., 2007. Immunomodulation of *Mytilus* hemocytes by individual estrogenic chemicals and environmentally relevant mixtures of estrogens: *In vitro* and *in vivo* studies. *Aquatic Toxicology* **812** 36-44.

Chait, B. T., 2006. Mass spectrometry: Bottom-up or top-down? *Science* **314**, 65-66.

Cavaletto, M., Ghezzi, A., Burlando, B., Evangelisti, V., Ceratto, N., Viarengo, A. , 2002. Effect of hydrogen peroxide on antioxidant enzymes and metallothionein level in the digestive gland of *Mytilus galloprovincialis*. *Comparative Biochemistry and Physiology* **131C**, 447-455.

Chelomin, V.P., Zakhartsev, M.P., Kurilenko, A.V., Belcheva, N.N., 2005. An *in vitro* study of the effect of reactive oxygen species on subcellular distribution of deposited cadmium in digestive gland of mussel *Crenomytilus grayanus*. *Aquatic Toxicology* **73**, 181-189

Cheung, M.S., Zhang, L.,Wang,W.-X., 2007. Transfer and efflux of cadmium and silver in marine snails and fish fed pre-exposed mussel prey. *Environmental Toxicology and Chemistry* **26**, 1172–1178.

Chouksey, M.K., Kadam, A.N., Zingde, M.D., 2004. Petroleum hydrocarbon residues in the marine environment of Bassein–Mumbai. *Marine Pollution Bulletin* **7–8**, 637–47.

Christiansen, T., Korsgaard, B., A. Jespersen, 1998a. Induction of vitellogenin synthesis by nonylphenol and 17 beta-estradiol and effects on the testicular structure in the eelpout *Zoarces viviparus*. *Marine Environmental Research* **46:** 141-144.

Christiansen,T., Korsgaard, B., A. Jespersen, 1998b. Effects of nonylphenol and 17 beta-oestradiol on vitellogenin synthesis, testicular structure and cytology in male eelpout *Zoarces viviparus*. *Journal of Experimental Biology* **201:** 179-192.

Christiansen, L. B., Pedersen, K. L., Korsgaard, B. and P. Bjerregaard, 1998c. Estrogenicity of xenobiotics in rainbow trout (*Oncorhynchus mykiss*), using *in vivo* synthesis of vitellogenin as a biomarker. *Marine Environmental Research* **46:** 137-140.

Chu, K.H., Wong, S.H., Leung, P.S., 2000. Tropomyosin Is the Major Mollusk Allergen: Reverse Transcriptase Polymerase Chain Reaction, Expression and IgE Reactivity. *Marine Biotechnology* **2**, 499–509.

Clark, R.B., 2001. *Marine Pollution*. 5th ed. Oxford University Press Inc, New York. 236 pp.

Coelho, M.R., Langstone, W.J., Bebianno, M.J., 2006. Effect of TBT on Ruditapes decussatus juveniles. *Chemosphere* **63**, 1499-1505.

Cohen, T., S.S. Que Hee and R.F. Ambrose, 2001. Comparison of trace metal concentrations in fish and invertebrates in three Southern California wetlands. *Marine Pollution Bulletin* **42:** 224-232.

Commandeur, J.N.M., Stijntjes, G.J., Vermeulen, N.P.E., 1995. Enzymes and transport systems involved in the formation and disposition of glutathione S-conjugates. Role in bioactivation and detoxication mechanisms of xenobiotics. *Pharmacology Reviews* **47**, 271-330.

Costa, V.M.V., Amorim, M.A., Quintanilha, A., Moradas-Ferreira, P., 2002. Hydrogen peroxide-induced carbonylation of key metabolic enzymes in *Saccharomyces cerevisiae*: the involvement of the oxidative stress response regulators Yap1 and Skn7. *Free Radical Biology and Medicine* **33**, 1507–1515.

Craig, P.J., 2003. *Organometallic compounds in the environment* (2nd edition). Wiley-VCH, 434 pp.

Dachs, J., Van Ry, D.A., Eisenreich, S.J., 1999. Occurrence of estrogenic nonylphenols in the urban and coastal atmosphere of the lower Hudson River estuary. *Environmental Science and Technology* **33**, 2676-2679.

Dalton, T.P., Shertzer, H.G., Puga, A., 1999. Regulation of gene expression by reactive oxygen. *Annual Review of Pharmacological Toxicology* **39**, 67-101.

Damiens, G., Mouneyrac, C., Quiniou, F., His, E., Gnassia-Barelli, M., 2006. Metal bioaccumulation and metallothionein concentrations in larvae of *Crassostrea gigas*. *Environmental Pollution* **3**, 492-499.

Davies, K.J. and Goldberg, A.L., 1987. Proteins damaged by oxygen radicals are rapidly degraded in extracts of red blood cells. *The Journal of biological chemistry* **262**, 8227–8234.

De la Torre, F.R., Salibián, A., Ferrari, L., 2007. Assessment of the pollution impact on biomarkers of effect of a freshwater fish. *Chemosphere* **68**,1582–90.

Delgado, M. and Pérez Camacho, A., 2002. Hermaphroditism in Ruditapes *decussatus* (L.) (Bivalvia) from the Galician coast (Spain). *Scientia Marina* **66**, 183–185.

Dellali, M., Roméo, M., Gnassia-Barelli, M., Aissa, P., 2004. A multivariate data analysis of the clam *Ruditapes decussatus* as sentinel organism of the Bizerta lagoon (Tunisia). *Water, Air, and Soil Pollution* **156**, 131-144.

Di Giulio, R.T., Washburn, P.C., Wenning, R.J., Winston, G.W., Jewell, C.S., 1989. Biochemical responses in aquatic animals: a review of determinants of oxidative stress. *Environmental Toxicology and Chemistry* **8**, 1103-1123.

Di Giulio, R.T., Benson, W.H., Sanders, B.M., van Veld, P.A., 1995. *Biochemical mechanisms: metabolism, adaptation, and toxicity.* In: Rand, G.M. (Ed.), Fundamentals of Aquatic Toxicology: Effects, Environmental fate, and Risk Assessment, second ed. Taylor and Francis, London, UK, pp. 523-562.

Dixon, D.R. and Pruski, A.M., 2002. Effects of cadmium on nuclear integrity and DNA repair efficiency in the gill cells of *Mytilus edulis*. *Aquatic Toxicology* **57,** 127–137.

Dondero, F., Dagnino, A., Jonsson, H., Capri, F., Gastaldi, L.,Viarengo, A., 2006.Assessing the occurrence of a stress syndrome in mussels (*Mytilus edulis*) using a combined biomarker/gene expression approach. *Aquatic Toxicology* **78**, S13–S24.

Dowling, V., Hoarau, P., Roméo, M., O'Halloran, J., Van Pelt, F., O'Brien, N., Sheehan, D., 2006. Protein carbonylation and heat shock response in *Ruditapes decussatus* following p,p′-dichlorodiphenyldichloroethylene (DDE) exposure: A proteomic approach reveals that DDE causes oxidative stress. *Aquatic Toxicology* **77**, 11-18.

Downs, C.A., Shigenaka, G., Fauth, J.E., Robinson, C.E., Huang, A. 2002. Cellular physiological assessment of bivalves after chronic exposure to spilled Exxon Valdez crude oil using a novel molecular diagnostic biotechnology. *Environmental Science and Technology* **36**, 2987-2993.

Dowsey, A.W., Dunn, M.J., Yang, G.Z., 2003. The role of bioinformatics in two-dimensional gel electrophoresis. *Proteomics* **3**, 1567-1596.

Doyen, P., Vasseur, P., Rodius, F., 2005. cDNA cloning and expression pattern of pi-class glutathione S-transferase in the freshwater bivalves *Unio tumidus* and *Corbicula fluminea*. *Comparative Biochemistry and Physiology C* **140**, 300-308.

Ekelund, R., Bergman, A., Granmo, A., Berggren, M., 1990. Bioaccumulation of 4-nonylphenol in marine animals a re-evaluation. *Environmental Pollution* **64**, 107-120.

Fiehn, O., Kopka, J., Dörmann, P., Altmann, T., Trethewey, R.N., Willmitzer L., 2000. Metabolite profiling for plant functional genomics. *Nature Biotechnology* **18**, 1157-1161.

Filho, D.W., Tribess, T., Gaspari, C., Claudio, F.D., Torres, M.A., Magalhaes, A.R.M., 2001. Seasonal changes in antioxidant defences of the digestive gland of the brown mussel (*Perna perna*). *Aquaculture* **203**, 149–158.

Filipic, M., Fatur, T., Vudrag, M., 2006. Molecular mechanisms of cadmium induced mutagenicity. *Human and experimental toxicology* **25**, 67–77.

Finne, E.F., Cooper, G.A., Koop, B.F., Hylland, K., Tollefsen, K.E., 2007. Toxicogenomic responses in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to model chemicals and a synthetic mixture. *Aquatic Toxicology* **81**, 293–303.

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

Foy, C.D., Chaney, R.L., White, M.C., 1978. The physiology of metal toxicity in plants. *Annual Review on Plant Physiology* **29**, 511–566.

Frank, R. and Hargreaves, R., 2003. Clinical biomarkers in drug discovery and development. *Nature Reviews. Drug Discovery* **2**, 566–580.

Friguet, B., 2006. Oxidized protein degradation and repair in ageing and oxidative stress. *FEBS Letters* **580**, 2910-2916.

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

Gagné, F., Blaise, C., Pellerin, J., Gauthier-Clerc, S., 2002. Alteration of the biochemical properties of female gonads and vitellins in the clam *Mya arenaria* at contaminated sites in the Saguenay Fjord. *Marine Environmental Research* **53**, 295-310.

Gagné, F. and Blaise, C., 2003. Effects of municipal effluents on serotonin and dopamine levels in the freshwater muss*el Elliptio complanata. Comparative Biochemistry and Physiology* **136C**, 117-125.

Gauthier-Clerc, S., Pellerin, J., Blaise, C., Gagné, F., 2002. Delayed gametogenesis of *Mya arenaria* in the Saguenay Fjord (Canada): a consequence of endocrine disruptors? *Comparative biochemistry and physiology* C 131, 457-467.

George, S.G., 1994. *Enzymology and molecular biology of phase II xenobiotic-conjugating enzymes in fish.* In: Malins, D.C., Ostrander, G.K. (Eds.), Aquatic Toxicology; Molecular, Biochemical and Cellular perspectives. Lewis Publishers, CRC press, pp. 37-85.

Geret, F., Serafim, Â., Barreira, L., Bebianno, M.J., 2002. Response of antioxidant systems to copper in the gills of the clam *Ruditapes decussatus*. *Marine Environmental Research* **54**, 413 –417.

Geret, F. and Bebianno, M.J., 2004. Does zinc produce reactive oxygen species in *Ruditapes decussatus*? *Ecotoxicology and Environmental Safety* **57(3)**, 399-409.

Gobas, F.A.P.C., Wilcockson, J.B., Russell, R.W., Haffner, G.D., 1999. Mechanisms of biomagnification in fish under laboratory and field conditions. *Environmental Science and Technology* **33**, 133-141.

Goeptar, A.R., Scheerens, H., Vermeulen, N.P.E., 1995. Oxygen reductase and substrate reductase activity of cytochrom P450. *Critical reviews in toxicology* **25**, 25-65.

Goldberg, ED, Koide, M., Hodge, V. Flegal, A.R., and Martin J., 1978. The mussel watch. *Environmental Conservation* **5**, 101-125.

Gorg, A., Weiss, W., Dunn, M.J., 2004. Current two-dimensional electrophoresis technology for proteomics. *Proteomics* **4**, 3665-3685.

Gracey, A.Y., Troll, J.V., Somero, G.N., 2001. Hypoxia-induced gene expression profiling in the euryoxid fish *Gillichthys mirabilis*. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 1993-1998.

Grøsvik, B.E., Jonsson, H., Rodríguez-Ortega, M.J., Roepstorff, P., Goksøyr, A., 2006.CYP1A-immunopositive proteins in bivalves identified as cytoskeletal and major vault proteins. *Aquatic Toxicology* **79**, 334–340.

Gueguen, Y., Cadoret, J.P., Flament, D., Barreau, C., 2003. Immune gene discovery by expressed sequence tags generated from hemocytes of the bacteria-challenged oyster, *Crassostrea gigas*. *Gene* **303**, 139-145.

Günzler, W.A. and Flohé, L., 1985. Glutathione peroxidase, in: Handbook of Methods for Oxygen Radical Research. Greenwald, R.A. (Ed.). CRC Press, Boca Raton, FL, pp. 285-290.

Halliwell, B. and Gutteridge, J.M.C., 1999. *Free radicals in biology and medicine*. 3 ed. New York: Oxford University Press Inc.

Hamza-Chaffai, A., Pellerin, J., Amiard, J.C., 2003. Health assessment of a marine bivalve *Ruditapes decussatus* from the Gulf of Gabès (Tunisia). *Environment International* **28**, 609– 617.

Hartwig, A., 1994. Role of DNA repair inhibition in lead and chromium-induced genotoxicity: a review. *Environmental Health Perspectives* **102**, 45-50.

Hassoun, E.A. and Stohs, S.J., 1996. Cadmium-induced production of superoxide anion and nitric oxide, DNA single strand breaks and lactate dehydrogenase leakage in J774A.1 cell cultures. *Toxicology* **112(3-2)**, 219-226.

Heijin, M., Oude, E., R.P.J., Jansen P.L.M., 1992. ATP-dependent multispecific organic anion transport system in rat erythrocyte membrane vesicles. *The American Journal of Physiology* **262**, C104-C110.

Hoarau, P., Garello, G., Gnassia-Barelli, M., Roméo, M., and Girard J. P., 2002, Purification and partial characterization of seven glutathione S-transferase isoforms from the clam *Ruditapes decussatus*. *European Journal of Biochemistry* **269,** 4359-4366.

Hoarau, P., Garello, G., Gnassia-Barelli, M., Roméo, M., and Girard, J. P., 2004, Effect of three xenobiotic compounds on Glutathione S-Transferase in the clam *Ruditapes decussatus*. *Aquatic Toxicology* **122**, 369-378.

Hook, S.E., Skillman, A.D., Small, J.A., Schultz, I.R., 2006. Gene expression patterns in rainbow trout, *Oncorhynchus mykiss*, exposed to a suite of model toxicants. *Aquatic Toxicology* **77**, 372–385.

Horning, E.C. and Horning, M.G., 1971. Metabolic Profiles - Gas-Phase Methods for Analysis of Metabolites. *Clinical Chemistry* **17**, 802–809.

Humphery-Smith, I., Cordwell, S. J., Blackstock, W., 1997. Proteome research: complementarity and limitations with respect to the RNA and DNA worlds. *Electrophoresis* **18**, 1217–1242.

Hyne, R.V. and Maher, A., 2003. Invertebrate biomarkers: links to toxicosis that predict population decline. *Ecotoxicology and environmental safety* **54**, 366–74.

Ishikawa**,** T., 1992. The ATP-dependent glutatione S-conjugate export pump. *Trends in Biochemical Sciences* **17**, 463-468

James, P., Quadroni, M., Carafoli, E., Gonnet, G., 1993. Protein identification by mass profile fingerprinting. *Biochemical and Biophysical Research Communications* **195**, 58-64.

Jenny, M.J., Ringwood, A.H., Schey, K.,Warr, G.W., Chapman, R.W., 2004. Diversity of metallothioneins in the American oyster *Crassostrea virginica*, revealed by transcriptomic and proteomic approaches. *European Journal of Biochemistry* **271**, 1702–1712.

Jonsson, H., Schiedek, D., Grosvik, B.E., Goksoyr, A. 2006. Protein responses in blue mussels (*Mytilus edulis*) exposed to organic pollutants: A combined CYP429 antibody/proteomic approach. *Aquatic Toxicology* **78(1)**, 42-48.

Kadiiska, M.B., Hanna, P.M., Jordan, S.J., Mason, R.P., 1993. Electron spin resonance evidence for free radical generation in copper-treated vitamin E- and selenium-deficient rats: *in vivo* spin-trapping investigation. *Molecular pharmacology* **44**, 222-227.

Kaloyianni, M., Dailianis, S., Chrisikopoulou, E., Zannou, A., Koutsogiannaki, S., Alamdari, D.H., Kang, Y.S., Kim, Y.M., Park, K.L., Cho, S.K., Choi, K.S., Cho, M., 2006. Analysis of EST and lectin expression in hemocytes of Manila clams (*Ruditapes phylippinarum)* (Bivalvia: Mollusca) infected with Perkinsus olseni. *Developmental and comparative immunology* **30**, 1119–1131.

Kehrer, J.P., 2000. The Haber-Weiss reaction and mechanisms of toxicity. *Toxicology*, **149**, 43-50.

Ketata, I., Guermazi, F., Rebai, T., Hamza-Chaffai, A., 2007a. Variation of steroid concentrations during the reproductive cycle of the clam *Ruditapes decussatus*: A one year study in the gulf of Gabès area. *Comparative Biochemistry and Physiology. Part A* **147**, 424-431.

Ketata, I., Smaoui-Damak, W., Guermazi, F., Rebai, T., Hamza-Chaffai, A., 2007 b. *In situ* endocrine disrupting effects of cadmium on the reproduction of *Ruditapes decussatus. Comparative Biochemistry and Physiology. Part C* **146**, 415-430.

Kettman, J.R., Frey, J.R., Lefkovits, I., 2001. Proteome, transcriptome and genome: top down or bottom up analysis? *Biomolecular Engineering* **18**, 207–212.

Khessiba, A., Roméo, M., Aïssa, P., 2005. Effects of some environmental parameters on catalase activity measured in the mussel (*Mytilus galloprovincialis*) exposed to lindane. *Environmental Pollution* **133,** 275-281.

Kirchin, M.A., Moore, M.N., Dean, R.T., Winston, G.W., 1992. The role of oxyradicals in intracellular proteolysis and toxicity in mussels. *Marine Environmental Research* **34**, 315–320.

Knigge, T., Monsinjon, T., Andersen, O.K., 2004. Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry approach to biomarker discovery in blue mussels (*Mytilus edulis*) exposed to polyaromatic hydrocarbons and heavy metals under field conditions. *Proteomics* **4**, 2722–2727.

Knight, J. A., Voorhees, R. P., 1990, Peroxidation of linolenic acid-catalysis by transition metal ions. *Annals of Clinical and Laboratory Science* **20**, 347–352.

Kobayashi, K., 1985. The effect of herbicide PCP on short necked clams and the process of detoxification. *In Fisheries in Japan, Bivalves* (T. Okutani, T. Tomiyama et T. Hibiya, eds.), pp. 161-165. Japan Marine Products Photo Materials Association.

Koliakos, G. and Dimitriadis, V.K., 2009. Oxidative effects of inorganic and organic contaminants on haemolymph of mussels. *Comparative Biochemistry and Physiology, Part C* **149**, 631-639.

Kopecka, J. and Pempkowiak, J., 2008. Temporal and spatial variations of selected biomarker activities in flounder (*Platichthys flesus*) collected in the Baltic. *Ecotoxicology and Environmental Safety* **70(3)**, 379-91.

Korsgaard, B. and K.L. Pedersen, 1998. Vitellogenin in *Zoarces viviparous*. Purification, quantification by ELISA and induction by estradiol-17 beta and 4-nonylphenol. *Endocrinology* **120**, 159-166.

Koskinen, H., Pehkonen, P., Vehniainen, E., Krasnov, A., Rexroad, C., Afanasyev, S., Molsa, H., Oikari, A., 2004. Response of rainbow trout transcriptome to model

chemical contaminants. *Biochemical and Biophysical Research Communications* **320**, 745–753.

Kruk, I., 1998. Environmental Toxicology and Chemistry of Oxygen Species. Berlin, Springer-Verlag.

Kumar, R., Asic, K., Agarwal, K. Seth, P.K., 1996. Oxidative stress-mediated neurotoxicity of cadmium. *Toxicology Letters* **89**, 65-69

LaBaer, J. and Ramachandran, N., 2005. Protein microarrays as tools for functional proteomics. *Current Opinion in Chemical Biology* **9**, 14-19.

Langston, W.J. and Bebianno, M.J., 1998 (eds). Metal metabolism in aquatic environments. London, Chapmann and Hall, pp. 209-283.

Lanne, B. and Panfilov, O., 2005. Protein staining influences the quality of mass spectra obtained by peptide mass fingerprinting after separation on 2-d gels. A comparison of staining with coomassie brilliant blue and sypro ruby. *Journal of Proteome Research* **4**, 175-179.

Layfield, R., Alban, A., Mayer, R.J., Lowe, J., 2001. The ubiquitin protein catabolic disorders. *Neuropathology and Applied Neurobiology* **27**, 171–179.

Lech, J.J. and Vodicnik, M.J., 1985. Biotransformation. *In*: Rand, G.M., Petrocelli, S.R. (eds.), *Fundamentals of Aquatic Toxicology; Methods and Applications*. Hemisphere Publishing Corporation, New York, USA, pp. 526-557.

Lee, B.G., Grimscom, S.B., Lee, J.S., Choi, H.J., Koh, C.H., Luoma, S.N., Fisher, N.S., 2000. Influences of dietary uptake and reactive sulfides on metal bioavailability from aquatic sediments. *Science* **287**, 282-284

Lemaire, P., Matthews, A., Foerlin, L., Livingstone, D.R., 1994. Stimulation of oxyradical production of hepatic microsomes of flounder (*Platichthys flesus*) and perch (*Perca fluviatilis*) by model and pollutant xenobiotics. *Archives of Environmental Contamination and Toxicology* **26**, 191-200

Lemaire, P. and Livingstone, D.R., 1997. Aromatic hydrocarbon quinone-mediated reactive oxygen species production in hepatic microsomes of the flounder (*Platichthys flesus* L.). *Comparative Biochemistry and Physiology* **117C**, 131-139

Lenaz, G., 1998. Role of mitochondria in oxidative stress and ageing. *Biochimica et Biophysica Acta* **1366**, 53-67.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., Stadtman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology* **186**, 464–478.

Liebler, D.C**.,** 2002. Proteomic approaches to characterize protein modifications: new tools to study the effects of environmental exposures. *Environmental Health Perspectives* **110(1)**, 3-9.

Lietti, E., Marin, M. G., Matozzo, V., Polesello, S., Valsecchi, S., 2007. Uptake and elimination of 4-nonylphenol by the clam *Tapes philippinarum*. *Archives of Environmental Contamination and Toxicology* **53**, 571-578.

Lindström-Seppä, P. and Oikari, A., 1991. Biotransformation activities of feral fish in waters receiving bleached pulp mill effluents. *Environmental Toxicology and Chemistry* **9**, 1415-1424.

Livingstone, D.R. and Ferrar, S.V., 1984. Tissue and subcellular distribution of enzyme activities of mixed-function-oxygenase an benzo(a)pyrene metabolism in the common mussel *Mytilus edulis. The Science of Total Environment* **39**, 209-235.

Livingstone, D.R., Garcia, P.M.,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 mollusks. *Funcional Ecology* **4**, 415- 424.

Livingstone, D.R., Mitchelmore, C.L., O'Hara, S.C.M., Lemaire, P., Sturve, J., Forlin, L., 2000. Increased potential for NAD(P)H-dependent reactive oxygen species production of hepatic subcellular fractions of fish species with in vivo exposure to contaminants. *Marine Environmental Research* **50**, 57-60.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Marine Pollution Bulletin* **42**, 656–666.

Lopez, J.L., Mosquera, E., Fuentes, J., Marina, A., Vazquez, J., Alvarez, G., 2001. Two-dimensional gel electrophoresis of *Mytilus galloprovincialis*: differences in protein expression between intertidal and cultured mussels. *Marine Ecology Progress Series* **224**, 149–156.

Lopez, J.L., Marina, A., Vázquez, J., Alvarez, G., 2002. A proteomic approach to the study of the marine mussels *Mytilus edulis* and *Mytilus galloprovincialis*. *Marine Biology* **141**, 217–223.

Lopez, M.F., Berggren, K., Chernokalskaya, E., Lazarev, A., Robinson, M., Patton, W.F., 2000. A comparison of silver stain and SYPRO Ruby Protein Gel Stain with respect to protein detection in two-dimensional gels and identification by peptide mass profiling. *Electrophoresis* **21**, 3673-3683.

López-Barea, J., 1995. Biomarkers in ecotoxicology: an overview. *Archives of Toxicology Supplement* **17**, 57-79.

Madsen, S. S., Mathiesen, A. B. and B. Korsgaard, 1997. Effects of 17 beta-estradiol and 4-nonylphenol on smoltification and vitellogenesis in Atlantic salmon (*Salmo salar*). *Fish Physiology Biochemistry* **17**, 303-312.

Mäenpää, K., Kukkonen, J. V. K., 2006. Bioaccumulation and toxicity of 4-nonylphenol (4-NP) and 4-(2-dodecyl)-benzene sulfonate (LAS) in *Lumbriculus variegatus* (Oligochaeta) and *Chironomus riparius* (Insecta). *Aquatic Toxicology* **77**, 329-338.

Manca, D., Richard, A.C., Van Tra, H. and Chevalier, G., 1994. Relation between lipid peroxidation and inflammation in the pulmonary toxicity of cadmium. *Archives of Toxicology* **68**, 364-369.

Manduzio, H., Cosette, P., Gricourt, L., Jouenne, T., Lenz, C., Andersen, O.K., Leboulenger, F., Rocher, B., 2005. Proteome modifications of blue mussel (*Mytilus edulis* L.) gills as an effect of water pollution. *Proteomics* **5**, 4958-4963.

Mann, M. and Wilm, M., 1994. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. *Analytical Chemistry* **66**, 4390-4399.

Marekov, L.N. and Steinert, P.M., 2003. Charge derivatization by 4- sulfophenyl isothiocyanate enhances peptide sequencing by post-source decay matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Journal of Mass Spectrometry* **38**, 373–377.

Marigómez, J.A., Vega, M.M., Cajaraville, M.P. and Angulo, E., 1989. Quantitative responses of the digestive lysosomal system of winkles to sublethal concentrations of cadmium. *Cellular and Molecular Biology* **35**: 555-562.

Marin, M. G., Rigato, S., Ricciardi, F., Matozzo, V., 2008. Lethal and estrogenic effects of 4-nonylphenol in the cockle *Cerastoderma glaucum*. *Marine Pollution Bulletin* **57**, 552-558.

Marques C, Pereira P, Taylor A, Liang JN, Reddy VN, Szweda LI, Shang F. 2004. Ubiquitin-dependent lysosomal degradation of the HNE modified proteins in lens epithelial cells. *Federation of American Societies for Experimental Biology Journal* **18**, 1424–1426.

Matozzo, V., Deppieri, M., Moschino, V., Marin, M.G., 2003. Evaluation of 4-nonylphenol toxicity in the clam *Tapes philippinarum*. *Environmental Research* **91**, 179-185.

Matozzo, V. and Marin, M.G., 2005. Can 4-nonylphenol induce vitellogenin-like proteins in the clam *Tapes philippinarum*? *Environmental Research* **97**, 43-49.

Matsumoto, I. and Kuhara, T., 1996. A new chemical diagnostic method for inborn errors of metabolism by mass spectrometry - Rapid, practical, and simultaneous urinary metabolites analysis. *Mass Spectrometry Reviews* **15**, 43–57.

McCarthy, J.F. and Shugart, L.R., 1990. Biological markers of environmental contamination. *In*: McCarthy, J.F., Shugart, L.R. (Eds.), Biomarkers of Environmental Contamination. Lewis Publishers, Boca Raton, FL, USA, pp. 3-16.

McDonagh, B., Tyther, R., Sheehan, D., 2005. Carbonylation and glutathionylation of specific proteins in the blue mussel *Mytilus edulis* detected by proteomic analysis and western blotting: actin as a target of oxidative stress. *Aquatic Toxicology* **73**, 315–326.

McDonagh, B. and Sheehan, D., 2006. Redox proteomics in the blue mussel *Mytilus edulis*: Carbonylation is not a pre-requisite for ubiquitination in acute free radical-mediated oxidative stress. *Aquatic Toxicology* **79**, 325–333.

McDonagh, B., Tyther, R., Sheehan, D., 2006. Redox proteomics in the mussel, *Mytilus edulis*. *Marine Environmental Research* **62**, 101–104.

McDonagh, B. and Sheehan, D., 2007. Effects of oxidative stress on protein thiols in the blue mussel *Mytilus edulis*: Proteomic identification of target proteins. *Proteomics* **7**, 3395-3403.

McDonagh, B. and Sheehan, D., 2008. Effects of oxidative stress on protein thiols and disulphides in *Mytilus edulis* revealed by proteomics: Actin and protein disulphide isomerase are redox targets. *Marine Environmental Research* **66**, 193-195.

Meneghini, R., 1988. Genotoxicity of active oxygen species in mammalian cells. *Mutation Research* **195**, 215-230.

Mezhoud, K., Praseuth, D., Puiseux-Dao, S., François, J., Bernard, C., Edery, M., 2008. Global quantitative analysis of protein expression and phosphorylation status in the liver of the medaka fish (*Oryzias latipes*) exposed to microcystin-LR.I. Balneation study. *Aquatic Toxicology* **86**, 166–175.

Mi, J., Orbea, A., Syme, N., Ahmed, M., Cajaraville, M. P., Cristobal, S., 2005. Peroxisomal proteomics, a new tool for risk assessment of peroxisome proliferating pollutants in the marine environment. *Proteomics* **5,** 3954–3965.

Miracle, A. L., Ankley, G. T., 2005. Ecotoxicogenomics: linkages between exposure and effects in assessing risks of aquatic contaminants to fish. *Reproductive Toxicology* **19**, 321-326.

Moens, L.N., van der Ven, K., Van Remortel, P., Del-Favero, J., De Coen, W.M., 2006. Expression profiling of endocrine-disrupting compounds using a customized *Cyprinus carpio* cDNA microarray. *Toxicological Sciences* **93(2)**, 298–310.

Monserrat, J.M., Martínez, P.E., Geracitano, L.A., Lund Amado, L., Martinez Gaspar Martins, C., Lopes Leães Pinho, G., Soares Chaves, I., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2007. Pollution biomarkers in estuarine animals: critical review and new perspectives. *Comparative Biochemistry and Physiology C* **146**, 221–234.

Monsinjon, T., Andersen, O.K., Leboulenger, F., Knigge, T., 2006. Data processing and classification analysis of proteomic changes: a case study of oil pollution in the mussel, *Mytilus edulis*. *Proteome Science* **4**(17), 1-13.

Moore, M.N., 2004. Diet restriction induced autophagy: A lysosomal protective system against oxidative- and pollutant-stress and cell injury. *Marine Environmental Research* **58**, 603-607.

Morcillo, Y. and Porte, C., 2000. Evidence of endocrine disruption in clams — *Ruditapes decussata* — transplanted to a tributyltin-polluted environment. *Environmental Pollution*, **107**, 47-52.

Morrison, H.A., Gobas, F.A.P.C., Lazar, R., Haffner, G.D., 1996. Development and verification of a bioaccumulation model for organic contaminants in benthic invertebrates. *Environmental Science and Technology* **30(11)**, 3377-3384.

Mulder, G.J., Coughty, M.W.H., Burchell, B., 1990. Glucuronidation. *In*: Mulder, G.J. (Ed.), *Conjugation Reactions in Drug Metabolism; an Integrated Approach*. Taylor and Francis, London.

Narbonne, J.F., Daubeze, M., Clerandeau, C., Garrigues, P., 1989. Scale of classification based on biochemical markers in mussels: application to pollution monitoring in European coasts. *Biomarkers* **4**, 415-25.

NAS, 1980. The international Mussel Watch. Washington (DC): *National Academy of Sciences of the* United States *of America*.

Nesatyy, V.J. and Suter, M.J.F., 2007. Proteomics for the analysis of environmental stress responses in organisms. *Environmental Science and Technology* **41**, 6891– 6900.

Nice, H. E., Thorndyke, M. C., Morritt, D., Steele, S., Crane, M., 2000. Development of *Crassostrea gigas* larvae is affected by 4-nonylphenol. *Marine Pollution Bulletin* **40**, 491-496.

NRC: Committee on Biological Markers of the National Research Council, 1987. Biological markers in environmental health research. *Environmental Health Perspectives* **74**, 3-9.

Nuwaysir, E.F., Bittner, M., Trent, J., Barrett, J.C., Afshari, C.A., 1999. Microarrays and toxicology: the advent of toxicogenomics. *Molecular Carcinogenesis* **24**, 153-9. Quality status report 2000: region IV—Bay of Biscay and Iberian coast. OSPAR Commission, London, 2000; 1–134.

Olsson, B., Bradley, B.P., Gilek, M., Reimer, O., Shepard, J.L., Tedengren, M., 2004. Physiological and proteomic responses in *Mytilus edulis* exposed to PCBs and PAHs extracted from Baltic Sea sediments. *Hydrobiologia* **514**, 15-27.

Pappin, D.J., Hojrup, P., Bleasby, A.J., 1993. Rapid identification of proteins by peptide-mass fingerprinting. *Current Biology* **3**, 327-332.

Patterson, S.D. and Aebersold, R.H., 2003. Proteomics: the first decade and beyond. *Nature Genetics* **33**, 311-323.

Peakall, D.B., 1994. The conceptual basis of the biomarker approach. *In*: Peakall DB, Shugart LR, editors. NATO Advanced Science Institutes Series. Berlin: Springer Verlag, 1993:15–29.

Pennie, W., Pettit, S.D., Lord, P.G., 2004. Toxicogenomics in risk assessment: an overview of an HESI collaborative research program. *Environmental Health Perspectives* **112**, 417–419.

Peters, L.D., O'Hara, S.C.M., Livingstone, D.R., 1996. Benzo[a]pyrene metabolism and xenobiotic stimulated reactive oxygen species generation by subcellular fraction of larvae of turbot (*Scophthalmus maximus* L.) *Comparative Biochemistry and Physiology* **114C**, 221-227.

Petricoin, E.E., Paweletz, C.P., Liotta, L.A., 2002. Clinical applications of proteomics: proteomic pattern diagnostics. *Journal of mammary gland biology and neoplasia* **7**, 433–40.

Pflieger, D., Le Caer, J.P., Lemaire, C., Bernard, B.A., Dujardin, G., 2000. Systematic identification of mitochondrial proteins by LCMS/ MS. *Analytical Chemistry* **74**, 2400-2406.

Phillips, D.J.H., 1986. The use of bio-indicators in monitoring conservative contaminants. Program design imperatives. *Marine Pollution Bulletin* **17**, 10-7.

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**, 27-33.

Porte, C., Janer, G., Lorusso, L.C., Ortiz-Zarragoitia, M., Cajaraville, M.P., Fossi, M.C., Canesi, L., 2006. Endocrine disruptors in marine organisms: approaches and perspectives. *Comparative Biochemistry and Physiology* **143C**, 303–315.

Prevodnik, A., Gardeström, J., Lilja, K., Elfwing, T., McDonagh, B., Petrović, N., Tedengren, M., Sheehan D., and Bollner T., 2007. Oxidative stress in response to xenobiotics in the blue mussel *Mytilus edulis* L.: Evidence for variation along a natural salinity gradient of the Baltic Sea. *Aquatic Toxicology* **82**, 63-71.

Prichard, A.K., Roby, D.D., Bowyer, R.T., Duffy, L.K., 1997. Pigeon guillemots as a sentinel species: a dose–response experiment with weathered oil in the field. *Chemosphere* **35(7)**, 1531–48.

Prusky, A.M. and Dixon, D.R., 2002. Effects of cadmium on nuclear integrity and DNA repair efficiency in the gill cells of *Mytilus edulis* L. *Aquatic Toxicology* **57**, 127-137.

Quinn, B., Gagné, F., Blaise, C., Costello, Wilson, J.G., Mothersill, C, 2006. Evaluation of the lethal and sub-lethal toxicity and potential endocrine disrupting effect of nonylphenol on the zebra mussel (*Dreissena polymorpha*). *Comparative Biochemistry and Physiology* **142C**, 118-127.

Rabilloud, T. (ed.), 2000. *Proteome research: Two-Dimensional Gel Electrophoresis and Identification Methods*. Springer Verlag, Heidelberg, 244 pp.

Regoli, F., Nigro, M., Orlando, E., 1998. Lysosomal and antioxidant responses to metals in the Antarctic scallop *Adamussium colbecki*. *Aquatic Toxicology* **40**, 375-392.

Remacle, J., Lambert, D., Raes, M., Pigeolet, E., Michiels, C., Toussaint, O., 1992. Importance of various antioxidant enzymes for cell stability Confrontation between theoretical and experimental data. Biochemical Journal **286**, 41-46.

Ricciardi, F., Matozzo, V., Marin, M.G., 2008. Effects of 4-nonylphenol exposure in mussels (*Mytilus galloprovincialis*) and crabs (*Carcinus aestuarii*) with particular emphasis on vitellogenin induction. *Marine Pollution Bulletin* **57**, 365-372.

Rodriguez-Ortega, M.J., Grosvik, B.E., Rodriguez-Ariza, A., Goksoyr, A., Lopez-Barea, J., 2003. Changes in protein expression profiles in bivalve molluscs (*Chamaelea gallina*) exposed to four model environmental pollutants. *Proteomics* **3**, 1535-1543.

Romero-Ruiz, A., Carrascal, M., Alhama, J., Gómez-Ariza, J. L., Abian, J., López-Barea, J., 2006. Environmental proteomics studies in clams from the Donaña bank of Guadalquivir Estuary (SW Spain). *Proteomics* **6**, 245-255.

Ruokojärvi, P., Jaana, K., Sirpa, H., Terttu, V., 2004. Mussel incubation in the monitoring of PCDDs, PCDFs, PCBs, PBDEs and PCNs in two Finnish watercources. *Organohalogen Compounds* **66,** 1829-1833.

Samuelsson, M.L., Forlin, L., Karlsson, G., Adolfsson-Erici, M., Larsson, D.G.J., 2006. Using NMR metabolomics to identify responses of an environmental estrogen in blood plasma of fish. *Aquatic Toxicology* **78**,341-349.

Satarug, S., Haswell-Elkins, M.R., Moore, M.R., 2000. Safe levels of cadmium intake to prevent renal toxicity in human subjects. Review article. *The British Journal of Nutrition* **84**, 791-802.

Schiedek, D., Broeg, K., Barsiene, J., Lehtonen, K.K., Gercken, J., Pfeifer, S., Vuontisjarvi, H., Vuorinen, P.J., Dedonyte, V., Koehler, A., Balk, L., Schneider, R., 2006. Biomarker responses as indication of contaminant effects in blue mussel (*Mytilus edulis*) and female eelpout (*Zoarces viviparus*) from the southwestern Baltic Sea. *Marine Pollution Bulletin* **53**, 387–405.

Schirle, M., Heurtier, M.A., Kuster, B., 2003. Profiling core proteomes of human cell lines by one-dimensional PAGE and liquid chromatography-tandem mass spectrometry. *Molecular & Cellular Proteomics* **2**, 1297-1305.

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., Gunten, U.V., Wehrli, B., 2006. The challenge of micropollutants in aquatic systems. *Science* **313**, 1072-1077.

Serafim, A. and Bebianno, M.J., 2007a. Involvement of MT in Zn accumulation and elimination strategies in *Ruditapes decussatus*. *Archives of Environmental Contamination and Toxicology* **52**, 189-199.

Serafim, A. and Bebianno, M.J., 2007b. Kinetic model of cadmium accumulation and elimination and metallothionein response in *Ruditapes decussatus*. *Environmental Toxicology and Chemistry* **26**, 960-969.

Serafim, A. and Bebianno, M.J., 2009. Metallothionein role in the kinetic model of copper accumulation and elimination in the clam *Ruditapes decussatus*. *Environmental Research* **109(4)**, 390-9.

Sheehan, D. and Power, A., 1999. Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. *Comparative Biochemistry and Physiology* **123C**, 193–199.

Shepard, J.L. and Bradley, B.P., 2000. Protein expression signatures and lysosomal stability in *Mytilus edulis* exposed to graded copper concentrations. *Marine Environmental Research* **50**, 457–463.

Shepard, J.L., Olsson, B., Tedengren, M., Bradley, B.P., 2000. Protein expression signatures identified in *Mytilus edulis* exposed to PCBs, copper and salinity stress. *Marine Environmental Research* **50**, 337–340.

Sherman, M.Y. and Goldberg, A.L., 2001. Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron* **29**, 15-32.

Shi, D., Blackmore, G., Wang, W.-X., 2003. Effects of aqueous and dietary preexposure and resulting body burden on silver biokinetics in the green mussel *Perma viridis*. *Environmental Science and Technology* **37**, 936–943.

Shimada, Y., Okuno, S., Kawai, A., Shinomiya, H., Saito, A., Suzuki, M., Omori, Y., Nishino, N., Kanemoto, N., Fujiwara, T., Horie, M., Takahashi, E., 1998. Cloning and chromosomal mapping of a novel ABC transporter gene (hABC7), a candidate for X-linked sideroblastic anemia with spinocerebellar ataxia. *Journal of Human Genetics* **43,** 115-122.

Shin, P.K.S. and Lam, W.K.C., 2001. Development of a marine sediment pollution index. *Environmental Pollution* **113**, 281-291.

Simes, D.C., Bebianno, M. J., Moura, J. J.G., 2003, Isolation and characterisation of metallothionein from the clam *Ruditapes decussatus*. *Aquatic Toxicology* **63**, 307-318.

Simkiss, K., Taylor,M., Mason, A.Z., 1982. Metal detoxification and bioaccumulation in molluscs. *Marine Biology Letters* **3**, 187-201.

Smaoui-Damak, W., Rebai, T., Berthet, B., Hamza-Chaffai, A., 2006. Does cadmium pollution affect reproduction in the clam *Ruditapes decussatus*? A one-year case study. *Comparative Biochemistry and Physiology* **143C**, 252-261.

Smital, T., Sauerborn, R., Hackenberger, B.K., 2003. Inducibility of the P-glycoprotein transport activity in the marine mussel *Mytilus galloprovincialis* and the freshwater mussel *Dreissena polymorpha*. *Aquatic Toxicology* **65**, 443-465.

Snape, J.R., Maund, S.J., Pickford, D.B., Hutchinson, T.H., 2004. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *Aquatic Toxicology* **67**, 143–154.

Sokolova, I.M., 2004. Cadmium effects on mitochondrial function are enhanced by elevated temperatures in a marine poikilotherm, *Crassostrea virginica* (Bivalvia: Ostreidae). *The Journal of Experimental Biology* **207**, 2639-48.

Spencer, J.P.E., Jenner, A., Aruoma, O.I., Cross, C.E., Wu, R., Halliwell, B., 1996. Oxidative DNA damage in human respiratory tract epithelial cells. Time of course in relation to DNA strand breakage. *Biochemical and Biophysical Research Communications* **224**, 17-22.

Stagg, R.M., 1998. The development of an international programme for monitoring the biological effects of contaminants in the OSPAR Convention area. *Marine Environmental Research* **46**, 307-313.

Staniek, K. and Nohl, H., 1999. H_2O_2 detection from intact mitochondria as a measure for one-electron reduction of dioxygen requires a non-invasive assay system. *Biochimica et Biophysica Acta* **1413**, 70-80.

Staniek, K. and Nohl H., 2000. Are mitochondria a permanent source of reactive oxygen species? *Biochimica et Biophysica Acta* **1460**, 268-75.

Staples, C., Mihaich, E., Carbone, J., Woodbrun, K., Klecka, G., 2004. A weight of evidence analysis of the chronic ecotoxicity of nonylphenol ethoxylates, nonylphenol ether carboxylates, and nonylphenol. *Human Ecology Risk Assessment* **10**, 999-1017.

Starita-Geribaldi, M., Roux, F., Garin, J., Chevallier, D., Fénichel, P., Pointis, G., 2003. Development of narrow immobilized pH gradients covering one pH unit for human seminal plasma proteomic analysis. *Proteomics* **3**, 1611–1619.

Stegeman, J.J., Brouwer, M., Richard, T.D.G., Förlin, L., Fowler, B.A., Sanders, B.M., van Veld, P.A., 1992. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. *In*: Huggett, R.J., Kimerly, R.A., Mehrle, P.M., Jr, Bergman, H.L. (Eds.), Biomarkers: Biochemical, Physiological and Histological markers of Anthropogenic Stress. Lewis Publishers, Chelsea, MI, USA, pp. 235-335.

Stegeman, J.J., Hahn, M.E., 1994. Biochemistry and molecular biology of monooxygenase: current perspective on forms, functions, and regulation of cytochrome P450 in aquatic species. *In*: Malins, D.C., Ostrander, G.K. (Eds.), *Aquatic toxicology; Molecular, Biochemical and Cellular Perspectives*. Lewis Publishers, CRC press, Boca Raton, pp. 87-206.

Stohs, S.J., Bagchi, D., Hassoun, E. and Bagchi, M., 2001. Oxidative mechanisms in the toxicity of chromium and cadmium ions. *Journal of Environmental Pathology, Toxicology and Oncology* **20**, 77-88.

Tanguy, A., Bierne, N., Saavedra, C., Pina, B., Bachère, E., Kube, M., Bazin, E., Bonhomme, F., Boudry, P., Boulo, V., Boutet, I., Cancela, L., Dossat, C., Favrel, P., Huvet, A., Jarque, S., Jollivet, D., Klages, S., Lapègue, S., Leite, R., Moal, J., Moraga, D., Reinhardt, R., Samain, J.F., Zouros, E., Canário, A., 2008. Increasing genomic information in bivalves through new EST collections in four species: Development of new genetic markers for environmental studies and genome evolution. *Gene* **408(1-2)**, 27-36.

Taris, N., Lang, R.P., Camara, M.D., 2008. Sequence polymorphism can produce serious artefacts in real-time PCR assays: hard lessons from Pacific oysters. *BMC Genomics* **9**, 234.

Tedengren, M., Olsson, B., Bradley, B. P., and Zhou, L., 1999. Heavy metal uptake, physiological response and survival of the blue mussel (*Mytilus edulis*) from marine and brackish waters in relation to the induction of heat-shock protein 70. *Hydrobiologia* **393**, 261–269.

Tedesco, S., Doyle, H., Redmond, G., Sheehan, D., 2008. Gold nanoparticles and oxidative stress in *Mytilus edulis*. *Marine Environmental Research* **66**, 131-133.

Thuvander, A., 1989. Cadmium exposure of rainbow trout, *Salmo gairdneri* Richardson: effects on immune functions. *Journal of Fish Biology* **35**, 521-529

Tilton, S.C., Givan, S.A., Pereira, C.B., Bailey, G.S., Williams, D.E., 2006. Toxicogenomic profiling of the hepatic tumor promoters indole-3-carbinol, 17beta-estradiol and beta-naphthoflavone in rainbow trout. *Toxicological Sciences* **90**, 61–72.

Timbrell, J., 2001 (ed.). Principles of Biochemical Toxicology. Taylor and Francis, London.

Trethewey, R.N., Krotzky, A.J., Willmitzer, L., 1999. Metabolic profiling: a Rosetta Stone for genomics? *Current opinion in plant biology* **2**, 83–85.

Trethewey, R.N., 2004. Metabolite profiling as an aid to metabolic engineering in plants. *Current Opinion in Plant Biology* **7**, 196-201.

Tweeddale, H., Notley-McRobb, L., Ferenci, T., 1998. Effect of slow growth on metabolism of *Escherichia coli*, as revealed by global metabolite pool ("Metabolome") analysis. *Journal of Bacteriology* **180**, 5109–5116.

UNEP (United Nations Environmental Programme), 2003. Global report: regionally based assessment of persistent toxic substances. UNEP chemicals.

UNEP/RAMOGE, 1999. Manual on the Biomarkers Recommended for the MED POL Biomonitoring Programme. UNEP, Athens.

Unlu, M., Morgan, M.E., Minden, J.S., 1997. Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. *Electrophoresis* **18**, 2071-2077.

Van der Oost, R., Opperhuizen, A., Satumalay, K., Heida, H., Vermeulen, N.P.E., 1996. Biomonitoring aquatic pollution with feral eel (*Anguilla anguilla*): I. Bioaccumulation: biota-sedimentratios of PCBs, OCPs, PCDDs and PCDFs. *Aquatic Toxicology* **35**, 21-46.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk. *Environmental Toxicology and Pharmacology* **13**, 57-149.

Vazquez-Duhalt, R., Marquez-Rocha, F., Licea, A.F., Viana, M.T., 2005. Nonyphenol, an integrated vision of a pollutant. *Applied Ecology and Environmental Research* **4**, 1-25.

Venier, P., De Pitta, C., Pallavicini, A., Marsano, F., Varotto, L., Romuali, C., Dondero, F., Viarengo, A., and Lanfranchi, G., 2006. Development of mussel mRNA profiling: can gene expression trends reveal coastal water pollution? *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **602 (1-2)**, 121–134.

Vermeulen, N.P.E., 1996. Role of metabolism in chemical toxicity. *In*: *Ioannides, C. (Ed.), Cytochromes P450: Metabolic and Toxicological Aspects*. CRC Press, Boca Raton, FL, USA, pp. 29-53.

Viarengo, A., 1989. Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. *Critical Reviews in Aquatic Sciences* **1**, 295–317.

Viarengo, A., Nott, J.A., 1993. Mechanism of heavy metal cation homeostasis in marine invertebrates. *Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology and Endocrinology* **104**, 355-372.

Viarengo, A., Burlando, B., Cavaletto, M., Marchi, B., Ponzano, E., Blasco, J., 1999. Role of metallothionein against oxidative stress in the mussel *Mytilus galloprovincialis*. *The American Journal of Physiology* **277**, R1612-1619.

Viarengo, A., Burlando, B., Ceratto, N., Panfoli, I., 2000. Antioxidant role of metallothioneins: a comparative overview. *Cellular and Molecular Biology* **46**, 407-417.

Waalkes, M.P. and R.R. Misra, 1996. Cadmium carcinogenicity and genotoxicity. *In Toxicology of Metals* (L. W. Chang, Ed.), pp. 231–244. CRC Press, Boca Raton, FL.

Wang, C.G., Zheng, R.H., Ding, X., Zuo, Z.H., Zhao, Y., Chen, Y.X., 2005. Effect of tributyltin, benzo[a]pyrene, and their mixture on the hepatic monooxygenase system in *Sebastiscus marmoratus*. *Bulletin of Environmental Contamination and Toxicology* **75**, 1214–9.

Wang, H. and Hanash, S., 2003. Multi-dimensional liquid phase based separations in proteomics. *Journal of Chromatography B* **787**, 11-18.

Washburn, M.P., Wolters, D., Yates, J.R. III, 2001. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nature Biotechnology* ***19***, 242-247.

Watanabe, M., Henmi, K., Ogawa, K. and Suzuki, T., 2003. Cadmium-dependent generation of reactive oxygen species and mitochondrial DNA breaks in photosynthetic and non-photosynthetic stains of *Euglena gracilis*. *Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology and Endocrinology* **134**, 227-234.

WHO, 1993. Guidelines for Drinking-Water Quality. Vol. 1. Recommendations. 2nd ed. WHO, Geneva, pp. 30–44.

Wilkins, M.R., Sanchez, J.-C., Williams, K.L., Hochstrasser, D.F., 1996. Current challenges and future applications for protein maps and post-translational vector maps in proteome projects. *Electrophoresis* **17**, 830–838.

Williams, T.D., Diab, A.M., George, S.G., Godfrey, R.E., Sabine, V., Conesa, A., Minchin, S.D., Watts, P.C., Chipman, J.K., 2006. Development of the GENIPOL European Flounder (*Platichthys flesus*) microarray and determination of temporal transcriptional responses to cadmium at low dose. *Environmental Science & Technology* **40**, 6479–6488.

Williams, T.D., Diab, A., Ortega, F., Sabine, V.S., Godfrey, R.E., Falciani, F., Chipman, J.K., George, S.G., 2008. Transcriptomic responses of European flounder (*Platichthys flesus*) to model toxicants. *Aquatic Toxicology* **90(2)**, 83-91.

Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., Mann, M., 1996. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379**, 466-469.

Winzer, K., 2001. Oxidative stress in the marine environment prognostic tools for toxic injury in fish liver cells. Academic Thesis, University of Amsterdam.

Wittke, S., Kaiser, T., Mischak, H., 2004. Differential polypeptide display: the search for the elusive target. *Journal of Chromatography B* **803**, 17-26.

Wulfkuhle, J.D., McLean, K.C., Paweletz, C.P., Sgroi, D.C., Trock, B.J., Steeg, P.S., Petricoin, E.F., 2001. New approaches to proteomic analysis of breast cancer. *Proteomics* **1**, 1205–1215.

Xia, K. and Chang, Y.J., 2004. Potodegradation of the Endocrine-Disrupting Chemical 4-Nonylphenol in Biosolids Applied to Soil. *Journal of Environmental Quality* **33,** 1568-1574.

Yamada, H., Miyahara, T. and Sasaki, Y., 1993. Inorganic cadmium increases the frequency of chemically induced chromosome aberrations in cultured mammalian cells. *Mutation Research* **302(3)**, 137-145.

Yergey, A.L., Coorssen, J.R., Backlund, P.S. Jr., Blank, P.S., Humphrey, G.A., Zimmerberg, J., Campbell, J.M., Vestal, M.L., 2002. *De novo* sequencing of peptides using MALDI/TOF-TOF. *Journal of the American Society for Mass Spectrometry* **13**, 784-791.

Zapata, M., Tanguy, A., David, E., Moraga, D., Riquelme, C., 2009. Transcriptomic response of *Argopecten purpuratus* post-larvae to copper exposure under experimental conditions. *Gene* **442**, 37–46.

Zhong, Z., Troll, W., Koenig, K.L. and Frenkel, K., 1990. Carcinogenic sulfide salts of nickel and cadmium induce H_2O_2 formation by human polymorphonuclear leukocytes. *Cancer Research* **50**, 7570.

Zorita I, Apraiz I, Ortiz-Zarragoitia M, Orbea A, Cancio I, Soto M, Marigomez, I., Cajaraville, M.P., 2007 Assessment of biological effects of environmental pollution along the NW Mediterranean Sea using mussels as sentinel organisms. *Environmental Pollution* **148**, 236–50.

Chapter 2.

Methodology

2.1. Exposure experiments

Clams were collected in the Ria Formosa Lagoon (southern coast of Portugal) (Figure 2.1).

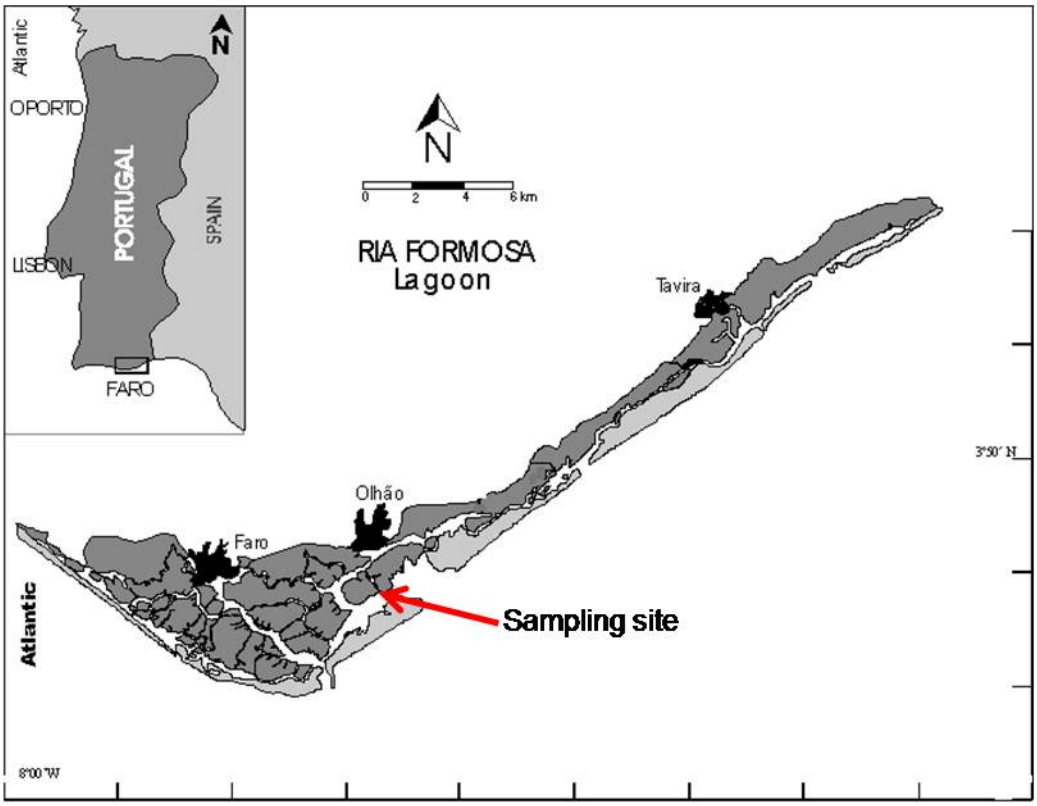

Figure 2.1 – Sampling site – Ria Formosa Lagoon, south of Portugal (adapted from Barreira *et al.*, 2007).

In the laboratory, clams were divided into six groups of 25 clams each (36.1 ± 1.6 mm), and kept in 25 L glass aquaria filled with continuously aerated seawater (35 psu, 18.0 ± 0.5 ºC). Following one week of acclimation, two groups were exposed to cadmium (Cd) (40 µg.l^{-1}) (stock solution 560 µg.mL^{-1}), two groups were exposed to nonylphenol (NP) (100 µg.l^{-1}) (stock solution of 1.4 mg NP.L^{-1} prepared in dimethyl sulfoxide, DMSO) whereas the remaining two groups were kept in seawater, as controls. Water was changed every other day providing clams with natural food,

avoiding starvation and any effect resulting from the food content. Cadmium and NP concentrations were re-established following each seawater change. Figure 2.2 summarizes the exposure experiment conducted mentioned above.

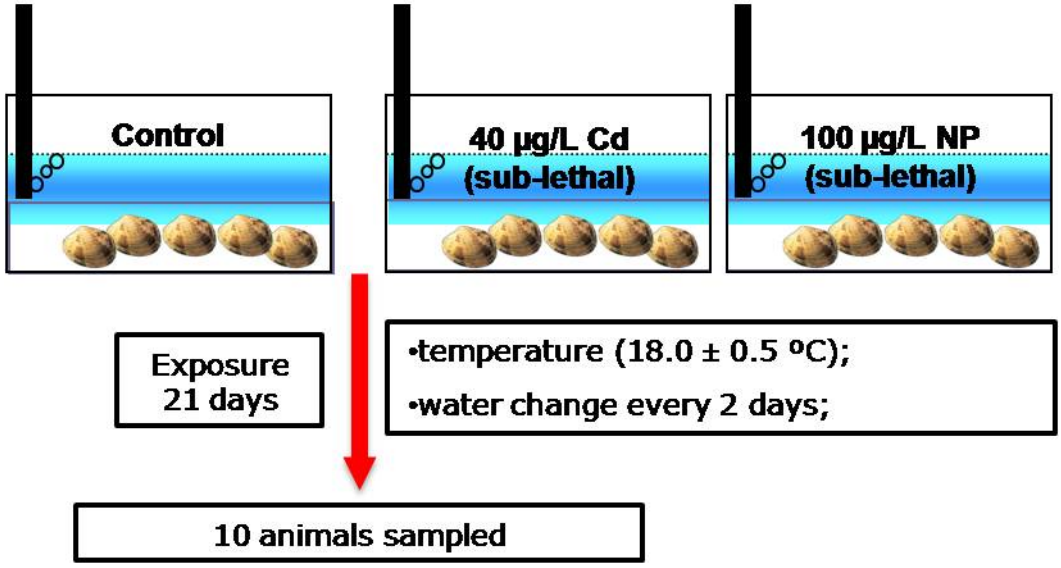

Figure 2.2 – Scheme illustrating the laboratory exposure experiments.

The exposures lasted 21 days and at the end of the experiment five controls and five exposed animals were collected from each condition, measured and dissected into gills and digestive gland. The two tissues were immediately frozen in liquid nitrogen and stored at - 80 °C.

2.2. Two-dimensional electrophoresis (2DE SDS-PAGE) protocol optimization

The two-dimensional electrophoresis allows to resolve several thousands of proteins from a sample on a 2DE polyacrylamide slab gel and is also a well-established, relatively easy to execute methodology (Wang and Hanash, 2003; Gorg *et al*., 2004). For this reason, in the present thesis, this methodology was selected for protein separation. The application of standard two-dimensional electrophoresis (2DE SDS-

PAGE) protocols developed for mammalian tissues proved not to be suited for marine bivalves, mainly due to the excess of salt water present and the lipid content of some of particular tissues. Salt interferes with the electrophoretic process because it migrates through the pH gradient and tends to accumulate at both sides of the immobilized pH gradient (IPG) strip, generating high conductivity zones. In these zones, the voltage drop and the electric field are very low, increasing the time required for the isoelectric focusing (IEF). Consequently, proteins are not well focused originating streaks or empty regions. Additionally, water movement can also occur causing one end of the strip to dry and the other end to swell. Several techniques could be used to remove salts or maintain them at low concentrations: dialysis, spin dialysis, gel filtration and precipitation (Rabilloud, 2000). The association of proteins and lipids, generally found in membranes, reduces the proteins solubility and affects isoelectric point (p*I*) and molecular mass (M*r*), thus affecting the electrophoresis process. Moreover, lipids can form complexes with detergents, decreasing the efficiency of detergents in protein solubilisation. The use of cold acetone in the precipitation process and the addition of high detergent content to the rehydration buffer minimizes protein-lipid interactions and helps removing some lipids. Furthermore, the TCA (trichloracetic acid)/acetone precipitation helps in the inactivation of proteases, minimizing protein degradation.

Several laboratory trials were conducted to optimize a 2DE SDS-PAGE protocol suitable to be applied to the clam *Ruditapes decussatus* gill and digestive gland. These trials were developed first using small gels and 7 cm strips, pH 3-10 (immobiline ® DryStrip, GE Healthcare) loaded with 80 μg of protein content of clam gill and digestive gland. Initially, in order to choose a suitable desalting

technique, dialysis and precipitation processes were tested. Since precipitation showed to be an efficient process that does not require large volumes of solution and is not time-consuming, *R. decussatus* samples were desalted by trichloroacetic acid (TCA) precipitation. Cold acetone was also used in this step for helping lipid removal.

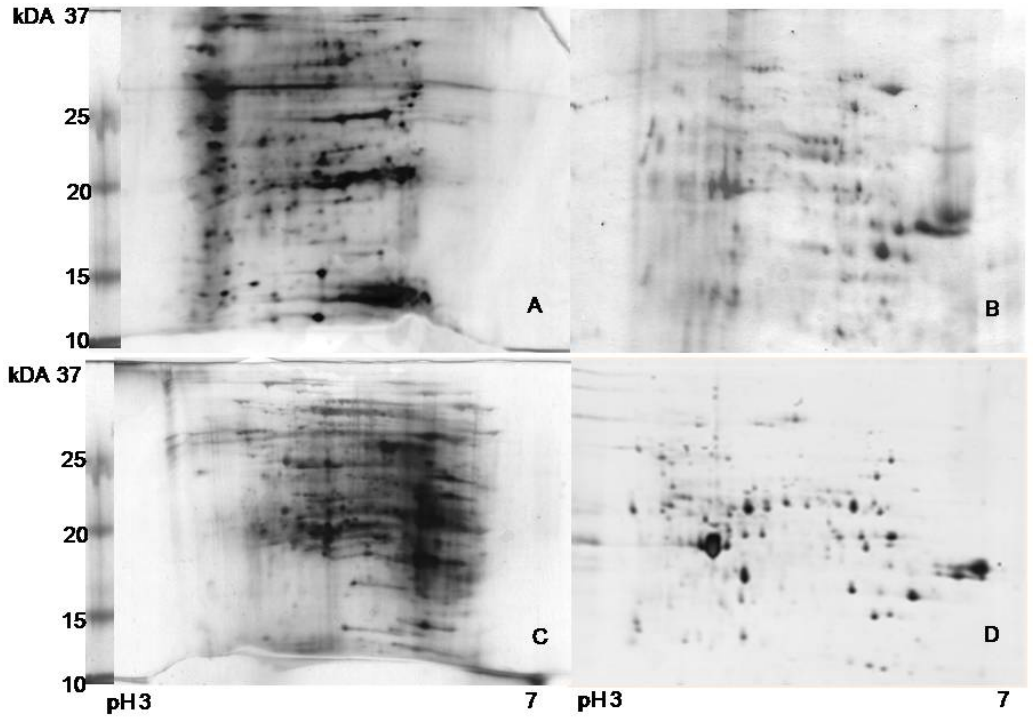

Figure 2.3 – 2DE 7 cm gels, loaded with 80 µg of protein content. Gill (A) and digestive gland (C) gels before 2DE SDS-PAGE protocol optimization; gill (B) and digestive gland (D) gels after 2DE SDS-PAGE protocol optimization.

Additionally, the rehydration buffer composition was tested several times. Its composition was modified, mainly by increasing the detergent content (CHAPS, 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate) in order to eliminate the lipids, until obtaining 2DE gels with spots well differentiated. To improve protein solubilization, thiourea and carrier ampholytes were included in rehydration buffer composition. The IEF program was then optimized for this size gels.

Afterwards, in order to obtain a better resolution, proteins were loaded in 18 cm Immobiline ® DryStrip (GE Healthcare), in the pH 4-7 range that enclose most of the spots of interest. Consequently, the IEF program was again optimized for this size gels (Figure 2.4).

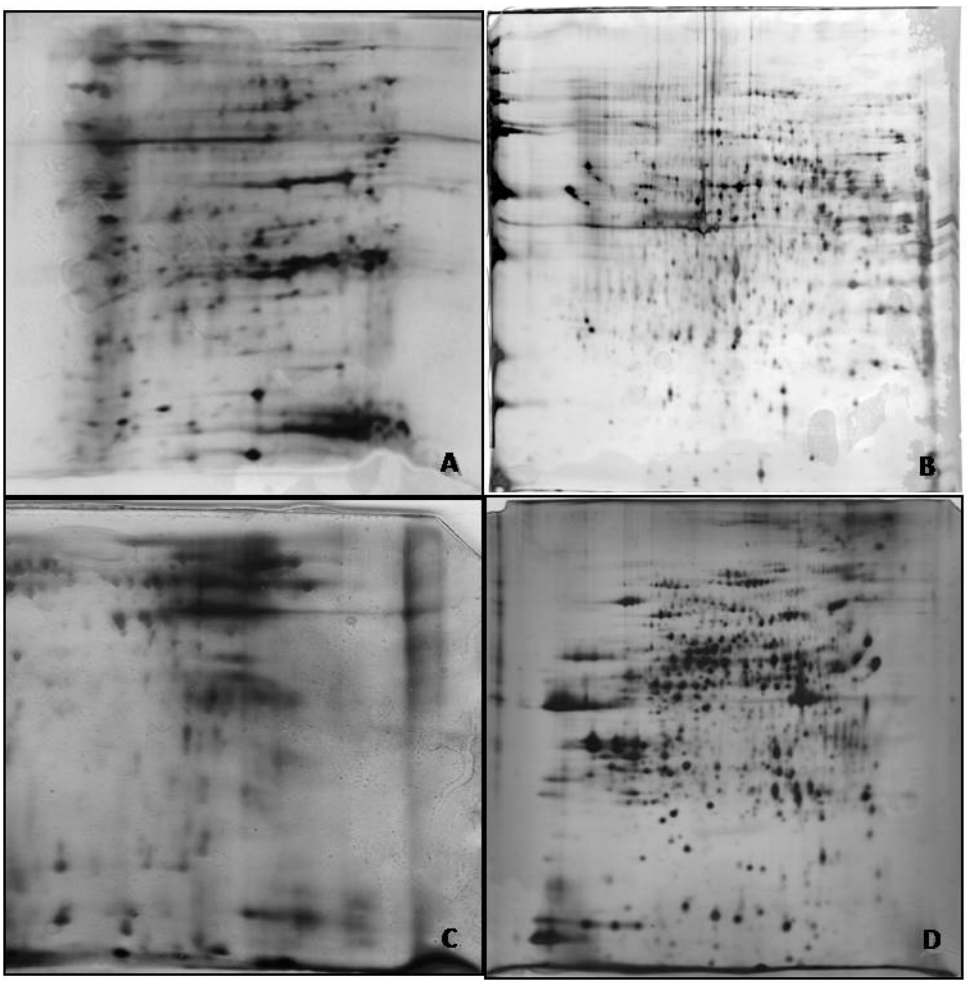

Figure 2.4 – Images of 2DE 18 cm gels, loaded with 150 μg of protein content. Gill (A) and digestive gland (C) gels before 2DE SDS-PAGE protocol optimization; gill (B) and digestive gland (D) gels after protocol optimization.

All these tests were essential to guarantee gel reproducibility that is essential to a good image analyses (Mi, 2005; Nesaty and Suter, 2008). The final 2DE optimized protocol for bivalve species is presented below.

2.2.1. Final protocol

Analysis of Proteins from Marine Molluscs

S. Chora, M.J. Bebianno and M. Roméo

***In*: D. Shehan and R.Tyther (eds.), Methods in Molecular Biology, Two-dimensional electrophoresis protocols, vol. 519. Humana Press, New York, 197-204 (2009).**

Chapter 12

Analysis of Proteins from Marine Molluscs

Suze Chora, Maria João Bebianno, and Michèle Roméo

Summary

Application of the two-dimensional gel electrophoresis (2DE) protocols which were developed for samples of mammalian origin gives unsatisfactory results when used in samples from marine molluscs. This chapter describes a detailed protocol of 2DE that can be applied to these organisms, especially for *Ruditapes decussatus* and *Bathymodiolus azoricus*.

Key words: "Two-dimensional electrophoresis (2DE)", Marine bivalves, *Ruditapes decussatus*, *Bathymodiolus azoricus*.

1. Introduction

Marine molluscs, particularly mussels, oysters, and clams, have been used worldwide as bioindicators to assess the impact of pollutants in coastal marine ecosystems and their health status. More recently, the discovery in 1997 of large mussel beds in deep sea hydrothermal vents *(1)*, particularly in the Mid-Atlantic Ridge, has attracted much scientific attention due to their capacity to live in one of the most extreme environments on Earth characterized by high temperature and pressure, low pH, enriched in toxic sulphide species *(2)*, radionuclides, and naturally high bioavailable metal concentrations that would be toxic or even lethal to coastal marine species *(3, 4)*. Therefore these species have been considered as models to assess pollution effects in natural contaminated environments *(5)*.

The isolation of proteins from marine bivalves from coastal or extreme environments, like deep sea hydrothermal vents, using two-dimensional electrophoresis (2DE) was proved not to be straightforward when using standard protocols developed for

David Sheehan and Raymond Tyther (eds.), *Methods in Molecular Biology, Two-Dimensional Electrophoresis Protocols, vol. 519*

DOI: 10.1007/978-1-59745-281-6_12

mammalian tissues mainly due to the excess of salt water and the lipid content of some of particular tissues studied. Recently, 2DE protocols were developed for bivalve species in particularly for the coastal clam *Ruditapes decussatus* and the deep sea hydrothermal vent mussel *Bathymodiolus azoricus*. Two tissues were used: gills and digestive gland. 2DE gels (18 × 18 cm) were able to separate more than 2,000 proteins.

Although the genome of these species is unknown, a number of proteins have already been sequenced. Some relevant proteins were separated from the spots and some of them sequenced. In bivalves, gill and digestive gland tissues usually do not provide good resolution 2DE gels because they may contain exogenous proteins. To overcome this problem in this protocol we describe the best technique for protein separation using 2DE.

2. Materials

2.1. Equipment

IKA, model Ultra-Turrax TD 25, Ettan IPGphor II (GE Healthcare), Protean II xi cell (Bio-Rad, Labs). Vertical system.

2.2. Solutions and Reagents

1. *Homogenization buffer*: 10 mM HEPES and 250 mM Sucrose, 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM PMSF, 10% protease inhibitors (protease inhibitor tablets, Sigma, Aldrich). DTT, PMSF, and protease inhibitors are added just prior to use. This can be stored at 4°C for 1 week.
2. *Precipitation solution*: 10% trichloroacetic acid (TCA) in acetone containing 20 mM DTT. DTT is added just prior to use. This can be stored at 4°C for 1 week.
3. *Sample dilution buffer*: 7 M urea (GE Healthcare), 2 M thiourea (GE Healthcare), amberlite MB-150 1% (Sigma, Aldrich) *(6)*. Filter solution through 0.20-µm filter. Add 4% CHAPS (GE Healthcare), 0.8% Pharmalyte (GE Healthcare), 65 mM DTT (GE Healthcare), 10% isopropanol *(6)*, and a few grains of bromophenol blue (BPB). Store in 2.5 mL aliquots at −70°C for 2 months.
4. *SDS equilibration buffer*: urea 6 M, Tris–HCl, pH 8.8, 75 mM, glycerol 3%, SDS 4%, and a few grains of bromophenol blue (BPB). Store aliquoted. Can be stored at −20°C for 2 months (*see* **Note 1**).
5. *Electrophoresis buffer*: SDS: 0.1%, Tris base: 25 mM, Glycine: 192 mM. Store at room temperature.
6. *10% SDS solution*: Filter solution through a 0.45-µm filter and store at room temperature.

7. *10% ammonium persulphate solution*: Prepare just prior to use.
8. *Agarose sealing solution*: 0.5% agarose. Add all ingredients into a 500 Erlenmeyer flask. Swirl to disperse. Heat on a heating stirrer until the agarose is completely dissolved (*see* **Note 2**). Dispense 2 mL aliquots and store at room temperature.
9. *EDTA 500 mM*: store at room temperature.
10. Immobiline DryStrip, pH 3–10 NL, 18 cm (GE Healthcare), immobilized pH-gradient isoelectric focusing gels for the first-dimension separation step.

2.3. Software

PdQuest (Bio-Rad, Laboratories, Hercules, CA).

3. Methods

3.1. Preparation of Animals

In contamination experiments animals should be acclimated in clean sea water for 7 days prior to the beginning of the experiment *(7)* (*see* **Note 3**).

3.2. Protein Sample Preparation

1. Dissect tissues at 4°C, using a scalpel cooled by dipping periodically into liquid nitrogen. It is convenient to use a pair of thin cotton gloves during these procedures to avoid transferring heat to the samples.
2. Collect tissues in 5-mL screw-capped plastic tubes on liquid nitrogen.
3. When exposed to the cold, the tissues immediately freeze and can subsequently be stored at –70°C or below (*see* **Note 4**).

3.3. Total Protein Extraction

Having obtained clean samples, it is very important to mince them effectively. Failure to do so will result in selective extraction of protein, which will distort the results of the experiment (*see* **Note 5**).

1. Suspend each sample in three volumes of homogenization buffer and homogenize in 15-mL tubes, using an Ultra-Turrax IKA-Werke homogenizer at 4°C in a cold room, on ice (*see* **Note 6** and Chapter "Difficult Proteins").
2. Centrifuge the homogenate at 15,000 × *g* for 2 h at 4°C.
3. Transfer the supernatant into 2.5-mL Eppendorf tubes for subsequent quantification of sample protein (*see* **Subheading 3.6**).
4. Prepare aliquots of sample so that the final quantity of protein is 300 µg.

3.4. Precipitation Procedure

Protein precipitation is an optional step in sample preparation for 2DE. Precipitation is generally employed selectively to separate proteins in the sample from contaminating species such as salts, detergents, nucleic acids, lipids, etc. that might otherwise interfere with the 2DE result *(8)*.

1. Suspend each sample in nine volumes of precipitation solution (*see* **Note 7**).
2. Precipitate proteins for at least 2 h at –20°C.
3. Pellet proteins by centrifugation at 10,000 × *g* for 30 min.
4. Wash pellet with cold acetone (*see* **Note 8**).
5. Remove residual acetone by air drying.

3.5. Protein Solubilization

1. Resuspend the precipitate in the sample dilution buffer. Use 300 µg in 300 µL, vortex, and leave for 30 min.
2. Centrifuge at 14,000 × *g* for 10 min (*see* **Note 9**).

3.6. Quantification of Sample Protein

Quantification of the proteins concentration was carried out using ovalbumin as a protein standard using the Bradford method *(9)*.

3.7. Isoelectric Focusing

Isoelectric focusing is an electrophoretic method that separates proteins according to their isoelectric points (pI). For this separation wide-range immobilized pH-gradient (IPG) gels, with pH values ranging from 3 to 10, in 18-cm length strips, were used (*see* **Note 10**).

About 300 µg of total extractable protein can be resolved by IEF on immobiline ® DryStrip, pH 3–10, NL, 18 cm (Pharmacia Biotech).:

1. Pipette the samples (prepared as described in the **Subheading3.5**) into each strip holder. Distribute the solution evenly over the channel length and remove any large bubbles.
2. Carefully remove the cover foil from the Immobiline DryStrip, starting from the anodic end (+ end).
3. Carefully place the Immobiline DryStrip into the holder channel, gel-side down (*see* **Note 11**).
4. Overlay the strip with Immobiline DryStrip Cover Fluid to minimize evaporation and prevent urea crystallization.
5. Apply the pressure blocks on the underside of the cover to ensure that the Immobiline DryStrip gel maintains good contact with the electrodes as the gel swells.
6. Ensure that the strip holders are properly positioned on the Ettan IPGphor II platform. Use the guide marks along the sides of the platform to position each strip holder and check that the pointed end is over the anode (pointing to the back of the unit) and the blunt end is over the cathode. (Please refer

to the Ettan IPGphor II user manual for complete details). Check that both external electrode contacts on the underside of each Strip Holder make metal-to-metal contact with the platform.

7. Close the Ettan IPGphor II lid. Program the first step of Ettan IPGphor II to make an active rehydration during 12 h under low voltage (20 V) at low current (50 µA/IPG strip), at 20°C (*see* **Note 12**).
8. Program the other steps of the IEF: 1,000 V, 1 h; 4,000 V, 1 h; 8,000 V, 1 h, and 8,000 V, until 50,000 V (~5 h) (*see* **Note 13**).
9. After IEF is complete, proceed to the second-dimension separation immediately or store the Immobiline DryStrip gels at –60°C or below in screw-capped tubes.

3.8. Second-Dimension SDS–PAGE

In the second-dimension separation, where proteins are separated by molecular weight, traditional SDS–PAGE is employed.

3.8.1. Equilibrating Immobiline DryStrip Gels

1. Place the IPG strips in individual tubes, with the support film towards the tube wall.
2. Add 5 mL of SDS equilibration buffer, 100 mg DTT per strip, 0.5 µL EDTA.
3. Cap or seal the tubes with flexible paraffin film and place them on their sides on a rocker for the equilibration process. Equilibrate for 15 min.
4. Pour off buffer from earlier step and add the appropriate volume of SDS equilibration buffer, 250 mg iodoacetamide, 0.5 µL EDTA to each strip. Again cap or seal the tubes with flexible paraffin film and place them on their sides on a rocker for the equilibration process. Equilibrate for an additional 15 min (*see* **Note 14**).

3.8.2. Electrophoresis Using a Vertical Electrophoresis System

Cast the 10% polyacrylamide gels using 30% Acrylamide/Bis solution (Bio-Rad Laboratories, Hercules, CA) in 1-mm cassettes (*see* **Note 15**).

1. Place the strip with the acidic end to the left, gel surface up onto the protruding edge of the longer glass plate.
2. With a thin plastic ruler, gently push the Immobiline dry strip gel down so that the entire lower edge of the Immobiline DryStrip gel is in contact with the top surface of the gel (*see* **Notes 16** and **17**).
3. Seal the Immobiline DryStrip gel in place with the agarose sealing solution (*see* **Note 18**).
4. Insert the cassettes into the tank and pour the electrophoresis buffer to the fill line.
5. Close the lid and connect the power leads to the power supply.

6. Electrophoresis is performed at constant current (90 A) in two steps. During the initial migration and stacking period (15 min), the current is 80 V, and during the separation we apply 200 V (*see* **Note 19**).
7. Stop electrophoresis when the dye front is approximately 1 mm from the bottom of the gel (*see* **Note 20**).

4. Notes

1. This is a stock solution. Just prior to use, add DTT or iodoacetamide, for first and second equilibration, respectively, and the EDTA 0.1 mM.
2. Do not allow the solution to boil over.
3. The animals should have the same size to ensure an equal sample.
4. It is very important that the tissues never defrost between collection and solubilization in electrophoresis buffer in order to avoid proteolysis.
5. Direct grinding of frozen tissues in liquid nitrogen does not result in a sufficiently fine mincing for efficient extraction.
6. Start 30 s with a low speed and two times 15 s in a high speed. In this step the homogenate can be frozen at –70°C.
7. This approach limits proteolysis and other protein modifications. Protease inhibitors were found to be efficient enough to stop the protease activities in the sample preparation process. A TCA–acetone treatment was employed in addition to PMSF where acid and organic solvent denature almost all proteins including proteases.
8. Residual TCA must be removed by extensive washing with acetone because extended exposure to this low pH solution may cause some protein degradation or modification.
9. This step helps to precipitate remaining salts.
10. These varied pH intervals allow fine-tuning of each separation strategy to increase first-dimension loading and resolve a greater number of spots from crowded areas, and they are available with strip lengths of 7, 11, 13, 18, and 24 cm. Choose shorter strips, i.e. up to 13 cm, for fast, cost-effective screening, or when the most abundant proteins are of highest interest. The shortest IPG strips give the fastest results, but their sample load is limited. Use longer strips, i.e. 18- and 24-cm strips, for maximal resolution and loading capacity. Longer strips allow detection of more spots and make it easier

to select and identify the proteins in the map, but require longer times in both the first- and the second-dimension separations.

11. To help coat the entire gel, gently lift and lower the strip and slide it back and forth along the surface of the solution. Be careful not to trap bubbles under the Immobiline dry strip gel.
12. The active rehydration facilitates the entry of high-molecular-weight proteins into the strips.
13. Many factors affect the amount of time required for complete focusing, and each specific set of conditions, e.g. sample and rehydration solution composition, Immobiline dry strip gel length, and pH gradient. Ramping the voltage slowly while the sample is entering the IPG strip improve results.
14. Be consistent with the timing of the equilibration steps.
15. The composition of this gel should be selected to resolve proteins in the MW range of interest. Thinner gels stain and destain more quickly and generally give less background staining. Thicker gels have a higher protein capacity. Thicker gels are also less fragile and easier to handle.
16. Ensure that no air bubbles are trapped between the Immobiline drystrip gel and the slab gel surface.
17. The MW marker proteins can be applied to a paper piece; then pick up the application piece with forceps and apply to the top surface of the gel next to one end of the Immobiline DryStrip gel.
18. The agarose sealing solution prevents the Immobiline dry strip gel from moving or floating in the electrophoresis buffer.
19. For these vertical systems, cooling is optional. However, temperature control improves gel-to-gel reproducibility, especially if the ambient temperature of the laboratory fluctuates significantly. For best results, gels should be run at 25°C.
20. After this step silver staining is the most sensitive staining technique (*see* Chapter "Silver Staining of Proteins in 2DE Gels"). For protein identification we use PDQuest, Bio-Rad, Laboratories, Hercules, CA.

References

1. Edmond, J.M., Measures, C., Magum, B.M., Grant, B., Sclater, F.R., et al. (1979) On the formation of metal-rich deposits at ridge crests. *Earth and Planetary Science Letter* 46, 19–30
2. Blum, J., and Fridovich, I. (1984) Enzymatic defenses against oxygen toxicity in the hydrothermal vent animals *Riftia pachyptila* and *Calyptogena magnifica*. *Archives of Biochemistry and Biophysics* 228, 617–620
3. Desbruyères, D., Biscoito, M., Caprais, J.C., Colaço, A., Comtet, T., Crassous, P., et al. (2001) Variations in deep-sea hydrothermal vent communities on the mid-atlantic ridge near the Azores plateau. *Deep Sea Research Part I: Oceanographic Research Paper* 48, 1325–1346

4. Géret, F., Rainglet, F., and Cosson, R.P. (1998) Comparison between isolation protocols commonly used for the purification of mollusc metallothioneins. *Marine Environmental Research* 46, 545–550
5. Bebianno, M.J., Company, R., Serafim, A., Camus, L., Cosson, R., and Fiala-Médoni, A. (2005) Antioxidant systems and lipid peroxidation in *Bathymodiolus Azoricus* from mid Atlantic ridge hydrothermal vent fields. *Aquatic Toxicology* 75, 354–373
6. Starita-Geribaldi, M., Roux, F., Garin, J., Chevallier, D., Fénichel, P., and Pointis, G., (2003) Development of narrow immobilized pH gradients covering one pH unit for human seminal plasma proteomic analysis. *Proteomics* 3, 1611–1619
7. Coelho, M. R., Bebianno, M.J., and Langston, W.J. (2002). Routes of TBT uptake in the clam *Ruditapes decussates* – Food as vector of TBT uptake. *Marine Environmental Research* 54, 193–207
8. Bjellqvist, B., Laird, N., McDowell, M., Olsson, I., and Westermeier, R. (1998). *2-D Electrophoresis, Principles and Methods.* Amersham-Biosciences 100 pp
9. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248–254

2.3. References

Barreira, L., Mudge, S. M.; Bebianno, M. J., 2007. Oxidative stress in the clam *Ruditapes decussatus* (Linnaeus, 1758) in relation to polycyclic aromatic hydrocarbon body burden. *Environmental Toxicology* **22**, 203-21.

Görg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., Weiss, W., 2000. The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* **21**, 1037-1053.

Mi, J., Orbea, A., Syme, N., Ahmed, M., Cajaraville, M.P., Cristobal, S., 2005, Peroxisomal proteomics, a new tool for risk assessment of peroxisome proliferating pollutants in the marine environment. *Proteomics* **5**, 3954-3965.

Nesatyy, V.J. and Suter, M.J.F., 2008. Analysis of environmental stress response on the proteome level. *Mass Spectrometry Reviews* **27**, 556-574.

Rabilloud, T. (ed.), 2000. *Proteome research: Two-Dimensional Gel Electrophoresis and Identification Methods*. Springer Verlag, Heidelberg, 244 pp.

Wang, H. and Hanash, S., 2003. Multi-dimensional liquid phase based separations in proteomics. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* **787**, 11-18.

Chapter 3.

Protein expression signatures (PESs) and single protein markers in R. decussatus

3. Introduction

The effect of cadmium in the clam *Ruditapes decussatus* assessed by proteomic analysis was already published in *Aquatic Toxicology* and is presented below (3.1). The responses of *Ruditapes decussatus* to nonylphenol exposure using a proteomic approach were also studied and the results are presented in a paper which was submitted to *Environmental pollution* that is shown afterwards (3.2). Comparison between Cd an NP effects in protein expression profiles (PEPs) are made in 3.3.

3.1. Effect of cadmium in the clam *Ruditapes decussatus* assessed by proteomic analysis

Suze Chora, Mireille Starita-Geribaldi, Jean-Marie Guigonis, Michel Samson, Michèle Roméo and Maria João Bebianno.

Aquatic Toxicology 94, 300 – 308 (2009).

Aquatic Toxicology 94 (2009) 300–308

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Effect of cadmium in the clam *Ruditapes decussatus* assessed by proteomic analysis

Suze Chora [a,b], Mireille Starita-Geribaldi [c], Jean-Marie Guigonis [d], Michel Samson [d,e], Michèle Roméo [b], Maria João Bebianno [a,*]

[a] University of Algarve, CIMA, Faculty of Marine and Environmental Sciences, Campus de Gambelas, 8000-139 Faro, Portugal
[b] University of Nice-Sophia Antipolis EA 4228 ECOMERS "Ecosystèmes Côtiers Marins et Réponses aux stress" Parc Valrose, BP 71, 06108 Nice cedex 2, France
[c] University of Nice-Sophia Antipolis, Laboratory of Oral Microbiology, URE 01., Faculty of Odontology, Pôle Universitaire Saint-Jean d'Angely, 24 avenue des Diables Bleus 06357 Nice cedex 4, France
[d] University of Nice-Sophia Antipolis, Plateforme Bernard Rossi - Protéome Métabolome IFR50, Nice, F-06107, France
[e] INSERM, Unité 638, Nice, F-06107, France

ARTICLE INFO

Article history:
Received 4 March 2009
Received in revised form 15 July 2009
Accepted 22 July 2009

Keywords:
Cadmium
Proteomic
Ruditapes decussatus
LC–MS/MS

ABSTRACT

Cadmium, an environmental stressor due to its toxicity, persistence and accumulation in biota, is widespread in the aquatic environment. Cadmium accumulation kinetics have revealed that *Ruditapes decussatus* has a high affinity to this metal. Proteomics is an effective tool to evaluate the toxic effects of contaminants. The aim of this study was to investigate the Cd effects in the gill and digestive gland of the sentinel species *R. decussatus*. Protein expression profiles (PEPs) in the clam tissues exposed to Cd (40 $\mu g\,l^{-1}$, 21 days) were compared to unexposed ones. Cd induces major changes in tissue-specific protein expression profiles in gill and digestive gland. This tissue dependent response results mainly from differences in Cd accumulation, protein inhibition and/or autophagy. An overall decrease of protein spots was detected in both treated tissues, being higher in gill. Some of the spots more drastically altered after pollutants exposure were excised and nine were identified by micro liquid chromatography tandem mass spectrometry (LC–MS/MS). Proteins identified by homology search in databases included: three proteins (8-fold) up-regulated, one down-regulated, four suppressed and one induced. Cd induces major changes in proteins involved in cytoskeletal structure maintenance (muscle-type actin, adductor muscle actin and β-tubulin), cell maintenance (Rab GDP) and metabolism (ALDH and MCAD, both identified by de novo sequencing) suggesting potential energetic change. They provide a valuable knowledge of Cd effects at biochemical and molecular levels in the gill and digestive gland of *R. decussatus*.

1. Introduction

Cadmium (Cd), a non-essential element, commonly detected in aquatic and terrestrial environments, is released both from natural sources (volcanic activities or leaching of Cd rich soils), and anthropogenic activities (*eg.* mining, smelting, electroplating, production and use of pigments, plastic stabilizers and nickel–cadmium batteries) (Crompton, 1997; Bargagli, 2000). This metal is an environmental stressor due to its toxicity, persistence and accumulation in the environment (Cole and Volpe, 1983; Herber, 2004) and, therefore, selected as a priority hazardous substance within the Water Framework Directive (EC, 2001). This pollutant is present at low concentrations in open seawater (0.01–0.25 $\mu g\,l^{-1}$). However, in coastal and estuarine areas its concentration reaches up to 5.38 $\mu g\,l^{-1}$ (Ferreira et al., 2004).

In marine organisms, Cd effects have been well documented specially in bivalve molluscs since the 80s. Recent papers underline some of these effects. Cd exposure inhibits ADP-stimulated respiration reducing mitochondrial efficiency and coupling, and stimulates proton leak inhibition in the mollusc *Mizuhopecten yessoensis* (Sokolova, 2004). Other Cd effects on marine bivalves were also reported, such as reduction of condition index and depressed growth (Geret et al., 2002). This metal can cause embryotoxicity in the oyster *Crassostrea gigas* (Beiras and Albentosa, 2004; Damiens et al., 2006) and is involved in endocrine disruption, interfering in the reproduction of *Ruditapes decussatus* (Smaoui-Damak et al., 2006; Ketata et al., 2007). In the bivalve mollusc *Mya arenaria* Cd exposure alters the chemical composition of the gonads and vitellin (Gagné et al., 2002), and reduces glycogen storage, essen-

Abbreviations: 2DE, two-dimensional electrophoresis; ALDH, aldehyde dehydrogenase; IEF, isoelectric focusing; LC–MS/MS, liquid chromatography tandem mass spectrometry; MCAD, medium chain-CoA dehydrogenase; MS, mass spectrometry; MTs, metallothioneins; NCBI, National Center of Biological Information; PAGE, polyacrylamide gel electrophoresis; PEPs, protein expression profiles; Rab GDP, Rab GDP dissociation inhibitor; ROS, reactive oxygen species.

* Corresponding author. Tel.: +351 289 800995.
E-mail address: mbebian@ualg.pt (M.J. Bebianno).

0166-445X/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2009.07.014

tial for reproduction (Gauthier-Clerc et al., 2002). These effects are linked to the uptake and accumulation of Cd into the cells of the organism.

Molluscs are known to accumulate cadmium in their tissues and the clams (*R. decussatus* and *Tapes philippinarum*) are considered good bioindicators to assess the contamination of the marine environment. Toxicity, kinetics of bioaccumulation and elimination, and biomarker responses to Cd exposure are well documented for *R. decussatus* (Serafim and Bebianno, 2007). Cd accumulation in *R. decussatus* is concentration, time and tissue dependent (Bebianno and Serafim, 1998; Serafim and Bebianno, 2007). Cd is mainly accumulated in the gill following transport to the digestive gland, where it is continuously accumulated (Bebianno and Serafim, 1998; Serafim and Bebianno, 2007). In *R. decussatus* exposed to Cd ($40\ \mu g\ l^{-1}$) the accumulation in the gill follows the model $[Cd] = 2.5t - 5.1$ ($r = 0.991$), where t is the time of exposure (Serafim and Bebianno, 2007) and once accumulated is mainly linked to metallothioneins (MTs) (Roméo and Gnassia-Barelli, 1995; Bebianno and Serafim, 1998; Hamza-Chaffai et al., 1999; Amiard et al., 2006; Romero-Ruiz et al., 2008). Cd elimination is, like the accumulation, tissue dependent, following the sequence gill > digestive gland and remaining tissues. The estimated half-life of Cd for the concentration used is 45 days and 55 days for gill and digestive gland, respectively (Serafim and Bebianno, 2007).

Cd is a non-redox metal unable to participate in Fenton-type reactions. In *R. decussatus*, however, it facilitates the formation of reactive oxygen species (ROS) and, consequently, stimulates the lipid peroxidation process by oxidation of polyunsaturated fatty acids (Geret et al., 2002). Therefore, antioxidant enzyme responses (SOD, CAT and GPx) are induced in the gill (Geret et al., 2002) and carbonylation (oxidation of proteins) and ubiquitination (degradation of proteins) in *R. decussatus* also occurs after Cd exposure (Chora et al., 2008). Cd also alters mitochondrial metabolism, membrane permeability, inhibits oxidative phosphorylation and protein synthesis, through binding to nucleic acid bases, modifying their structures. Such changes are most likely associated with changes in the protein expression profile in this species (Geret et al., 2002).

Integrated approaches have been carried out to combine the responses of well-established ("core") biomarkers in response to pollutants. However, recent progress in proteomics indicates that proteome modifications in molluscs are more useful to evaluate the effect of water pollution (Manduzio et al., 2005; Romero-Ruiz et al., 2006; Amelina et al., 2007). Therefore the aim of this paper was to assess the effect of Cd ($40\ \mu g\ l^{-1}$) on protein expression signatures in the gill and digestive gland of the clam *R. decussatus* exposed for 21 days, using two-dimensional gel electrophoresis (2-DE). Significantly affected proteins were identified by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

2. Materials and methods

2.1. Reagents

Reagents were from GE Healthcare (Uppsala, Sweden) (agarose, ammonium persulfate, bromophenol blue, CHAPS, DTT, glycerol, glycine, HEPES, Immobiline DryStrips, iodoacetamide, pharmalyte 4–6.5, SDS, Tris, TEMED, thiourea, urea) and from Sigma (St Louis, MO) (acetone, EDTA, formaldehyde, technical grade nonylphenol, PMSF, P8340 Protease Inhibitor cocktail, trichloroacetic acid).

2.2. Cd exposure

R. decussatus clams were collected in the "Ria Formosa" lagoon (southern coast of Portugal), Ria Formosa Natural Park, and taken alive to the laboratory.

In the laboratory, 4 groups of 25 clams, of approximately the same size (36.1 ± 1.6 cm valve size), were kept in 25 l glass aquaria filled with continuously aerated seawater (35 psu, 18.0 ± 0.5 °C). After one week of acclimation, 2 groups were exposed to Cd ($40\ \mu g\ l^{-1}$) (stock solution 560 μg/mL) and the other group kept in seawater, as control. Animals were only fed with natural seawater which was changed every other day providing animals with natural food to avoid starvation and any effects resulting from the food. After seawater renewal Cd concentration was re-established. The exposure lasted 21 days and at the end of the experiment 5 controls and 5 exposed animals were collected from each condition. Clams were dissected into gills and digestive gland, immersed in liquid nitrogen and stored at −80 °C.

2.3. Cell-free extract preparation and protein assay

Pools of three tissues were weighed, suspended in 20% (w/v) buffer (10 mM HEPES and 250 mM Saccharose solution containing 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1/1000° P8340 SIGMA) and homogenised using a Ultra-Turrax IKA-Werke homogenizer at 4 °C.

Cell-free extracts were collected by centrifugation at $15{,}000 \times g$ for 2 h. The soluble protein extract was frozen (−80 °C) for further use. The protein content was determined by Bradford method (1976) using bovine serum albumin (BSA) as standard. Aliquots of protein content (150 μg each) were suspended in nine volumes of precipitation solution (10% trichloroacetic acid in cold acetone containing 20 mM DTT) for 2 h, at −20 °C, centrifuged at $10{,}000 \times g$ for 30 min, at 4 °C, and washed with cold acetone. The residual acetone was removed by air drying.

2.4. 2-Dimensional electrophoresis (2-DE)

Proteins were first separated by isoelectric focusing (IEF) followed by SDS-PAGE. Each sample, containing 150 μg of protein, was incubated for 30 min in 300 μL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.8% pharmalyte, 65 mM DTT and bromophenol blue traces), centrifuged ($14{,}000 \times g$, 10 min, 4 °C) and loaded on Immobiline® DryStrip (pH 4–7, 18 cm). After 6 h of passive and 6 h of active (50 V) rehydration, IEF was carried out (20 °C, 50 μA/strip) in a Protean IEF Cell using a five-step program: 1000 V, 1 h; 4000 V, 1 h; 8000 V, 1 h and 8000 V, to reach a total of 50,000 Vh. The strips were frozen at −80 °C. Before the second dimension, strips were equilibrated for 15 min in equilibration mixture (6 M urea, 75 mM Tris, pH 8.8, 4% SDS, 29.3% glycerol, and bromophenol blue traces) containing 2% DTT and then equilibrated for 15 min in buffer containing 2.5% iodoacetamide. SDS-PAGE was done in 10% polyacrylamide gels using the Protean Cell XL Cell Format vertical system (20 °C) in two steps: 90 V, 30 min and 300 V until separation was finished (~5 h). All 2-DE equipment was from BIORAD, Hercules, CA. Gels were silver stained by a protocol compatible with MS analysis (Blum et al., 1987, modified). To ensure the reproducibility of the gels, four replicates of each condition, control and treatment with Cd, were prepared.

2.5. Image acquisition and analysis

After staining, gels were scanned on a GS-800 densitometer (Bio-Rad) and data were analyzed including spot detection, quantification and normalization, data analysis and statistics using the PDQuest software (V8.0, Bio-Rad). All the 2-DE map analyses were performed with identical background subtraction (floating ball method) (10-fold) directly after the spot detection. A master gel was constructed by combining the 2-DE maps (quadruplicates) from the control condition. The 2-DE maps from the exposed tissues were matched to the reference 2-DE map. To accurately compare the measurements of spots in different gels, a normalization step was

used. In this step, the normalized volume for a spot was obtained by dividing its volume by the total volume of the detected spots on the image. Normalized volumes from different spots on sample from the exposed tissues were compared against the corresponding spots from the reference gel. The number of valid protein spots was determined for each gel, as well as the number of proteins matched to every gel, and qualitative and quantitative differences in the protein patterns between the treatment and control group were determined. In this study, spots with 8-fold or greater variation expression common in the control and contaminated tissues, new and suppressed spots were retained. The selection of 8-fold expression change was based on proteomic results on in bivalve species (Amelina et al., 2007).

The protein intensity of each spot was normalized to the total intensity of each gel image. Non-parametric Mann–Whitney *U*-rank test was applied for comparison between control and the exposed group. Values given are means ± standard deviation (M±). $p < 0.05$ was considered significant.

2.6. In-gel tryptic digestion and protein identification by mass spectrometry

Spots of interest were manually excised from the gels and destained with a solution of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate according to the method described by Gharahdaghi et al. (1999). Gel pieces were crushed in Eppendorf tubes, dehydrated with acetonitrile for 5 min and vacuum-dried. Gel pieces were first fully rehydrated with 15–50 μl of ammonium bicarbonate:acetonitrile (25 mM:10%) supplemented with trypsin (5 ng μl^{-1}, Promega, Madison, WI, USA) and then overlayed with an equal volume of buffer without trypsin. After 20–24 h incubation at 37 °C, the incubation volume was adjusted to 100 μl with ultra-pure water and acidified with 25 μl of formic acid at 25% (5% final concentration). Tryptic peptides were extruded from the gel by adding 125 μl of acetonitrile. After a 15 min incubation at room temperature, the gel pieces were spun down (8000 × *g*, 5 min, room temperature), the supernatant collected and the pellet overlaid with 50 μl of acetonitrile and vortexed to complete peptide extrusion. The extracted material was pooled with the supernatant and vacuum-dried. Lysates were solubilized in 10 μl 5% formic acid/20% methanol and stored at −20 °C until used. All samples were analyzed by micro LC/ESI/MS/MS on a LTQ/FT-Orbitrap mass spectrometer (Thermo Fisher, Waltham, MA, USA) coupled with pumps and auto-sampler under standard conditions: capillary temperature, 275 °C; source voltage, 4500 V. Helium was used as collision gas. Experiments were done in parallel mode (survey at 30,000 resolution and 5 data dependent ion trap MS/MS (Top 5)). The MS/MS parameters were: isolation width, 3; collision energy, 35%. Micro-HPLC SURVEYOR (Thermo Fisher) 30 min gradient, BioBasic C18 (Thermo Scientific, Waltham, MA, USA) column (100 mm × 0.18 mm). Such high mass accuracy on the precursor ion allowed the elimination of virtually any false positive peptide identifications, suggesting that peptides that do not match the specificity of the protease used in the digestion should not automatically be considered as false positives. Acquired MS/MS spectra were interpreted using MASCOT Peptide Mass Fingerprint software version 2.2.0 (MatrixScience, London) in house software. Search parameters were set as follows: enzyme specificity: trypsin; 1 missed cleavage permitted; fixed modification: carbamidomethylation of cysteine; variable modification: methionine oxidation; mass tolerance for precursor ions: 10 ppm; mass tolerance for fragment ions: 0.6 Da. Significance threshold $p < 0.05$ and score above 50. Both b and y ion series were used to search against UniProt KB/Swiss-Prot/TrEMBL or NCBI National Center for Biotechnology Information (Bethesda, MD, USA) nr (non-redundant) databases. No species restriction was applied to allow protein identification by sequence homologies conserved through the phylogenesis. In case of peptides matching to multiple members of a protein family, the presented protein was selected based on both the highest score and the highest number of matching peptides. The tblastn algorithm (all taxa excluding man and mouse: NCBI-EST others and with mollusks as optional organisms) was used to try a search with the matched peptides. De novo sequencing was performed on spots 3412 and 7228 using PEAKS Studio 4.5 (Ma et al., 2003). The de novo sequence from LC–MS/MS spectra was then used to search the sequence databases (NCBI), using BLAST (Basic Local Alignment Tool).

3. Results

Protein expression profiles (PEPs) were studied by 2-DE in cell extracts of *R. decussatus* gills and digestive gland, after optimizing conditions for protein extraction and separation. Proteins were first loaded in IPG strips of pH (3–10 NL) range. Because most of proteins were detected in the pH range 4–7, strips with 4–7 pH range were used loaded with 150 μg protein. The PEPs for gill and digestive gland of unexposed clams were different. The electrophoresis and data analysis allowed discriminating 816 ± 46 average protein spots from the gill and 922 ± 17 from the digestive gland of non-exposed clams (Figs. 1 and 2).

The overall quantity of proteins and the pattern of protein expression changed as a result of Cd exposure. Table 1 summarizes the 8-fold up-/down-regulated proteins after Cd exposure. In the gill, 790 spots were differently expressed (Mann–Whitney *U*-rank test, $p < 0.05$) after Cd exposure. Among them 15 were 8-fold over-expressed, 59 were newly detected and 5 decreased by 8-fold (Table 1; Fig. 3). Around 65% of polypeptides disappeared and only 263 spots were common to both control and treated tissues (Fig. 3). The newly detected proteins were situated in the central and the lower-half part of the 2-DE map with p*I* ranging from 4 to 6 and Mr from 75 to 25 kDa. The spots that disappeared were well distributed, with p*I* values between 4 and 7 and Mr values between 150 and 15 kDa. The down-regulated spots were situated in the upper-half part of the 2-DE map with p*I* ranging from 5.4 to 6.1 and Mr from 89.7 to 41.0 kDa (Fig. 1). The up-regulated spots were concentrated

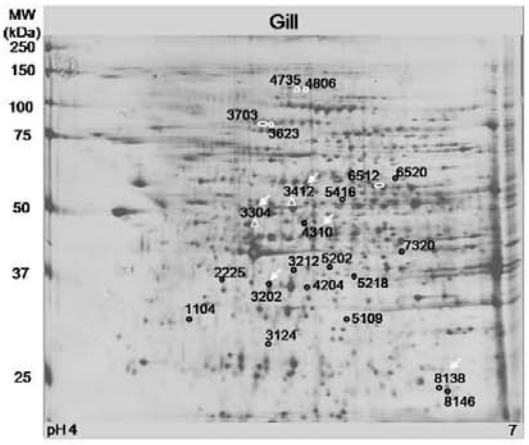

Fig. 1. *Ruditapes decussatus* gill representative control 2-DE maps ($n = 4$). One hundred fifty micrograms of protein content was separated on 18 cm IPG strips, in 4–7 pH gradients. The second dimension was performed in 10% SDS-PAGE gels. Spots 8-fold up-/down-regulated after Cd exposure are circled in black/white, respectively; suppressed spots are indicated by white triangles. Spots are indicated by PDQuest numbers. The protein names of the identified spots are listed in Table 2.

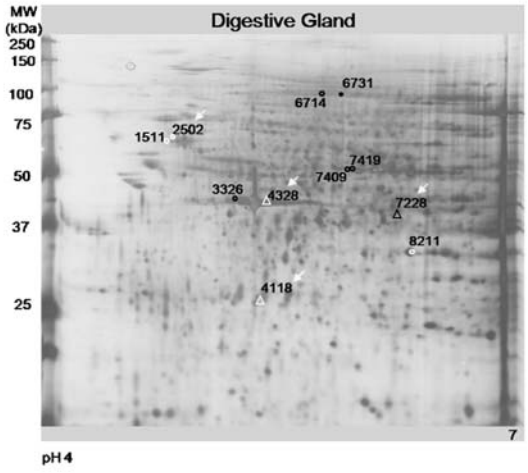

Fig. 2. *Ruditapes decussatus* digestive gland representative control 2-DE maps ($n = 4$). One hundred fifty micrograms of protein content was separated on 18 cm IPG strips, in 4–7 pH gradients. The second dimension was performed in 10% SDS-PAGE gels. Spots 8-fold up-/down-regulated after Cd exposure are circled in black/white, respectively; induced/suppressed spots are indicated by black/white triangles, respectively. Spots are indicated by PDQuest numbers. The protein names of the identified spots are listed in Table 2.

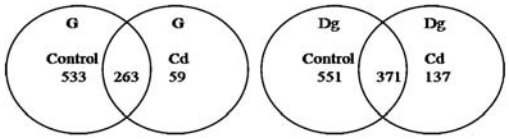

Fig. 3. Venn diagram showing the differences between spots from Cd exposed and control samples of gill (G) and digestive gland (Dg).

in the central part of the 2-DE map with p*I* from 5.1 to 6.4 and Mr from 59.1 to 23.4 kDa (Fig. 1).

The digestive gland exhibited 562 protein spots differentially expressed (Mann–Whitney *U*-rank test, $p < 0.05$): 5 were 8-fold over-expressed, 137 new, 3 were 8-fold down-regulated, 60% disappeared, while 371 spots were common between conditions (Fig. 3; Table 1). The newly detected proteins and proteins that disappeared were well distributed with p*I* ranging from 4 to 7 and M*r* ranging from 150 to 250 kDa. The down-regulated proteins were concentrated in the central part of the 2-DE map (p*I* 4.7–6.2, *Mr* 64.46–30.57 kDa) (Fig. 2). The majority of up-regulated proteins were distributed in the upper-half part (p*I* 5.3–5.9, *Mr* 107.6–42.8 kDa, Fig. 2).

Since *R. decussatus* is a non-model organism, most of the protein sequences are absent from databases. Consequently, protein identification was done by homology. The identified proteins in gill and digestive gland are summarized in Table 2 (A: gill; B: digestive gland). The species found in last column of Table 2A and B is given by NCBI blast (Mascot search results). In the case of same amino acid sequence and same access number, NCBI blast gives the species with the best score and homology.

A good agreement between predicted and observed p*I* and *Mr* was achieved. Table 2 shows that suppressed proteins were observed in control gels, these suppressed proteins corresponded to spots 3304 and 3412 in the gill (Table 2A) and 4118 and 4328 in the digestive gland (Table 2B). Three proteins were up-regulated in Cd treated gill, they corresponded to spots 3202, 8138 and 4310 (Table 2A). In digestive gland one protein, spot 2502, was down-regulated and protein 7228 was induced in the presence of Cd (Table 2B).

In gill of exposed clams (Fig. 1 and Table 2A), among the three proteins up-regulated: spot 3202 (5.4/35.3) was identified as actin adductor muscle, obtained from *Placopecten magellanus*, spot 8138 (6.4/23.7) was homologous to actin, muscle-type of *Molgula oculata* with an identical sequence as that found in spot 3202, and spot 4310 (5.7/46.4) was identified as Rab GDP dissociation inhibitor alpha by homology with a protein of *Bos taurus*. In the digestive gland (Fig. 2;

Table 1
Gill and digestive gland spots 8-fold up↑-/down↓-regulated after Cd exposure and respective p*I*/*Mr* (obtained from gel migration).

Tissue	8-Fold	Spot	p*I*	*Mr* ($\times 10^3$)
Gill	↑	1104	5.07	30.76
	↑	2225	5.22	35.89
	↑	3124	5.40	27.75
	↑	3202	5.41	35.34
	↑	3212	5.59	37.06
	↑	4204	5.69	34.96
	↑	4310	5.66	46.36
	↑	5109	5.92	30.80
	↑	5202	5.81	37.56
	↑	5218	5.97	36.52
	↑	5416	5.89	53.59
	↑	6520	6.19	59.09
	↑	7320	6.23	40.26
	↑	8138	6.37	23.72
	↑	8146	6.39	23.41
	↓	3623	5.42	77.92
	↓	3703	5.36	77.71
	↓	4735	5.62	89.71
	↓	4806	5.67	89.78
	↓	6512	6.11	57.35
Digestive gland	↑	3326	5.28	42.77
	↑	6714	5.75	107.62
	↑	6731	5.85	101.31
	↑	7409	5.88	53.67
	↑	7419	5.91	53.11
	↓	1511	4.73	62.43
	↓	2502	4.79	64.46
	↓	8211	6.23	30.57

Table 2
LC–MS/MS identification of marked spots on the representative gill and digestive gland 2-DE gels (Figs. 1 and 2).

Spot no.	Obs.[a] pI/Mr (kDa)	Theo.[b] pI/Mr (kDa)	Protein identification	A. no.	Biological function	Peptide sequence	Sco[c]	Pep.	Cov. %	Species	Exp.[d]
(A) Gill											
3202	5.4/35.3	5.3/41.9	Actin	Q26065	Cytoskeletal structure/function	GYSFTTTAER (1131.5), SYELPDGQVITIGNER (1789.8), VAPEEHPVLLTEAPLNPK (1953.0), DLYANTVLSGGSTMYPGIADR (2200.0), LCYVALDFEQEMSTAASSSSLEK (2565.1)	433	12	37	*Placopecten magellanus*	↑ (11.38)
3304	5.4/48.4	4.7/50.5	Tubulin β chain (β-tubulin)	P11833	Cytoskeletal structure/function	INVYYNEATGGK (1327.6), IMNTFSVVPSPK (1334.6)	239	7	16	*Paracentrotus lividus*	0 (0.03)
8138	6.4/23.7	6.4/23.7	Actin, muscle-type	Q26065	Cytoskeletal structure/function	EITALAPSTMK (1176.6), SYELPDGQVITIGNER (1789.8), VAPEEHPVLLTEAPLNPK (1953.0), DLYANTVLSGGSTMYPGIADR (2200.0), LCYVALDFEQEMSTAASSSSLEK (2565.1)	282	9	33	*Molgula oculata*	↑ (13.73)
3412	5.8/57.2	6.6/57.5	ALDH member B1	Q9CZS1	Amino acid Metabolism	ADVDLAVK (829.4) YYAGWADK (972.4), ELGEYGLQAYTEVK (1599.7), *De novo* sequencing (see Table 3)	72	5	6	*Mus musculus*	0 (0.02)
4310	5.7/46.4	5.0/51.1	Rab GDP dissociation inhibitor alpha Rab GDI α	P21856	Cell maintenance	GRDWNVDLIPK (1311.6), SPYLYPLYGLGELPQGFAR (2140.0)	71	3	6	*Bos taurus*	↑ (8.77)
(B) Digestive gland											
2502	5.4/35.3	5.3/41.9	Actin adductor muscle	Q26065	Cytoskeletal structure/function	IIAPPERK (922.5), AGFAGDDAPR (975.4), GYSFTTTAER (1131.5), EITALAPSTMK (1160.6), HQGVMVGMGQK (1170.5), DSYVGDEAQSK (1197.5), AVFPSIVGRPR (1197.5), SYELPDGQVITIGNER (1789.8), VAPEEHPVLLTEAPLLPK (1953.0)	137	11	28	*Placoplecten magellanicus*	↓ (35.44)
4118	5.5/40.7	5.3/42.1	Actin, muscle-type	Q25472	Cytoskeletal structure/function	SYELPDGQVITIGNER (1789.8), VAPEEHPVLLTEAPLNPK (1953.0), DLYANTVLSGGSTMYPGIADR (2200.0)	196	9	29	*Molgula oculata*	0 (0.00)
4328	5.5/42.6	5.1/42.5	Actin muscle-type	Q25472	Cytoskeletal structure/function	AGFAGDDAPR (975.4), GYSFTTTAER (1131.5), EITALAPSTMK (1176.6), DSYVGDEAQSKR (1353.6), SYELPDGQVITIGNER (1789.8), VAPEEHPVLLTEAPLNPK (1953.0), DLYANTVLSGGTTMYPGIADR (2230.0), LCYVALDFEQEMATAASSSSLEK (2565.1)	764	21	51	*Molgula oculata*	0 (0.02)
7228	6.1/40.4	8.5/46.8	Medium chain specific acyl CoA dehydrogenase (MCAD)	P41367	Fatty acid β-oxidation	AAWEIDQGR (1044.4), TRPPVAAGAVGLAQR (1462.8), IYQIYEGTAQIQR (1581.8146), *De novo* sequencing (see Table 3)	170	4	9	*Sus scrofa*	I (233.8)

Spot no.: spot number; A. no.: accession number of NCBI database of matched protein; Pep: peptides matched. Cov: amino acid sequence coverage for the identified proteins. Exp.: protein expression after Cd exposure.↓: ≤8-fold down-regulated; ↑: ≥8-fold up-regulated; 0: suppressed spots; I: induced spot.
Peptides sequences: The above table gives the peptide sequences with the highest individual ion scores indicating identity or extensive homology ($p < 0.05$), the number in parentheses are the experimental *Mr*, the sequence underlined is the one with the best score.

[a] Observed (Obs.) isoelectric point (*pI*) and molecular mass (*Mr*) were obtained from gel migration.

[b] Theoretical (Theo.) isoelectric point (*pI*) and molecular mass (*Mr*) were obtained from Swiss Prot./TrEMBL database.

[c] Scores of the matches using MASCOT version 2.2.0 (Matrix Science, London) software.

[d] The spot intensity ratio between control and Cd treated samples is shown in brackets. Ratios are calculated by PDQuest using the average of four replicates in each group. For all comparisons the *p*-value is <0.05. Tblastn spot 3202: AJ626410, *Mytilus galloprovincialis* haemolymph, gills, digestive gland, foot, adductor muscles and mantle *Mytilus galloprovincialis*, cDNA clone GPPD05884, mRNA sequence. Tblastn spot 3304: MUW04-E19.x1d-t SHGC-MUW *Mytilus californianus* cDNA 5′, mRNA sequence. Tblastn spot 3412 AJ624775 *Mytilus galloprovincialis* haemolymph, gills, digestive gland, foot, adductor muscles and mantle *Mytilus galloprovincialis*, cDNA clone GPPD03500, mRNA sequence. Tblastn spot 4118 MUS21-P10.x1d-t SHGC-MUS *Mytilus californianus* cDNA 5′, mRNA sequence. Tblastn spot 4310: AJ624260, *Mytilus galloprovincialis* haemolymph, gills, digestive gland, foot, adductor muscles and mantle *Mytilus galloprovincialis*, cDNA clone GPPD02787, mRNA sequence.

Table 3
R. decussatus proteins identified by *de novo* sequencing by PEAKS Studio 4.5.

Spot no.	Obs.[a] pI/*Mr* (kDa)	Theo.[b] pI/*Mr* (kDa)	Protein identification	A. no.	Biological function	Peptide sequence	Sco[c]	Pep.	Cov. %	Species	Exp.[d]
3412	5.8/57.2	6.6/57.5	ALDH member B1	Q9CZS1	Amino acid metabolism	ELGEYGLQAYTEVK, Other sequences: ADVDLAVK, YYAGWADK, LAVK, AD, AYTEVK	72	5	6	*Mus musculus*	0 (0.00)
			De novo sequencing:								
7228	6.1/40.4	8.5/46.8	Medium chain specific acyl CoA dehydrogenase (MCAD)	P41367	Fatty acid β-oxidation	TRPPVAAGAVGLAQR, Other sequences: AAWELDQGR, IYQTYEGTAQIQR, AAWELDQGR, AAWELDQGR, TRPGVASAAVGLAQR	170	4	9	*Sus scrofa*	1 (23.8)
			De novo sequencing: NCBI EST others: acyl CoA dehydrogenase C-4–C-12 straight chain	XP002165179						*Hydra magnipapillata*	

Spot no.: spot number; A. no.: accession number of NCBI database of matched protein; Pep.: peptides matched; Cov.: amino acid sequence coverage for the identified proteins. Exp.: protein expression after Cd exposure. ↓: suppressed spot; ↑: induced spot.

[a] Observed (Obs.) isoelectric point (pI) and molecular mass (*Mr*) were obtained from gel migration.
[b] Theoretical (Theo.) isoelectric point (pI) and molecular mass (*Mr*) were obtained from Swiss Prot./TrEMBL database.
[c] Scores of the matches using MASCOT version 2.2.0 (Matrix Science, London) software.
[d] The spot intensity ratio between control and Cd treated samples is shown in brackets. Ratios are calculated by PDQuest using the average of four replicates in each group. For all comparisons the *p*-value is <0.05.

Table 2B) the down-regulated spot 2502 (4.8/64.5) was identified as actin adductor muscle by homology with a protein from *Placopecten magellanicus* (Fig. 2; Table 2B).

Among suppressed proteins in the gill identified by homology (Table 2A): spot 3304 was homologous to β-Tubulin of *Paracentrotus lividus* and spot 3412 (5.8/57.2) was similar to aldehyde dehydrogenase X, mitochondrial precursor (ALDH family 1 member B1) of *Mus musculus*. ALDH was unambiguously identified by de novo (Table 3) sequencing. Two small sequences (LAVK and AD) and one bigger (AYTEVK) were generated. However, these sequences were not long enough to provide results in databases searching. In the pair of proteins from the digestive gland of unexposed clams (Table 2B): spots 4118 (5.5/40.7) and 4328 (5.5/42.6) were similar to actin muscle-type of *M. oculata*. The spot 7228 (40.4/6.1), only in the digestive gland of treated clams, was homologous to medium chain specific acyl-CoA dehydrogenase (MCAD) of *Sus scrofa*. MCAD was also unambiguously identified by de novo (Table 3) sequencing. The generated sequence AAWELDQGR was searched in databases, exactly the same alignment was found as well as another sequence TRPGVASAAVGLAQR (very near to the original sequence with only 3 different AA among 15 AA). Both fragments belong to XP002165179 similar to Acyl CoA dehydrogenase C-4 to C-12 straight chain (in the invertebrate *Hydra magnipapillata*).

The majority of the identified proteins have cytoskeletal and structural functions.

4. Discussion

R. decussatus is a bio-indicator species. Biomarkers (classical or proteomic ones) measured in the exposed clams give an indication of the quality of the environment since pollutants affect their physiology. Proteomics, which was mainly developed in medical research, was applied in this study to environmental problems and more particularly the alteration of protein expression profile by Cd. Dowling and Sheehan (2006) demonstrated that proteomics could be a route to identification of toxicity targets in environmental toxicology.

The sublethal cadmium concentration used (40 $\mu g l^{-1}$) was chosen since Cd accumulation pattern was already known (Serafim and Bebianno, 2007; Geret et al., 2002). The health of exposed animals was checked: they were regularly filtering and both siphons were pumping in and out, this behavior is consistent with the animals' healthy state. Previous experiments on clams demonstrated that, at the concentration used, metallothioneins (MTs) were induced protecting clams from metallic damage (Serafim and Bebianno, 2007). This study focused in gill and in digestive gland due to their important role in initial-/long-term Cd storage (Roméo and Gnassia-Barelli, 1997; Geret et al., 2002; Serafim and Bebianno, 2007). Previous studies have shown that Cd is accumulated in both tissues of Cd exposed clams with concentrations reaching ca 400 nmol/g and 750 nmol/g in gill and digestive gland, respectively, after 21 days of exposure (Serafim and Bebianno, 2007). This difference is thought to be related to tissue adsorption surface in contact with the dissolved Cd (higher in gill) and/or with the available ligands within cells (higher in digestive gland) resulting in moderately higher Cd accumulation in the digestive gland (Park et al., 2001; Serafim and Bebianno, 2007).

The results of the present paper show that Cd exposure causes changes in protein expression profiles (PEPs) in gill and digestive gland. The tissues exhibited different protein expression profiles due to their different functions in clams (Figs. 1 and 2). An overall decrease of protein spots was detected in both treated tissues. Lysosomal autophagy could explain this decrease since it is considered a survival strategy (Cuervo, 2004; Moore, 2004). Autophagy confers advantages to organisms, especially molluscs exposed to

pollutants, by protecting the cells against the harmful effects of damaged and malfunctioning proteins (Moore, 2004; Moore et al., 2006, 2007). Carbonylation and/or ubiquitination of proteins were already detected in *R. decussatus* exposed to DDE and a similar Cd concentration, demonstrating tissue-specific redox requirements (Dowling et al., 2006; Chora et al., 2008). Ubiquitinated proteins are removed from cells by proteolysis via the ubiquitin-proteasome pathway (UPP) (Marques et al., 2004). Therefore, this removal could also explain the high protein spot reduction detected in treated samples (Fig. 3). Exposure of haemocytes of *Mytilus galloprovincialis in vitro* to Cd concentrations above 100 μM (1.12 102 μg/L) stimulate phagocytic and lysosomal activities (Olabarrieta et al., 2001).

Gills are the first site of Cd accumulation, following then transport to the digestive gland (Roméo and Gnassia-Barelli, 1995, Bebianno and Serafim, 1998). The digestive gland has a great number of lysosomes, which trap Cd for long-term storage/elimination (Marigómez et al., 2002), and higher MT levels compared to the gill. Since lysosomes and MTs are thought to have a protective role against Cd effects, this could explain the smallest protein spots reduction detected in digestive gland (Serafim and Bebianno, 2007; Marigómez et al., 2002). The higher degree of basal peroxidation obtained in gill after Cd exposure (Roméo and Gnassia-Barelli, 1997) could also explain the higher protein spot reduction detected in this tissue.

Several proteins were identified by LC–MS/MS, in particular a considerable number of cytoskeletal proteins (tubulin and actin). This could be due to their abundance, prevalence in mollusc databases, or to the role as major targets of pollutant-related oxidative stress (Rodriguez-Ortega et al., 2003; Miura et al., 2005). β-tubulin is involved in the structure and function of the cytoskeleton and a decrease in its level results from oxidative stress effects (Miura et al., 2005). The decrease in the level of this protein in clam gill could be compared to a down-regulation of a similar protein reported for *Mytilus edulis* (digestive gland) from polluted sites (Apraiz et al., 2006). Actin is involved inthe structure and function of the cytoskeleton, being associated with protein synthesis since it inhibits eukaryotic elongation factors, raising the possibility of "cross-talk" between protein synthesis, the cytoskeleton and cell redox status (Dalle-Donne et al., 2001; Bektas et al., 2004). Most actins have 43 kDa molecular mass, so the different spots identified in the present paper could be actin isoforms. In the gill of *R. decussatus*, actin isoforms (adductor muscle actin and muscle-type actin) were up-regulated. In the digestive gland, actin isoforms (adductor muscle and muscle-type) were down-regulated/suppressed. Rodriguez-Ortega et al. (2003) reported this variable modulation in the whole soft body of *Chamalea gallina*, where in clams experimentally exposed to pollutants an actin isoform (5.45/38) was decreased by Aroclor and Cu(II) but increased by TBT and As(III) and another actin isoform (6.30/12) was up-regulated by Aroclor and Cu(II) but down-regulated by TBT and As(III). Rodriguez-Ortega et al. (2003) hypothesized that damage to cytoskeletal proteins could promote their increased expression. This could explain that only two up-regulated proteins were found in the gill of *R. decussatus* rather than in the digestive gland where Cd remains stored. Actin down-regulation has already been reported in *M. edulis* (gill, Manduzio et al., 2005 and digestive gland, Amelina et al., 2007) from contaminated sites. Exposure of haemocytes of *M. galloprovincialis in vitro* to Cd cause disruption of actin cytoskeleton (Olabarrieta et al., 2001) and it was suggested that Cd could bind directly to cytoskeletal proteins followed by their denaturation (Matozzo et al., 2001). These results point also to the decline of the condition of animals probably due to oxidative stress that alters Ca^{2+} homeostasis (Gómez-Mendikute and Cajaraville, 2003). This decline was not obvious in the present study since animals had a normal behavior. High concentrations of Ca^{2+} activate proteases, which might hydrolyze the actin filaments and the proteins fixing them to the cellular membranes. It has been hypothesized that the ability to resist a toxicant may be expensive in terms of energy, involving a decrease in the energy available for allocation to other processes (Holloway et al., 1990), such as basal metabolism, growth, reproduction.

Rab GDP dissociation inhibitor α was up-regulated in clam gill. Rab GDPases (Rabs) are a large family of small molecular weight proteins (Stenmark and Olkkonen, 2001), mediators of vesicle formation, trafficking, and fusion (Zerial and McBride, 2001; Slimane et al., 2003). These molecules have key roles in many vesicular transport pathways (Zerial and McBride, 2001; Slimane et al., 2003) and are related to granule organization and release (Zerial and McBride, 2001; Lippolis and Reinhardt, 2005). Pan and Wang (2008) demonstrated that Cd in scallops is stored in the non-toxic forms both in MTs and vesicles. This could explain the Rab GDP dissociation inhibitor alpha up-regulation, detected in the clam gill by increasing vesicular formation and transport in response to Cd exposure. Aldehyde dehydrogenase (ALDH) was suppressed in treated gill. This enzyme catalyzes oxidation (dehydrogenation) of aldehydes to carboxylic acids and acts on primary unbranched aldhehydes. ALDH was down-regulated in *M. edulis* from contaminated sites (Apraiz et al., 2006; Amelina et al., 2007). ALDH has been used as biochemical marker in *M. galloprovincialis* (Nasci et al., 2002; Nesto et al., 2004).

Medium chain specific MCAD was induced in clam digestive gland exposed to cadmium. This protein was up-regulated in *M. edulis* digestive gland from polluted sites (Amelina et al., 2007). MCAD is an enzyme responsible for the metabolism of medium chain fatty acids. It catalyzes the first step in each cycle of fatty acid β-oxidation in mitochondria (Crane et al., 1956). This enzyme induction is connected to an adaptive response to pollutant exposure. Muller and Muller (1998) showed that sponges develop an efficient defense system against environmental stressors, having genes involved in an adaptive response against xenobiotics. Among these genes, a protein homologous to the mammalian MCAD was identified (Muller and Muller, 1998). De novo sequencing was performed on ALDH and MCAD, the only induced peptide (results in Table 3). These two peptides were unambiguously identified in the gill and digestive gland, respectively, of *R. decussatus*. As regards the other peptides identified with UniProt KB/Swiss-Prot/TrEMBL, they were blasted using tblastn search (results in the footnotes of Table 2). All were homologous to mRNA sequences of genes belonging to molluscs and particularly *M. galloprovincialis* and *M. californianus*, which is important since for the moment no protein databases exist for *R. decussatus*.

The PEPs detected and identified in this study provide new information on the possible mechanisms of toxicity of Cd in *R. decussatus* gill and digestive gland. They are more reliable than a few unique biomarkers, which are measured independently, since they may reflect the complexity of a toxicological response (Monsinjon and Knigge, 2007). The identification of putative proteins from PEPs provides additional knowledge of potential Cd effects at biochemical and molecular level.

5. Conclusions

Overall, these results reveal that Cd induces major changes in PEPs in gill and digestive gland. This tissue dependent response results mainly from differences in Cd accumulation, protein expression inhibition and induction due to Cd exposure. Identified proteins indicate that some changes in PEPs induced by Cd exposure include cytoskeletal structure maintenance (actin, tubulin), cell maintenance (Rab GDP dissociation inhibitor α), metabolism (MCAD and ALDH) suggesting potential alteration in energetics. Clams could cope with cadmium and survive at the expense of cell and cytoskeletal structure maintenance. The proteins identified in

this paper belong to the major protein classes that Monsinjon and Knigge (2007) identified in environmental proteomic studies.

Acknowledgements

This work was supported by a PhD grant from the FCT, Portugal.

References

Amelina, H., Apraiz, I., Sun, W., Cristobal, S., 2007. Proteomics-based method for the assessment of marine pollution using liquid chromatography coupled with two-dimensional electrophoresis. J. Proteome Res. 6, 2094–2104.

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

Apraiz, I., Mi, J., Cristobal, S., 2006. Identification of proteomic signatures of exposure to marine pollutants in mussels (*Mytilus edulis*). Mol. Cell. Proteomics 5, 1274–1285.

Bargagli, R., 2000. Trace metals in Antarctica related to climate change and increasing human impact. Rev. Environ. Contam. Toxicol. 166, 129–173.

Bebianno, M.J., Serafim, M.A., 1998. Comparison of metallothionein induction in response to cadmium in the gills of the bivalve molluscs *Mytilus galloprovincialis* and *Ruditapes decussatus*. Sci. Total Environ. 214, 123–131.

Beiras, R., Albentosa, M., 2004. Inhibition of embryo development of the commercial bivalves *Ruditapes decussatus* and *Mytilus galloprovincialis* by trace metals; implications for the implementation of seawater quality criteria. Aquaculture 230, 205–213.

Bektas, M., Guncer, B., Guven, C., Nurten, R., Bermek, E., 2004. Actin—an inhibitor of eukaryotic elongation factor activities. Biochem. Biophys. Res. Commun. 317, 1061–1066.

Blum, H., Biere, H., Gross, H.J., 1987. Improved silver staining of plant proteins. RNA and DNA in polyacrylamide gels. Electrophoresis 8, 93–99.

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

Chora, S., McDonagh, B., Starita-Geribaldi, M., Roméo, M., Bebianno, M.J., 2008. Ubiquitination and carbonylation as markers of oxidative-stress in *Ruditapes decussatus*. Mar. Environ. Res. 66, 95–97.

Cole, J.F., Volpe, R., 1983. The effect of cadmium in the environment. Ecotoxicol. Environ. Saf. 7, 151–159.

Crane, F.L., Mii, S., Hauge, J.G., Green, D.E., Beinert, H., 1956. On the mechanism of dehydrogenation of fatty acyl derivatives of coenzyme A. I. The general fatty acyl coenzyme A dehydrogenase. J. Biol. Chem. 218, 701–706.

Crompton, T.R., 1997. Toxicants in Aqueous Ecosystem. John Wiley and Sons, Chichester, New York.

Cuervo, A.M., 2004. Autophagy: in sickness and in health. Trends Cell. Biol. 14, 70–77.

Dalle-Donne, I., Rossi, R., Milzani, A., Di Simplicio, P., Colombo, R., 2001. The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself. Free Radic. Biol. Med. 31, 1624–1632.

Damiens, G., Mouneyrac, C., Quiniou, F., His, E., Gnassia-Barelli, M., Roméo, M., 2006. Metal bioaccumulation and metallothionein concentrations in larvae of *Crassostrea gigas*. Environ. Pollut. 140, 492–499.

Dowling, V.A., Sheehan, D., 2006. Proteomics as a route to identification of toxicity targets in environmental toxicology. Proteomics 6, 5597–5604.

Dowling, V., Hoarau, P., Roméo, M., O'Halloran, J., Frank Van Pelt, O'Brien, N., Sheehan, D., 2006. Protein carbonylation and heat shock response in *Ruditapes decussatus* following p,p'dichlorodiphenyldichloroethylene (DDE) exposure: a proteomic approach reveals that DDE causes oxidative stress. Aquat. Toxicol. 77, 11–18.

EC, 2001. Decision No. 2455/2001/EC of the European Parliament and of the Council of 20 November 2001 establishing the list of priority substances in the field of water policy and amending Directive 2000/60/EC. Official Journal of the European Communities, L 331, pp. 1–5.

Ferreira, A.C., Costa, A.C.S., Korn, M.G.A., 2004. Preliminary evaluation of the cadmium concentration in seawater of the Salvador city, Brazil. Microchem. J. 78, 77–83.

Gagné, F., Blaise, C., Pellerin, J., Gauthier-Clerc, S., 2002. Alteration of the biochemical properties of female gonads and vitellins in the clam *Mya arenaria* at contaminated sites in the Saguenay Fjord. Mar. Environ. Res. 53, 295–310.

Gauthier-Clerc, S., Pellerin, J., Blaise, C., Gagné, F., 2002. Delayed gametogenesis of (*Mya arenaria*) in the Saguenay Fjord (Canada): a consequence of endocrine disruptors? Comp. Biochem. Physiol. C 131, 457–467.

Geret, F., Serafim, A., Barreira, L., Bebianno, M.J., 2002. Effect of Cd on antioxidant enzymes in the gills of the clam *Ruditapes decussatus*. Biomarkers 7, 242–256.

Gharahdaghi, F., Weinberg, C.R., Meagher, D.A., Imai, B.S., Mishe, S.M., 1999. Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. Electrophoresis 20, 601–605.

Gómez-Mendikute, A., Cajaraville, M.P., 2003. Comparative effects of cadmium, copper, paraquat and benzo[a]pyrene on the actin cytoskeleton and production of reactive oxygen species (ROS) in mussel haemocytes. Toxicol. In Vitro 17, 539–546.

Hamza-Chaffai, A., Amiard, J.C., Cosson, R.P., 1999. Relationship between metallothionein and metals in a natural population of clam *Ruditapes decussatus* from Sfax coast, a non-linear model using Box–Cox transformation. Comp. Biochem. Physiol. C 123, 153–163.

Herber, R.F.M., 2004. Cadmium. In: Merian, E., Anke, M., Ihnat, M., Stoeppler, M. (Eds.), Elements and their Compounds in the Environment. Metals and Their Compounds, vol. 2. Wiley-VCH, Germany, pp. 698–708.

Holloway, G.J., Sibly, R.M., Povey, S.R., 1990. Evolution in toxin-stressed environments. Funct. Ecol. 4, 289–294.

Ketata, I., Smaoui-Damak, W., Guermazi, F., Rebai, T., Hamza-Chaffai, A., 2007. In situ endocrine disrupting effects of cadmium on the reproduction of *Ruditapes decussatus*. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 146, 415–430.

Lippolis, J., Reinhardt, T., 2005. Proteomic survey of bovine neutrophils. Vet. Immunol. Immunopathol. 103, 53–65.

Ma, B., Zhang, K., Hendrie, C., Liang, C., Li, M., et al., 2003. PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry. Rapid Commun. Mass Spectrom. 17, 2337–2342.

Manduzio, H., Cosette, P., Gricourt, L., Jouenne, T., Lenz, C., Andersen, O.K., Leboulenger, F., Rocher, B., 2005. Proteome modifications of blue mussel (*Mytilus edulis* L.) gills as an effect of water pollution. Proteomics 5, 4958–4963.

Marigómez, I., Soto, M., Cajaraville, P.M., Angulo, E., Giamberini, L., 2002. Cellular and subcellular distribution of metals in molluscs. Microsc. Res. Technol. 56, 358–392.

Marques, C., Pereira, P., Taylor, A., Liang, J.N., Reddy, V.N., Szweda, L.I., Shang, F., 2004. Ubiquitin-dependent lysosomal degradation of the HNE modified proteins in lens epithelial cells. FASEB J. 18, 1424–1426.

Matozzo, V., Ballarin, L., Pamparin, D.M., Marin, M.G., 2001. Effects of copper and cadmium exposure on functional responses of hemocytes in the clam, *Tapes philippinarum*. Arch. Environ. Contam. Toxicol. 41, 163–170.

Miura, Y., Kano, M., Abe, K., Urano, S., Suzuki, S., Toda, T., 2005. Age-dependent variations of cell response to oxidative stress: proteomic approach to protein expression and phosphorylation. Electrophoresis 26, 2786–2796.

Monsinjon, T., Knigge, T., 2007. Proteomic applications in ecotoxicology. Proteomics 7, 2997–3009.

Moore, M.N., 2004. Diet restriction induced autophagy: lysosomal protective system against oxidative- and pollutant-stress and cell injury. Mar. Environ. Res. 58, 603–607.

Moore, M.N., Icarus, A.J., Somerfield, P.J., 2006. Autophagy: role in surviving environmental stress. Mar. Environ. Res. 62, S420–S425.

Moore, M.N., Viarengo, A., Donkin, P., Hawkins, J.S., 2007. Autophagic and lysosomal reactions to stress in the hepatopancreas of blue mussels. Aquat. Toxicol. 84, 80–91.

Muller, W., Muller, I., 1998. In: Wells, P.G., Lee, K.H., Blaise, C. (Eds.), Microscale Aquatic Toxicology: Advance, Techniques and Practise. CRC Lewis, Boca Raton, FL, pp. 97–112.

Nasci, C., Nesto, N., Monteduro, R.A., Da Ros, L., 2002. Field application of biochemical markers and a physiological index in the mussel. *Mytilus galloprovincialis*: transplantation and biomonitoring studies in the lagoon of Venice (NE Italy). Mar. Environ. Res. 54, 811–816.

Nesto, N., Bertoldo, M., Nasci, C., Da Ros, L., 2004. Spatial and temporal variation of biomarkers in mussels (*Mytilus galloprovincialis*) from the Lagoon of Venice, Italy. Mar. Environ. Res. 58, 287–291.

Olabarrieta, I., L'Azou, B., Yuric, S., Cambar, J., Cajaraville, M.P., 2001. In vitro effects of cadmium on two different animal cell models. Toxicol. In Vitro 15, 511–517.

Pan, K., Wang, W., 2008. The subcellular fate of cadmium and zinc in the scallop *Chlamys nobilis* during waterborne and dietary metal exposure. Aquat. Toxicol. 90, 253–260.

Park, J.D., Liu, Y., Klaassen, C.D., 2001. Protective effect of metallothionein against the toxicity of cadmium and other metals. Toxicology 163, 93–100.

Rodriguez-Ortega, M.J., Grosvik, B.E., Rodriguez-Ariza, A., Goksoyr, A., Lopez-Barea, J., 2003. Changes in protein expression profiles in bivalve molluscs (*Chamaelea gallina*) exposed to four model environmental pollutants. Proteomics 3, 1535–1543.

Roméo, M., Gnassia-Barelli, M., 1995. Metal distribution in different in tissues and in subcellular fractions of the Mediterranean clam *Ruditapes decussatus* treated with cadmium, copper or zinc. Comp. Biochem. Physiol. 111C, 457–463.

Roméo, M., Gnassia-Barelli, M., 1997. Effect of heavy metals on lipid peroxidation in the mediterranean clam *Ruditapes decussatus*. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 118, 33–37.

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

Romero-Ruiz, A., Carrascal, M., Alhama, J., Gómez-Ariza, J.L., Abian, J., López-Barea, J., 2006. Environmental proteomics studies in clams from the Donaña bank of Guadalquivir estuary (SW Spain). Proteomics 6 (Suppl. 1), S245–S255.

Serafim, A., Bebianno, M.J., 2007. Kinetic model of cadmium accumulation and elimination and metallothionein response in *Ruditapes decussatus*. Environ. Toxicol. Chem. 26, 960–969.

Slimane, T.A., Trugnan, G., Van IJzendoorn, S.C.D., Hoekstra, D., 2003. Raft-mediated trafficking of apical resident proteins occurs in both direct and transcytotic pathways in polarized hepatic cells: role of distinct lipid microdomains. Mol. Biol. Cell. 14 (2), 611–624.

Smaoui-Damak, W., Rebai, T., Berthet, B., Hamza-Chaffai, A., 2006. Does cadmium pollution affect reproduction in the clam *Ruditapes decussatus*? A one-year case study. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 143, 252–261.
Sokolova, I.M., 2004. Cadmium effects on mitochondrial function are enhanced by elevated temperatures in a marine poikilotherm. Crassostrea virginica Gmelin (Bivalvia: Ostreidae). J. Exp. Biol. 207, 2639–2648.
Stenmark, H., Olkkonen, V.M., 2001. The Rab GTPase family. Genome Biol. 2 (5), 3007.1–3007.7.
Zerial, M., McBride, H., 2001. Rab proteins as membrane organizers. Nat. Rev. Mol. Cell Biol. 2, 107–117.

3.2. Responses of *Ruditapes decussatus* to nonylphenol exposure using a proteomic approach

Suze Chora, Mireille Starita-Geribaldi,Jean-Marie Guigonis, Michel Samson, Michèle Roméo and Maria João Bebianno

Environmental Pollution (*submitted*)

Abstract

Nonylphenol (NP) has great toxicity due to its accumulation in the biota, estrogenic properties and persistence in the environment. Proteomics is an effective tool to evaluate the effects of toxic responses. The aim of this study was to investigate the NP effects in the gill and digestive gland of the sentinel species *Ruditapes decussatus.* Protein expression profiles (PEPs) in the clam tissues exposed to NP (100 µg.l^{-1}, 21 days) were compared to unexposed ones. NP was accumulated in both clam tissues, although higher in the digestive gland. In NP treated clams less protein spots were observed. Five 8 fold up/down-regulated and 7 suppressed proteins were identified by homology. NP induced changes in proteins involved in cytoskeletal structure maintenance (actin, tubulin), cell maintenance (Hsc70-4), metabolism (enolase, ALDH), suggesting energetic potential change, and in signal transducer transcription regulation (histone binding protein, identified by *de novo* sequencing). They provide a valuable knowledge of NP effects at biochemical and molecular level in the gill and digestive gland of *R. decussatus.*

3.2.1. Introduction

There is a growing concern over the potential effects of chemicals in the marine wildlife. The alkylphenol ethoxylates (APEOs) constitute a group of new contaminants widely used as non-ionic surfactants representing 6 % of the total world production (Nimrod and Benson, 2004). APEOs are degraded into nonylphenol polyethoxylates (NPEs), which are frequently used in detergents owing to their rapid biodegradability (Abad *et al.*, 2005). They are also used in plastics, latex paints, lubricating oils, emulsifiers, household and industrial detergents, paper, cosmetics and textile industries

(Nimrod and Benson, 2004). In sewage treatment plants NPEs are degraded into nonylphenol (NP) (Abad *et al*., 2005); consequently, a large quantity of NP is released into the environment from municipal and industrial sources (Nice *et al*., 2005). NP was detected in a variety of fresh and marine waters from concentrations below detection limit to high concentrations (95 $\mu g.l^{-1}$, Dachs *et al*., 1999).

Since NP is a lipophilic substance, it is easily accumulated in a wide scope of aquatic and marine organisms (Staples *et al*., 2004) including bivalve molluscs (Ekelund *et al*., 1990, Lietti *et al*., 2007; Ricciardi *et al*., 2008). This compound has great persistence and recognized aquatic toxicity. Acute and chronic toxicity of NP in aquatic organisms was recently reviewed (Staples *et al*., 2004). The negative effects of this compound were reported for marine invertebrates including: changes in population growth rates (Matozzo *et al*., 2003; Mäenpää *et al*., 2006), re-burrowing capacity of *Tapes philipinarum* (Matozzo *et al*., 2004) and reduction of the stability of lysosomal membranes of *Mytilus galloprovincialis* (Canesi *et al*., 2004, 2007). NP is also known to influence hormonal functions in various aquatic organisms due to its capacity to mimic the action of endogenous estrogens by binding to estrogen receptors, and leading to the disruption of several reproduction processes (Butwell *et al*., 2002; Vazquez-Duhalt *et al*., 2005). Endocrine disruption effects of NP were detected in *Elliptio complanata*, where the normal metabolism of serotonin and dopamine, involved in sexual differentiation in bivalves, was changed (Gagné *et al*., 2003). Moreover the exposure to 4-NP (1 and 100 $\mu g.l^{-1}$) at early life stages of *C. gigas* altered the sex ratio towards females, increased the percentage of functional hermaphrodites in adults and reduced gamete viability in the subsequent generation (Nice *et al*., 2000). NP may also induce vitellogenin (Vtg) or Vtg-like proteins synthesis of male marine bivalves, both

in vitro and *in vivo* (*T. philippinarum*, Matozzo and Marin, 2005; *Dreissena polymorpha*, Quinn *et al*., 2006; *Saccostrea glomerata*, Andrew *et al*., 2008; *Cerastoderma glaucum,* Marin *et al*., 2008 and *Mytilus galloprovincialis*, Ricciardi *et al*., 2008). Biomarkers also provide early warning information about bioavailability of pollutants and their potential damage. Nevertheless, the utility of these biomarkers have been biased by the requirement of a deep knowledge of their mechanism of toxicity. New technologies such as transcriptomics and proteomics are considered promising tools to evaluate the relationship between toxicity and gene or protein changes (Bebianno *et al*., 2004). Proteomics has the advantage of providing global information about fingerprint protein changes induced by pollutants without requiring any previous knowledge of their toxic mechanisms (Miracle and Ankley, 2005). Some relevant field and laboratory studies using proteomics on bivalves showed different PEPs and identified proteins altered by pollutant exposure (Aroclor 1254, Cu(II), TBT and As(III), Rodriguez-Ortega *et al*., 2003; diallyl phthalate, PBDE-47, and bisphenol-A, Apraiz *et al*., 2006; DDE, Dowling *et al*., 2006; Fe, Zn, Mn, Cu, Pb, Ni, Cr, Cd and As, Romero-Ruiz *et al*., 2006; organic chlorines, polychlorobiphenyls, petroleum hydrocarbons, polyaromatic hydrocarbons, furans, dioxins, metals Amelina *et al*., 2007; Cd, Chora *et al.,* 2009, *see Chapter 3.1*). The clam *Ruditapes decussatus*, (Linnaeus, 1758) (Veneroida, Veneridae) is widely distributed in Atlantic and Mediterranean coastal waters. This species is used as a bioindicator due to its sessile nature, filter-feeding habits and the ability to accumulate contaminants (Bebianno *et al*., 2004). Many biomarkers were measured in this sentinel species (MT, SOD, CAT, GPX, GST, AChE, LPO) in different laboratory and field conditions (Bebianno *et al*., 2004; Dellali *et al*., 2004; Barreira *et al*., 2007; Ketata et al., 2007a,b; Serafim and Bebianno, 2007a,b). However, there are few proteomic studies using this species. These include protein

carbonylation and HSP response due to p,p'-dichlorodiphenyldichloroethylene (DDE) exposure and ubiquitination and carbonylation as markers of oxidative-stress as a result of cadmium exposure (Dowling *et al*., 2006, Chora *et al*., 2008, *see Chapter 4.1*). Therefore the aim of this paper was to assess the effect of NP (100 $\mu g.l^{-1}$) on protein expression signatures in the gills and digestive gland of the clam *R. decussatus* using two-dimensional gel electrophoresis (2-DE). Significant proteins were sequenced by capillary liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and subsequently identified.

3.2.2. Materials and methods

3.2.2.1. Reagents

Reagents were purchased from GE Healthcare (Uppsala, Sweden) (agarose, ammonium persulfate, bromophenol blue, CHAPS, DTT, glycerol, glycine, HEPES, Immobiline DryStrips, iodoacetamide, pharmalyte 4-6.5, SDS, tris, TEMED, thiourea, urea), from Sigma-Aldrich Sigma (St Louis, MO) (acetone, acetonitril, EDTA, formaldehyde, formic acid, methanol, technical grade nonylphenol, PMSF, protease inhibitor P8340, trichloroacetic acid) and from Promega (Madison, WI, USA) (trypsin).

3.2.2.2. NP exposure

Clams were collected in the "Ria Formosa" lagoon (southern coast of Portugal), Ria Formosa Natural Park, and taken alive to the laboratory. In the laboratory, 4 groups of 25 clams, of about the same size (36.1 ± 1.6 mm), were maintained in 25 L glass containing natural aerated seawater (35 psu, 18.0 ± 0.5ºC). Following one week of

acclimation, 2 groups were exposed to NP (100 µg.l^{-1}) (stock solution of 1.4 mgNP.l^{-1} prepared in DMSO) and the remaining groups kept in seawater, as controls (*see Chapter 2.1*, Figure 2.2). Water was changed every other day providing clams with natural food to avoid starvation and any effect resulting from the food content and NP concentration re-established. During the exposure time (21 days) 2 and 5 individuals of control and exposed, respectively, died. Following the exposure, 10 animals, 5 controls and 5 NP exposed, were collected and dissected into gills and digestive gland. Samples were immersed in liquid nitrogen and stored at -80 °C until further use.

3.2.2.3. Cell-free extract preparation and protein assay

Each pool of three tissues was weighed, suspended in 20 % (w/v) buffer (10 mM HEPES and 250 mM Saccharose solution with 1 mM DTT, 1mM EDTA, 1mM PMSF, 1/1000º protease inhibitor P8340 SIGMA) and homogenised at 4 ºC employing a Ultra-Turrax IKA-Werke homogenizer. Cell-free extracts were obtained by centrifugation at 15 000 *g* for 2 h and the soluble protein extract was immediately frozen (-80 °C) until further use. Quantification of the protein content was made by Bradford method (1976) using bovine serum albumin (BSA) as standard. It was checked that nonylphenol did not altered protein assay. Each aliquot of 150 µg protein was suspended in nine volumes of precipitation solution (10% trichloroacetic acid in cold acetone containing 20 mM DTT) during 2h, at -20 ºC, centrifuged at 10 000 *g* for 30 min, at 4 ºC, and washed with cold acetone. The remaining acetone was eliminated by air drying (Chora *et al*., 2009, *see Chapter 2.2.1*).

3.2.2.4. 2DE-SDS PAGE

Proteins were initially separated by isoelectric focusing (IEF) followed by SDS PAGE. All the samples, containing 150 μg of protein each, were incubated during 30 min in 300 μL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.8 % pharmalyte, 65 mM DTT and bromophenol blue traces), centrifuged (14000 *g*, 10 min, 4 ºC) and loaded on Immobiline ® DryStrip (pH 4-7, 18 cm) (Chora *et al*., 2009, *see Chapter 2.2.1*). Following 6h of passive and 6h active (50 V) rehydration, proteins were focused (20 ºC, 50 μA/strip) in a Protean IEF Cell through a five steps program with linear voltage increases: 1000 V(1h); 4000 V (1h); 8000 V (1h) and 8000 V, until reaching a total of 50 000 Vh. The strips were then frozen at -80 °C. After IEF strips were equilibrated for 15 min in equilibration buffer (6 M urea, 75 mM Tris, pH 8.8, 4% SDS, 29.3% glycerol, and bromophenol blue traces, containing 2 % DTT). After that strips were equilibrated during 15 min in buffer containing 2.5% iodoacetamide. Two-dimensional SDS PAGE was performed using 10% polyacrylamide gels in a Protean Cell XL Cell Format vertical system (20 ºC) through two steps: 90 V (30 min) and 300 V until the end of separation (~5h). The 2DE equipment was purchased from Bio-Rad, Hercules, CA, USA. Two-dimensional gels were visualized by silver staining using a protocol compatible with MS analysis (Blum *et al*., 1987). To guarantee the reproducibility of the 2DE gels, four replicates of each condition, control and treatment with NP, were produced.

3.2.2.5. Image acquisition and analysis

Following staining, gels were scanned on a GS-800 calibrated densitometer (Bio-Rad Laboratories) and the optical density from each lane was measured by PDQuest

software (V8.0, Bio-Rad) (Bio-Rad). Data were analyzed including spot detection, quantification and normalization, data analysis and statistics. An average of at least three replicates from three different extracts for each condition and tissue studied was determined. All the 2-DE gel analysis were performed with the same background subtraction (floating ball method) (10-fold) directly after the spot detection. The 2DE gels from the exposed tissues were then matched to the control 2DE gels. To allow a good comparison between the measurements of spots in different gels, a normalization step was performed: the normalized volume for a spot was obtained by dividing its volume by the total volume of the detected spots on the image. The amount of valid protein spots was calculated for each gel, as well as the number of proteins matched to all the gels. Qualitative and quantitative differences in the protein patterns between the treatment and control group were assessed. Spots eightfold (or higher) differentially expressed common to control and contaminated tissues, new and missing were selected. Nonparametric Mann–Whitney U-rank test was used to compare control and exposed groups. Values were expressed as means ± standard error (S.E.) performed in quadruplicate. A significance of $p < 0.05$ was considered.

3.2.2.6. NP Analysis

NP concentration in clam tissues was extracted by microwave using dichloromethane. The extract was reduced with petroleum ether to 1 ml and NP detection was made using gas chromatography (GC) coupled with mass spectrometry equipped with simple quadrupole. An aliquot of 1 µl was injected in the GC column (type 5MS 25m*0.25*0.25) and temperature programmed. Detection was made by SIM (selected ion monitoring) and validated by identification of the three characteristic ions of the NP molecule. Only 4-NP was identified and quantified (ISO norm 18857-1).

3.2.2.7. In-gel tryptic digestion, MS analysis and protein identification

Spots were manually excised from the gels and destained as described by Gharahdaghi *et al*. (1999). Gel pieces were crushed in Eppendorf tubes, dehydrated with acetonitril (ACN) for 5 min and vacuum-dried. After, gel pieces were rehydrated with 15-50 µl of ammonium bicarbonate acetonitril, (25 mM : 10%), complemented with trypsin (5 $ng.\mu l^{-1}$, Promega, Madison, WI, USA) and then overlayed with the same volume of buffer without trypsin. Following incubation (20-24 h, 37 °C), the volume was adjusted to 100 µl with ultra-pure water and acidified with 25 µl of formic acid at 25 % (5 % final concentration). Tryptic peptides were extruded from the gel through adding 125 µl of ACN (15 min at room temperature). The gel pieces were spun down (8000 *g*, 5 min, room temperature) and the supernatant was collected. The pellet was overlaid with 50 µl of acetonitrile and vortexed to complete peptide extrusion. The extracted material was pooled with the supernatant and vacuum-dried. Lysates were solubilised in 10 µl 5% formic acid / 20% methanol and stored at –20 °C until further use. All samples were analysed by micro LC/ESI/MS/MS on a LTQ / FT-Orbitrap mass spectrometer (Thermo Fisher, Waltham, MA, USA) coupled with pumps and auto-sampler under standard conditions: capillary temperature, 275 °C; source voltage, 4500 V. Helium was used as collision gas. Experiments were done in parallel mode (survey at 30000 resolution and 5 data dependent ion trap MS/MS (Top 5). The MS/MS parameters were: isolation width, 3; and collision energy, 35%. Micro-HPLC SURVEYOR (Thermo Fisher) 30 min. gradient, BioBasic C18 (Thermo Scientific, Waltham, MA, USA) column (100 x 0.18 mm). This high mass accuracy on the precursor ion allowed the elimination of any false positive peptide identifications, suggesting that peptides that do not match the specificity of the protease used in the digestion must not automatically be considered as

false positives. Acquired MS/MS spectra were analysed by the Mascot version 2.2.0 (Matrix Science, London) in house software. Search parameters were set as follows: enzyme specificity: trypsin; 1 missed cleavage permitted; fixed modification: carbamidomethylation of cysteine; variable modification: methionine oxidation; mass tolerance for precursor ions: 10 ppm; mass tolerance for fragment ions: 0.6 Da. Significance thresholds were at $p< 0.05$ and score above 50. Both b and y ion series were used to search against UniProt KB / Swiss-Prot/TrEMBL or NCBI National Center for Biotechnology Information (Bethesda, MD, USA) nr (non-redundant) databases. No species restriction was applied to allow protein identification by sequence homologies conserved through the phylogenesis. In case of peptides matching to multiple members of a protein family, the presented protein was selected based on both the highest score and the highest number of matching peptides. The tblastn algorithm (all taxa less human and mouse: NCBI-EST others and with molluscs as optional organisms) was used to try a search with the matched peptides. *De novo* sequencing was made using PEAKS Studio 4.5 (Ma *et al*., 2003). The *de novo* sequence from LC MS/MS spectra was then used to search the sequence databases (NCBI), using BLAST (Basic Local Alignment Tool).

3.2.3. Results

3.2.3.1. NP content in the gill and digestive gland

Nonylphenol concentrations in tissues from unexposed clams were similar between the two tissues (0.11 ± 0.02 $\mu g.g^{-1}$ dw in gill and 0.13 ± 0.02 $\mu g.g^{-1}$ dw in digestive gland, n= 3). After exposure to 100 $\mu g.l^{-1}$ for 21 days, NP was considerably accumulated in

both tissues (43.52 ± 0.53 µg.g^{-1} dw in gill and 55.29 ± 0.60 µg.g^{-1} dw in digestive gland, n= 3).

3.2.3.2. Protein expression patterns in the gill and digestive gland

Protein expression profiles (PEPs) were obtained by 2DE SDS-PAGE in the cytosolic fractions of *R. decussatus* gill and digestive gland. Initially, proteins were loaded in IPG strips of pH (3–10 NL) range.

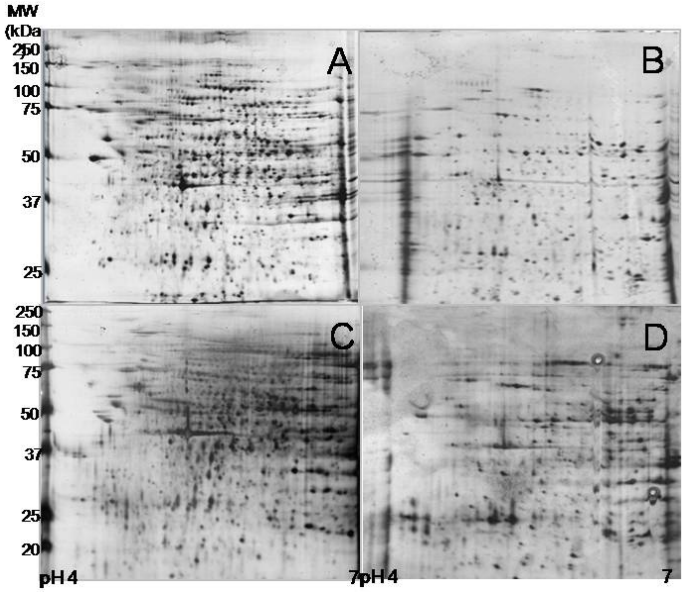

Figure 3.2.1 - Representative 2DE gels, loaded with 150 µg protein: A- control gill; B – NP exposed gill; C – control digestive gland; D- exposed digestive gland.

Nevertheless, since most of proteins were found in the pH range 4-7, strips with 4-7 pH range were used loaded with 150 µg protein from each tissue. The PEPs obtained for gill and digestive gland of unexposed clams were distinct. The electrophoresis and data analysis allowed discriminating 816 ± 46 average protein spots from the gill and 922 ± 17 from the digestive gland of control clams (Figures 3.2.1 A-C). The total number of proteins and the pattern of PEPs changed after NP exposure (Figures 3.2.1 B-D).

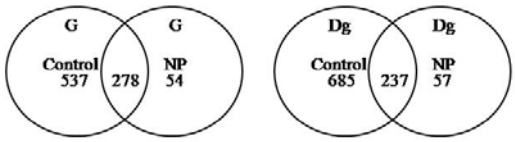

Figure 3.2.2 - Venn diagram showing the differences between spots from NP exposed and control samples of gill (G) and digestive gland (Dg).

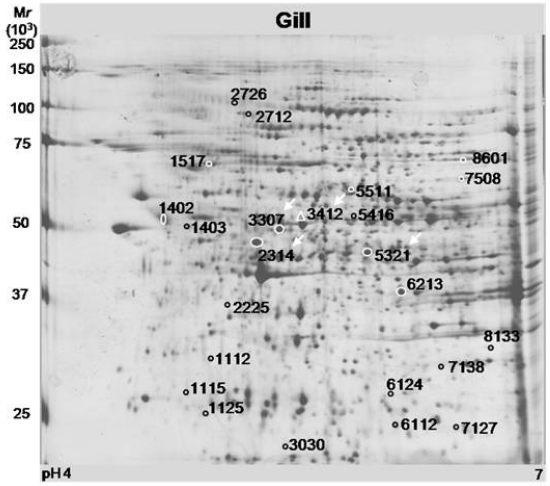

Figure 3.2.3 - *Ruditapes decussatus* gill representative control 2DE gel (n = 4). One hundred fifty micrograms of protein content were separated on 18 cm IPG strips, in 4–7 pH gradients. The second dimension was performed in 10% SDS-PAGE gels. Spots 8-fold up/down-regulated after NP exposure are circled white; suppressed spots are indicated by white triangles. Spots are indicated by PDQuest numbers. The protein names of the identified spots are listed in Table 3.2.1.

In the gill, 473 spots were differently expressed after NP exposure (Mann–Whitney U-rank test, $p < 0.05$). Fourteen were 8-fold up-regulated, 54 (7 %) (Figure 3.2.2) were newly detected and 10 decreased by 8-fold (Figure 3.2.3). Approximately 66 % of proteins disappeared and 278 spots were common to control and treated gels (Figure 3.2.2). The down-regulated proteins were concentrated in the centre of the 2DE map (p*I* 4.9 - 6.52, *M*r 67.1 - 37.5 kDa, Figure 3.2.3). The majority of up-regulated proteins were distributed in the lower-half part (p*I* 5.5 - 6.5, *M*r 35.9 - 22.3 kDa, Figure 3.2.3).

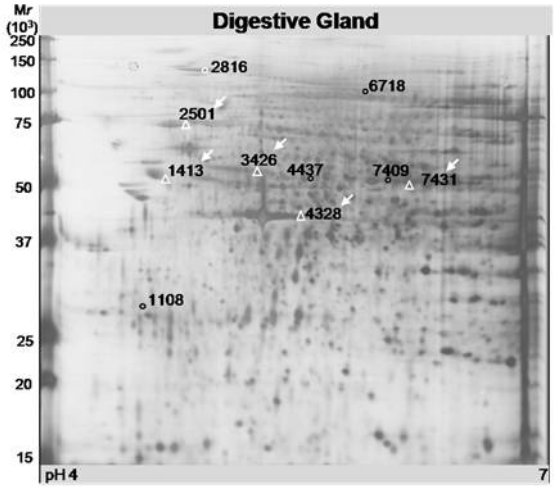

Figure 3.2.4 - *Ruditapes decussatus* digestive gland representative control 2DE gel (n = 4). One hundred fifty micrograms of protein content were separated on 18 cm IPG strips, in 4–7 pH gradients. The second dimension was performed in 10% SDS-PAGE gels. Spots 8-fold up/down-regulated after NP exposure are circled in black/white, respectively; induced/suppressed spots are indicated by black/white triangles, respectively. Spots are indicated by PDQuest numbers. The protein names of the identified spots are listed in Table 3.2.1.

In the digestive gland 701 protein spots were differentially expressed (Mann–Whitney U-rank test, $p < 0.05$). Five were 8-fold up-regulated (Figure 3.2.4), 57 new (6 %), 1 down-regulated by 8-fold, 74 % disappeared, whereas 237 spots were common to both conditions (Figure 3.2.2). The down-regulated spot (SSP 2816) was situated in the upper part of the 2-DE map while the up-regulated spots varied with p*I* and *M*r (p*I* 4.5 - 5.8, *M*r 111.1 - 22.1 kDa (Figure 3.2.4).

Table 3.2.1 - LC-MS/MS identifijcation of marked spots on the representative gill and digestive gland 2DE gels (Figures 3.2.3 and 3.2.4). Spot nº.: spot number; A. no.: accession number of NCBI database of matched protein; Pep: peptides matched. Cov: amino-acid sequence coverage for the identified proteins. Exp.: protein expression after NP exposure.↓: ≤ 8-fold down-regulated; ↑: ≥ 8-fold up-regulated; 0: suppressed spots.

Spot No.	A.n.	Obs./Theo[a] *Mr* (kDa)	Obs./Theo[b] p*I*	Protein identity	Peptide sequence	Biological function	Score[c]	Pep.	Cov (%)	Species	Exp.[d]
					A – Gill						
2314	P18288	46.4/46.4	5.3/5.3	Tubulin α-2/α-4 chain	TIQFVDWCPTGFK (1597.7) AVFVDLEPTVVDEVR (1686.8) NLDIERPTYTNLNR (1717.8) VGINYQPPTVVPGGDLAK (1823.9) AVCMLSNTTAIAEAWAR (1863.8)	Cytoskeletal structure/function	152	6	18	*Patella vulgata*	↓ (-8)
3307	P11833	49.8/49.7	5.3/5.4	Tubulin ß chain (ß tubulin)	LAVNMVPFPR (1142.6) NSSYFVEWIPNNV (1695.8)	Cytoskeletal structure/function	239	2	16	*Paracentrotus lividus*	↓ (-17)
5321	O02654	43.9/47.7	5.9/5.8	Enolase	HIADLAGNK (937.4) AAVPSGASTGIYEALEMR (1837.8) SGETEDTFIADLVVGLCTGQIK (2352.1)	Glycolysis	106	5	14	*Loligo palei*	↓ (-9)
3412[e]	Q9CZS1	57.2/58.1	5.8/6.6	ALDH member B1	ADVDLAVK (829.4) YYAGWADK (972.4) ELGEYGLQAYTEVK (1599.7)	Amino acid Metabolism	72	5	6	*Mus musculus*	0 (-85)
					B - Digestive gland						

2501	P11147	71.0/71.4	4.8/5.4	Heat shock 70 kDa protein cognate 4	ITITNDKGR (1016.5) LLQDFFNGK (1080.5) VEIIANDQGNR (1227.6) FEELNADLFR (1252.6) ARFEELNADLFR (1479.7) IINEPTAAAIAYGLDK (1658.8) QTQTFTTYSDNQPGVLIQVYEGE R (2773.3)	Cell maintenance	551	14	16	*Drosophila melanogaster*	0 (-103)
3426	P41383	50.5/50.9	5.0/4.9	Tubulin α -2/α -4 chain	VGINYQPPTVVFGGDLAK (912.9) TIGGGDDSFNTFFSETGAGK (1004.4) FDGALNVDLTEFQTNLVPYPR (1205.1) QLFHPEQLITGKEDAANNYAR (1208.1)	Cytoskeletal structure/function	965	40	47	*Patella vulgata*	0 (-146)
4328 [e]	Q25472	42.6/42.5	5.5/5.1	Actin, muscle-type	AGFAGDDAPR (975.4) GYSFTTTAER (1131.5) EITALAPSTMK (1176.6) DSYVGDEAQSKR (1353.6) SYELPDGQVITIGNER (1789.8) VAPEEHPVLLTEAPLNPK (1953.0) DLYANTVLSGGTTMYPGIADR (2230.0) LCYVALDFEQEMATAASSSSLEK (2565.1) (2200.0)	Cytoskeletal structure/function	764	21	51	*Molgula oculata*	0 (-971)
7431	O02654	50.5/47.7	6.8/5.8	Enolase	HIADLAGNK (937.4) AAVPSGASTGIYEALEMR (1837.8) SGETEDTFIADLVVGLCTGQIK (2352.1)	Glycolysis	294	3	9	*Loligo pealei*	0 (-391)
1413	Q5M7K4	51.2/48.0	4.6/4.8	Histone-binding protein (RBBP7)	VINEEYKIWK (1320.7) YMPQNPCIIATK(1450.7) TPSSDVLVFDYTK (1470.7) *de novo* sequencing (*see* Table 3.2.2)	Signal transducers transcription factor regulators	263	8	17	*Xenopus (Silurana) tropicalis*	0 (-254)

Peptides sequences: The above table gives the peptide sequences with the highest individual ion scores indicating identity or extensive homology ($p<0.05$), the number in parentheses are the experimental *M*r, the sequence underlined is the one with the best score.

[a]Observed (Obs.) isoelectric point (p*I*) and molecular mass (*M*r) were obtained from gel migration. [b]Theoretical (Theo.) isoelectric point (p*I*) and molecular mass (*M*r) were obtained from Swiss Prot./TrEMBL database.

[c]Scores of the matches using MASCOT version 2.2.0 (Matrix Science, London) software.

[d]The spot intensity ratio between control and NP treated samples is shown in brackets. Ratios are calculated by PDQuest using the average of four replicates in each group. For all comparisons the *p*-value is <0.05.

[e]These Spots were already identified in Chora *et al*., 2009 (*see Chapter 3.1*).

Tblastn spot 2314 FG596303.1 *Pinctata martensi,* cDNA clone, CHIP_05_liver_12_70.PT_F09.ab1, mRNA sequence.

Blastp spot 2314: general database - Alpha tubulin, putative of *Schistosoma mansoni;* Blastp mollusc database: BAD88768,Tubulin of *Crassostrea gigas.*

Blastp spot 3307: molluscs database, AAU93877, ß tubulin of *Crassostrea gigas.*

Tblastn spot 3412: AJ624775 *Mytilus galloprovincialis* haemolymph, gills, digestive gland, foot, adductor muscles and mantle *Mytilus galloprovincialis,* cDNA clone GPPD03500, mRNA sequence.

Blastp spot 3412: molluscs database: AAF73122.1 Aldehyde dehydrogenase, *Placopecten magellanicus.*

Tblastn spot 2501: Mg_Nor01_32l15 nor01 *Mytilus galloprovincialis,* cDNA 3', mRNA sequence.

Blastp spot 2501: general database – ACO72585, Heat shock protein, *Argopecten purpuratus.* Blastp mollusc database: ACF31553, Heat shock protein 70, *Pincata fucata*¸ AAS17723, Heat shock protein 70, *Argo. Irradians.*

Tblastn spot 3426: GE757990, MUW08-N05.y1d-s SHGC-MUW *Mytilus californianus* cDNA 3', mRNA sequence.

Blastp spot 3426: molluscs database, BAD88768, tubulin, *Crassostrea gigas.*

Tblastn spot 7431: ABM46846, alpha enolase *Echiurus echiurus.*

Blastp spot 7431: general database: ABM46843 alpha enolase, *Leptochiton sp.*

Considering that *R. decussatus* is a non-model organism, most protein sequences are absent from databases. Therefore, protein identification by LC MS/MS was done by homology. The identified proteins are presented in Table 3.2.1 (A: gill; B: digestive gland). The species presented in last column of Table 3.2.1 A and B is provided by NCBI blast (Mascot search results). When the same aminoacid sequence and the same access number are obtained, NCBI blast stated the species with the best score and homology. Figure 3.2.5 shows close-up views of the spots identified.

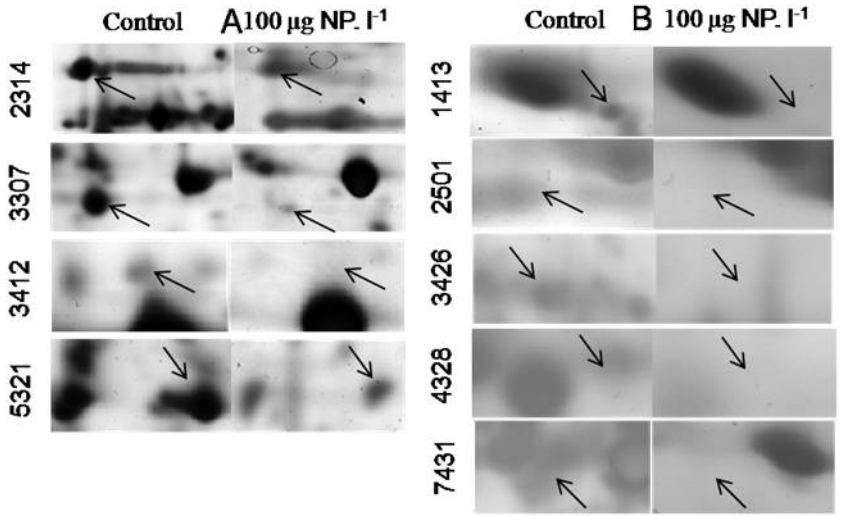

Figure 3.2.5 - Differential expression of the proteins identified. Arrows on cropped images of 2DE gels represent protein spots that showed significantly different changes in gill (A) and digestive gland (B) between control and NP treatment.

A good agreement between predicted and observed p*I* and *M*r was obtained. Three 8-fold down-regulated proteins were identified in the gill: spot 2314 (5.3/46.4) as tubulin α-2/α-4 chain, obtained from *Patella vulgata*; spot 3307 (5.3/49.8) was similar to a putative ß tubulin chain of *Paracentrotus lividus* and spot 5321 (5.9/43.9) was homologous to enolase of *Loligo palei*.

Eight proteins, which disappeared in treated samples, were also identified: 7 existing only in digestive gland and 1 only in gill (Figures 3.2.3 - 3.2.4, Table 3.2.1). Spot 3412 (5.8/57.2), suppressed in treated gill, was similar to aldehyde dehydrogenase X, mitochondrial precursor (ALDH family 1 member B1) of *Mus musculus*. In digestive gland controls: spot 2501 (4.8/71.0) was similar to heat shock 70 kDa protein cognate 4 (Hsc70-4) of *Drosophila melanogaster*; spot 3426 (5.0/50.5), corresponded to tubulin α-2/α-4 chain of *Patella vulgata*; spot 4118 (5.5/40.7) was identified as actin-15A by homology with a protein from *Strongylocentrotus franciscanus*; spot 4328 (5.5/42.6) was homologous to actin, muscle-type (A2) of *Molgula oculata*; spot 5413 (5.7/55.0) was identified as enolase, obtained from *Loligo palei*; spot 7431 (5.8/47.7) was identified as enolase by homology with a protein from *L. palei* and spot 1413 (4.6/51.2) homologous to histone-binding protein (RBBP7) of *Xenopus (Silurana) tropicalis*. RBBP7 was unambiguously identified by *de novo* sequencing (Table 2). The generated sequence, SSDVLVFDYT, was searched in databases: this fragment belongs to ACO15352.1, similar to probable histone-binding protein Caf1 of *Caligus clemensi*.

The majority of the identified proteins have cytoskeletal and structural functions.

Spot 1413 was unambiguously identified, by *de novo* (Table 3.2.2), as probable histone-binding protein Caf1 from *Caligus clemensi*.

Table 3.2.2 – *R. decussatus* proteins identified by *de novo* sequencing by PEAKS-STUDIO 4.5. Spot nº.: spot number; A. nº.: accession number of NCBI database of matched protein; Pep.: peptides matched; Cov: amino-acid sequence coverage for the identified proteins. Exp.: protein expression after NP exposure. 0: suppressed spot.

Spot Nº	Obs.[a] pI/ *M*r(kDa)	Theor.[b] pI/ *M*r(kDa)	Protein identification	A. nº	Biological function	Peptide Sequence	Sco[c]	Pep.	Cov %	Species	Exp.[d]
1413	4.6/51.2	4.8/48.0	Histone-binding protein RBBP7	Q5M7K4	Signal transducers transcription factor regulators	TPSSDVLVFDYTK	263	8	17	*Xenopus (Silurana) tropicalis*	0 (0.00)
			De novo sequencing: EST others: probable histone-binding protein Caf1	ACO15352.1		SSDVLVFDYT SSDVLVFDYT				*Caligus clemensi*	

[a]Observed (Obs.) isoelectric point (p*I*) and molecular mass (*M*r) were obtained from gel migration.

[b]Theoretical (Theo.) isoelectric point (p*I*) and molecular mass (*M*r) were obtained from Swiss Prot./TrEMBL database.

[c]Scores of the matches using MASCOT version 2.2.0 (Matrix Science, London) software.

[d]The spot intensity ratio between control and NP treated samples is shown in brackets. Ratios are calculated by PDQuest using the average of four replicates in each group. For all comparisons the *p*-value is <0.05.

3.2.4. Discussion

The nonylphenol (NP) concentration used (100 µg.l^{-1}) was chosen as a function of the highest NP concentration found in the environment (95 µg.l^{-1}) (Dachs *et al*., 1999). This concentration is sub-lethal since mortality was not affected as well as siphon extension and therefore filtration in the clams. No mortality occurred in molluscs such as *Dreissena polymorpha*, *Cerastoderma glaucum* and *Mytilus galloprovincialis* (Quinn *et al*., 2006; Marin *et al*., 2008; Ricciardi *et al*., 2008, respectively) at this NP concentration. However, a decrease in respiration rate, clearance and scope for growth was reported for the clam *Tapes philippinarum* exposed to 100 µg NP.l^{-1} (Matozzo *et al*., 2003). In the present paper, experiments lasted 21 days and the clams were not fed and water was changed every other day providing animals with natural food; low mortality was observed both in controls and treated animals. NP was accumulated in *R. decussatus* gill and digestive gland. The highest concentration was in the digestive gland, which is in accordance with previous data with *Tapes philippinarum* (ratio between NP concentrations, after 14 days of exposure to 25 µg NP.l^{-1}, was. 3:2, Lietti *et al*., 2007). Exposure to NP caused changes in protein expression profiles (PEPs) obtained after 2DE SDS-PAGE and image analysis. It was confirmed that these modifications not resulted from NP or its metabolites influence in the protein assay by dyes (Bradford method). Different protein expression profiles (PEPs) were obtained for the two tissues, due to their different functions in the clam. An overall decrease of protein spots (> 50 %) was detected in both treated tissues. Lysosomal autophagy could explain this decrease since it is considered as a survival strategy (Cuervo, 2004; Moore, 2004). This process has some advantages to organisms, especially molluscs, exposed to pollutants, by protecting the cell against the harmful effects of damaged and

malfunctioning proteins (Moore, 2004; Moore *et al*., 2006). The higher protein reduction detected in digestive gland (detoxification tissue) compared to the gill may be the result of high NP bioaccumulation and/or slower NP elimination from the digestive tract (Lietti *et al*., 2007). Gill, in direct contact with the external medium, exhibits multi-xenobiotic defense or resistance proteins contributing to the removal of organic pollutants from this tissue (Kurelec *et al*., 1995).

Some authors (Okai *et al*., 2004; Gong and Han 2006) demonstrated that nonylphenol exerts oxidative stress by producing reactive oxygen species, in particular H_2O_2 and $O_2^{\bullet-}$ (superoxide anion). In *Tapes philippinarum* exposed to NP, an inhibition of antioxidant enzymes was attributed to oxidative stress (Matozzo *et al*., 2004). Oxidative damage can lead to oxidation, glutathionylation, glycation, carbonylation and/or ubiquitination of proteins. Carbonylation and/or ubiquitination of proteins were already shown in *R. decussatus* exposed to DDE pollutants demonstrating tissue-specific redox requirements (Dowling *et al*., 2006, Cd Chora *et al*., 2008, *see Chapter 4.1*). Ubiquitinated proteins may be removed from cells by proteolysis via the ubiquitin-proteasome pathway (UPP) (Marques *et al*., 2004). This removal may also explain the high protein spot reduction in treated samples.

In this study several proteins were identified by LC MS/MS, in particular, a considerable number of cytoskeletal proteins (tubulin and actin). This could be due to their abundance, prevalence in mollusc databases, or to the role as major targets of pollutant-related OS (Rodriguez-Ortega *et al*., 2003; Miura *et al*., 2005). Tubulins and actin were down-regulated in NP treated clams compared to controls. Alpha-tubulins, that form tubulin heterodimers, get assembled into linear proto-filaments that pull together into microtubules. An important function of these microtubules is to move

around cellular structures such as chromosomes, mitotic spindles, and other organelles inside the cells (Howard and Hyman, 2003). Since bis-phenols could affect both polymerization and inhibition of microtubule assembly in culture cells (Sakakibara *et al*., 1990), NP could have affected the gill and digestive gland of *R. decussatus*, inducing the α-tubulin down-regulation/suppression. The down-regulation of ß-tubulin found in clam gill and also reported in *M. edulis* (digestive gland) from polluted sites (Apraiz *et al*., 2006) is related to the structure and function of the cytoskeleton and to oxidative stress effects (Miura *et al*., 2005). Actin (15 A and muscle type) was suppressed in clam digestive gland. An actin down-regulation was already reported in the gill and digestive gland of *M. edulis* (Manduzio *et al*., 2005; Amelina *et al*., 2007) and in the whole soft body of *Chamaelea gallina* (Rodriguez-Ortega *et al*., 2003) from contaminated sites. These results pointed out to the decline of the condition state of animals probably due to oxidative stress that alters Ca^{2+} homeostasis (Gomez-Mendikute *et al*., 2002). High concentrations of Ca^{2+} activate proteases, which might hydrolyze the actin filaments and the proteins fixing them to the cellular membranes. More generally, different authors pointed out the down-regulation of proteins from the cytoskeleton in molluscs exposed to pollutants aroclor 1254, Cu(II), TBT and As(III) (Rodriguez-Ortega *et al*., 2003) and diallyl phthalate, PBDE-47, and bisphenol-A (Apraiz *et al*., 2006).

The protein Hsc70-4 was suppressed in digestive gland. Hsc70-4 is the constitutive protein of heat shock protein 70 (HSP70) family, with a high sequence homology with HSP70 protein. HSP70 was up-regulated in *M. edulis* from polluted sites (Manduzio *et al*., 2005) whereas Hsc70 was down-regulated in *M. edulis* (digestive gland) from contaminated areas (Amelina *et al*., 2007). HSP70 is a ubiquitous chaperone family that

co-operates with various co-chaperones and serves several cellular functions (Mayer and Bukau, 2005). A direct actin-small HSP interaction may inhibit actin polymerization and participate in the *in vivo* regulation of actin filament dynamics. In such a way, the cytoskeleton is protected against the disruption induced by various stressful conditions (Arrigo *et al*., 2002). In agreement with this, phosphorylation of small HSP was described as causally related to the regulation of microfilament dynamics following oxidative stress, and α-tubulin promotes phosphorylation to prevent the formation of microtubules under oxidative stress (Miura *et al*., 2005). However, NP is thought to inactivate phosphorylation in *Mytilus galloprovincialis* (Canesi *et al*., 2004). This could explain the Hsc70-4, actin and tubulin down-regulation/suppression. The use of HSPs as biomarkers was assessed in laboratory for marine bivalves: HSPs were induced in *M. edulis* whole body exposed to Aroclor1248 (Olsson *et al*., 2004); in *R. decussatus* HSP-60 was induced in gill exposed to TBT (Solé, 2000) and HSP-60 and HSP-70 were induced in the digestive gland exposed to DDE (Dowling *et al*., 2006).

Aldehyde dehydrogenase (ALDH) was suppressed in treated clam gill. This enzyme is involved in oxidation (dehydrogenation) of aldehydes to carboxylic acid and acts on primary unbranched aldehydes. ALDH was also down-regulated in *Mytilus edulis* from contaminated sites (Amelina *et al*., 2007) or exposed to diallyl phthalate and bisphenol-A (Apraiz *et al*., 2006). This enzyme was employed as a biochemical marker of pro-oxidants in *M. galloprovincialis* from polluted sites (Nasci *et al*., 2002; Nesto *et al*., 2004).

Enolase, an enzyme involved in glycolysis and energy yield, was also down-regulated/suppressed in gill and digestive gland of *R. decussatus*, respectively. This suggests that NP exposure can cause energetic potential change in this species. In *T.*

philipinarum, NP exposure induced a delay in its re-burrowing capacity (Matozzo *et al*., 2004) and reduction in respiration rate and scope for growth (Matozzo *et al*., 2003). Enolase, also known as phosphopyruvate dehydratase, is a metalloenzyme responsible for the catalysis of 2-phosphoglycerate to phosphoenolpyruvate, the penultimate step of glycolysis. Tubulin α -2/ α-4 chain identified in both tissues were down-regulated in gill and suppressed in digestive gland. This could be due to higher NP accumulation in the digestive gland and to different detoxification pathways between the two tissues.

RBBP7, which was suppressed, was unambiguously identified by *de novo* sequencing in the digestive gland of *R. decussatus* (Table 3.2.2). This protein is a member of retinoblastoma-binding proteins (RBPs). These structural proteins participate in chromatin packing, being considered general markers of DNA synthesis. Nevertheless, since RBPs are involved in gene transcription modulation, their participation in stress response processes was suggested (Dondero *et al*., 2006). This could explain its suppression as a response to stress caused by NP exposure. However, histone-binding gene was up-regulated in *Crassotrea gigas*, mantle and gill, under hypoxia and in the digestive gland of *M. edulis* exposed to copper (Dondero *et al*., 2006). In conclusion, PES (protein expression signatures) obtained and identified in this work provides novel information on the possible mechanisms of toxicity of NP in *R. decussatus* gill and digestive gland and is a more robust technique than single biomarker analysis. Proteomics have several advantages compared to the well-established biomarkers since 2DE SDS-PAGE allows detection and quantification of a great amount of spots (Vioque-Fernández *et al*., 2009). The identification of putative proteins from PES provides additional knowledge of the effect of NP at biochemical and molecular levels.

Overall, these results reveal that NP induces major changes in PEPs in gill and digestive gland, mainly protein suppression. The surfactant nature of this compound may induce proteolysis. This tissue dependent response results mainly from protein inhibition and/or autophagy induction due to NP exposure. Identified proteins are involved in cytoskeletal structure maintenance (actin, tubulin), cell maintenance (Hsc70-4), metabolic activities (enolase, ALDH) and a signal transducers transcription factor regulator (RBBP7). Histone is not included in the "hit parade" of repeatedly identified differentially expressed proteins as referred by Petrak *et al*. (2008) from literature data.

The deep up- or down-regulation of those proteins confers them high potential to become possible biomarkers of NP exposure.

3.3. Comparison between protein expression profiles (PEPs) of gill and digestive gland in the clam *Ruditapes decussatus*, after cadmium and nonylphenol exposure

Cadmium (Cd) and nonylphenol (NP) are two pollutants found in marine ecosystems that could affect marine bivalves, mainly by causing endocrine disruption (Gagné and Blaise, 2003; Ketata b *et al.*, 2007) and enhancing ROS production (*see Chapter 4*). These compounds are easily accumulated by the clam *R. decussatus* (*see 3.1* and *3.2*) and in Chapter 3.1 and Chapter 3.2 of the present thesis proteomics was applied to assess their effects in this species. These studies revealed major changes in gill and digestive gland proteomes after Cd and NP exposure, mainly protein suppression. Cadmium induced changes in proteins involved in: cytoskeletal structure maintenance (actin muscle-type, actin adductor muscle and ß-tubulin), cell maintenance (Rab GDP) and metabolism (ALDH and MCAD, both identified by *de novo* sequencing). Nonylphenol caused also changes in proteins concerning: cytoskeletal structure maintenance (actin, tubulin), cell maintenance (Hsc70-4), metabolism (enolase, ALDH), suggesting energetic potential change, and in signal transducer transcription regulation (histone binding protein, identified by *de novo* sequencing). The aim of this Chapter is to compare the PEPs of gill and digestive gland after 21 days exposure to Cd (40 $\mu g.l^{-1}$) (Chapter 3.1) and NP (100 $\mu g.l^{-1}$) (Chapter 3.2), to characterize PESs and key protein signatures as well as to suggest possible new biomarkers for Cd and NP exposure.

Comparison between the proteomes after Cd and NP, (section 3.1 and 3.2, respectively) exposure was made using the PDQuest software (V8.0, Bio-Rad). All the 2-DE maps were performed with identical background subtraction (floating ball method) (10-fold) directly after the spot detection. The master gel constructed by combining the 2-DE gels (quadruplicates) from the control condition was matched with the 2-DE gels from the

Cd and NP exposed tissues. New and suppressed spots and spots with two-fold or greater variation expression common and non common to Cd and NP contaminated tissues were highlighted.

In gill and digestive gland, the quantity of significantly differential expressed proteins (Mann–Whitney U-rank test, $p < 0.05$) was high after exposure to both contaminants (*see* 3.1 and 3.2). The percentage of suppressed proteins was greater in NP exposed clams, in both tissues. In the gill, the number of new proteins was ca 60 (< 10 % total proteins) for Cd (59) and NP (54) in exposed clams; the number of suppressed proteins was much higher > 65% total proteins (533 for Cd and 537 for NP, respectively). In the digestive gland Cd induced much more proteins (137) than NP (57), and the effect of NP accumulation suppressed more proteins (685: 74 %) than effect of Cd (551: 60%) (Table 3.3.1). Change in protein expression more than 2-fold is in Table 3.3.2, including some of the 8-fold proteins already reported in 3.1 and 3.2 of the present chapter.

Thirty proteins from gill and 9 from digestive gland are common to both treatments and exhibited the same trend (up- or down-regulated, Figures 3.3.1 A-B). In the gill 20 proteins were up-regulated and 10 down-regulated. In the digestive gland 7 proteins were up-regulated and 2 down-regulated. These two groups of proteins are suggested as the minimal PESs of exposure to a mixture of the two pollutants.

Table 3.3.1 - New detected proteins; suppressed proteins; 2 fold up (↑) or down (↓) regulated proteins common between the two groups (Cd and NP); 2 fold non common proteins up- (↑) or down-regulated (↓) classified in different ranges (2– 4 fold, 4–8 fold, and more than 8 fold). N = 4 gels for each group.

Treatment	New spots (n)	Suppressed spots (n)	2 fold common spots (n)		2 fold non common spots (n)			
			↑	↓	2 fold	2-4 fold	4-8 fold	8 fold
Gill								
Cd	59 (7%)	533 (65%)	20	10	↑78 ↓37	↑46 ↓23	↑24 ↓9	↑9 ↓4
NP	54 (7%)	537 (66%)			↑73 ↓62	↑38 ↓37	↑25 ↓18	↑10 ↓8
Digestive gland								
Cd	137 (15%)	551 (60%)	7	2	↑109 ↓42	↑78 ↓33	↑27 ↓7	↑4 ↓2
NP	57 (6%)	685 (74%)			↑56 ↓37	↑31 ↓23	↑20 ↓13	↑5 ↓1

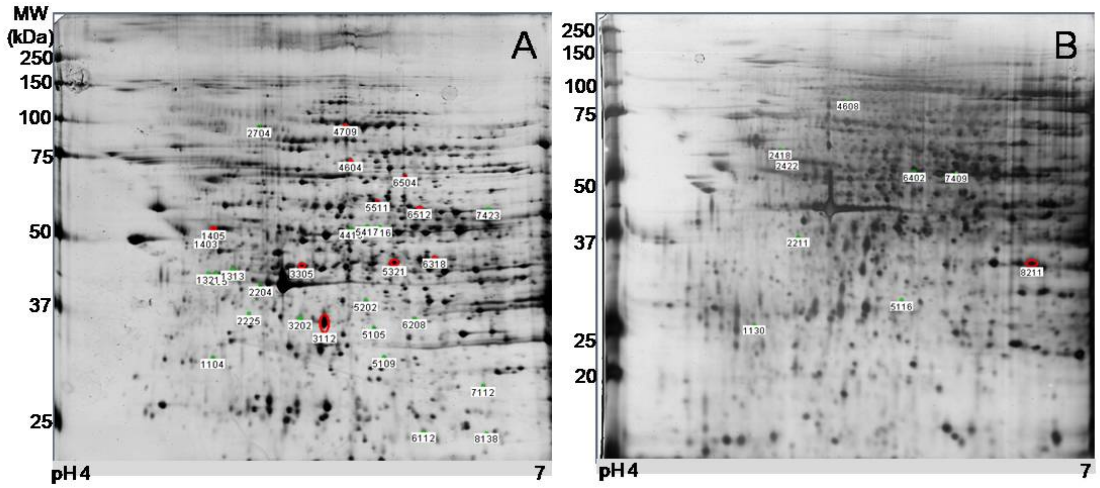

Figure 3.3.1 - Representative 2DE gels from gill (A) and digestive gland (B): proteins exhibiting the same trend after Cd and NP exposure (the minimal PESs of exposure to a mixture of both pollutants): green – up-regulated; red – down-regulated.

The characteristics of these 39 proteins (*M*r, p*I* and average ratio of expression of exposed/control group obtained by PDQuest, $p < 0.05$) and the average ratio variation between the two treatments are shown in Table 3.3.2.

In gill these proteins exhibited p*I* values between 5.04 and 6.37 and M*r* between 82.87 and 23.84 kDa. In the case of Cd exposure (Table 3.3.2), the average ratio was higher for up-regulated proteins nº 1104 and nº 5202 (Figure 3.3.2 A). Spots number 3202 and 8138 were previously identified as actin and actin muscle-type, respectively (*see* 3.1, Table 2). In response to NP exposure (Table 3.3.2), the highest average ratio corresponded to spots nº 1403, nº 2225 and nº 5416. Among the down-regulated proteins, NP induced the highest average ratio of proteins nº 5321 and 5511, while for Cd the highest average ratio corresponded to spot nº 6512 (Figure 3.3.2 A).

In the digestive gland, the set of common proteins up/down-regulated exhibited p*I* values between 4.75 and 6.23 and *M*r values between 85.30 and 22.08 kDa (Table 3.3.2).

Table 3.3.2 – Isoelectric point (p*I*) and molecular mass (*M*r) identification of the 2-fold, or higher, differentially expressed proteins common to the two groups (Cd and NP), and their average ratios of expression. Exp.: changes in protein expression 2-fold up-regulated (↑); 2-fold down-regulated (↓). Spot nº: spot number. The higher average ratios are underscored.

Gill						Digestive gland					
Exp.	Spot nº.	pI (Obs[a])	Mr (kDa) (Obs[a])	Average ratio[b]		Exp.	Spot nº	pI (Obs[a])	Mr (kDa) (Obs[a])	Average ratio[b]	
				Cd	NP					Cd	NP
↑	1104	5.07	30.76	<u>41.15</u>	7.33	↑	1130	4.75	22.08	2.32	2.04
↑	1205	5.09	41.45	3.48	3.13	↑	2211	5.07	35.76	2.19	4.89
↑	1313	5.17	42.53	4.38	3.36	↑	2418	4.96	59.29	3.29	2.21
↑	1321	5.05	41.47	4.74	4.65	↑	4608	5.40	85.30	4.76	7.69
↑	1403	5.04	50.34	4.45	<u>13.78</u>	↑	5116	5.66	24.78	2.44	3.33
↑	2204	5.25	39.71	3.02	5.70	↑	6402	5.70	53.43	2.33	2.71
↑	2225	5.22	35.89	12.36	<u>11.16</u>	↑	7409	5.88	53.67	<u>11.31</u>	<u>10.78</u>
↑	2704	5.24	82.14	2.24	3.18	↓	2422	4.99	55.90	2.36	<u>7.52</u>
↑	3202[c]	5.42	35.34	11.38	2.37	↓	8211	6.23	30.57	<u>24.31</u>	2.77
↑	4413	5.73	52.41	7.09	2.91						
↑	5105	5.86	34.15	3.60	3.99						
↑	5109	5.92	30.80	14.27	5.99						
↑	5202	5.81	37.56	<u>56.28</u>	3.43						
↑	5416	5.89	53.59	9.41	<u>12.67</u>						
↑	5417	5.83	53.25	4.69	3.19						
↑	6112	6.13	23.84	4.76	10.59						
↑	6208	6.09	35.32	4.15	3.94						
↑	7112	6.36	27.83	7.22	3.16						
↑	7423	6.37	57.06	3.17	2.96						
↑	8138[c]	6.37	23.72	13.73	8.12						
↓	1405	5.07	52.36	2.08	3.44						
↓	3112	5.55	34.71	5.51	6.1						
↓	3305	5.41	43.36	2.66	2.46						
↓	4604	5.71	72.09	2.44	4.73						
↓	4709	5.68	82.87	2.34	2.02						
↓	5321	5.97	43.99	6.28	<u>9.04</u>						
↓	5511	5.87	59.21	5.61	<u>10.38</u>						
↓	6318	6.17	45.24	4.98	2.01						
↓	6504	6.03	67.1	2.85	2.27						
↓	6512[c]	6.11	57.35	<u>10.78</u>	3.78						

[a]Observed (Obs.) isoelectric point (p*I*) and molecular mass (*M*r) were obtained from gel migration.

[b]Spot intensity ratio between control, Cd and NP treated samples. Ratios are calculated by PDQuest using the average of four replicates in each group. For all comparisons the *p*-value is <0.05.

[c]Proteins identified in 3.1.: nº 3202 – actin; nº 8138 – actin muscle-type.

The highest average ratio was similar for both contaminants in digestive gland and corresponded to the up-regulated protein nº 7409, whereas the down-regulated protein with the highest average ratio was nº 8211 for Cd and nº 2422 for NP (Figure 3.3.2 B). When comparing 2-fold or more of non common proteins (Table 3.3.1), results showed that, in general, proteins were up-regulated after exposure to both Cd and NP, for both tissues.

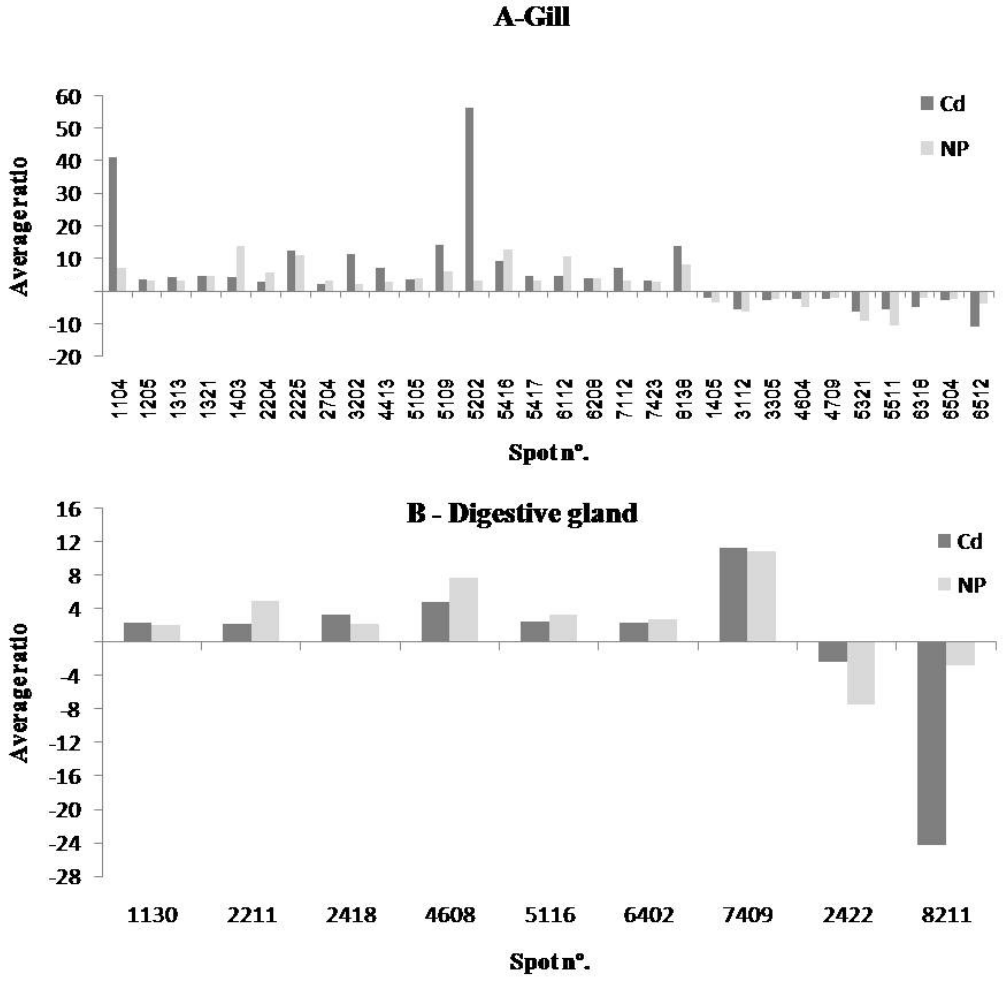

Figure 3.3.2 – Proteins with the same trend regulated in the two groups (Cd and NP) in comparison with the control. The *vertical* axis corresponds to the average ratio of protein expression. The up-regulated proteins are above while the down-regulated ones are below the *vertical* axis.

In Cd treated gill, 79 (46 by 2 to 4-fold; 24 by 4 to 8-fold; 9 by 8-fold, or more) proteins among a total 115 of proteins differentially expressed, were up-regulated (Figure 3.3.3 A) while 36 (23 decreased by 2-4-fold; 9 by 4-8-fold and 4 decreased by 8-fold, or more) were down-regulated (Figure 3.3.3 C).

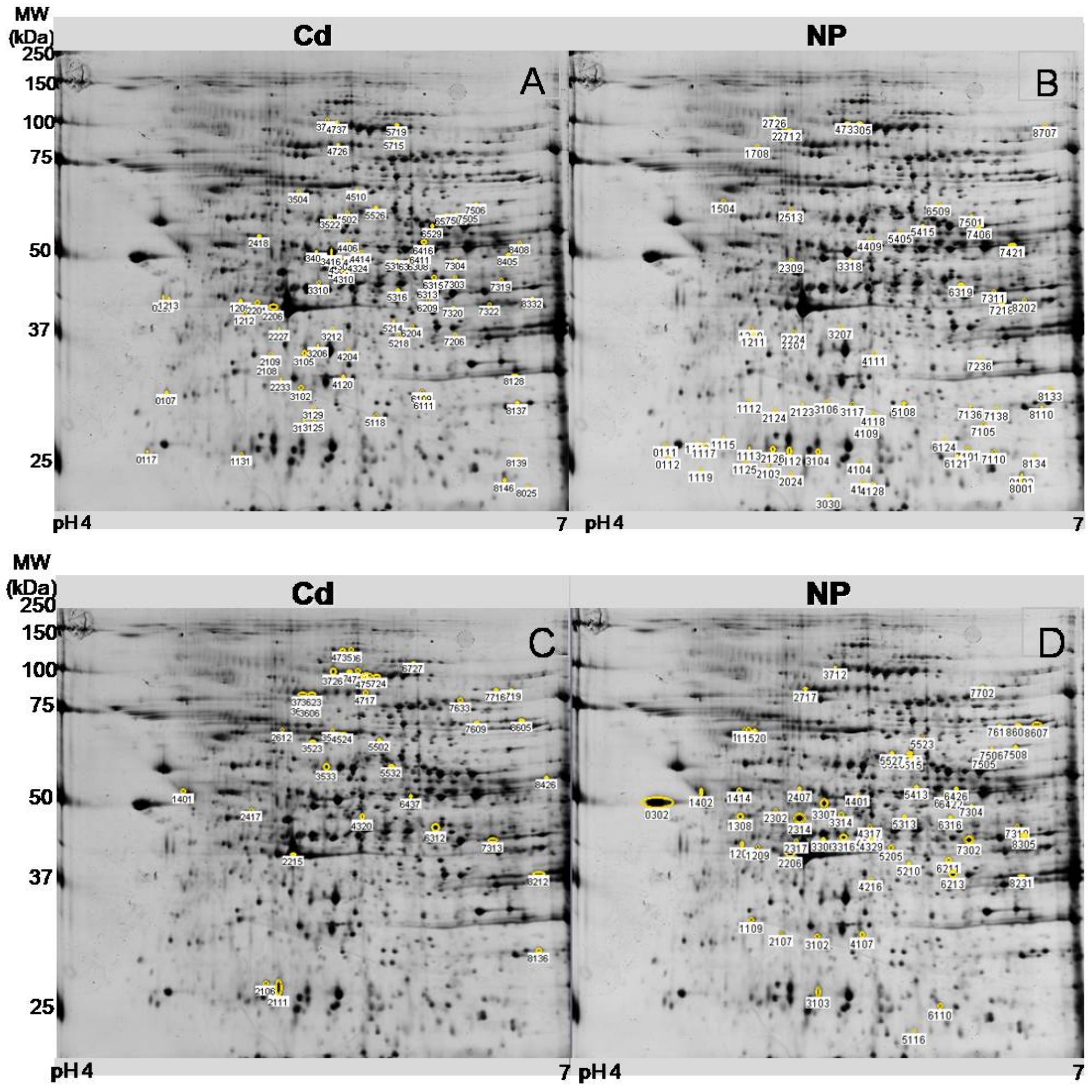

Figure 3.3.3 - Representative 2-DE gels from clam gill. PESs characterized by proteins 2-fold up-regulated specific of Cd (A) and specific of NP (B) exposed clams. PESs composed by proteins 2-fold down-regulated specific of Cd (C) and of NP (D) exposed clams.

These groups of proteins represent possible PESs specific for Cd exposure. In NP treated gill, 73 (38 by 2-4-fold, 25 by 4-8-fold and 10 higher than 8-fold) (Figure 3.3.3

B) proteins, out of 136 proteins differentially expressed, were up-regulated while 63 proteins (37 by 2-4-fold; 18 by 4-8-fold and 8 were inhibited by 8-fold, or more) (Figure 3.3.3 D) were down-regulated, corresponding to PESs for NP exposed gill.

In the digestive gland, the number of the up-regulated proteins was higher after Cd exposure (Table 3.3.1).

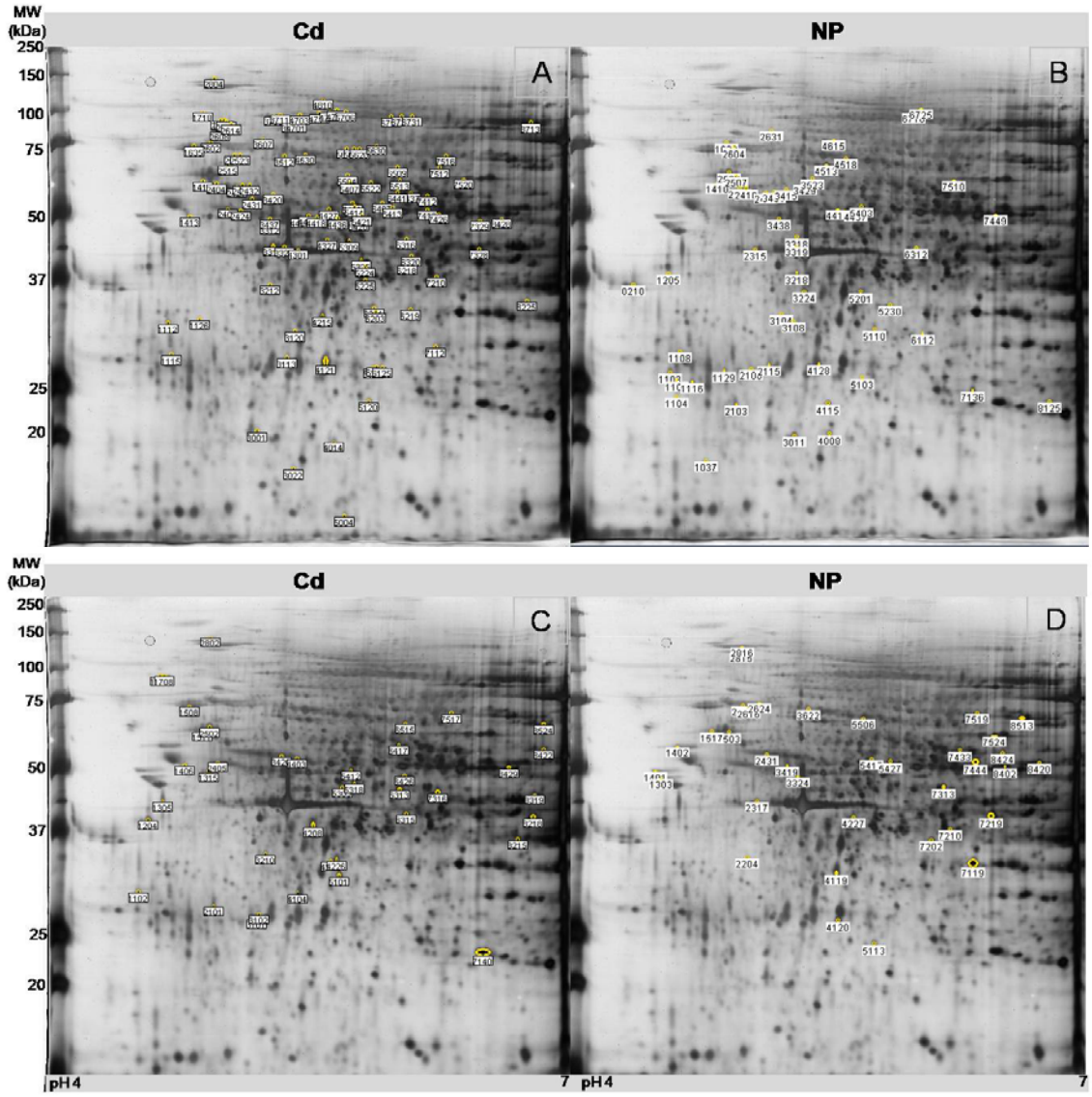

Figure 3.3.4 -Representative 2-DE gels from clam digestive gland. PESs composed of proteins 2-fold up-regulated for Cd (A) and for NP (B) exposed clams; PESs composed of proteins 2-fold down-regulated existing only in Cd (C) and only in NP (D) exposed clams.

One hundred and nine (78 proteins by 2 to 4-fold; 27 by 4 -8-fold; 4 by 8-fold, or more) (Figure 3.3.4 A) proteins, out of a total of 151 proteins differentially expressed (Table 3.3.1), were up-regulated, while 42 (33 by 2 to 4-fold; 7 by 4 to 8-fold; 2 by 8-fold or more) were down-regulated (Figure 3.3.4 C). Again, this set of proteins has potential as PESs for Cd exposition. In NP exposed digestive gland 56 (31 proteins by 2-4-fold; 20 by 4-8-fold and 5 proteins 8-fold, or more) (Figure 3.3.4 B), out of 93 spots, were up-regulated, while 37 proteins (23 by 2-4-fold; 13 by 4 to 8-fold and one more than 8-fold) were down-regulated (Figure 3.3.4 D), representing PESs for NP exposure.

Seventy five common, newly detected or suppressed proteins were excised but only 18 were identified by LC-MS/MS: 7 from gill (Figure 3.3.5 A) and 11 from digestive gland (Figure 3.3.5 B).

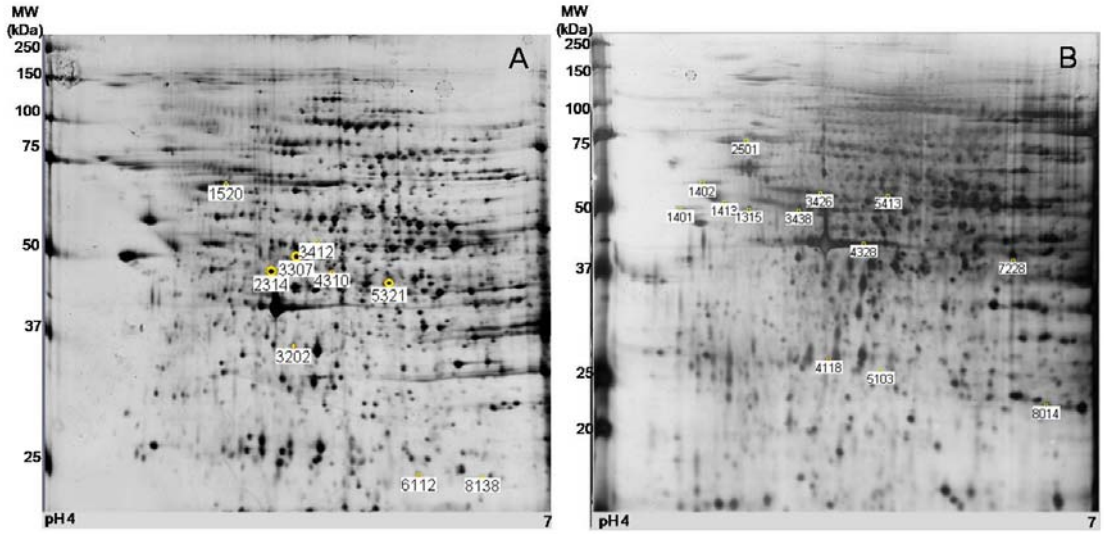

Figure 3.3.5 - Representative 2-DE gels: proteins identified by LC-MS/MS (including proteins identified in 3.1 and 3.2) in the gill (A) and digestive gland (B).

Table 3.3.3 - LC-MS/MS identification of marked spots on the representative gill and digestive gland 2DE gels. Spot. Nº.: spot number; A. Nº.: accession number of NCBI database of matched protein; Pep: peptides matched. Cov: amino-acid sequence coverage for the identified proteins. Exp.: protein expression after Cd/NP exposure.↓: ≤ 2-fold down-regulated; ↑: ≥ 2-fold up-regulated; s: suppressed spots; n: new spot.

Spot Nº	Obs.[a] p*I*/ M*r*(kDa)	Theo[b] p*I*/ M*r*(kDa)	Protein identification	A Nº	Biological function	Peptide Sequence	Sco [c]	Pep	Cov %	Species	Exp.	
											Cd	NP
Gill												
[d]3202	5.4/35.3	5.3/41.9	Actin	Q26065	Cytoskeletal structure/function	GYSFTTTAER (1131.5) SYELPDGQVITIGNER (1789.8) VAPEEHPVLLTEAPLNPK (1953.0) DLYANTVLSGGSTMYPGIADR (2200.0) LCYVALDFEQEMSTAASSSSLEK (2565.1)	433	12	37	*Placopecten magellanus*	↑	↑
6112	6.1/23.8	5.2/42.9	actin, alpha skeletal muscle A	P68140	Cytoskeletal structure/function	AVFPSIVGRPR (1197.7) SYELPDGQVITIGNER (1789.8) VAPEEHPTLLTEAPLNPK (1955.0)	109	6	17	*Fugu rubripes*	↑	↑
[d]8138	6.4/23.7	6.4/23.7	Actin, muscle-type (A2)	Q26065	Cytoskeletal structure/function	EITALAPSTMK (1176.6) SYELPDGQVITIGNER (1789.8) VAPEEHPVLLTEAPLNPK (1953.0) DLYANTVLSGGSTMYPGIADR (2200.0) LCYVALDFEQEMSTAASSSSLEK (2565.1)	282	9	33	*Molgula oculata*	↑	↑

1520[f]	5.2/66.9	5.8/76.2	Heat shock 70 kDa protein II (HSP70II)	P22623	Cell maintenance and stress response	IINEPTAAAIAYGLDK (1658.9)	54	1	4	*Paracentrotus lividus*	**s**	↓
[d]4310	5.7/46.4	5.0 /51.1	Rab GDP dissociation inhibitor alpha, (Rab GDI α)	P21856	Cell maintenance and stress response	GRDWNVDLIPK (1311.6) SPYLYPLYGLGELPQGFAR (2140.0)	71	3	6	*Bos taurus*	↑	**S**
[d]3412	5.8/57.2	6.6/57.5	Aldehyde dehydrogenase (ALDH) member B1	Q9CZS1	Metabolism	ADVDLAVK (829.4) YYAGWADK (972.4) ELGEYGLQAYTEVK (1599.7) *de novo* sequencing (*see* Table 3, Chapter 3.1)	72	5	6	*Mus musculus*	**S**	**S**
[e]5321	5.9/43.9	5.8/47.7	Enolase	O02654	Metabolism	HIADLAGNK (937.4) AAVPSGASTGIYEALEMR (1837.8) SGETEDTFIADLVVGLCTGQIK (2352.1)	106	5	14	*Loligo palei*	↓	↓
						Digestive gland						
[e]3426	5.0/50.5	4.9/50.8	Tubulin α -2/ α -4 chain	P41383	Cytoskeletal structure/function	VGINYQPPTVVFGGDLAK (912.9) TIGGGDDSFNTFFSETGAGK (1004.4) FDGALNVDLTEFQTNLVPYPR (1205.1) QLFHPEQLITGKEDAANNYAR (1208.1)	965	40	47	*Patella vulgata*	↓	**S**
3438	5.1/50.0	4.7/50.5	Tubulin ß chain (ß tubulin)	P11833.1	Cytoskeletal structure/function	AVLVDLEPGTMDSVR (1616.8) ALTVPELTQQMFDAK (1706.8) MSATFIGNSTAIQELFK (1872.9) MSMKEVDEQMLNVQNK (1970.9)	647	19	30	*Paracentrotus lividus*	-	↑

[d]4118	5.5/40.7	5.3/42.1	Actin, muscle-type	Q25472	Cytoskeletal structure/function	SYELPDGQVITIGNER (1789.8) VAPEEHPVLLTEAPLNPK (1953.0) DLYANTVLSGGSTMYPGIADR (2200.0)	196	9	29	*Molgula oculata*	**S**	**S**
[d]4328	5.5/42.6	5.1/42.5	Actin, muscle-type	Q25472	Cytoskeletal structure/function	SYELPDGQVITIGNER (1789.8) VAPEEHPVLLTEAPLNPK (1953.0) DLYANTVLSGGSTMYPGIADR (2200.0)	764	21	51	*Molgula oculata*	**S**	**S**
1402	4.5/57.9	4.3/48.3	Calreticulin precursor (CRP55)	P27797	Cell maintenance and stress response	VHVIFNYK (1018.6) KVHVIFNYK (1146.7) HEQNIDCGGGYVK (1475.6)	78	4	5	*Mus musculus*	-	↓
5103 [f]	5.6/22.6	5.9/16.0	Superoxide dismutase, (Cu-Zn) Cu/ZnSOD	P28755	ROS	TIVVHADPDDLGK (1378.7)	58	1	8	*Ceratitis capitata*	-	↑
1315	4.7/49.9	5.6/56.7	ATP synthase subunit beta. Mitochondrial precursor	Q5ZLC5	Metabolism	VALTGLTVAEYFR (1438.8) VALVYGQMNEPPGAR (1616.8) RAAGPSHGFLPLLLSR (1690.9) IPSAVGYQPTLATDMGTMQER (2297.1)	520	22	23	*Gallus gallus*	↓	-
5413	5.7/55.0	5.8/47.7	Enolase	O02654	Metabolism	HIADLAGNK (937.4) AAVPSGASTGIYEALEMR (1837.8) SGETEDTFIADLVVGLCTGQIK (2352.1)	116	3	7	*Loligo pealei*	↑	**S**
8014 [f]	6.0/32.7	6.7/36.3	Glyceraldehyde -3-phosphate dehydrogenase (GAPDH)	P19089	Metabolism	TAAQNIIPSSTGAAK (1428.7)	47	1	4	*Cryphonectria parasitica*	-	**S**

1401	4.4/51.0	5.8/71.2	Serum albumin precursor	P02769	Transport	FKDLGEEHFK (1248.6) HLVDEPQNLIK (1304.7) RHPEYAVSVLLR (1438.8) DAFLGSFLYEYSR(1566.7)	207	5	9	*Bos taurus*	-	↓
1413[e]	4.6/51.2	4.8/48.0	Histone-binding protein (RBBP7)	Q5M7K4	Signal transducers transcription factor regulators	VINEEYKIWK (1320.7) YMPQNPCIIATK (1450.7) TPSSDVLVFDYTK (1470.7) *de novo* sequencing (*see* Table 3.2.2, Chapter 3.2)	263	8	17	*Xenopus (Silurana) tropicalis*	↑	**S**

Peptides sequences: The above table gives the peptide sequences with the highest individual ion scores indicating identity or extensive homology ($p<0.05$), the number in parentheses are the experimental *M*r, the sequence underlined is the one with the best score.

[a]Observed (Obs.) isoelectric point (p*I*) and molecular mass (*M*r) were obtained from gel migration.

[b]Theoretical (Theo.) isoelectric point (p*I*) and molecular mass (*M*r) were obtained from Swiss Prot./TrEMBL database.

[c]Scores of the matches using MASCOT version 2.2.0 (Matrix Science, London) software.

[d]Proteins 8-fold up/down-regulated after Cd exposure identified in Chapter 3.1.

[e]Proteins 8-fold up/down-regulated after NP exposure identified in Chapter 3.2.

[f]Proteins homologous to just one sequence tag.

The LC-MS/MS identification of proteins from gill and digestive gland is in Table 3.3.3. The accession number of NCBI database of matched protein is given. The suppressed and new proteins are indicated as well as the 2-fold up/down-regulated ones. The proteins identified in gill were as follows: three involved in cytoskeletal structure/function (actin, actin alpha skeletal muscle A and actin muscle-type (A2)), one involved in cell maintenance and stress response (RabGDP dissociation inhibitor alpha) and two in the metabolism (aldehyde dehydrogenase (ALDH) member B1 and enolase) (Table 3.3.1). In the digestive gland, the identified proteins were: four with cytoskeletal structure/function (tubulin α-2, ß tubulin and 2 actin muscle-type); two proteins participating in cell maintenance and stress response (calreticulin precursor (CRP) 55, heat shock 70 kDa protein cognate (Hsc70-4)); three involved in metabolism (ATP synthase subunit beta mitochondrial precursor, enolase and medium-chain acyl-CoA dehydrogenase (MCAD)); one transport protein (serum albumin percursor) and one signal transduction protein (histone binding protein) (Table 3.3.1). Three more proteins are indicated in this Table: one concerned with cell maintenance and stress response (heat shock protein 70 kDa (HSP70II), one participating in the metabolism (glyceraldehyde-3-phosphate dehydrogenase (GADPH)) and one involved in the response to ROS (superoxide dismutase (Cu-Zn), (Cu/ZnSOD)). However, these three identifications were considered not valid since were based in only one protein sequence (Romero-Ruiz *et al.,* 2006). Cytoskeletal structure/function of proteins identified showed the same trend for both contaminants. In gill, actins (actin (Nº3202) alpha skeletal muscle A (Nº6112) and muscle-type (Nº8138)) increased. In digestive gland, tubulin α-2 (Nº3426) and actin muscle type (A2) (Nº 4118 and Nº4328) decreased,

whereas ß tubulin (Nº3438) expression changed only after Cd exposure, having increased.

Among proteins involved in cell maintenance and stress response, in the gill, Rab GDP dissociation inhibitor alpha (Nº4310), increased in Cd and decreased in NP treated clams. In digestive gland, calreticulin precursor (CRP55) (Nº1402) decreased after NP exposure. Some of the identified proteins are involved in glycolysis, fatty acid and energetic metabolism. In gill, ALDH member B1 (Nº3412) and enolase (Nº5321) were both down-regulated after Cd and NP exposure. In the digestive gland, enolase (Nº5413) was up-regulated in Cd and suppressed in NP exposed clams. ATP synthase subunit beta (Nº1315) expression changed only in Cd exposed clams, being down-regulated. Moreover, transport protein (serum albumin precursor (Nº1401)) expression changed only after NP exposure, being up-regulated, whereas the signal transduction protein (histone-binding RBBP7 (Nº1413)) was up-regulated after Cd exposure and suppressed in Np exposed clams.

The application of proteomics in environmental research provides not only the identification of single protein markers, but also generates PESs specific of particular pollutants (Knigge *et al.*, 2004). Significant variations in proteins of bivalves exposed to chemical pollutants and collected in a polluted environment have been reported (Chapter 1, Table 1.5). However, few works identified PESs in bivalves exposed to individual pollutants: PESs were described in *Mytilus edulis* exposed to metals and poly aromatic hydrocarbons (PAHs) (Knigge *et al.*, 2004) and to diallyl phthalate, PBDE-47, and bisphenol-A (Apraiz *et al.*, 2006). Since biotic or abiotic factors do not produce a unique kinetic response in all the proteins, PESs could provide a wider range of information. The goal of this Chapter was to define PESs with potential to characterize

exposure of *R. decussatus* to Cd (40 µg.l^{-1}, 21 days) and NP (100 µg.l^{-1}, 21 days) (3.1 and 3.2). Most of the proteins in these PESs and single key proteins did not possess extreme p*I*/M*r* values, indicating that the 2-DE-based proteomic analysis was appropriate and the results reliable. Cadmium and NP induced major changes in protein expression profiles (PEPs) in gill and digestive gland that were tissue dependent. These tissue dependent responses are mainly due to their different functions in clams and differences in Cd and NP accumulation (Roméo and Gnassia-Barelli, 1995; Serafim and Bebianno 2007 b; Lietti *et al.*, 2007). The comparison of all the PEPs allowed to define a set of 2-fold or more regulated proteins with the same trend that compose the minimal PESs for exposure to a mixture of the two pollutants (Chapter 3.3, Figure 3.3.1). A general tendency for these proteins up-regulation was observed in both tissues. Nevertheless, in gill exposed to Cd a higher over expression was observed when compared to NP. This could be due to the fact that in *R. decussatus* gill are the first site of Cd accumulation (Roméo and Gnassia-Barelli, 1995; Bebianno and Serafim, 1998).

Moreover Cd induced new proteins particularly in the digestive gland which is the principal site for Cd storage and detoxification in *R. decussatus*. The proposed PESs specific of Cd and NP exposure reflect differences between effects of exposure to each pollutant.

Therefore, these PESs could be useful in future field studies for detecting the presence of Cd or NP by comparing the PESs specific or common to these pollutants with the ones obtained in field clams. The proteins identified in 3.1 and 3.2 (Tables 2 and 3.2.1) indicate that Cd and NP induced major changes in proteins involved: in cytoskeletal structural/functional; cell maintenance and stress response; metabolism and signal transduction. These results reflect mainly a structural debilitation and an energetic

potential change after Cd and NP exposure. Furthermore, since actins are major targets of pollutant-related to oxidative stress, these results also points out to OS induction by Cd and NP (Rodriguez-Ortega *et al.*, 2003; Miura *et al.*, 2005). The new proteins, identified in Table 3.3.3, are involved in cytoskeletal structural/functional (actin, alpha skeletal muscle A, tubulin ß chain); cell maintenance and stress response (CRP55); metabolism (ATP synthase subunit beta, Enolase); transport (serum albumin precursor). In the new identified proteins, actin, actin alpha skeletal muscle A, was up-regulated in the gill exposed to both pollutants. CRP55 is a calreticulin protein (CRT) located in the sarcoplasmic/endoplasmic reticulin (ER), cell surface, nucleus, bloodstream and the extracellular matrix (Weimin *et al.*, 2008). CRT are calcium-binding proteins functioning as a chaperone of Ca^{2+} regulation levels (Luana *et al.*, 2007). Since calreticulin are stress-protein members of the family of molecular chaperones, the increase of their levels confers stress tolerance to the organism by preventing aggregation of stress-damage proteins, helping in the correct folding of nascent polypeptides and targeting damaged protein for proteolytic destruction (Hofmann and Somero, 1996; Chapple *et al.*, 1998). CRT is also involved in the regulation of the immune system responses (Luana *et al.*, 2007). The down-regulation of CRP55 after NP may be due to stress. This is in agreement with the calreticulin gene down-regulation in gill and mantle *Crassotrea gigas* submitted to hypoxia (David *et al.*, 2005). Mitochondrial membrane ATP synthase produces ATP from ADP in the presence of a proton gradient across the membrane, which is generated by electron transport complexes of the respiratory chain. ATP synthase, in the digestive gland, was down-regulated in Cd exposed clams. Its down-regulation was previously reported in the digestive gland of *M. edulis* exposed to 2,2',4,4—tetrabromodiphenyl ether (PBDE-47) and bisphenol-A (Apraiz *et al.*, 2006) and in the gill of the same species from sites

contaminated with polycyclic aromatic hydrocarbons (PAHs), metals, and chemical additives such as alkylphenols (Manduzio *et al.*, 2005). These results show that Cd change the energetic balance of *R. decussatus*. Cadmium affects mitochondrial bioenergetics of oysters *in vitro* and *in vivo* at concentrations as low as 10^{-7}–10^{-6}M leading to reduced coupling and impaired ability to produce ATP (Skulachev *et al.*, 1967; Kesseler and Brand, 1994, 1995; Ye *et al.*, 2001; Sokolova, 2004). Enolase was identified in both tissues. This enzyme, essential in glycolysis because it catalyzes the 2-phosphoglycerate to phosphoenolpyruvate, was down-regulated in the gill after exposure to Cd and NP. In the digestive glands this protein was up-regulated after Cd exposure and suppressed in NP exposed clams. This indicates an energetic potential change in this clam due to both pollutants exposure. In agreement with this, in *T. philipinarum* NP exposure induced a delay in its re-burrowing capacity (Matozzo *et al.*, 2004) and reduction in respiration rate and scope for growth (Matozzo *et al.*, 2003). Serum albumin, the main plasma protein, has a good binding capacity for water, Ca^{2+}, Na^{+}, K^{+}, fatty acids, hormones, billirubin and drugs and, in mussels, this protein is associated with energy production and storage (Lopez *et al.*, 2002). Serum albumin precursor was down-regulated in digestive gland exposed NP.

The PESs characterized and partially identified give new information about differences and similarities in the mechanisms of Cd and NP toxicity in *R. decussatus*, interfering in cytoskeletal structure/function, cell maintenance and stress response, metabolism and signal transduction. This Chapter highlights five proteins: ALDH, MCAD, serum albumin precursor, and RBBP7, which do not belong to the "hit parade" of repeatedly, identified differentially expressed proteins mentioned by Petrak *et al.* (2008).

Some of these proteins have potential for biomarkers of Cd or Np exposure. Therefore, and taken into account the identified proteins whose expression changed more drastically: RabGDP and RBBP7 are suggested as potential biomarkers for both contaminants exposure, being up-regulated after Cd exposure and suppressed in clams NP exposed; CRP55 and serum albumin precursor are indicated as possible biomarker specific for NP exposure, being down-regulated; ATP synthase subunit beta is proposed as biomarker for Cd exposure, being down-regulated, whereas MCAD is suggested as Cd biomarker being newly detected in Cd exposed digestive gland. Nonetheless, validation should be made prior to consider these proteins as adequate biomarkers of Cd and NP exposure.

3.4. References

Abad, E., Martínez, K., Planas, C., Palacios, O., Caixach, J., Rivera, J. 2005. Priority organic pollutant assessment of sludges for agricultural purposes. *Chemosphere* **61**, 1358-1369.

Amelina, H., Apraiz, I., Sun, W., Cristobal, S., 2007. Proteomics-based method for the assessment of marine pollution using liquid chromatography coupled with two-dimensional electrophoresis. *Journal of Proteomic Research* **6**, 2094-2104.

Andrew, M. N., Dunstan, R. H., O'Connor, W. A., Van Zwieten, L., Nixona, B., MacFarlanea, G.R., 2008., Effects of 4-nonylphenol and 17-ethynylestradiolexposure in the Sydney rock oyster, *Saccostrea glomerata*: Vitellogenin induction and gonadal development. *Aquatic Toxicology* **88**, 39-47.

Apraiz, I., Mi, J., Cristobal, S., 2006. Identification of proteomic signatures of exposure to marine pollutants in mussels (*Mytilus edulis*). *Molecular and Cellular Proteomics* 5, 1274-1285.

Arrigo, A. P., Paul, C., Ducasse, C., Sauvageot, O., Kretz-Remy, C., 2002. Small stress proteins: modulation of intracellular redox state and protection against oxidative stress. *Progress in Molecular and Subcellular Biology* **28**, 171-184.

Barreira, L., Mudge, S. M.; Bebianno, M. J., 2007. Oxidative stress in the clam *Ruditapes decussatus* (Linnaeus, 1758) in relation to polycyclic aromatic hydrocarbon body burden. *Environmental Toxicology* **22**, 203-21.

Bebianno, M.J., Serafim, M.A., 1998. Comparison of metallothionein induction in response to cadmium in the gills of the bivalve molluscs *Mytilus galloprovincialis* and *Ruditapes decussatus*. *The Science of Total Environment* **214**, 123–131.

Bebianno, M.J., Geret, F., Hoarau, P., Serafim, M.A., Coelho, M.R., Gnassia-Barelli, M., Roméo, M., 2004. Biomarkers in *Ruditapes decussatus*: a potential bioindicator species. *Biomarkers* **9**, 305-330.

Blum, H., Biere, H., Gross, H. J., 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis 8*, 93-99.

Bradford, M., 1976. A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-Dye binding. *Analytical Biochemistry* 72, 248-254.

Butwell, A. J., Hetheridge, M., James, H. A., Johnson, A. C., Young, W. F., (eds.) 2002. *Endocrine disrupting chemicals in wastewater: A review of occurrence and removal*, UK Water Industry Research Limited, London.

Canesi, L., Lorusso, L. C., Ciacci, C., Betti, M., Gallo, G., 2004. Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways. *General and Comparative Endocrinology*, **138**, 58-69.

Canesi, L., Lorusso, L. C., Ciacci, C., Betti, M., Rocchi, M., Pojana, G., Marcomini, A., 2007. Immunomodulation of *Mytilus* hemocytes by individual estrogenic chemicals and environmentally relevant mixtures of estrogens: *In vitro* and *in vivo* studies. *Aquatic Toxicology* **812**, 36-44.

Chapple, J.P., Smerdon, G.R., Berry, R.J., Hawkins, A.J.S., 1998. Seasonal changes in stress-70 protein levels reflect thermal tolerance in the marine bivalve *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology* **229**, 53−68.

Chora, S., Bebianno, M.J., and Roméo, M., 2009. *Analysis of Proteins from Marine Molluscs.* In: Shehan, D. and Tyther, R. (eds.), Methods in Molecular biology, Two-dimensional electrophoresis protocols, vol. 519. Humana Press, New York, pp. 197-204.

Chora, S., McDonagh, B., Starita-Geribaldi, M., Roméo, M., Bebianno, M.J., 2008. Ubiquitination and carbonylation as markers of oxidative-stress in *Ruditapes decussatus*. *Marine Environmental Research* **66**, 95-97.

Cuervo, A.M., 2004. Autophagy: in sickness and in health. *Trends in Cell Biology* **14**, 70-77.

Dachs, J., Van Ry, D.A., Eisenreich, S.J., 1999. Occurrence of estrogenic nonylphenols in the urban and coastal atmosphere of the lower Hudson River estuary. *Environmental Science and Technology*, **33**, 2676-2679.

David, E., Tanguy, A., Pichavant, K., Moraga, D., 2005. Response of the Pacific oyster *Crassostrea gigas* to hypoxia exposure under experimental conditions. *The FEBS Journal* **272**, 5635–5652.

Dellali, M., Roméo, M., Gnassia-Barelli, M., Aissa, P., 2004. A multivariate data analysis of the clam *Ruditapes decussatus* as sentinel organism of the Bizerta lagoon (Tunisia). *Water, Air, and Soil Pollution* **156**, 131-144.

Dondero, F., Dagnino, A., Jonsson, H., Capri, F., Gastaldi, L.,Viarengo, A., 2006. Assessing the occurrence of a stress syndrome in mussels (*Mytilus edulis*) using a combined biomarker/gene expression approach. *Aquatic Toxicology* **78**, S13–S24.

Dowling, V., Hoarau, P., Roméo, M., O'Halloran, J., Van Pelt, F., O'Brien, N., Sheehan, D., 2006. Protein carbonylation and heat shock response in *Ruditapes decussatus* following p,p′-dichlorodiphenyldichloroethylene (DDE) exposure: A proteomic approach reveals that DDE causes oxidative stress. *Aquatic Toxicology* **77**, 11-18.

Ekelund, R., Bergman, A., Granmo, A., Berggren, M., 1990. Bioaccumulation of 4-nonylphenol in marine animals a re-evaluation. *Environmental Pollution* **64**, 107-120.

Gagné, F., Blaise, C., 2003. Effects of municipal effluents on serotonin and dopamine levels in the freshwater muss*el Elliptio complanata. Comparative Biochemistry and Physiology* **136C**, 117-125.

Gharahdaghi, F., Weinberg, C.R., Meagher, D.A., Imai, B.S., Mishe, S.M., 1999. Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. *Electrophoresis* **20**, 601-605.

Gomez-Mendikute, A., Etxeberria, A., Olabarrieta, I., Cajaraville, M. P., 2002. Oxygen radicals production and actin filament disruption in bivalve haemocytes treated with benzo(a)pyrene. *Marine Environmental Research*, **54**, 431-436.

Gong, Y. and Han, X. D., 2006. Nonylphenol-induced oxidative stress and cytotoxicity in testicular *Sertoli* cells. *Reproductive Toxicology* **22**, 623-630.

Hofmann, G.E., Somero, G.N., 1996. Interspecific variation in thermal denaturation of proteins in the congeneric mussels *Mytilus trossulus* and *M. galloprovincialis*: evidence from the heat-shock response and protein ubiquitination. *Marine Biology* **126**, 65−75.

Howard, J., Hyman, A. A., 2003. Dynamics and mechanics of the microtubule plus end. *Nature* **422**, 753-758.

Kesseler, A., Brand, M.D., 1994. Localisation of the sites of action of cadmium on oxidative phoshorylation in potato tuber mitochondria using top-down elasticity analysis. *European Journal of Biochemistry* **225**, 897–906.

Kesseler, A., Brand, M.D., 1995. The mechanism of the stimulation of State-4 respiration by cadmium in potato-tuber (*Solanum tuberosum*) mitochondria. *Plant Physiology and Biochemistry* **33**, 519–528.

Ketata, I., Guermazi, F., Rebai, T., Hamza-Chaffai, A., 2007a. Variation of steroid concentrations during the reproductive cycle of the clam *Ruditapes decussatus*: A one year study in the gulf of Gabès area. *Comparative Biochemistry and Physiology. Part A* **147**, 424-431.

Ketata, I., Smaoui-Damak, W., Guermazi, F., Rebai, T., Hamza-Chaffai, A., 2007 b. *In situ* endocrine disrupting effects of cadmium on the reproduction of *Ruditapes decussatus. Comparative Biochemistry and Physiology. Part C* **146**, 415-430.

Knigge, T., Monsinjon, T., Andersen, O.K., 2004. Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry approach to biomarker discovery in blue mussels (*Mytilus edulis*) exposed to polyaromatic hydrocarbons and heavy metals under field conditions. *Proteomics* **4**, 2722–2727.

Kurelec, B., 1995. Reversion of the multixenobiotic resistance mechanism in gills of a marine mussel *Mytilus galloprovincialis* by a model inhibitor and environmental modulators of P170-glycoprotein. *Aquatic Toxicology* **33**, 93-103.

Lietti, E., Marin, M. G., Matozzo, V., Polesello, S., Valsecchi, S., 2007. Uptake and elimination of 4-nonylphenol by the clam *Tapes philippinarum*. *Archives of Environmental Contamination and Toxicology* **53**, 571-578.

Lopez, J.L., Marina, A., Vázquez, J., Alvarez, G., 2002. A proteomic approach to the study of the marine mussels *Mytilus edulis* and *M. galloprovincialis*. *Marine Biology* **141**, 217−223.

Luana, W., Li, F., Wang, B., Zhang, X., Liu, Y., Xiang, J., 2007. Molecular characteristics and expression analysis of calreticulin in Chinese shrimp *Fenneropenaeus chinensis*. *Comparative Biochemistry and Physiology Part B* **147**, 482–491.

Ma, B., Zhang, K., Hendrie, C., Liang, C., Li, M., Doherty-Kirby, A. Lajoie, G., 2003. PEAKS: Powerful software for peptide de novo sequencing by tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **17**, 2337–2342.

Mäenpää, K., Kukkonen, J. V. K., 2006. Bioaccumulation and toxicity of 4-nonylphenol (4-NP) and 4-(2-dodecyl)-benzene sulfonate (LAS) in *Lumbriculus variegatus* (Oligochaeta) and *Chironomus riparius* (Insecta). *Aquatic Toxicology* **77**, 329-338.

Manduzio, H., Cosette, P., Gricourt, L., Jouenne, T., Lenz, C., Andersen, O.K., Leboulenger, F., Rocher, B., 2005. Proteome modifications of blue mussel (*Mytilus edulis* L.) gills as an effect of water pollution. *Proteomics* 5, 4958-4963.

Marin, M. G., Rigato, S., Ricciardi, F., Matozzo, V., 2008. Lethal and estrogenic effects of 4-nonylphenol in the cockle *Cerastoderma glaucum*. *Marine Pollution Bulletin* **57**, 552-558.

Marques C, Pereira P, Taylor A, Liang JN, Reddy VN, Szweda LI, Shang F. 2004. Ubiquitin-dependent lysosomal degradation of the HNE modified proteins in lens epithelial cells. *Federation of American Societies for Experimental Biology Journal* **18**, 1424–1426.

Matozzo, V., Deppieri, M., Moschino, V., Marin, M.G., 2003. Evaluation of 4-nonylphenol toxicity in the clam *Tapes philippinarum*. *Environmental Research* **91**, 179-185.

Matozzo, V., Ballarin, L., Marin, M., 2004. Exposure of the clam *Tapes philippinarum* to 4-nonylphenol: changes in anti-oxidant enzyme activities and re-burrowing capability. *Marine Pollution Bulletin* **48**, 563-571.

Matozzo, V., Marin, M.G., 2005. Can 4-nonylphenol induce vitellogenin-like proteins in the clam *Tapes philippinarum*? *Environmental Research* **97**, 43-49.

Mayer, M. P., Bukau, B., 2005. Hsp70 chaperones: cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences* **62**, 670-684.

Miracle, A. L., Ankley, G. T., 2005. Ecotoxicogenomics: linkages between exposure and effects in assessing risks of aquatic contaminants to fish. *Reproductive Toxicology* **19**, 321-326.

Miura, Y., Kano, M., Abe, K., Urano, S., Suzuki, S., Toda, T., 2005. Age-dependent variations of cell response to oxidative stress: proteomic approach to protein expression and phosphorylation. *Electrophoresis* 26, 2786-2796.

Moore, M.N., 2004. Diet restriction induced autophagy: A lysosomal protective system against oxidative- and pollutant-stress and cell injury. *Marine Environmental Research* **58**, 603-607.

Moore, M.N., Icarus, A. J., Somerfield, P. J., 2006. Autophagy: Role in surviving environmental stress. *Marine Environmental Research* **62**, S420-S425.

Nasci, C., Nesto, N., Monteduro, R. A., Da Ros, L., 2002. Field application of biochemical markers and a physiological index in the mussel, *Mytilus galloprovincialis*: transplantation and biomonitoring studies in the lagoon of Venice (NE Italy). *Marine Environmental Research* **54**, 811-816.

Nesto, N., Bertoldo, M., Nasci, C., Da Ros, L., 2004. Spatial and temporal variation of biomarkers in mussels (*Mytilus galloprovincialis*) from the Lagoon of Venice, Italy. *Marine Environmental Research* **58**, 287-291.

Nice, H. E., Thorndyke, M. C., Morritt, D., Steele, S., Crane, M., 2000. Development of *Crassostrea gigas* larvae is affected by 4-nonylphenol. *Marine Pollution Bulletin* **40**, 491-496.

Nice, H.E., Thorndyke, M. C., Morritt, D., Steele, S., Crane, M., 2005. Sperm motility in the Pacific oyster (*Crassostrea gigas*) is affected by nonylphenol. *Marine Pollution Bulletin*, **50***,* 1668-1674.

Nimrod, A. C., Benson, W. H., 2004. Estrogenic responses to xenobiotics in channel catfish (*Ictalwus punctatus*). *Marine Environmental Research*, **42***,* 155-160.

Okai, Y., Sato, E., Higashi-Okai, K., Inoue, M., 2004. Effect of endocrine disruptor para-nonylphenol on the cell growth and oxygen radical generation in *Escherichia coli* mutant cells deficient in catalase and superoxide dismutase. *Free Radical Biology and Medicine* **37**, 1412-1418.

Olsson, B., Bradley, B.P., Gilek, M., Reimer, O., Shepard, J.L., Tedengren, M., 2004. Physiological and proteomic responses in *Mytilus edulis* exposed to PCBs and PAHs extracted from Baltic Sea sediments. *Hydrobiologia*, *514*, 15-27.

Petrak, J., Ivanek, R., Toman, O., Cmejla, R., Cmejlova J., Vyoral, D., Zivny, J., Vulpe, C., 2008. Déjà vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. *Proteomics* **8**, 1744–1749.

Quinn, B., Gagné, F., Blaise, C., Costello, Wilson, J.G., Mothersill, C-, 2006. Evaluation of the lethal and sub-lethal toxicity and potential endocrine disrupting effect of nonylphenol on the zebra mussel (*Dreissena polymorpha*). *Comparative Biochemistry and Physiology C* **142**, 118-127.

Ricciardi, F., Matozzo, V., Marin, M.G., 2008. Effects of 4-nonylphenol exposure in mussels (*Mytilus galloprovincialis*) and crabs (*Carcinus aestuarii*) with particular emphasis on vitellogenin induction. *Marine Pollution Bulletin*, **57***,* 365-372.

Rodriguez-Ortega, M. J., Grosvik, B. E., Rodriguez-Ariza, A., Goksyr, A., López-Barea, J., 2003. Changes in protein expression profiles in bivalve molluscs (*Chamaelea gallina*) exposed to four model environmental pollutants. *Proteomics* **3**, 1535-1543.

Roméo, M., Gnassia-Barelli, M., 1995. Metal distribution in different in tissues and in subcellular fractions of the Mediterranean clam *Ruditapes decussatus* treated with cadmium, copper or zinc. *Comparative Biochemistry Physiology* **111C**, 457–463.

Romero-Ruiz, A., Carrascal, M., Alhama, J., Gómez-Ariza, J. L., Abian, J., López-Barea, J., 2006. Environmental proteomics studies in clams from the Donaña bank of Guadalquivir Estuary (SW Spain). *Proteomics* **6**, S245-S255.

Sakakibara, Y., Hasegawa, K., Oda, T., Saito, H., Hirata, A., Matsuhashi, M., Sato, Y., 1990. Effects of synthetic estrogens, (*R*,*R*)-(+)-, (*S*,*S*)-(-)-, *dl*- and *meso*-hexestrol stereoisomers on microtubule assembly. *Biochemical Pharmacology* **39**, 167-172.

Serafim, A., Bebianno, M.J., 2007a. Involvement of MT in. Zn accumulation and elimination strategies in *Ruditapes decussatus*. *Archives of Environmental Contamination and Toxicology* **52**, 189-199.

Serafim, A., Bebianno, M.J., 2007b. Kinetic model of cadmium accumulation and elimination and metallothionein response in *Ruditapes decussatus*. *Environmental Toxicology and Chemistry* **26**, 960-969.

Skulachev, V.P., Chistyakov, V.V., Jasaitis, A.A., Smirnova, E.G., 1967. Inhibition of the respiratory chain by zinc ions. *Biochemical and Biophysical Research Communications* **26**, 1–6.

Sokolova, I.M., Evans, S., Hughes, F.M., 2004. Cadmium-induced apoptosis in oyster hemocytes involves disturbance of cellular energy balance but no mitochondrial permeability transition. *The Journal of Experimental Biology* **207**, 3369–3380.

Solé, M., 2000, Evidence of tributyltin on the MFO system of the clam *Ruditapes decussatus*: a laboratory and field approach. *Comparative Biochemistry and Physiology C* **125**, 93-101.

Staples, C., Mihaich, E., Carbone, J., Woodbrun, K., Klecka, G., 2004. A weight of evidence analysis of the chronic ecotoxicity of nonylphenol ethoxylates, nonylphenol ether carboxylates, and nonylphenol. *Human Ecology Risk Assessment* **10**, 999-1017.

Vazquez-Duhalt, R., Marquez-Rocha, F., Licea, A. F., Viana, M. T., 2005. Nonyphenol, an integrated vision of a pollutant. *Applied Ecology and Environmental Research* **4**, 1-25.

Vioque-Fernández, A., Alves de Almeida, E., López-Barea, J., 2009. Assessment of Doñana National Park contamination in *Procambarus clarkii*: Integration of conventional biomarkers and proteomic approaches. *Science of the Total Environment* **407**, 1784-1797.

Weimin, F., Yilin, H., Changzhong, L., Liping, X., Rongqing Z., 2008. Cloning, Characterization, and Expression Analysis of Calreticulin from Pearl Oyster *Pinctada fucata*. Tsinghua Science and Technology **13**, 466-473.

Ye, B., Maret,W., Vallee, B.L., 2001. Zinc metallothionein imported into liver mitochondria modulates respiration. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 2317–2322.

Chapter 4.

Redox proteomics:

Carbonylation and ubiquitination

of proteins as markers of oxidative stress

4. Introduction

The carbonylation and ubiquitination of proteins in clams *Ruditapes decussatus* exposed to cadmium is already published in *Marine Environmental Research* and is presented below (4.1).

Carbonylation and ubiquitination in nonylphenol exposed clams was studied and the results are presented in a manuscript which was submitted to *Comparative Biochemistry and Physiology - Part C* (4.2).

4.1. Ubiquitination and carbonylation as markers of oxidative-stress in *Ruditapes decussatus*

Suze Chora, Brian McDonagh, David Sheehan, Mireille Starita-Geribaldi, Michèle Romèo, Maria João Bebianno.

Marine Environmental Research 66 (2008): 95-97.

Marine Environmental Research 66 (2008) 95–97

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Ubiquitination and carbonylation as markers of oxidative-stress in *Ruditapes decussatus*

Suze Chora [a,b], Brian McDonagh [c], David Sheehan [c], Mireille Starita-Geribaldi [d], Michèle Roméo [b], Maria J. Bebianno [a,*]

[a] University of Algarve, CIMA, Faculty of Marine and Environmental Sciences, Campus de Gambelas, 8000-139 Faro, Portugal
[b] University of Nice-Sophia E.A. 4228 ECOMERS "Ecosystèmes Côtiers Marins et Réponses aux Stress", Faculty of Sciences, Parc Valrose BP 71, 06102 Nice cedex 2, France
[c] University College Cork, Proteomics Research Group, Environmental Research Institute, Ireland
[d] University of Nice-Sophia Antipolis, INSERM U.670 "Physiopathologie de la prolifération germinale", Faculty of Medicine, Avenue de Valombrose, 06107 Nice cedex 2, France

ARTICLE INFO

Keywords:
Reactive oxygen species
Proteomics
Ruditapes decussatus
Ubiquitination
Carbonylation

ABSTRACT

Environmental pollutants, such as metals, are widespread in aquatic environments and can lead to the formation of reactive oxygen species (ROS). ROS are highly toxic in marine species since they can cause serious reversible and irreversible changes in proteins including ubiquitination and modifications such as carbonylation. This study aimed to confirm the potential of ubiquitination and carbonylation as markers of oxidative stress in the clam *Ruditapes decussatus* (Veneroida, Veneridae) exposed to cadmium (40 μg/L). After 21 days of exposure clams were dissected into gills and digestive gland. Cytosolic proteins of both tissues were separated by two-dimensional electrophoresis (2-D SDS-PAGE) and analysed by immunoblotting. Higher ubiquitination and carbonylation levels were in digestive gland of contaminated organisms. These results confirm the potential of ubiquitination and carbonylation as a sensitive and specific marker of oxidative stress in marine bivalves. In this approach, changes in protein structure provide options for affinity selection of sub-proteomes for 2D SDS-PAGE, simplifying the detection of protein biomarkers using proteomic approach.

Metals, including cadmium, can generate oxidative stress in marine molluscs (McDonagh et al., 2005; Dowling et al., 2006). Changes in protein ubiquitination and carbonylation processes are two biochemical perturbations resulting from oxidative stress (McDonagh and Sheehan, 2006). As with many marine bivalves, *R. decussatus* is able to accumulate relatively high concentrations of Cd and induce changes in other antioxidant defences, such as superoxide dismutase, catalase and decrease glutathione peroxidase activity (Bebianno and Serafim, 2003; Géret et al., 2002). Therefore, this study aimed to determine the levels of protein carbonylation and to analyse ubiquitination to investigate whether these two biomarkers of oxidative damage are affected in *R. decussatus* after Cd exposure.

Clams were collected at "Ria Formosa" lagoon (Bebianno and Serafim, 2003) and exposed to Cd (40 μg/L) for 21 days. The sub-lethal Cd concentration used was based on previous studies with the same species (Géret et al., 2002; Serafim and Bebianno, 2007). Clams were measured and dissected into gills and digestive gland. These tissues were homogenised and centrifuged (15,000 g, 2 h) and the protein content determined by the Bradford method. Aliquots of 80 μg of protein content were precipitated in cold acetone and loaded on 7 cm strips pH 3–10 NL, added to 125 μL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.8% Pharmalyte; 65 mM DTT, 10% isopropanol, and bromophenol blue traces). After 12 h of passive rehydration, proteins were focused (250 V, 15 min; 1000 V, 30 min; 4000 V, 4 h, until 20,000 Vh) on a Protean IEF Cell (BioRad). Post IEF, only the strips for carbonylation were derivatized (10 mM 2,4-dinitrophenylhydrazine in 10% trifluoroacetic acid) for 20 min and then placed in neutralisation buffer (2M Tris Base/30% glycerol) for 20 min. All strips were equilibrated prior to SDS PAGE, loaded on 10% polyacrylamide gels and run at a constant voltage (200 V) and temperature (20 °C). Three replicates of each gel were run. Once electrophoresis was completed, gels were transferred to nitrocellulose membranes (0.2 μM) during 1 h at 100 mA per blot. Protein expression profiles (PEPs) were visualised by silver staining (Rabilloud, 2000). Membranes were blocked 1 h with 1.0% BSA in PBS-Tween, washed and incubated with the first anti-body diluted in 1.0% BSA (1/5000 Anti DNP by Sigma; 1/2000 Polyclonal rabbit anti-ubiquitin Dako Ref: Z0458) overnight, washed again and incubated with the second anti-body (1/1000 Polyclonal goat Anti rabbit Immunoglobulins HRP DakoCytomation 1/1000 anti-rabbit A-9169 Sigma) for 1 h. Each blot was treated with chemiluminescent reagent,

* Corresponding author.
E-mail address: mbebian@ualg.pt (M.J. Bebianno).

0141-1136/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marenvres.2008.02.034

S. Chora et al. / Marine Environmental Research 66 (2008) 95–97

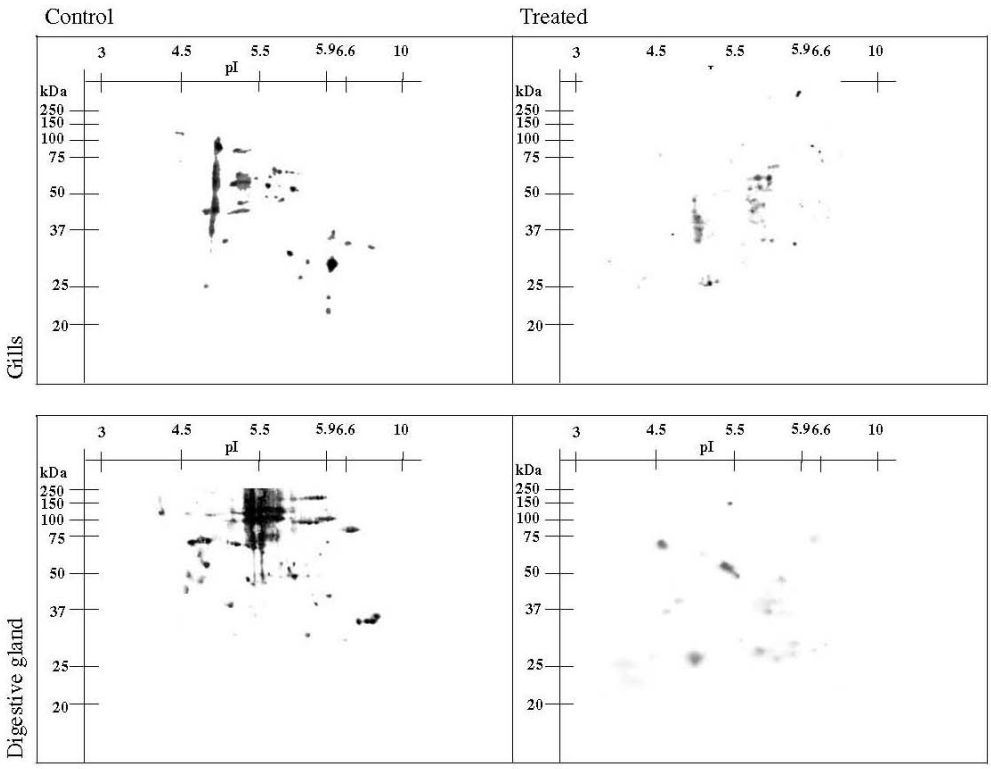

Fig. 1. Immunoblots of ubiquitinated proteins in 2D separations of digestive gland and gill proteins from control and cadmium exposed clams.

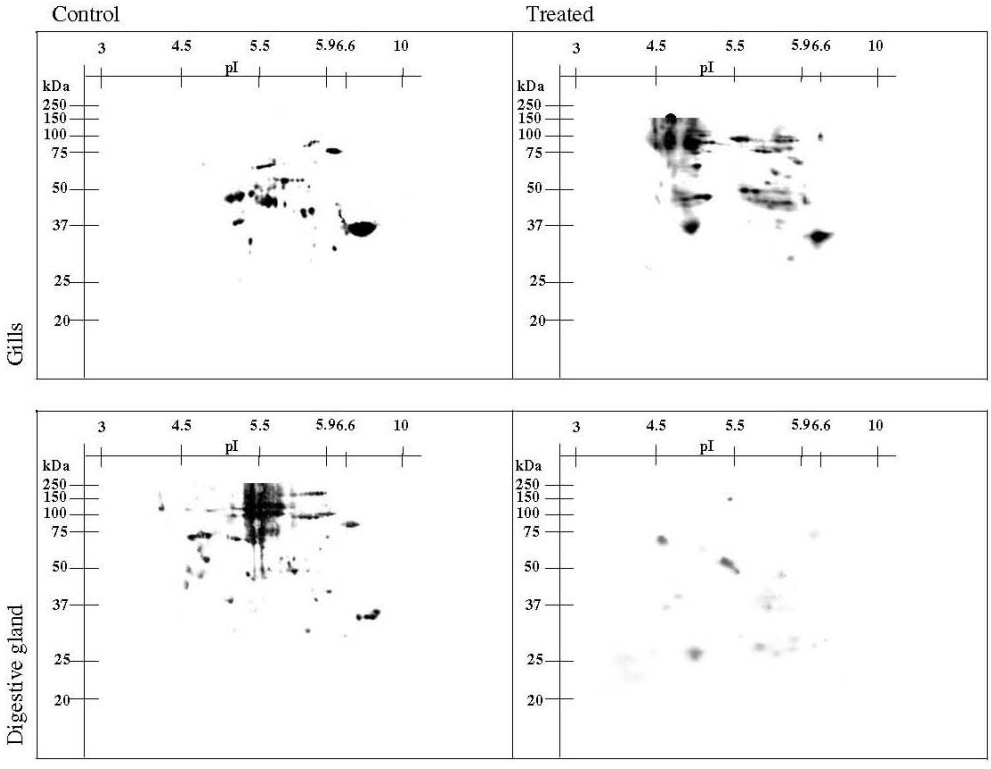

Fig. 2. Immunoblots of carbonylated proteins in 2D separations of digestive gland and gill proteins from control and cadmium exposed clams.

exposed to film, and developed. X-ray films of immunoblots were scanned and quantification of ubiquitinated and carbonylated proteins performed with the 2D gels and blots using ImageMaster2D version 3.1 (Amersham).

PEPs in 2-D SDS PAGE gels for gill and digestive gland are different, demonstrating that intensity and pattern of protein expression change due to Cd exposure. In the corresponding immunoblots both tissues present quite distinct profiles for ubiquitination and carbonylation and between tissues (Figs. 1 and 2). In immunoblots probed with anti-ubiquitin, the level of ubiquitination was lower than that of carbonylation and both tissues presented a different pattern and intensity of spots. The apparently stronger staining in the control blots could be due to a residual non-specific binding or/and to a higher load in control samples. Ubiquitination earmarks damaged or short-lived proteins for proteolysis in the 20S core of the 26S proteasome (Davies, 2001; Friguet, 2006). The different patterns attained show that some proteins of gill and digestive gland are specifically targeted for ubiquitination in response to Cd treatment. In both tissues the ubiquitination pattern is different from the carbonylation one demonstrating that ubiquitination and carbonylation are independent (McDonagh and Sheehan, 2006). Immunoblots probed with anti-DNP revealed lower levels of carbonylation in controls (Fig. 2). In Cd-treated clams, the number of carbonylated proteins increased in both tissues and in the digestive gland were almost three-fold that of gills (214 vs. 78) (results not shown). This confirms that ROS are produced in these tissues as a response to Cd exposure. Gills are in direct contact with the surrounding environment and reflect short-term metal exposure, whereas the digestive gland acts as a storage organ reflecting long-term metal exposure (Bebianno and Serafim, 2003). Phase I detoxification often results in formation of ROS (Zangar et al., 2004) and this may explain the elevated carbonylation in the digestive gland (Dowling et al., 2006). Higher ROS levels in this organ in *R. decussatus* may be due to tissue-specific accumulation of Cd (Géret et al., 2002; Serafim and Bebianno, 2007). These results confirm the potential of ubiquitination and carbonylation as sensitive and specific markers of oxidative stress in this species.

Acknowledgement

S. Chora was supported by an FCT grant SFRH/BD/19477/2004.

References

Bebianno, M.J., Serafim, M.A., 2003. Archives of Environmental Contamination and Toxicology 44, 53–66.
Davies, K.J.A., 2001. Biochimie 83, 301–310.
Dowling, V., Hoarau, P.C., Roméo, M., O'Halloran, J., Van Pelt, F., O'Brien, N., et al., 2006. Aquatic Toxicology 77, 11–18.
Friguet, B., 2006. FEBS Letters 580, 2910–2916.
Géret, F., Serafim, A., Barreira, L., Bebianno, M.J., 2002. Biomarkers 7, 242–256.
McDonagh, B., Sheehan, D., 2006. Aquatic Toxicology 79, 325–333.
McDonagh, B., Tyther, R., Sheehan, D., 2005. Aquatic Toxicology 73, 315–326.
Rabilloud, T., 2000. Proteome research: two-dimensional gel electrophoresis and identification methods. p. 244.
Serafim, A., Bebianno, M.J., 2007. Environmental Toxicology and Chemistry 26, 960–969.
Zangar, R.C., Varnum, S.M., Covington, C.Y., Smith, R.D., 2004. Disease Markers 20, 135–148.

4.2. Effects of nonylphenol exposure on ubiquitination and carbonylation of proteins in the clam *Ruditapes decussatus*

Suze Chora, Brian McDonagh, David Sheehan, Mireille Starita-Geribaldi, Michèle Romèo, Maria João Bebianno

Comparative Biochemical and Toxicology: Part C (*submitted*)

Abstract

Nonylphenol (NP) is an intermediate in the production of nonylphenol ethoxylates, widely employed in plastics, household/industrial detergents, and the paper/textile industries. NP is lipophilic and does not degrade easily under anaerobic conditions and therefore can be accumulated by a wide range of marine life including molluscs. Once accumulated in tissues, NP induces production of reactive oxygen species (ROS) that cause reversible and irreversible modifications of proteins, such as protein ubiquitination and carbonylation. These are considered sensitive and specific markers of oxidative stress in marine bivalves. The aim of this study was to investigate ubiquitination and carbonylation of proteins in gill and digestive gland of *Ruditapes decussatus* exposed to NP (100 $\mu g.l^{-1}$, 21 days) using a proteomic approach. Protein expression profiles obtained for gill and digestive gland were different and NP-treated samples contained less protein spots than controls. Corresponding immunoblots revealed tissue-specific differences and similar ubiquitination and carbonylation patterns between conditions. However, in NP-treated samples, more spots were present and the majority of proteins were more intense. This work showed that ROS are produced in gill and digestive gland in response to NP exposure and that ubiquitination and carbonylation are independent processes with potential as markers of oxidative stress in *R. decussatus*.

4.2.1. Introduction

Nonylphenol (NP) is used as an intermediate in the production of nonylphenol ethoxylates (NPEs), a large group of non-ionic surfactants widely employed in the plastics, latex paints, lubricating oils, emulsifiers, household and industrial detergents, paper and textile industries (Lee, 1999). Owing to their widespread use, large quantities of NPEs are discharged into the aquatic environment, either directly from untreated effluents or indirectly from sewage-treatment plants (STPs) (Ekelund *et al*., 1993, Maguire 1999). In aquatic ecosystems, NPEs biodegrade to de-ethoxylated intermediates ($NP_{(n\_1)}Es$) of which NP is the final product (Maguire 1999). NP was detected in a variety of fresh and marine waters at concentrations of 95 $\mu g\ l^{-1}$ (Dachs *et al*., 1999). Since NP is lipophilic, it is easily accumulated, has great persistence and causes recognized aquatic toxicity in a wide range of aquatic and marine organisms (Staples *et al*., 2004), particularly bivalve molluscs (Ekelund *et al*., 1990). Acute and chronic toxicity of NP in aquatic organisms has recently been reviewed (Servos, 1999; Staples *et al*., 2004). The negative effects of this compound have been reported in both fish and marine invertebrates: NP has been shown to affect population growth rates (Bechmann, 1999; Hansen *et al*., 1999, Mäenpää and Kukkonen, 2006), re-burrowing capacity (Matozzo *et al*., 2004), to reduce the stability of lysosomal membranes (Canesi *et al*., 2004) and to interfere with the androgenic gland of crustaceans (Brown *et al*., 1999). NP also has estrogenic properties (Vazquez-Duhalt *et al*., 2005; Marin *et al*., 2008). Endocrine disruption effects were detected in *Elliptio complanata*, where NP caused changes in the normal metabolism of serotonin and dopamine, involved in sexual differentiation in bivalves (Gagné and Blaise, 2003). Moreover, exposure to NP (1 and 100 $\mu g.l^{-1}$) at early life stages of *Crassostrea gigas* resulted in an altered sex ratio towards females, a higher percentage of functional hermaphrodites in adults and

reduced gamete viability in the subsequent generation (Nice *et al*., 2000). NP may also induce vitellogenin (Vtg) or Vtg-like protein (biomarkers of estrogenic effects) synthesis in marine bivalves, both *in vitro* and *in vivo* (*Dreissena polymorpha,* Quinn *et al*., 2006; *Tapes philippinarum* Matozzo and Marin, 2005, *Saccostrea glomerata* Andrew *et al*., 2008; *Cerastoderma glaucum* Marin *et al*., 2008).

It is well-known that both organic and inorganic contaminants can induce oxidative stress in *Ruditapes decussatus* by producing reactive oxygen species (ROS) such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Geret *et al*., 2002; Bebianno and Serafim, 2003). NP cause oxidative stress in *T. philippinarum* by inhibiting superoxide dismutase (SOD) activity (Matozzo *et al*., 2004). The main protein redox lesions are: direct oxidation of hydroxyl radicals, formation of aldehyde/ketones (carbonylation), oxidation of S-containing residues (such as methionine and cysteine), ubiquitination and effects on disulphide patterns, like glutathionylation, and on protein thiol status (Takata *et al*., 2005; Biswas *et al*., 2006; McDonagh *et al*., 2005, 2006; McDonagh and Sheehan, 2006, 2007, 2008). Carbonylation is an irreversible modification of amino acid residue side-chains into aldehyde or ketone groups, which can lead to protein aggregation, inactivation or degradation (Levine *et al*., 2000; Costa *et al*., 2002). Ubiquitin is a highly–conserved protein (20 kDa) that flags damaged cytosolic and nuclear proteins for transport to the proteasome for degradation via the ubiquitin-proteasome pathway (UPP) (Marques *et al*., 2004). Damaged proteins are removed from cells by proteolysis, mainly *via* UPP. Therefore, this process is essential for normal cell growth and viability (Sherman and Goldberg, 2001). In bivalves, carbonylation and ubiquitination of proteins assessed through a proteomic approach (redox proteomics) have recently been applied as biomarkers of oxidative stress inflicted by several environmental stressors (Dowling *et al*., 2006; McDonagh *et al*., 2005, 2006;

McDonagh and Sheehan, 2006; Prevodnik *et al*., 2007; McDonagh and Sheehan 2008; Chora *et al*., 2008; Tedesco *et al*., 2008).

Therefore, the aim of this study was to determine the levels of protein carbonylation and to analyse ubiquitination, through redox proteomics, to investigate whether these two biomarkers of oxidative damage are affected in the sentinel *R. decussatus* due to NP exposure.

4.2.2. Materials and Methods

4.2.2.1. Animals and nonylphenol exposure

Clams were collected at "Ria Formosa" lagoon (Bebianno and Serafim, 2003) and acclimatised in the laboratory for 7 days before exposure. In the laboratory, 4 groups of 25 clams were kept in 25 L glass aquaria filled with continuously aerated seawater (35 psu, 18.0 ± 0.5 ºC). One group of clams was exposed to NP (100 $\mu g.l^{-1}$) (stock solution of 1.4 mg NP/L prepared in DMSO), and the other kept in seawater, as control. Animals were not fed to avoid any confounding effects resulting from food content. Water was renewed every two days, providing natural food, and NP concentration re-established (*see* Figure 2.2, Chapter 2). After 21 days exposure, 3 controls and 3 exposed animals were collected from each condition. Clams were dissected into gill and digestive gland, pooled (3 tissues in each pool), immersed in liquid nitrogen and stored at -80 °C.

4.2.2.2. Cell-free extract preparation and protein assay

Samples were weighed, suspended in 20 % buffer (10 m*M* HEPES and 250 m*M* saccharose solution containing 1 m*M* DTT, 1m*M* EDTA, 1m*M* PMSF, 1/1000º P8340 SIGMA) and homogenised using a Ultra-Turrax IKA-Werke homogenizer at 4 ºC. Cell-free extracts were collected by centrifugation at 15 000 *g* for 2 h. The soluble protein extract was frozen (-80 °C) for further use. Protein content was determined by the

Bradford method (1976) using bovine serum albumin (BSA) as standard. Aliquots of protein (80 µg each) were suspended in nine volumes of precipitation solution (10 % trichloroacetic acid in cold acetone containing 20 mM DTT) for 2h, at -20 ºC, centrifuged at 10 000 *g* for 30 min, at 4 ºC, and washed with cold acetone. Residual acetone was removed by air drying.

4.2.2.3. Electrophoresis methods

Proteins were first separated by isoelectric focusing (IEF) followed by SDS-PAGE in two-dimensional electrophoresis (2DE) (Görg *et al*., 2004). Each sample (80 µg protein) was incubated for 30 min in 125 µL of rehydration buffer (7M urea, 2 M thiourea, 4 % CHAPS, 0.8 % pharmalyte; 65 mM DTT and bromophenol blue traces), centrifuged (14 000 *g*, 10 min, 4 ºC) and loaded on Immobiline ® DryStrip (pH 3-10 NL, 7 cm) (Chora *et al*., 2008). After 12h of passive rehydration, proteins were focused as previously described (Chora *et al*., 2008). After IEF, strips were derivatized in 10 % TFA with 10 mM DNPH for 20 min. Samples were washed in 2 M Tris-Base/30% glycerol for 15min and then equilibrated as normal prior to SDS-PAGE separation. Strips were loaded on 10 % polyacrylamide gels and run at a constant voltage (200 V) and temperature (20 ºC). All 2DE equipment was from Bio-Rad, Hercules, CA, USA. Gels were silver stained (Blum *et al*., 1987, modified). To ensure reproducibility of gels, three replicates of each condition, control and treatment with NP, were prepared.

4.2.2.4. Immunoblotting and quantification of proteins

Following 2DE, proteins were transferred to nitrocellulose membranes (0.2 µM, ProTran, Schleicher and Schuell, Dasel, Germany), stained using BLOT-*FastStain*™ (Geno Technology, St. Louis, MO USA) to ensure equal protein loading and blotting efficiency and scanned (GS-800 scanner, Bio-Rad Laboratories) (McDonagh and

Sheehan, 2006). Membranes were blocked and probed as previously described (Chora *et al*., 2008). Each blot was treated with chemiluminescence reagent, exposed to film and developed. X-ray films of immunoblots were scanned (GS-800 scanner, Bio-Rad Laboratories) and quantification of carbonylated and ubiquitinated proteins performed with the 2DE gels and blots using PDQuest software (V8.0, Bio-Rad Laboratories), where the treatment with the highest valid spot intensity was used as the master image and compared against other blots (McDonagh and Sheehan, 2006).

4.2.2.5. Statistical Analysis

Determinations were performed in triplicate. Values were expressed as means ± standard error (S.E.) where n=3.

Differences in spot numbers in gill and digestive gland from treated animals were compared with controls using Student's one-tailed t-test and Mann-Whitney Rank Sum Test (non-parametric test), p-values < 0.05 were accepted as representing statistical significance, SigmaStat® version 3.5 (Sistat software, CA, USA) .

4.2.3. Results

Protein expression profiles (PEPs) obtained for gill and digestive gland of exposed clams differed from controls suggesting that the overall quantity and pattern of protein expression changed after NP exposure (Figure 4.2.1).

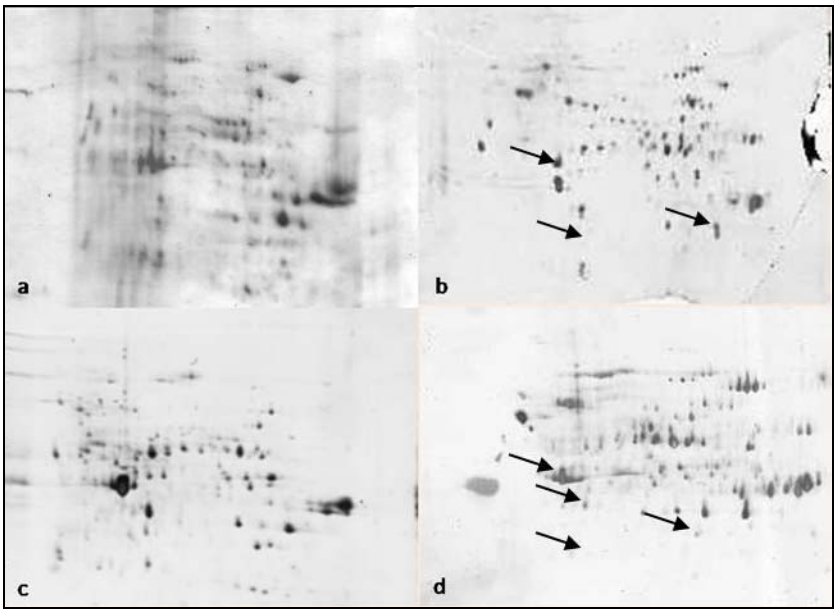

Figure 4.2.1 - Representative 2DE gels, 80 µg protein loading. a: gill control; b: gill NP-treated; c: digestive gland control and d: digestive gland NP-treated. Arrows highlight spots more drastically altered.

In both tissues, gels from NP-treated animals contained less protein spots than controls (gill had < 34 % and digestive gland < 50 %).

4.2.3.1. Ubiquitination of proteins

Immunoblots of 2DE gels showed tissue-specific differences in protein ubiquitination (Figure 4.2.2). In gill and digestive gland, a considerable number of ubiquitinated proteins were more intense in immunoblots as a result of NP exposure (Figure 4.2.2 b-d) and gill exhibited a statistically-significant increase in the number of ubiquitinated proteins (Table 4.2.1).

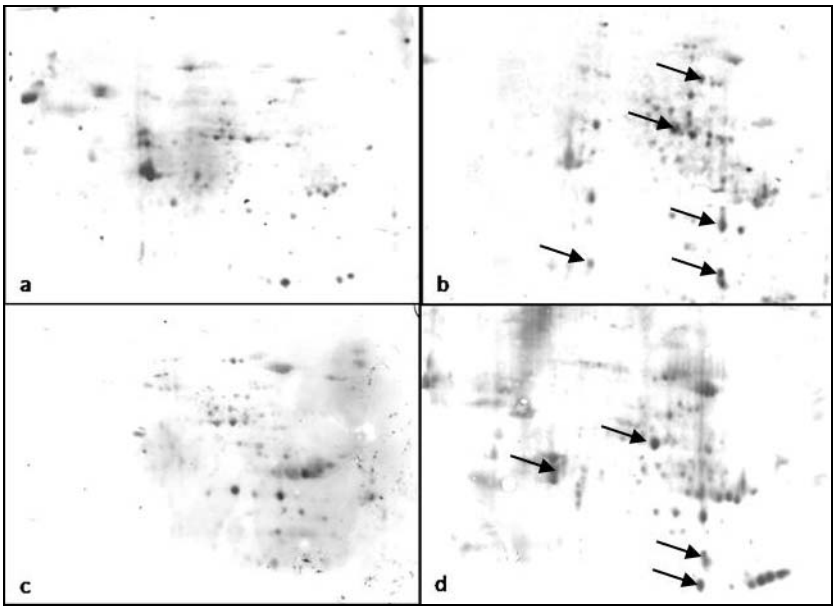

Figure 4.2.2 - Representative 2D anti-ubiquitin immunoblots, 80 µg protein loading. a: gill control samples; b: gill NP-treated; c: digestive gland control and d: digestive gland NP-treated. Arrows highlight spots more drastically altered.

Table 4.2.1 - Quantification of 2D SDS-PAGE immunoblots and anti-ubiquitin and anti-DNP. The number of spots identified with PDQuest are tabulated for each group (n=3). * $p < 0.05$ when compared to control.

	Control	**Np**
2D SDS-PAGE gels		
Gill	159 ± 15	105 ± 7
Digestive gland	231 ± 11	119 ± 9
Immunoblots		
Ubiquitination		
Gill	40 ± 2	82 ± 3*
Digestive gland	68 ± 18	81 ± 6
Carbonylation		
Gill	81 ± 4	84 ± 12
Digestive gland	51 ± 4	83 ± 12*

Among all immunoblots of gill and digestive gland clams treated with anti-ubiquitin, 22 (55 %) and 16 control spots (24 %), respectively, were common to treated samples, revealing that additional proteins, not visible in controls, were specifically ubiquitinated in response to NP exposure.

4.2.3.2. Carbonylation of proteins

Immunoblots of 2DE separations for carbonylated proteins revealed tissue-specific differences (Figure 4.2.3). Apparently stronger staining in gill control blots could be due to residual non-specific binding and/or to a higher loading in control samples. In digestive gland, some carbonylated protein spots were more intense in response to NP exposure (Figures 4.2.3 b-d).

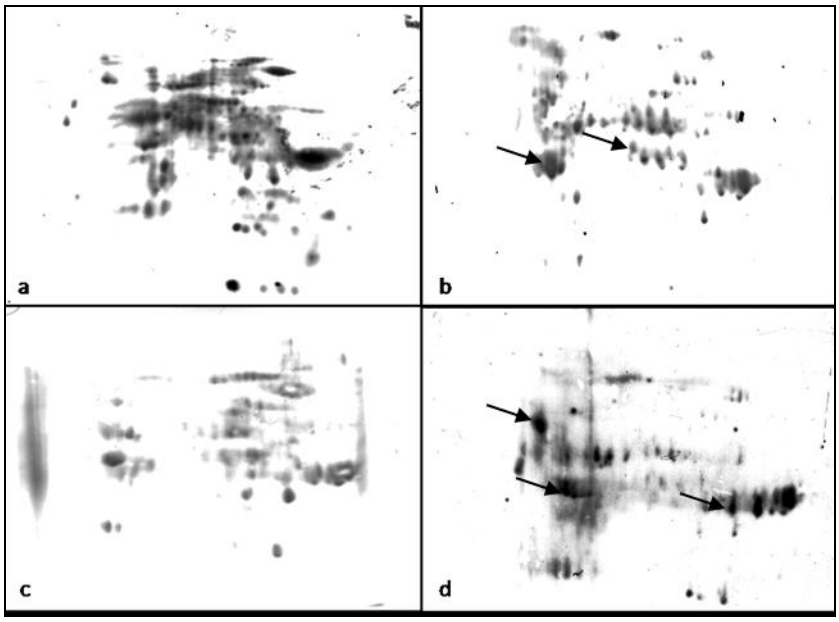

Figure 4.2.3 - Representative 2D carbonylation immunoblots, 80 µg protein loading. a: gill control samples; b: gill NP-treated; c: digestive gland control and d: digestive gland NP-treated. Arrows highlight spots more drastically altered.

Digestive gland showed a statistically-significant increase in number of carbonylated protein spots after NP exposure (Table 4.2.1).In immunoblots (2DE) for control gill and

digestive gland carbonylation, 23 (28 %) and 16 (31 %) spots, respectively, were common to corresponding NP immunoblots. These percentages suggest that a considerable number of additional proteins, not displayed in control immunoblots, were specifically carbonylated in response to NP exposure. For both tissues, the ubiquitinated spot pattern did not match that of the carbonylated one.

4.2.4. Discussion

NP has significant toxicity potential as a result of its persistence in the environment, bioaccumulation in biota (Staples *et al*., 2004) and estrogenic properties (Vazquez-Duhalt *et al*., 2005). Previous studies have shown that NP accumulation in *T. philipinarum* occurs mainly in gill and digestive gland (Lietti *et al*., 2007). In *R. decussatus* exposed to sublethal NP concentrations (100 $\mu g.l^{-1}$) for 21 days, NP was accumulated in two tissues (43.52 ± 0.53 $\mu g.g^{-1}$ in gill and 55.29 ± 0.60 $\mu g.g^{-1}$ in digestive gland; Chora *et al*., *unpublished results*). For these reasons it was of interest to study these two tissues using a concentration comparable to the highest NP concentrations reported in the environment (Dachs *et al*., 1999).The ‘‘oxygen paradox” is that molecular oxygen is essential to aerobic life whilst, simultaneously, oxygen can form toxic reactive oxygen species (ROS) (Halliwell and Gutteridge, 2007). Antioxidant defenses protect biological systems against ROS. However, if these defenses are compromised, or if the levels of ROS exceed them, oxidative stress (OS) results (Halliwell and Gutteridge, 2007). ROS are highly toxic to marine species since they cause serious reversible and irreversible changes in proteins including ubiquitination and modifications such as carbonylation (Chora *et al*., 2008, Tedesco *et al*., 2008), resulting in significant alterations in the proteome (McDonagh *et al*., 2006; McDonagh and Sheehan, 2006). Ubiquitination of proteins increases in the presence of

OS (McDonagh *et al*., 2006; McDonagh and Sheehan, 2006). In the carbonylation process amino acid side-chains can be irreversibly modified into aldehyde or ketone groups leading to protein aggregation, inactivation or degradation (Levine *et al*., 2000). The use of protein carbonyl groups as biomarkers of OS has advantages when compared to the quantification of other oxidation products, due to the early formation and stability of carbonylated proteins (Davies and Goldberg, 1987; Augustin *et al*., 1997). Proteomics techniques are promising tools in evaluating changes in subproteomes helping to assess environmental pollution. The integration of gel electrophoresis and western blot (redox proteomics) could be helpful for the detection of ROS formation (Nesatyy and Suter, 2008; Dorts *et al.,* 2009). Use of redox proteomics has been successfully applied in the assessment of protein ubiquitination and carbonylation as specific early biomarkers of OS in bivalve species exposed to several environmental stressors (Dowling *et al*., 2006; McDonagh and Sheehan, 2006; Tedesco *et al*., 2008). Concerning *R. decussatus*, changes in protein ubiquitination and carbonylation occur in response to cadmium (Chora *et al*., 2008) and *p,p*-dichlorodiphenyldichloroethylene (DDE) exposure (Dowling *et al*., 2006). NP induced OS in marine bivalves (Matozzo *et al*., 2004) but the clam, *R. decussatus*, widely used as a sentinel species in ecotoxicology, has not previously been extensively studied in this regard (Bebianno *et al*., 2004).

In the present study, effects of NP exposure on protein ubiquitination and carbonylation patterns were assessed, for the first time, in *R. decussatus*. Distinct PEPs were obtained for the two tissues (Figure 4.2.1 a, c). In gill, a superoxide dismutase isoform can be induced in response to OS (Geret *et al*., 2002) whereas digestive gland is the major site of both Phase I and II detoxification in clams (De Luca-Abbott *et al*., 2005). Therefore, tissue-specific 2DE profiles may be due to differences in the detoxification machinery

of the tissues studied and may reflect the individual redox requirements of each tissue (McDonagh and Sheehan, 2006 , Chora *et al*., 2008) and/or tissue-specific accumulation of NP (Chora *et al*., *unpublished results*).

Exposure to NP caused changes to 2DE PEPs (Figure 4.2.1 b,d) of both tissues, mainly a significant decrease in the number of spots ($p < 0,05$ when compared to control). This could be due to modifications such as carbonylation (McDonagh and Sheehan, 2008), ubiquitination of some proteins and their removal from cells by proteolysis via the ubiquitin-proteasome pathway (UPP) (Marques *et al*., 2004), or to lysosomal autophagy protecting the cell against harmful effects of damaged and malfunctioning proteins (Moore, 2004; Moore *et al*., 2006). Tissue- and treatment-distinct ubiquitination and carbonylation patterns were found (Figures 4.2.2 and 4.2.3), showing that some gill and digestive gland proteins are specifically targeted for either ubiquitination or carbonylation in response to NP exposure. This is in accordance with previous studies (McDonagh and Sheehan, 2006; Chora *et al*., 2008; Tedesco *et al* 2008), suggesting the distinctness of these processes. Immunoblots of contaminated samples showed an increase in the number and intensity of proteins becoming ubiquitinated or carbonylated (Table 4.2.1). The same trend was obtained in gill of *M edulis* exposed to menadione and H_2O_2 (McDonagh and Sheehan, 2006). Apparently stronger staining in the gill control blots for carbonylation (Figures 4.2.3 a) could be due to residual non-specific binding and/or to a higher load in control samples.

The decrease in spot number and high degree of carbonylation observed in digestive gland could result from higher NP accumulation and/or slower NP elimination from the clam´s digestive tract, owing to binding to glycogen or lipid, which is thought to be responsible for high toxicant bioaccumulation potential (Lietti *et al*., 2007). In *R.*

decussatus exposed to DDE, a higher number and intensity of carbonylated proteins was also detected in digestive gland (Dowling *et al*., 2006). Since phase I detoxification often results in formation of ROS (Zangar *et al*., 2004), this may explain the elevated degree of carbonylation in this tissue.

Ubiquitinated spot patterns did not generally match with those for carbonylation, which is in accordance with previous studies (McDonagh and Sheehan, 2006; Chora *et al*., 2008) suggesting that ubiquitination and carbonylation are independent processes and that carbonylated proteins in clams are not strongly targeted for ubiquitination.

4.2.5. Conclusions

This work showed that ROS are produced in the gill and digestive gland of *R. desussatus* in response to NP exposure and confirms that proteomics methodologies are suitable to detect this. In NP exposed tissues, PEPs were strongly affected and ubiquitination and carbonylation of proteins were both evident. Therefore, NP toxicity acts partly by altering protein expression and by ROS generation. This study confirms the utility of redox proteomics in detection of protein carbonylation and ubiquitination and supports exploration of ubiquitination and carbonylation as potentially informative biomarkers of oxidative stress in environmental toxicology.

4.3. References

Andrew, M.N., Dunstan, R.H., O'Connor, W.A., Van Zwieten, L., Nixon, B., MacFarlane G.R., 2008. Effects of 4- nonylphenol and 17 – ethynylestradiol exposure in the Sydney rock oyster, *Saccostrea glomerata*: Vitellogenin induction and gonadal development. *Aquatic Toxicology* **88**, 39-47

Augustin, W.,Wiswedel, I., Noack, H., Reinheckel, T., Reichelt, O., 1997. Role of endogenous and exogenous antioxidants in the defence against functional damage and lipid peroxidation in rat liver mitochondria. *Molecular and Cellular. Biochemistry* **174**, 199–205.

Bebianno, M.J. and Serafim, M.A., 2003. Variation of metal and metallothionein Concentrations in a Natural Population of *Ruditapes decussatus*. *Archives of Environmental Contamination and Toxicology* **44**, 53-66.

Bebianno, M.J., Geret, F., Hoarau, P., Serafim, M.A., Coelho, M.R., Gnassia-Barelli, M., Roméo, M., 2004. Biomarkers in *Ruditapes decussatus*: a potential bioindicator species. *Biomarkers* **9**, 305-330.

Bechmann, R.K., 1999. Effect of the endocrine disrupter nonylphenol on the marine copepod *Tisbe battagliai*. *Science of the Total Environment* **233**, 33–46.

Biswas, S., Chida, A.S., Rahnam, I., 2006. Redox modifications of protein thiols: Emerging roles in cell signalling. *Biochemistry and Pharmacology* **71**, 551-564.

Blum, H., Biere, H., Gross, H.J., 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93-99

Brown, R.J., Conradi, M., Depledge, M.H., 1999. Long-term exposure to 4-nonylphenol affects sexual differentiation and growth of the amphipod *Corophium volutator* (Pallas, 1766). *Science of the Total Environment* **233**, 77–88.

Canesi, L., Lorusso, L.C., Ciacci, C., Betti, M., Gallo, G., 2004. Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways. *General and Comparative Endocrinology* **138**, 58–69.

Chora, S., McDonagh, B., Starita-Geribaldi, M., Roméo, M., Bebianno, M.J., 2008. Ubiquitination and carbonylation as markers of oxidative-stress in *Ruditapes decussatus*. *Marine Environmental Research* **66**, 95-97.

Costa, V.M., Amorim, M.A., Quintanilha, A., Moradas-Ferreira, P., 2002. Hydrogen peroxide induced carbonylation of key metabolic enzymes in *Saccharomyces cerevisiae*: the involvement of the oxidative stress response regulators Yap1 and Skn7. *Free Radical Biology and Medicine* **33**, 1507–1515.

Dachs, J., Van Ry, D.A., Eisenreich, S.J., 1999. Occurrence of estrogenic nonylphenols in the urban and coastal atmosphere of the lower Hudson River estuary. *Environmental Science and Technology* **33**, 2676-2679.

Davies, K.J. and Goldberg, A.L., 1987. Proteins damaged by oxygen radicals are rapidly degraded in extracts of red blood cells. *Journal of Biological Chemistry* **262**, 8227– 8234.

De Luca-Abbott, S.B., Richardson, B.J., McLellan, K.E., Zheng, G.J., Martin, M., Lam, P.K.S., 2005. Field validation of antioxidant enzyme biomarkers in mussels (*Perna viridis*) and clams (*Ruditapes philippinarium*) transplanted in Hong Kong coastal waters. *Marine Pollution Bulletin* **51**, 694–707.

Dorts, J., Silvestre, F., Tu, H.T., Tyberghein, A., Phuong, N.T., Kestemont, P., 2009. Oxidative stress, protein carbonylation and heat shock proteins in the black tiger

shrimp, *Penaeus monodon*, following exposure to endosulfan and deltamethrin. *Environmental Toxicology and Pharmacology* **28**, 302-310.

Dowling, V., Hoarau, P., Roméo, M., O'Halloran, J., Van Pelt, F., O'Brien, N., Sheehan, D., 2006. Protein carbonylation and heat shock response in *Ruditapes decussatus* following p,p′-dichlorodiphenyldichloroethylene (DDE) exposure: A proteomic approach reveals that DDE causes oxidative stress. *Aquatic Toxicology* **77**, 11-18.

Ekelund, R., Bergman, A., Granmo, A., Berggren, M., 1990. Bioaccumulation of 4-nonylphenol in marine animals a re-evaluation. *Environmental Pollution* **64**, 107-120.

Ekelund, R., Granmo, A., Magnusson, K., Berggren, M., Bergman, A., 1993. Biodegradation of 4-nonylphenol in seawater and sediment. *Environmental Pollution* **79**, 59–61.

Gagné, F. and Blaise, C., 2003. Effects of municipal effluents on serotonin and dopamine levels in the freshwater mussel *Elliptio complanata*. *Comparative Biochemistry and Physiology C* **136**, 117–125.

Geret, F., Serafim, A., Barreira L., Bebianno, M.J., 2002. Response of antioxidant systems to copper in the gill of the clam *Ruditapes decussatus*. *Biomarkers* **7**, 242-256.

Görg, A., Weiss, W., Dunn, M.J., 2004. Current two-dimensional electrophoresis technology for proteomics. *Proteomics* **4**, 3665-3685.

Halliwell, B. and Gutteridge, J., 2007. *Free Radicals in Biology and Medicine*. Oxford University Press, London, England.

Hansen, F.T., Forbes, V.E., Forbes, T.L., 1999. Effects of 4-nonylphenol on life-history traits and population dynamics of a polychaete. *Ecological Applications* **9**,482–495.

Lee, H.B., 1999. Review of analytical methods for the determination of nonylphenol and related compounds in environmental samples. *Water Quality Research Journal Canada* **34**, 3–35.

Levine, R.L., Wehr, N., Williams, J.A., Stadtman, E.R., Shacter, E., 2000. Determination of carbonyl groups in oxidized proteins. *Methods of Molecular Biology* **99**, 15–24.

Lietti, E., Marin, M.G., Matozzo, V., Polesello, S., Valsecchi, S., 2007. Uptake and elimination of 4-nonylphenol by the clam *Tapes philippinarum*. *Archives of Environmental Contamination and Toxicology* **53**, 571–578.

Mäenpää, K. and Kukkonen, J.V.K., 2006. Bioaccumulation and toxicity of 4-nonylphenol (4-NP) and 4-(2-dodecyl)-benzene sulfonate (LAS) in *Lumbriculus variegatus* (Oligochaeta) and *Chironomus riparius* (Insecta). *Aquatic Toxicology* **77**, 329-338.

Maguire, R.J., 1999. Review of the persistence on nonylphenol and nonylphenol ethoxylates in aquatic environments. *Water Quality Research Journal of Canada* **34**, 37–78.

Marin, M.G., Rigato, S., Ricciardi, F., Matozzo, V., 2008. Lethal and estrogenic effects of 4-nonylphenol in the cockle *Cerastoderma glaucum*. *Marine Pollution Bulletin*, **57**, 552-558.

Marques, C., Pereira, P., Taylor, A., Liang, J.N., Reddy, V.N., Szweda, L.I., Shang, F., 2004. Ubiquitin-dependent lysosomal degradation of the HNE modified proteins in lens epithelial cells. *Federation of American Societies for Experimental Biology Journal* **18**, 1424–1426.

Matozzo, V., Ballarin, L., Marin, M., 2004. Exposure of the clam *Tapes philippinarum* to 4-nonylphenol: changes in anti-oxidant enzyme activities and re-burrowing capability. *Marine Pollution Bulletin* **48**, 563-571.

Matozzo, V. and Marin, M., 2005. Can 4-nonylphenol induce vitellogenin-like proteins in the clam *Tapes philippinarum*? *Environmental Research* **97**, 43-49.

McDonagh, B., Tyther, R., Sheehan, D., 2005. Carbonylation and glutathionylation of specific proteins in the blue mussel *Mytilus edulis* detected by proteomic analysis and western blotting: actin as a target of oxidative stress. *Aquatic Toxicology* **73**, 315–326.

McDonagh, B. and Sheehan, D., 2006. Redox proteomics in the blue mussel *Mytilus edulis*: Carbonylation is not a pre-requisite for ubiquitination in acute free radical-mediated oxidative stress. *Aquatic Toxicology* **79**, 325–333.

McDonagh, B., Tyther, R., Sheehan, D., 2006. Redox proteomics in the mussel, *Mytilus edulis*. *Marine Environmental Research* **62**, 101–104.

McDonagh, B. and Sheehan, D., 2007. Effects of oxidative stress on protein thiols in the blue mussel *Mytilus edulis*: Proteomic identification of target proteins. *Proteomics* **7**, 3395-3403.

McDonagh, B. and Sheehan, D., 2008. Effects of oxidative stress on protein thiols and disulphides in *Mytilus edulis* revealed by proteomics: Actin and protein disulphide isomerase are redox targets. *Marine Environmental Research* **66**, 193-195.

Moore, M.N., 2004. Diet restriction induced autophagy: lysosomal protective system 615 against oxidative- and pollutant-stress and cell injury. *Marine Environmental Research* **58**, 603–607.

Moore, M.N., Icarus, A.J., Somerfield, P.J., 2006. Autophagy: role in surviving environmental stress. *Marine Environmental Research* **62**, S420–S425.

Nesatyy, V.J. and Suter, M.J.F., 2008. Analysis of environmental stress response on the proteome level. *Mass Spectrometry Reviews* **27**, 556-574.

Nice, H.E., Thorndyke, M.C., Morritt, D., Steele, S., Crane, M., 2000. Development of *Crassostrea gigas* larvae is affected by 4-nonylphenol. *Marine Pollution Bulletin* **40**, 491–496.

Prevodnik, A., Gardeström, J., Lilja, K., Elfwing, T., McDonagh, B., Petrović, N., Tedengren, M., Sheehan D., and Bollner T., 2007. Oxidative stress in response to xenobiotics in the blue mussel *Mytilus edulis* L.: Evidence for variation along a natural salinity gradient of the Baltic Sea. *Aquatic Toxicology* **82**, 63-71.

Quinn, B, Gagnè, F., Costello, M., McKenzie, C., Wilson, J., Mothersill, C., 2006. The endocrine disrupting effect of municipal effluent on the zebra mussel (*Dreissena polymorpha*). *Aquatic Toxicology* **66**, 279–292.

Servos, M.R., 1999. Review of the aquatic toxicity, estrogenic responses and bioaccumulation of alkylphenols and alkylphenol polyethoxylates. *Water Quality Research Journal of Canada* **34**, 123–177.

Sherman, M.Y. and Goldberg, A.L., 2001. Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron* **29**, 15-32.

Staples, C., Mihaich, E., Carbone, J., Woodbrun, K., Klecka, G., 2004. A weight of evidence analysis of the chronic ecotoxicity of nonylphenol ethoxylates, nonylphenol ether carboxylates, and nonylphenol. *Human Ecological Risk Assessment* **10**, 999–1017.

Takata, T., Shimo-Oka, T., Miki, K., Fujii, N., 2005. Characterization of new D-β-aspartate-containing proteins in a lens-derived cell line. *Biochemical and Biophysical Research Communications* **334**, 1022-1031.

Tedesco, S., Doyle, H., Redmond, G., Sheehan, D., 2008. Gold nanoparticles and oxidative stress in *Mytilus edulis*. *Marine Environmental Research* **66**, 131-133.

Vazquez-Duhalt, R., Marquez-Rocha, F., Licea, A.F., Viana, M-T., 2005. Nonyphenol, an integrated vision of a pollutant. *Applied Ecology and Environmental Research* **4**, 1-25.

Zangar, R.C., Davydov, D.R., Verma, S., 2004. Mechanisms that regulate production of reactive oxygen species by cytochrome P-450. *Toxicology and Applied Pharmacology* **199**, 316–331.

Chapter 5.

General Discussion

Since the beginning of application of biomonitoring programs for marine pollution assessment, a wide range of biomarkers was tested, providing specific and fast responses to several stresses. However, the conventional biomarkers have some disadvantages. One of their main problems is that they do not give specific information on the mechanism of the pollutant toxicity. This gap can be bridged by the application of multi-endpoint approaches such as proteomics that, additionally, have good capacity in finding new sensitive biomarkers (Dowling *et al.*, 2006; Lopez-Barea and Gomez-Ariza, 2006; Monsinjon and Knigge, 2007). Proteomics has capacity to detect and quantify hundreds to thousands of proteins in a single experiment, providing qualitative and quantitative assessment of changes in protein expression between different tissues or between conditions (e.g. healthy/disease, presence/absence of drugs). Furthermore, this approach allows following the responses of different stress stages over time, helping to understand the kinetics of specific stress effects (Shepard and Bradley, 2000). Recently, the proteome analysis has become a promising tool in ecotoxicology in the assessment of pollutant effects (Monsinjon and Knigge, 2007). Nevertheless, there are few proteomic studies involving bivalves, considered as good biological indicators (Table 1.5). In some of these studies redox proteomics, a useful tool in assessing oxidative stress caused by several stresses was applied (McDonagh *et al.*, 2005; Dowling *et al.*, 2006; McDonagh and Sheehan, 2006, 2007; Tedesco *et al.*, 2008).

The pollutants cadmium (Cd) and nonylphenol (NP) have great persistence in the environment, recognized aquatic toxicity and are easily bioaccumulated by marine organisms. Therefore, the health effects caused by exposure to these compounds are of great concern. In this context, the present dissertation highlighted the proteomic approach application in the assessment of cadmium and nonylphenol effects in the sentinel species *Ruditapes decussatus*. Since reproducible high-resolution separation of

protein mixtures is vital for successful proteomics, and the existing 2-D SDS PAGE protocols were not appropriate for protein separation in this species, the primary task of this thesis was to develop a 2DE SDS-PAGE protocol suitable for proteomic application in bivalves (Chapter 2.2).

5.1. 2DE SDS-PAGE protocol optimization

Both salts and lipid are known to disturb the electrophoresis process. Salts are the most frequent cause of insufficient focusing of protein spots whereas lipid reduces protein solubilization. Since bivalve gill and digestive gland possess high salt and lipid content, it was necessary to remove the excess of these compounds by TCA and ice-cold precipitation and enhance the rehydration of the detergent content (Rabilloud, 2000).

To improve protein solubilization, thiourea and carrier ampholytes were included in rehydration buffer composition. Reproducibility could also be a major problem since comparison between PEPs could be difficult, limiting the application of 2DE SDS-PAGE (Mi, 2005; Nesatyy and Suter, 2008). The 2D SDS-PAGE protocol suitable for proteomic application in bivalves (Chapter 2.1) allowed to obtain well resolved 2-DE gels under reproducible controlled situations, even when generated in different laboratories (Chapter 2.1, Figures 2.3-2.4). In addition, despite the difficulty of obtaining good 2-DE gels for digestive gland due to its high content of proteases the optimized methodology allowed also to obtain 2-DE gels well resolved for this tissue. Furthermore, this protocol was successfully applied to other mollusc species: *Bathymodiolus azoricus* (Company *et al.*, data not published); *Scrobicularia plana* and *Mytilus edulis* (*Gomes et al.*, data not published) and *Strombus latus* (*Castro et al.*, data not published) and the polychaete *Nereis diversicolor* (*Gomes et al.*, data not published).

5.2. Protein expression signatures (PESs) and single protein markers in *R. decussatus*

The aim of this study was to detect differences in protein expression profiles (PEP) and to identify PESs and single protein markers that could characterize the exposure of *R. decussatus* to cadmium (40 µg.l^{-1}, 21 days) and nonylphenol (100 µg.l^{-1}, 21 days) (3.1 and 3.2). The 2-DE maps obtained from gill and digestive gland exposed to Cd (3.1) and NP (3.2) were compared between them and to control gels. Cadmium and NP induced considerable changes in the PEPs of gill and digestive gland that were tissue dependent, mainly a decrease in the total number of proteins. These differences are the consequence of these tissues distinct roles in clams and differences in Cd and NP accumulation (Roméo and Gnassia-Barelli, 1995, Bebianno and Serafim, 1998, Lietti *et al.*, 2007). The results demonstrated that Cd showed a higher tendency toward up-regulation while NP tends to suppress proteins. Moreover Cd induced more new proteins, particularly in the digestive gland which is the principal site of storage and detoxification of metals in *R. decussatus*. Although, as stated by Petrak *et al.* (2008), the observed changes in PEPs of both tissues represent cellular stress response, they could also reflect the technical limitations of 2DE SDS-PAGE, mainly the percentage of polyacrylamide used. In both tissues, a trend for proteins up-regulation was detected in the proposed PESs for exposure to a mixture of the two pollutants, mainly in the gill exposed to Cd. This could be due to the fact that this organ is the first site of Cd accumulation in clams (Roméo and Gnassia-Barelli, 1995; Bebianno and Serafim, 1998). The PESs associated to each one of the pollutants revealed differences between effects of exposure to Cd and NP. The PESs defined have great potential to become biomarkers of exposure to a combined mixture of Cd and NP (PESs common to both pollutants) or to each pollutant (specific PESs). The identified proteins provide a

valuable knowledge to better understand the toxicological effects of Cd and NP exposure (Tables 2, 3.2.1 and 5.3.1).

Table 5.3.1 - Proteins identified in Cd and NP gels 2-fold up/down-regulated (Table 3.3.3) grouped by biological function. Spot Nº.: spot number; A. Nº.: accession number of NCBI database of matched protein; Exp.: protein expression after Cd/NP exposure.↓: ≤ 2-fold down-regulated; ↑: ≥ 2-fold up-regulated; S: suppressed spots; n: new spot.

Sopt Nº	**Protein identification**	**A Nº**	**Exp.**	
			Cd	**NP**
	Gill			
	Cytoskeletal structure/function			
[d]3202	Actin	Q26065	↑	↑
6112	Actin, alpha skeletal muscle A	P68140	↑	↑
[d]8138	Actin, muscle-type (A2)	Q26065	↑	↑
	Cell maintenance and stress response			
[d]4310	Rab GDP dissociation inhibitor alpha, (Rab GDI α)	P21856	↑	**S**
	Metabolism			
[d]3412	Aldehyde dehydrogenase (ALDH) member B1	Q9CZS1	**S**	**S**
[e]5321	Enolase	O02654	↓	↓
	Cytoskeletal structure/function			
[e]3426	Tubulin α -2/ α -4 chain	P41383	↓	**S**
[d]4118	Actin, muscle-type	Q25472	**S**	**S**
[d]4328	Actin, muscle-type	Q25472	**S**	**S**
	Cell maintenance and stress response			
1402	Calreticulin precursor (CRP55)	P27797	-	↓
	Metabolism			
1315	ATP synthase subunit beta mitochondrial precursor	Q5ZLC5	↓	-

5413	Enolase	O02654	↑	**S**
	Transport			
1401	Serum albumin precursor	P02769	-	↓
	Signal transducers transcription factor regulators			
[e]1413	Histone-binding protein (RBBP7)	Q5M7K4	↑	**S**

[d]Proteins identified in Chapter 3.1.

[e]Proteins identified in Chapter 3.2.

The main changes caused by Cd and NP involved proteins related to: cytoskeletal structural/functional; cell maintenance and stress response; metabolism; transport and signal transduction. The PESs described and partially identified provide new information about mechanisms of toxicity of Cd and NP in *R. decussatus*, interfering in cytoskeletal structure/function, cell maintenance and stress response, metabolism and signal transduction. Four of the identified proteins (aldehyde dehydrogenase (ALDH), medium-chain acyl-CoA dehydrogenase (MCAD), serum albumin precursor, and histone-binding protein (RBBP7)) are not included in the "hit parade" of repeatedly, identified differentially expressed proteins (Petrak *et al.,* 2008) and three of them were identified using *de novo* sequencing (MCAD, ALDH and RBBP7). RabGDP dissociation inhibitor alpha and RBBP7 are suggested as potential biomarkers for both contaminants exposure, being up-regulated after Cd exposure and suppressed in clams NP exposed; calreticulin precursor (CRP55) and serum albumin precursor are indicated as possible biomarker specific for NP exposure, being down-regulated, whereas ATP synthase subunit beta is proposed as biomarker for Cd exposure, being down-regulated as well as the MCAD, that is induced.

These results will reinforce the importance of proteomics in ecotoxicology, not only for finding new single protein biomarkers but also for establishing PESs specific and

common to Cd and NP that could be useful tools to monitor the pollution status of marine ecosystem.

5.3. Redox proteomics – carbonylation and ubiquitination of proteins as markers of oxidative stress

As focused in Chapter 1, exposure to pollutants can lead to the formation of reactive oxygen species (ROS). In response to ROS, organisms developed antioxidant defense systems (Chapter 1.3.1). However, when the generation of ROS exceeds the ability to neutralize and eliminate them, oxidative stress (OS) occurs and several damages may happen: proteins inactivation/modification, lipid peroxidation, DNA damage, apoptosis or necrosis (Chapter 1.3). Since Cd and NP produce ROS in bivalves and proteins are one major target of ROS, one of the aims of this thesis was to apply redox proteomics to investigate the potential of both protein redox lesions (ubiquitination and carbonylation) as markers of OS in *R. decussatus* gill and digestive gland. Results showed that, after Cd and NP exposure, both gill and digestive gland presented quite distinct profiles for ubiquitination and carbonylation, displaying distinct pattern and intensity of spots (Figures 1 and 2 Chapter 4.1 and figures 2 and 3 Chapter 4.2). These differences reflect that detoxification machinery of gill and digestive gland has specific redox requirements associated with tissue-specific accumulation for Cd and NP. In fact, digestive gland is the major site of both Phase I and Phase II detoxification in clams (De Luca-Abbott *et al.*, 2005). For both contaminants, the increase of carbonylated proteins was especially high in digestive gland. This is in agreement with the known higher accumulation and slower elimination of Cd and NP in this organ (Serafim and Bebianno, 2007 and Chapter 3.2). Besides tissue dependent, ubiquitination and carbonylation also showed to be treatment specific. Therefore, these results demonstrated that some proteins of gill

and digestive gland were specifically targeted for ubiquitination/ carbonylated in response to Cd and NP exposure. Furthermore, ubiquitinated spot patterns did not generally matched with those for carbonylation, which is in accordance with previous studies suggesting that ubiquitination and carbonylation are independent processes and that carbonylated proteins in clams are not strongly targeted for ubiquitination (McDonagh and Sheehan, 2006). The specificity and independency of ubiquitination and carbonylation confers them good capacity to be biomarkers for ROS. The distinct patterns for ubiquitinated and carbonylated proteins obtained for both tissues exposed to each one of the pollutants show that ROS caused by Cd and NP exposure inflict damage in different proteins, having potential to be considered biomarkers for exposure to Cd and NP.

This work showed that ROS are produced in the gill and digestive gland of *R. decussatus* in response to Cd and NP exposure and confirms that redox proteomics methodologies are suitable to detect this. Therefore, the mechanisms of Cd and NP toxicity act via ROS generation and, consequently, protein alteration and ubiquitination and carbonylation are independent process having great potential as markers of oxidative stress in this species.

5.4. References

Bebianno, M.J. and Serafim, M.A., 1998. Comparison of metallothionein induction in response to cadmium in the gills of the bivalve molluscs *Mytilus galloprovincialis* and *Ruditapes decussatus*. *The Science of Total Environment* **214**, 123-131.

De Luca-Abbott, S.B., Richardson, B.J., McLellan, K.E., Zheng, G.J., Martin, M., Lam, P.K.S., 2005. Field validation of antioxidant enzyme biomarkers in mussels (*Perna viridis*) and clams (*Ruditapes philippinarium*) transplanted in Hong Kong coastal waters. *Marine Pollution Bulletin* **51**, 694–707.

Dowling, V., Hoarau, P., Roméo, M., O'Halloran, J., Van Pelt, F., O'Brien, N., Sheehan, D., 2006. Protein carbonylation and heat shock response in *Ruditapes*

decussatus following p,p′-dichlorodiphenyldichloroethylene (DDE) exposure: A proteomic approach reveals that DDE causes oxidative stress. *Aquatic Toxicology* **77**, 11-18.

Lietti, E., Marin, M. G., Matozzo, V., Polesello, S., Valsecchi, S., 2007. Uptake and elimination of 4-nonylphenol by the clam *Tapes philippinarum*. *Archives of Environmental Contamination and Toxicology* **53**, 571-578.

López-Barea, J. and Gómez-Ariza, J.L., 2006. Environmental proteomics and metallomics. *Proteomics* **6**, S51-S62.

McDonagh, B. and Sheehan, D., 2006. Redox proteomics in the blue mussel *Mytilus edulis*: Carbonylation is not a pre-requisite for ubiquitination in acute free radical-mediated oxidative stress. *Aquatic Toxicology* **79**, 325-333.

McDonagh, B. and Sheehan, D., 2007. Effects of oxidative stress on protein thiols in the blue mussel *Mytilus edulis*: Proteomic identification of target proteins. *Proteomics*, **7**, 3395-3403.

McDonagh, B., Tyther, R., Sheehan, D., 2005. Carbonylation and glutathionylation of specific proteins in the blue mussel *Mytilus edulis* detected by proteomic analysis and western blotting: actin as a target of oxidative stress. *Aquatic Toxicology*, **73**, 315-326.

McDonagh, B., Tyther, R., Sheehan, D., 2006. Redox proteomics in the mussel, *Mytilus edulis*. *Marine Environmental Research*, **62**, 101-104.

Mi, J., Orbea, A., Syme, N., Ahmed, M., Cajaraville, M.P., Cristobal, S., 2005, Peroxisomal proteomics, a new tool for risk assessment of peroxisome proliferating pollutants in the marine environment. *Proteomics* **5**, 3954-3965.

Monsinjon, T. and Knigge, T., 2007. Proteomic applications in ecotoxicology. *Proteomics* **7**, 2997-3009.

Nesatyy, V.J. and Suter, M.J.F., 2008. Analysis of environmental stress response on the proteome level. *Mass Spectrometry Reviews* **27**, 556-574.

Petrak, J., Ivanek, R., Toman, O., Cmejla, R., Cmejlova J., Vyoral, D., Zivny, J., Vulpe, C., 2008. Déjà vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. *Proteomics* **8,** 1744-1749.

Rabilloud, T. (ed.), 2000. *Proteome research: Two-Dimensional Gel Electrophoresis and Identification Methods*. Springer Verlag, Heidelberg, 244 pp.

Roméo, M. and Gnassia-Barelli, M., 1995. Metal distribution in different in tissues and in subcellular fractions of the Mediterranean clam *Ruditapes decussatus* treated with cadmium, copper or zinc. *Comparative Biochemistry Physiology* **111C**, 457-463.

Serafim, A. and Bebianno, M.J., 2007. Kinetic model of cadmium accumulation and elimination and metallothione in response in *Ruditapes decussatus*. *Environmental Toxicology and Chemistry* **26**, 960-969.

Shepard, J.L. and Bradley, B.P., 2000. Protein expression signatures and lysosomal stability in *Mytilus edulis* exposed to graded copper concentrations. *Marine Environmental Research* **50**, 457-463.

Tedesco, S., Doyle, H., Redmond, G., Sheehan, D., 2008. Gold nanoparticles and oxidative stress in *Mytilus edulis*. *Marine Environmental Research* **66**, 131-133.

Chapter 6.

Conclusions

6. Conclusions of the thesis

The final conclusions of this dissertation on "Study of contaminant effects in a sentinel species, the clam *Ruditapes decussatus*, through protein expression analysis: monitoring in marine environment" can be summarized as follows:

- The development of a 2DE SDS-PAGE protocol suitable for bivalves was crucial the for successful of proteomics analysis;
- Proteomics tools have a good capacity in assessing effects in gill and digestive gland of *R. decussatus* exposed to cadmium (Cd) and nonylphenol (NP);
- Cd and NP cause changes in protein expression profiles (PEPs) of *R. decussatus*, mainly a decrease in the total number of spots;
- PESs obtained could be useful in field studies for detecting the presence of Cd and NP, being suggested as potential biomarkers;
- Cd shows a higher tendency toward up-regulation while NP tends to suppress proteins.
- Cd and NP induce changes in proteins involved in cytoskeletal structural/functional; cell maintenance; stress response, metabolism, transport and signal transduction;
- RabGDP dissociation inhibitor alpha and RBBP7 are suggested as potential biomarkers for both contaminants; calreticulin precursor (CRP55) and serum albumin precursor are indicated as possible biomarkers specific for NP exposure; ATP synthase subunit beta and MCAD are proposed as biomarkers for Cd exposure;
- Ubiquitination and carbonylation are specific and independent processes and distinct patterns for ubiquitinated and carbonylated proteins were obtained after Cd and

NP exposure. Therefore they constitute good markers of oxidative stress in the clam *R. decussatus* exposed to Cd and NP.

6.1. Future perspectives

- To follow the responses to other Cd and NP concentrations, particularly lower concentrations, over time, could help to better understand the kinetics of both pollutants effects;
- In order to better understand the mechanisms of toxicity of Cd and NP further research should be developed applying “omics” tools. Since transcriptome is considered a sensitive read-out of the proteome or the biochemical state of the cell, genomic techniques, mainly “arrays”, could be a good complement, allowing the study of a high number of responses to pollutant exposure. Additionally, metabolomics could be also applied, providing specific metabolic footprints;
- Since larvae are very sensitive to pollutant exposure, proteomics will be applied to understand how larvae cope with pollutants exposure.

Chapter 7.

References

Abad, E., Martínez, K., Planas, C., Palacios, O., Caixach, J., Rivera, J. 2005. Priority organic pollutant assessment of sludges for agricultural purposes. *Chemosphere* **61**, 1358-1369.

Achard, M., Baudrimont, M., Boudou, A., Bourdineaud, J.P., 2004. Induction of a multixenobiotic resistance protein (MXR) in the Asiatic clam *Corbicula fluminea* after heavy metals exposure. *Aquatic Toxicology* **67**, 347–357.

Ahmad, S., 1995. *Oxidative Stress and Antioxidant Defenses in Biology*. Chapman and Hall. New York. pp. 457.

Ahokas, J.T., Holdway, D.A., Brennan, S.E., Goudey, R.W., Bibrowska, H.B., 1994. MFO activity in carp (*Cyprinus carpio*) exposed to treated pulp and paper mill effluent in lake Coleman, Victoria, Australia, in relation to AOX, EOX and muscle PCDD/PCDF. *Environmental Toxicology and Chemistry* **13**, 41-50.

Alban, A., David, S.O., Bjorkesten, L., Andersson, C., Sloge, E., Lewis, S., Currie, I., 2003. A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics* **3**, 36-44, 2003.

Ali, M. and Sreekrishnan, T.R., 2001. Aquatic toxicity from pulp and paper mill effluents: a review. *Advances in Environmental Research* **2**, 175–96.

Allen, J.I. and M. N. Moore, 2004. Environmental prognostics: Is the current use of biomarkers appropriate for environmental risk evaluation? *Marine Environmental Research* **58**, 227-232.

Amelina, H., Apraiz, I., Sun, W., Cristobal, S., 2007. Proteomics-based method for the assessment of marine pollution using liquid chromatography coupled with two-dimensional electrophoresis. *Journal of Proteomic Research* **6**, 2094-2104.

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

Andrew, M. N., Dunstan, R. H., O'Connor, W. A., Van Zwieten, L., Nixona, B.,, MacFarlanea, G.R., 2008. Effects of 4- nonylphenol and 17_ ethynylestradiolexposure in the Sydney rock oyster, *Saccostrea glomerata*: Vitellogenin induction and gonadal development. *Aquatic Toxicology* **88**, 39-47.

Apraiz, I., Mi, J., Cristobal, S., 2006. Identification of proteomic signatures of exposure to marine pollutants in mussels (*Mytilus edulis*). *Molecular and Cellular Proteomics* **5**, 1274-1285.

Armenta, J.M., 2009. Microfluidic chips for protein differential expression profiling. *Electrophoresis* **30**, 1145-1156.

Arrigo, A. P., Paul, C., Ducasse, C., Sauvageot, O., Kretz-Remy, C., 2002. Small stress proteins: modulation of intracellular redox state and protection against oxidative stress. *Progress in Molecular and Subcellular Biology* **28**, 171-184.

Arukwe, A., Knudsen, F. R. and A. Goksoyr, 1997. Fish zona radiata (eggshell) protein: A sensitive biomarker for environmental estrogens. *Environmental Health Perspectives* **105,** 418-422.

Ashfield, L. A., Pottinger, T. G., J. P. Sumpter, 1998. Exposure of female juvenile rainbow trout to alkylphenolic compounds results in modifications to growth and ovosomatic index. *Environmental Toxicology Chemistry* **17:** 679-686.

Askenov, M., Askenova, M., Butterfield, D., Geddes, J., Markesbery, W., 2001. Protein oxidation in the brain in Alzheimer's disease. *Neuroscience* **103**, 373–383.

Atiezar, F.A., Billinghust, Z., Depledge, M.H., 2002. 4-n-nonylphenol and 17- β estradiol may induce common DNA effects in developing barnacle larvae. *Environmental Pollution* **120,** 735-738.

Augustin, W.,Wiswedel, I., Noack, H., Reinheckel, T., Reichelt, O., 1997. Role of endogenous and exogenous antioxidants in the defence against functional damage and lipid peroxidation in rat liver mitochondria. *Molecular and cellular biochemistry* **174**, 199–205.

Bargagli, R., 2000.Trace metals in Antarctica related to climate change and increasing human impact. *Reviews of Environmental Contamination and Toxicology* **166**, 129–173.

Barreira, L., Mudge, S. M.; Bebianno, M. J., 2007. Oxidative stress in the clam *Ruditapes decussatus* (Linnaeus, 1758) in relation to polycyclic aromatic hydrocarbon body burden. *Environmental Toxicology* **22**, 203-21.

Barreira, L. and Bebianno, M.J., 2009. Polycyclic aromatic hydrocarbons concentrations and biomarker responses in the clam *Ruditapes decussatus* transplanted in the Ria Formosa lagoon. *Ecotoxicology and Environmental Safety* **72(7)**, 1849-60.

Bebianno, M.J. and Serafim, M.A., 1998. Comparison of metallothionein induction in response to cadmium in the gills of the bivalve molluscs *Mytilus galloprovincialis* and *Ruditapes decussatus*. *The Science of the Total Environment* **214(1-3)**, 123-131.

Bebianno, M.J. and Serafim, M.A., 2003. Variation of metal and metallothionein Concentrations in a Natural Population of *Ruditapes decussatus*. *Archives of Environmental Contamination and Toxicology* **44**, 53-66.

Bebianno, M.J., Geret, F., Hoarau, P., Serafim, M.A., Coelho, M.R., Gnassia-Barelli, M., Roméo, M., 2004. Biomarkers in *Ruditapes decussatus*: a potential bioindicator species. *Biomarkers* **9**, 305-330.

Bebianno, M.J., Company, R., Serafim, A., Camus, L., Cosson R., Fiala-Médoni, A., 2005.Antioxidant systems and lipid peroxidation in Bathymodiolus Azoricus from Mid Atlantic Ridge Hydrothermal vent fields. *Aquatic Toxicology* **75**, 354-373.

Bechmann, R.K., 1999. Effect of the endocrine disrupter nonylphenol on the marine copepod *Tisbe battagliai*. *Science of the Total Environment* **233**, 33–46.

Beiras, R., Albentosa, M., 2004. Inhibition of embryo development of the commercial bivalves *Ruditapes decussatus* and *Mytilus galloprovincialis* by trace metals; implications for the implementation of seawater quality criteria. *Aquaculture* 230, 205-213.

Bektas, M., Guncer, B., Guven, C., Nurten, R., Bermek, E., 2004. Actin-an inhibitor of eukaryotic elongation factor activities. *Biochem. Biophys. Res. Commun.* 317, 1061-1066.

Belfroid, A.C., Sijm, D.T.H.M., Van Gestel, C.A.M., 1996. Bioavailability and toxicokinetics of hydrophobic aromatic compounds in benthic and terrestrial invertebrates. *Environmental Reviews* **4**, 276-299.

Beninger, P.G. and Stephan, G., 1985. Seasonal variations in a fatty acids of the triacylglycerols and phospholipids of two populations of adult clam (*Tapes decussatus* L. and *Tapes philippinarum*) reared in a common habitat. *Comparative Biochemistry and Physiology* **81B**, 591-601.

Beyersmann, D. and Hechtenberg, S., 1997. Cadmium, gene regulation and cellular signaling in mammalian cells. *Toxicology and Applied Pharmacology* **144**: 247-261.

Bigot, A., Doyen, P., Vasseur, P., Rodius, F., 2009. Metallothionein coding sequence identification and seasonal mRNA expression of detoxification genes in the bivalve *Corbicula fluminea. Ecotoxicology and Environmental Safety* **72**, 382-387.

Biswas, S., Chida, A.S., Rahman, I., 2006. Redox modifications of protein-thiols: emerging roles in cell signaling. *Biochemical pharmacology* **71**,551-564.

Bjellqvist, B., Laird, N., McDowell, M., Olsson, I., R. Westermeier, 1998. 2-D Electrophoresis, Principles and Methods. Amersham-Biosciences 100 pp.

Blum, J. and Fridovich, 1984. Enzymatic defenses against oxygen toxicity in the hydrothermal vent animals Riftia pachyptila and Calyptogena magnifica. *Archives of Biochemistry and Biophysics* **228**, 617-620.

Blum, H., Biere, H., Gross, H.J., 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93-99

Bonacci, S., Iacocca, A., Fossi, S., Lancini, L., Caruso, T., Corsi, I., 2007. Biomonitoring aquatic environmental quality in a marine protected area: a biomarker approach. *AMBIO* **36**, 308–15.

Bradford, M. 1976 A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry* **72**, 248-254.

Bremmer, I., 1998. Manifestations of copper excess. *The American Journal of Clinical Nutrition* **67**, 1069-1073.

Brown, R.J., Conradi, M., Depledge, M.H., 1999. Long-term exposure to 4-nonylphenol affects sexual differentiation and growth of the amphipod *Corophium volutator* (Pallas, 1766). *Science of the Total Environment* **233**, 77–88.

Brown, M., Davies, I.M., Moffat, C.F., Craft, J.A., 2006. Application of SSH and a macroarray to investigate altered gene expression in Mytilus edulis in response to exposure to benzo[a]pyrene. *Marine Environmental Research* **62**, S128–S135.

Bryan, G.W. and Langston, W.J., 1992. Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. *Environmental Pollution* **76,** 89-131

Butwell, A. J., Hetheridge, M., James, H. A., Johnson, A. C., Young, W. F., (eds.) 2002. *Endocrine disrupting chemicals in wastewater: A review of occurrence and removal*, UK Water Industry Research Limited, London.

Cajaraville, M.P., Marigómez, J.A., Angulo, E., 1989. A stereological survey of lysosomal structure alterations in *Littorina littorea* exposed to 1-naphthol. *Comparative biochemistry and physiology* **93C**, 231-237.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C. and Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *The Science of the Total Environment* **247(2-3)**, 295-311.

Canesi, L., Lorusso, L. C., Ciacci, C., Betti, M., Gallo, G., 2004. Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways. *General and Comparative Endocrinology* **138**, 58-69.

Canesi, L., Lorusso, L. C., Ciacci, C., Betti, M., Rocchi, M., Pojana, G., Marcomini, A., 2007. Immunomodulation of *Mytilus* hemocytes by individual estrogenic chemicals and environmentally relevant mixtures of estrogens: *In vitro* and *in vivo* studies. *Aquatic Toxicology* **812** 36-44.

Cavaletto, M., Ghezzi, A., Burlando, B., Evangelisti, V., Ceratto, N., Viarengo, A. , 2002. Effect of hydrogen peroxide on antioxidant enzymes and metallothionein level in the digestive gland of *Mytilus galloprovincialis*. *Comparative Biochemistry and Physiology* **131C**, 447-455.

Chait, B. T., 2006. Mass spectrometry: Bottom-up or top-down? *Science* **314**, 65-66.

Chapple, J.P., Smerdon, G.R., Berry, R.J., Hawkins, A.J.S., 1998. Seasonal changes in stress-70 protein levels reflect thermal tolerance in the marine bivalve *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology* **229**, 53–68.

Chelomin, V.P., Zakhartsev, M.P., Kurilenko, A.V., Belcheva, N.N., 2005. An *in vitro* study of the effect of reactive oxygen species on subcellular distribution of deposited cadmium in digestive gland of mussel *Crenomytilus grayanus*. *Aquatic Toxicology* **73**, 181-189

Cheung, M.S., Zhang, L.,Wang,W.-X., 2007. Transfer and efflux of cadmium and silver in marine snails and fish fed pre-exposed mussel prey. *Environmental Toxicology and Chemistry* **26**, 1172–1178.

Chora, S., McDonagh, B., Starita-Geribaldi, M., Roméo, M., Bebianno, M.J., 2008. Ubiquitination and carbonylation as markers of oxidative-stress in *Ruditapes decussatus*. *Marine Environmental Research* **66**, 95-97.

Chora, S., Bebianno, M.J., and Roméo, M., 2009. *Analysis of Proteins from Marine Molluscs.* In: Shehan, D. and Tyther, R. (eds.), Methods in Molecular biology, Two-dimensional electrophoresis protocols, vol. 519. Humana Press, New York, pp. 197-204.

Chouksey, M.K., Kadam, A.N., Zingde, M.D., 2004. Petroleum hydrocarbon residues in the marine environment of Bassein–Mumbai. *Marine Pollution Bulletin* **7–8**, 637–47.

Christiansen, T., Korsgaard, B., A. Jespersen, 1998a. Induction of vitellogenin synthesis by nonylphenol and 17 beta-estradiol and effects on the testicular structure in the eelpout *Zoarces viviparus*. *Marine Environmental Research* **46,** 141-144.

Christiansen,T., Korsgaard, B., A. Jespersen, 1998b. Effects of nonylphenol and 17 beta-oestradiol on vitellogenin synthesis, testicular structure and cytology in male eelpout *Zoarces viviparus*. *Journal of Experimental Biology* **201,** 179-192.

Christiansen, L. B., Pedersen, K. L., Korsgaard, B., P. Bjerregaard, 1998c. Estrogenicity of xenobiotics in rainbow trout (*Oncorhynchus mykiss*), using *in vivo* synthesis of vitellogenin as a biomarker. *Marine Environmental Research* **46,** 137-140.

Chu, K.H., Wong, S.H., Leung, P.S., 2000. Tropomyosin Is the Major Mollusk Allergen: Reverse Transcriptase Polymerase Chain Reaction, Expression and IgE Reactivity. *Marine Biotechnology* **2**, 499–509.

Clark, R.B., 2001. *Marine Pollution*. 5th ed. Oxford University Press Inc, New York. 236 pp.

Coelho, M. R., Bebianno, M.J. and W.J. Langston, 2002. Routes of TBT uptake in the clam Ruditapes decussatus- Food as vector of TBT uptake. *Marine Environmental Research* **54**, 193–207.

Coelho, M.R., Langstone, W.J., Bebianno, M.J., 2006. Effect of TBT on Ruditapes decussatus juveniles. *Chemosphere* **63**, 1499-1505.

Cohen, T., S.S. Que Hee and R.F. Ambrose, 2001. Comparison of trace metal concentrations in fish and invertebrates in three Southern California wetlands. *Marine Pollution Bulletin* **42:** 224-232.

Cole, J.F., Volpe, R., 1983. The effect of cadmium in the environment. *Ecotoxicol. Environ. Saf.* 7, 151–159.

Commandeur, J.N.M., Stijntjes, G.J., Vermeulen, N.P.E., 1995. Enzymes and transport systems involved in the formation and disposition of glutathione S-conjugates. Role in bioactivation and detoxication mechanisms of xenobiotics. *Pharmacology Reviews* **47**, 271-330.

Costa, V.M., Amorim, M.A., Quintanilha, A., Moradas-Ferreira, P., 2002. Hydrogen peroxide induced carbonylation of key metabolic enzymes in *Saccharomyces cerevisiae*: the involvement of the oxidative stress response regulators Yap1 and Skn7. *Free Radical Biology and Medicine* **33**, 1507–1515.

Craig, P.J., 2003. *Organometallic compounds in the environment* (2nd edition). Wiley-VCH, 434 pp.

Crane, F. L., Mii, S., Hauge, J. G., Green, D. E., Beinert, H., 1956. On the mechanism of dehydrogenation of fatty acyl derivatives of coenzyme A. I. The general fatty acyl coenzyme A dehydrogenase. *J. Biol. Chem.* 218, 701-706.

Crompton, T.R., 1997. Toxicants in Aqueous Ecosystem. John Wiley and Sons, Chichester, New York.

Cuervo, A.M., 2004. Autophagy: in sickness and in health. *Trends in Cell Biology* **14**, 70-77.

Dachs, J., Van Ry, D.A., Eisenreich, S.J., 1999. Occurrence of estrogenic nonylphenols in the urban and coastal atmosphere of the lower Hudson River estuary. *Environmental Science and Technology* **33**, 2676-2679.

Dalle-Donne, I., Rossi, R., Milzani, A., Di Simplicio, P., Colombo, R., 2001. The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself. *Free Radic. Biol. Med.* 31, 1624-1632.

Dalton, T.P., Shertzer, H.G., Puga, A., 1999. Regulation of gene expression by reactive oxygen. *Annual Review of Pharmacological Toxicology* **39**, 67-101.

Damiens, G., Mouneyrac, C., Quiniou, F., His, E., Gnassia-Barelli, M., 2006. Metal bioaccumulation and metallothionein concentrations in larvae of *Crassostrea gigas*. *Environmental Pollution* **3**, 492-499.

David, E., Tanguy, A., Pichavant, K., Moraga, D., 2005. Response of the Pacific oyster *Crassostrea gigas* to hypoxia exposure under experimental conditions. *The FEBS Journal* **272**, 5635–5652.

Davies, K.J. and Goldberg, A.L., 1987. Proteins damaged by oxygen radicals are rapidly degraded in extracts of red blood cells. *The Journal of biological chemistry* **262**, 8227–8234.

Davies, K.J.A., 2001. Degradation of oxidized proteins by the 20S proteasome. *Biochimie* **83**, 301-310.

De la Torre, F.R., Salibián, A., Ferrari, L., 2007. Assessment of the pollution impact on biomarkers of effect of a freshwater fish. *Chemosphere* **68**,1582–90.

De Luca-Abbott, S.B., Richardson, B.J., McLellan, K.E., Zheng, G.J., Martin, M., Lam, P.K.S., 2005. Field validation of antioxidant enzyme biomarkers in mussels (*Perna viridis*) and clams (*Ruditapes philippinarium*) transplanted in Hong Kong coastal waters. *Marine Pollution Bulletin* **51**, 694–707.

Delgado, M. and Pérez Camacho, A., 2002. Hermaphroditism in Ruditapes *decussatus* (L.) (Bivalvia) from the Galician coast (Spain). *Scientia Marina* **66**, 183–185.

Dellali, M., Roméo, M., Gnassia-Barelli, M., Aissa, P., 2004. A multivariate data analysis of the clam *Ruditapes decussatus* as sentinel organism of the Bizerta lagoon (Tunisia). *Water, Air, and Soil Pollution* **156**, 131-144.

Desbruyères, D., Biscoito, M., Caprais, J.C., Colaço, A., Comtet, T., Crassous, P., Fouquet, Y., Khripounoff, A., Le Bris, N., Olu, K., Riso, R., Sarradin, P.M., Ségonzac, M., A. Vangriesheim, 2001.Variations in deep-sea hydrothermal vent communities on the Mid-Atlantic Ridge near the Azores plateau. *Deep Sea Research Part I: Oceanographic Research Paper* **48**, 1325-1346.

Di Giulio, R.T., Washburn, P.C., Wenning, R.J., Winston, G.W., Jewell, C.S., 1989. Biochemical responses in aquatic animals: a review of determinants of oxidative stress. *Environmental Toxicology and Chemistry* **8**, 1103-1123.

Di Giulio, R.T., Benson, W.H., Sanders, B.M., van Veld, P.A., 1995. *Biochemical mechanisms: metabolism, adaptation, and toxicity.* In: Rand, G.M. (Ed.), Fundamentals of Aquatic Toxicology: Effects, Environmental fate, and Risk Assessment, second ed. Taylor and Francis, London, UK, pp. 523-562.

Dixon, D.R. and Pruski, A.M., 2002. Effects of cadmium on nuclear integrity and DNA repair efficiency in the gill cells of *Mytilus edulis*. *Aquatic Toxicology* **57,** 127–137.

Dondero, F., Dagnino, A., Jonsson, H., Capri, F., Gastaldi, L.,Viarengo, A., 2006. Assessing the occurrence of a stress syndrome in mussels (*Mytilus edulis*) using a combined biomarker/gene expression approach. *Aquatic Toxicology* **78**, S13–S24.

Dorts, J., Silvestre, F., Tu, H.T., Tyberghein, A., Phuong, N.T., Kestemont, P., 2009. Oxidative stress, protein carbonylation and heat shock proteins in the black tiger shrimp, *Penaeus monodon*, following exposure to endosulfan and deltamethrin. *Environmental Toxicology and Pharmacology* **28**, 302-310.

Dowling V, Hoarau P, Roméo M, O'Halloran J, Frank Van Pelt, O'Brien N, Sheehan D. 2006. Protein carbonylation and heat shock response in *Ruditapes decussatus* following p,p′dichlorodiphenyldichloroethylene (DDE) exposure: A proteomic approach reveals that DDE causes oxidative stress. *Aquatic Toxicology* **77**, 11-18.

Dowling, V.A. and Sheehan, D., 2006. Proteomics as a route to identification of toxicity targets in environmental toxicology. *Proteomics* **6**, 5597-5604.

Downs, C.A., Shigenaka, G., Fauth, J.E., Robinson, C.E., Huang, A. 2002. Cellular physiological assessment of bivalves after chronic exposure to spilled Exxon Valdez crude oil using a novel molecular diagnostic biotechnology. *Environmental Science and Technology* **36**, 2987-2993.

Dowsey, A.W., Dunn, M.J., Yang, G.Z., 2003. The role of bioinformatics in two-dimensional gel electrophoresis. *Proteomics* **3**, 1567-1596.

Doyen, P., Vasseur, P., Rodius, F., 2005. cDNA cloning and expression pattern of pi-class glutathione S-transferase in the freshwater bivalves *Unio tumidus* and *Corbicula fluminea*. *Comparative Biochemistry and Physiology C* **140**, 300-308.

Edmond, J.M., Measures, C., Magum, B.m Grant, B., Sclater, F:R:, Collier, R., Hudson, A., Gordon, I., J:B: Corliss, 1979. On the formation of metal-rich deposits at ridge crests. *Earth and Planetary Science Letter* **46**, 19-30.

Ekelund, R., Bergman, A., Granmo, A., Berggren, M., 1990. Bioaccumulation of 4-nonylphenol in marine animals a re-evaluation. *Environmental Pollution* **64**, 107-120.

Ekelund, R., Granmo, A., Magnusson, K., Berggren, M., Bergman, A., 1993. Biodegradation of 4-nonylphenol in seawater and sediment. *Environmental Pollution* **79**, 59–61.

Ferreira, A.C., Costa, A.C.S., Korn, M.G.A., 2004. Preliminary evaluation of the cadmium concentration in seawater of the Salvador City, Brazil. *Microchem. J.* 78, 77-83.

Fiehn, O., Kopka, J., Dörmann, P., Altmann, T., Trethewey, R.N., Willmitzer L., 2000. Metabolite profiling for plant functional genomics. *Nature Biotechnology* **18**, 1157-1161.

Filho, D.W., Tribess, T., Gaspari, C., Claudio, F.D., Torres, M.A., Magalhaes, A.R.M., 2001. Seasonal changes in antioxidant defences of the digestive gland of the brown mussel (*Perna perna*). *Aquaculture* **203**, 149–158.

Filipic, M., Fatur, T., Vudrag, M., 2006. Molecular mechanisms of cadmium induced mutagenicity. *Human and experimental toxicology* **25**, 67–77.

Finne, E.F., Cooper, G.A., Koop, B.F., Hylland, K., Tollefsen, K.E., 2007. Toxicogenomic responses in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to model chemicals and a synthetic mixture. *Aquatic Toxicology* **81**, 293–303.

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

Foy, C.D., Chaney, R.L., White, M.C., 1978. The physiology of metal toxicity in plants. *Annual Review on Plant Physiology* **29**, 511–566.

Frank, R. and Hargreaves, R., 2003. Clinical biomarkers in drug discovery and development. *Nature Reviews. Drug Discovery* **2**, 566–580.

Friguet, B., 2006. Oxidized protein degradation and repair in ageing and oxidative stress. *FEBS Letters* **580**, 2910-2916.

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

Gagné, F., Blaise, C., Pellerin, J., Gauthier-Clerc, S., 2002. Alteration of the biochemical properties of female gonads and vitellins in the clam *Mya arenaria* at contaminated sites in the Saguenay Fjord. *Marine Environmental Research* **53**, 295-310.

Gagné, F. and Blaise, C., 2003. Effects of municipal effluents on serotonin and dopamine levels in the freshwater muss*el Elliptio complanata. Comparative Biochemistry and Physiology* **136C**, 117-125.

Gauthier-Clerc, S., Pellerin, J., Blaise, C., Gagné, F., 2002. Delayed gametogenesis of *Mya arenaria* in the Saguenay Fjord (Canada): a consequence of endocrine disruptors? *Comparative biochemistry and physiology* **131C**, 457-467.

George, S.G., 1994. *Enzymology and molecular biology of phase II xenobiotic-conjugating enzymes in fish.* In: Malins, D.C., Ostrander, G.K. (Eds.), Aquatic Toxicology; Molecular, Biochemical and Cellular perspectives. Lewis Publishers, CRC press, pp. 37-85.

Geret, F., Rainglet, F. and R.P. Cosson, 1998. Comparison between isolation protocols commonly used for the purification of mollusc metallothioneins. *Marine Environmental Research* **46**, 545-550.

Geret, F., Serafim, Â., Barreira, L., Bebianno, M.J., 2002. Response of antioxidant systems to copper in the gills of the clam *Ruditapes decussatus*. *Marine Environmental Research* **54**, 413 –417.

Geret, F. and Bebianno, M.J., 2004. Does zinc produce reactive oxygen species in *Ruditapes decussatus*? *Ecotoxicology and Environmental Safety* **57(3)**, 399-409.

Gharahdaghi, F., Weinberg, C.R., Meagher, D.A., Imai, B.S., Mishe, S.M., 1999. Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. *Electrophoresis* **20**, 601-605.

Gobas, F.A.P.C., Wilcockson, J.B., Russell, R.W., Haffner, G.D., 1999. Mechanisms of biomagnification in fish under laboratory and field conditions. *Environmental Science and Technology* **33**, 133-141.

Goeptar, A.R., Scheerens, H., Vermeulen, N.P.E., 1995. Oxygen reductase and substrate reductase activity of cytochrom P450. *Critical reviews in toxicology* **25**, 25-65.

Goldberg, ED, Koide, M., Hodge, V. Flegal, A.R., and Martin J., 1978. The mussel watch. *Environmental Conservation* **5**, 101-125.

Gomez-Mendikute, A., Cajaraville, M.P., 2002. Comparative effects of cadmium, copper, paraquat and benzo[a]pyrene on the actin cytoskeleton and production of reactive oxygen species (ROS) in mussel haemocytes. *Toxicol In Vitro* 17, 539-546.

Gomez-Mendikute, A., Etxeberria, A., Olabarrieta, I., Cajaraville, M. P., 2002. Oxygen radicals production and actin filament disruption in bivalve haemocytes treated with benzo(a)pyrene. *Marine Environmental Research*, **54**, 431-436.

Gong, Y. and Han, X. D., 2006. Nonylphenol-induced oxidative stress and cytotoxicity in testicular *Sertoli* cells. *Reproductive Toxicology* **22**, 623-630.

Görg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., Weiss, W., 2000. The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* **21**, 1037-1053.

Görg, A., Weiss, W., Dunn, M.J., 2004. Current two-dimensional electrophoresis technology for proteomics. *Proteomics* **4**, 3665-3685.

Gracey, A.Y., Troll, J.V., Somero, G.N., 2001. Hypoxia-induced gene expression profiling in the euryoxid fish *Gillichthys mirabilis*. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 1993-1998.

Grøsvik, B.E., Jonsson, H., Rodríguez-Ortega, M.J., Roepstorff, P., Goksøyr, A., 2006.CYP1A-immunopositive proteins in bivalves identified as cytoskeletal and major vault proteins. *Aquatic Toxicology* **79**, 334–340.

Gueguen, Y., Cadoret, J.P., Flament, D., Barreau, C., 2003. Immune gene discovery by expressed sequence tags generated from hemocytes of the bacteria-challenged oyster, *Crassostrea gigas*. *Gene* **303**, 139-145.

Günzler, W.A. and Flohé, L., 1985. Glutathione peroxidase, in: Handbook of Methods for Oxygen Radical Research. Greenwald, R.A. (Ed.). CRC Press, Boca Raton, FL, pp. 285-290.

Halliwell, B. and Gutteridge, J.M.C., 1999. *Free radicals in biology and medicine*. 3 ed. New York: Oxford University Press Inc.

Halliwell, B. and Gutteridge, J., 2007. *Free Radicals in Biology and Medicine*. Oxford University Press, London, England.

Hamza-Chaffai, A., Amiard, J.C., Cosson, R.P., 1999. Relationship between metallothionein and metals in a natural population of clam *Ruditapes decussatus* from Sfax coast, a non-linear model using Box–Cox transformation. *Comp. Biochem. Physiol.* 123,153– 63.

Hamza-Chaffai, A., Pellerin, J., Amiard, J.C., 2003. Health assessment of a marine bivalve *Ruditapes decussatus* from the Gulf of Gabès (Tunisia). *Environment International* **28**, 609– 617.

Hansen, F.T., Forbes, V.E., Forbes, T.L., 1999. Effects of 4-nonylphenol on life-history traits and population dynamics of a polychaete. *Ecological Applications* **9**,482–495.

Hartwig, A., 1994. Role of DNA repair inhibition in lead and chromium-induced genotoxicity: a review. *Environmental Health Perspectives* **102**, 45-50.

Hassoun, E.A. and Stohs, S.J., 1996. Cadmium-induced production of superoxide anion and nitric oxide, DNA single strand breaks and lactate dehydrogenase leakage in J774A.1 cell cultures. *Toxicology* **112(3-2)**, 219-226.

Heijin, M., Oude, E., R.P.J., Jansen P.L.M., 1992. ATP-dependent multispecific organic anion transport system in rat erythrocyte membrane vesicles. *The American Journal of Physiology* **262**, C104-C110.

Herber, R.F.M. 2004. Cadmium, *In*: Merian, E., Anke, M., Ihnat, M., Stoeppler, M. (Eds.), *Elements and their Compounds in the Environment. Metals and Their Compounds*, vol. 2. Wiley-VCH, Germany, pp. 698–708.

Hoarau, P., Garello, G., Gnassia-Barelli, M., Roméo, M., and Girard J. P., 2002, Purification and partial characterization of seven glutathione S-transferase isoforms from the clam *Ruditapes decussatus*. *European Journal of Biochemistry* **269,** 4359-4366.

Hoarau, P., Garello, G., Gnassia-Barelli, M., Roméo, M., and Girard, J. P., 2004, Effect of three xenobiotic compounds on Glutathione S-Transferase in the clam *Ruditapes decussatus*. *Aquatic Toxicology* **122**, 369-378.

Hofmann, G.E., Somero, G.N., 1996. Interspecific variation in thermal denaturation of proteins in the congeneric mussels *Mytilus trossulus* and *M. galloprovincialis*: evidence from the heat-shock response and protein ubiquitination. *Marine Biology* **126**, 65–75.

Holloway, G.J., Sibly, R.M., Povey, S.R., 1990. Evolution in toxin-stressed environments. *Funct Ecol* 4, 289-294.

Hook, S.E., Skillman, A.D., Small, J.A., Schultz, I.R., 2006. Gene expression patterns in rainbow trout, *Oncorhynchus mykiss*, exposed to a suite of model toxicants. *Aquatic Toxicology* **77**, 372–385.

Horning, E.C. and Horning, M.G., 1971. Metabolic Profiles - Gas-Phase Methods for Analysis of Metabolites. *Clinical Chemistry* **17**, 802–809.

Howard, J., Hyman, A. A., 2003. Dynamics and mechanics of the microtubule plus end. *Nature* **422**, 753-758.

Humphery-Smith, I., Cordwell, S. J., Blackstock, W., 1997. Proteome research: complementarity and limitations with respect to the RNA and DNA worlds. *Electrophoresis* **18**, 1217–1242.

Hyne, R.V. and Maher, A., 2003. Invertebrate biomarkers: links to toxicosis that predict population decline. *Ecotoxicology and environmental safety* **54**, 366–74.

Ishikawa**,** T., 1992. The ATP-dependent glutatione S-conjugate export pump. *Trends in Biochemical Sciences* **17**, 463-468

James, P., Quadroni, M., Carafoli, E., Gonnet, G., 1993. Protein identification by mass profile fingerprinting. *Biochemical and Biophysical Research Communications* **195**, 58-64.

Jenny, M.J., Ringwood, A.H., Schey, K.,Warr, G.W., Chapman, R.W., 2004. Diversity of metallothioneins in the American oyster *Crassostrea virginica*, revealed by transcriptomic and proteomic approaches. *European Journal of Biochemistry* **271**, 1702–1712.

Jonsson, H., Schiedek, D., Grosvik, B.E., Goksoyr, A. 2006. Protein responses in blue mussels (*Mytilus edulis*) exposed to organic pollutants: A combined CYP429 antibody/proteomic approach. *Aquatic Toxicology* **78(1)**, 42-48.

Kadiiska, M.B., Hanna, P.M., Jordan, S.J., Mason, R.P., 1993. Electron spin resonance evidence for free radical generation in copper-treated vitamin E- and selenium-deficient rats: *in vivo* spin-trapping investigation. *Molecular pharmacology* **44**, 222-227.

Kaloyianni, M., Dailianis, S., Chrisikopoulou, E., Zannou, A., Koutsogiannaki, S., Alamdari, D.H., Kang, Y.S., Kim, Y.M., Park, K.L., Cho, S.K., Choi, K.S., Cho, M., 2006. Analysis of EST and lectin expression in hemocytes of Manila clams (*Ruditapes phylippinarum)* (Bivalvia: Mollusca) infected with Perkinsus olseni. *Developmental and comparative immunology* **30**, 1119–1131.

Kehrer, J.P., 2000. The Haber-Weiss reaction and mechanisms of toxicity. *Toxicology*, **149**, 43-50.

Kesseler, A., Brand, M.D., 1994. Localisation of the sites of action of cadmium on oxidative phoshorylation in potato tuber mitochondria using top-down elasticity analysis. *European Journal of Biochemistry* **225**, 897–906.

Kesseler, A., Brand, M.D., 1995. The mechanism of the stimulation of State-4 respiration by cadmium in potato-tuber (*Solanum tuberosum*) mitochondria. *Plant Physiology and Biochemistry* **33**, 519–528.

Ketata, I., Guermazi, F., Rebai, T., Hamza-Chaffai, A., 2007a. Variation of steroid concentrations during the reproductive cycle of the clam *Ruditapes decussatus*: A one year study in the gulf of Gabès area. *Comparative Biochemistry and Physiology. Part A* **147**, 424-431.

Ketata, I., Smaoui-Damak, W., Guermazi, F., Rebai, T., Hamza-Chaffai, A., 2007b. *In situ* endocrine disrupting effects of cadmium on the reproduction of *Ruditapes decussatus. Comparative Biochemistry and Physiology. Part C* **146**, 415-430.

Kettman, J.R., Frey, J.R., Lefkovits, I., 2001. Proteome, transcriptome and genome: top down or bottom up analysis? *Biomolecular Engineering* **18**, 207–212.

Khessiba, A., Roméo, M., Aïssa, P., 2005. Effects of some environmental parameters on catalase activity measured in the mussel (*Mytilus galloprovincialis*) exposed to lindane. *Environmental Pollution* **133,** 275-281.

Kirchin, M.A., Moore, M.N., Dean, R.T., Winston, G.W., 1992. The role of oxyradicals in intracellular proteolysis and toxicity in mussels. *Marine Environmental Research* **34**, 315–320.

Knigge, T., Monsinjon, T., Andersen, O.K., 2004. Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry approach to biomarker discovery in blue mussels (*Mytilus edulis*) exposed to polyaromatic hydrocarbons and heavy metals under field conditions. *Proteomics* **4**, 2722–2727.

Knight, J. A., Voorhees, R. P., 1990, Peroxidation of linolenic acid-catalysis by transition metal ions. *Annals of Clinical and Laboratory Science* **20**, 347–352.

Kobayashi, K., 1985. The effect of herbicide PCP on short necked clams and the process of detoxification. *In Fisheries in Japan, Bivalves* (T. Okutani, T. Tomiyama et T. Hibiya, eds.), pp. 161-165. Japan Marine Products Photo Materials Association.

Koliakos, G. and Dimitriadis, V.K., 2009. Oxidative effects of inorganic and organic contaminants on haemolymph of mussels. *Comparative Biochemistry and Physiology, Part C* **149**, 631-639.

Kopecka, J. and Pempkowiak, J., 2008. Temporal and spatial variations of selected biomarker activities in flounder (*Platichthys flesus*) collected in the Baltic. *Ecotoxicology and Environmental Safety* **70(3)**, 379-91.

Korsgaard, B. and K.L. Pedersen, 1998. Vitellogenin in *Zoarces viviparous*. Purification, quantification by ELISA and induction by estradiol-17 beta and 4-nonylphenol. *Endocrinology* **120**, 159-166.

Koskinen, H., Pehkonen, P., Vehniainen, E., Krasnov, A., Rexroad, C., Afanasyev, S., Molsa, H., Oikari, A., 2004. Response of rainbow trout transcriptome to model chemical contaminants. *Biochemical and Biophysical Research Communications* **320**, 745–753.

Kruk, I., 1998. Environmental Toxicology and Chemistry of Oxygen Species. Berlin, Springer-Verlag.

Kumar, R., Asic, K., Agarwal, K. Seth, P.K., 1996. Oxidative stress-mediated neurotoxicity of cadmium. *Toxicology Letters* **89**, 65-69

Kurelec, B., 1995. Reversion of the multixenobiotic resistance mechanism in gills of a marine mussel *Mytilus galloprovincialis* by a model inhibitor and environmental modulators of P170-glycoprotein. *Aquatic Toxicology* **33**, 93-103.

LaBaer, J. and Ramachandran, N., 2005. Protein microarrays as tools for functional proteomics. *Current Opinion in Chemical Biology* **9**, 14-19.

Langston, W.J. and Bebianno, M.J., 1998 (eds). Metal metabolism in aquatic environments. London, Chapmann and Hall, pp. 209-283.

Lanne, B. and Panfilov, O., 2005. Protein staining influences the quality of mass spectra obtained by peptide mass fingerprinting after separation on 2-d gels. A comparison of staining with coomassie brilliant blue and sypro ruby. *Journal of Proteome Research* **4**, 175-179.

Layfield, R., Alban, A., Mayer, R.J., Lowe, J., 2001. The ubiquitin protein catabolic disorders. *Neuropathology and Applied Neurobiology* **27**, 171–179.

Lech, J.J. and Vodicnik, M.J., 1985. Biotransformation. *In*: Rand, G.M., Petrocelli, S.R. (eds.), *Fundamentals of Aquatic Toxicology; Methods and Applications*. Hemisphere Publishing Corporation, New York, USA, pp. 526-557.

Lee, H.B., 1999. Review of analytical methods for the determination of nonylphenol and related compounds in environmental samples. *Water Quality Research Journal Canada* **34**, 3–35.

Lee, B.G., Grimscom, S.B., Lee, J.S., Choi, H.J., Koh, C.H., Luoma, S.N., Fisher, N.S., 2000. Influences of dietary uptake and reactive sulfides on metal bioavailability from aquatic sediments. *Science* **287**, 282-284

Lemaire, P., Matthews, A., Foerlin, L., Livingstone, D.R., 1994. Stimulation of oxyradical production of hepatic microsomes of flounder (*Platichthys flesus*) and perch (*Perca fluviatilis*) by model and pollutant xenobiotics. *Archives of Environmental Contamination and Toxicology* **26**, 191-200

Lemaire, P. and Livingstone, D.R., 1997. Aromatic hydrocarbon quinone-mediated reactive oxygen species production in hepatic microsomes of the flounder (*Platichthys flesus* L.). *Comparative Biochemistry and Physiology* **117C**, 131-139

Lenaz, G., 1998. Role of mitochondria in oxidative stress and ageing. *Biochimica et Biophysica Acta* **1366**, 53-67.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., Stadtman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology* **186**, 464–478.

Levine, R.L., Wehr, N., Williams, J.A., Stadtman, E.R., Shacter, E., 2000. Determination of carbonyl groups in oxidized proteins. *Methods of Molecular Biology* **99**, 15–24.

Liebler, D.C**.,** 2002. Proteomic approaches to characterize protein modifications: new tools to study the effects of environmental exposures. *Environmental Health Perspectives* **110(1)**, 3-9.

Lietti, E., Marin, M. G., Matozzo, V., Polesello, S., Valsecchi, S., 2007. Uptake and elimination of 4-nonylphenol by the clam *Tapes philippinarum*. *Archives of Environmental Contamination and Toxicology* **53**, 571-578.

Lindström-Seppä, P. and Oikari, A., 1991. Biotransformation activities of feral fish in waters receiving bleached pulp mill effluents. *Environmental Toxicology and Chemistry* **9**, 1415-1424.

Lippolis, J. and Reinhardt, T., 2005. Proteomic survey of bovine neutrophils. *Vet Immunol Immunopathol* **103**, 53–65.

Livingstone, D.R. and Ferrar, S.V., 1984. Tissue and subcellular distribution of enzyme activities of mixed-function-oxygenase an benzo(a)pyrene metabolism in the common mussel *Mytilus edulis. The Science of Total Environment* **39**, 209-235.

Livingstone, D.R., Garcia, P.M.,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 mollusks. *Funcional Ecology* **4**, 415- 424.

Livingstone, D.R., Mitchelmore, C.L., O'Hara, S.C.M., Lemaire, P., Sturve, J., Forlin, L., 2000. Increased potential for NAD(P)H-dependent reactive oxygen species production of hepatic subcellular fractions of fish species with in vivo exposure to contaminants. *Marine Environmental Research* **50**, 57-60.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Marine Pollution Bulletin* **42**, 656–666.

Lopez, J.L., Mosquera, E., Fuentes, J., Marina, A., Vazquez, J., Alvarez, G., 2001. Two-dimensional gel electrophoresis of *Mytilus galloprovincialis*: differences in protein

expression between intertidal and cultured mussels. *Marine Ecology Progress Series* **224**, 149–156.

Lopez, J.L., Marina, A., Vázquez, J., Alvarez, G., 2002. A proteomic approach to the study of the marine mussels *Mytilus edulis* and *Mytilus galloprovincialis*. *Marine Biology* **141**, 217–223.

Lopez, M.F., Berggren, K., Chernokalskaya, E., Lazarev, A., Robinson, M., Patton, W.F., 2000. A comparison of silver stain and SYPRO Ruby Protein Gel Stain with respect to protein detection in two-dimensional gels and identification by peptide mass profiling. *Electrophoresis* **21**, 3673-3683.

López-Barea, J., 1995. Biomarkers in ecotoxicology: an overview. *Archives of Toxicology Supplement* **17**, 57-79.

López-Barea, J. and Gómez-Ariza, J.L., 2006. Environmental proteomics and metallomics. *Proteomics* **6**, S51-S62.

Luana, W., Li, F., Wang, B., Zhang, X., Liu, Y., Xiang, J., 2007. Molecular characteristics and expression analysis of calreticulin in Chinese shrimp *Fenneropenaeus chinensis*. *Comparative Biochemistry and Physiology Part B* **147**, 482–491.

Ma, B., Zhang, K., Hendrie, C., Liang, C., Li, M. et al., 2003. PEAKS:Powerful software for peptide de novo sequencing by tan- dem mass spectrometry. *Rapid Communications in Mass Spectrometry* **17**, 2337–2342.

Madsen, S. S., Mathiesen, A. B. and B. Korsgaard, 1997. Effects of 17 beta-estradiol and 4-nonylphenol on smoltification and vitellogenesis in Atlantic salmon (*Salmo salar*). *Fish Physiology Biochemistry* **17**, 303-312.

Mäenpää, K. and Kukkonen, J.V.K., 2006. Bioaccumulation and toxicity of 4-nonylphenol (4-NP) and 4-(2-dodecyl)-benzene sulfonate (LAS) in *Lumbriculus variegatus* (Oligochaeta) and *Chironomus riparius* (Insecta). *Aquatic Toxicology* **77**, 329-338.

Maguire, R.J., 1999. Review of the persistence on nonylphenol and nonylphenol ethoxylates in aquatic environments. *Water Quality Research Journal of Canada* **34**, 37–78.

Manca, D., Richard, A.C., Van Tra, H. and Chevalier, G., 1994. Relation between lipid peroxidation and inflammation in the pulmonary toxicity of cadmium. *Archives of Toxicology* **68**, 364-369.

Manduzio, H., Cosette, P., Gricourt, L., Jouenne, T., Lenz, C., Andersen, O.K., Leboulenger, F., Rocher, B., 2005. Proteome modifications of blue mussel (*Mytilus edulis* L.) gills as an effect of water pollution. *Proteomics* **5**, 4958-4963.

Mann, M. and Wilm, M., 1994. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. *Analytical Chemistry* **66**, 4390-4399.

Marekov, L.N. and Steinert, P.M., 2003. Charge derivatization by 4- sulfophenyl isothiocyanate enhances peptide sequencing by post-source decay matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Journal of Mass Spectrometry* **38**, 373–377.

Marigómez, J.A., Vega, M.M., Cajaraville, M.P. and Angulo, E., 1989. Quantitative responses of the digestive lysosomal system of winkles to sublethal concentrations of cadmium. *Cellular and Molecular Biology* **35**: 555-562.

Marigómez, I., Soto, M., Cajaraville, P.M., Angulo, E., Giamberini, L., 2002. Cellular and subcellular distribution of metals in molluscs. *Microsc. Res. Tech.* 56, 358–392.

Marin, M. G., Rigato, S., Ricciardi, F., Matozzo, V., 2008. Lethal and estrogenic effects of 4-nonylphenol in the cockle *Cerastoderma glaucum*. *Marine Pollution Bulletin* **57**, 552-558.

Marques C, Pereira P, Taylor A, Liang JN, Reddy VN, Szweda LI, Shang F. 2004. Ubiquitin-dependent lysosomal degradation of the HNE modified proteins in lens epithelial cells. *Federation of American Societies for Experimental Biology Journal* **18**, 1424–1426.

Matozzo, V., Ballarin, L., Pamparin, D.M., Marin, M.G., 2001. Effects of copper and cadmium exposure on functional responses of hemocytes in the clam, *Tapes philippinarum*. *Arch. Environ. Contam. Toxicol.* 41, 163–170.

Matozzo, V., Deppieri, M., Moschino, V., Marin, M.G., 2003. Evaluation of 4-nonylphenol toxicity in the clam *Tapes philippinarum*. *Environmental Research* **91**, 179-185.

Matozzo, V., Ballarin, L., Marin, M., 2004. Exposure of the clam *Tapes philippinarum* to 4-nonylphenol: changes in anti-oxidant enzyme activities and re-burrowing capability. *Marine Pollution Bulletin* **48**, 563-571.

Matozzo, V. and Marin, M., 2005. Can 4-nonylphenol induce vitellogenin-like proteins in the clam *Tapes philippinarum*? *Environmental Research* **97**, 43-49.

Matsumoto, I. and Kuhara, T., 1996. A new chemical diagnostic method for inborn errors of metabolism by mass spectrometry - Rapid, practical, and simultaneous urinary metabolites analysis. *Mass Spectrometry Reviews* **15**, 43–57.

Mayer, M. P., Bukau, B., 2005. Hsp70 chaperones: cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences* **62**, 670-684.

McCarthy, J.F. and Shugart, L.R., 1990. Biological markers of environmental contamination. *In*: McCarthy, J.F., Shugart, L.R. (Eds.), Biomarkers of Environmental Contamination. Lewis Publishers, Boca Raton, FL, USA, pp. 3-16.

McDonagh, B., Tyther, R., Sheehan, D., 2005. Carbonylation and glutathionylation of specific proteins in the blue mussel *Mytilus edulis* detected by proteomic analysis and western blotting: actin as a target of oxidative stress. *Aquatic Toxicology* **73**, 315–326.

McDonagh, B. and Sheehan, D., 2006. Redox proteomics in the blue mussel *Mytilus edulis*: Carbonylation is not a pre-requisite for ubiquitination in acute free radical-mediated oxidative stress. *Aquatic Toxicology* **79**, 325–333.

McDonagh, B. and Sheehan, D., 2007. Effects of oxidative stress on protein thiols in the blue mussel *Mytilus edulis*: Proteomic identification of target proteins. *Proteomics* **7**, 3395-3403.

McDonagh, B. and Sheehan, D., 2008. Effects of oxidative stress on protein thiols and disulphides in *Mytilus edulis* revealed by proteomics: Actin and protein disulphide isomerase are redox targets. *Marine Environmental Research* **66**, 193-195.

Meneghini, R., 1988. Genotoxicity of active oxygen species in mammalian cells. *Mutation Research* **195**, 215-230.

Mezhoud, K., Praseuth, D., Puiseux-Dao, S., François, J., Bernard, C., Edery, M., 2008. Global quantitative analysis of protein expression and phosphorylation status in the liver of the medaka fish (*Oryzias latipes*) exposed to microcystin-LR.I. Balneation study. *Aquatic Toxicology* **86**, 166–175.

Mi, J., Orbea, A., Syme, N., Ahmed, M., Cajaraville, M. P., Cristobal, S., 2005. Peroxisomal proteomics, a new tool for risk assessment of peroxisome proliferating pollutants in the marine environment. *Proteomics* **5,** 3954–3965.

Miracle, A. L., Ankley, G. T., 2005. Ecotoxicogenomics: linkages between exposure and effects in assessing risks of aquatic contaminants to fish. *Reproductive Toxicology* **19**, 321-326.

Miura, Y., Kano, M., Abe, K., Urano, S., Suzuki, S., Toda, T., 2005. Age-dependent variations of cell response to oxidative stress: proteomic approach to protein expression and phosphorylation. *Electrophoresis* **26**, 2786-2796.

Moens, L.N., van der Ven, K., Van Remortel, P., Del-Favero, J., De Coen, W.M., 2006. Expression profiling of endocrine-disrupting compounds using a customized *Cyprinus carpio* cDNA microarray. *Toxicological Sciences* **93(2)**, 298–310.

Monserrat, J.M., Martínez, P.E., Geracitano, L.A., Lund Amado, L., Martinez Gaspar Martins, C., Lopes Leães Pinho, G., Soares Chaves, I., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2007. Pollution biomarkers in estuarine animals: critical review and new perspectives. *Comparative Biochemistry and Physiology C* **146**, 221–234.

Monsinjon, T., Andersen, O.K., Leboulenger, F., Knigge, T., 2006. Data processing and classification analysis of proteomic changes: a case study of oil pollution in the mussel, *Mytilus edulis*. *Proteome Science* **4(17)**, 1-13.

Monsinjon, T. and Knigge, T., 2007. Proteomic applications in ecotoxicology. *Proteomics* **7**, 2997-3009.

Moore, M.N., 2004. Diet restriction induced autophagy: A lysosomal protective system against oxidative- and pollutant-stress and cell injury. *Marine Environmental Research* **58**, 603-607.

Moore, M.N., Icarus, A.J., Somerfield, P.J., 2006. Autophagy: role in surviving environmental stress. *Marine Environmental Research* **62**, S420–S425.

Moore, M.N., Viarengo, A., Donkin, P., Hawkins, J.S., 2007. Autophagic and lysosomal reactions to stress in the hepatopancreas of blue mussels. *Aquatic Toxicology* **84**, 80-91.

Morcillo, Y. and Porte, C., 2000. Evidence of endocrine disruption in clams — *Ruditapes decussata* — transplanted to a tributyltin-polluted environment. *Environmental Pollution*, **107**, 47-52.

Morrison, H.A., Gobas, F.A.P.C., Lazar, R., Haffner, G.D., 1996. Development and verification of a bioaccumulation model for organic contaminants in benthic invertebrates. *Environmental Science and Technology* **30(11)**, 3377-3384.

Mulder, G.J., Coughty, M.W.H., Burchell, B., 1990. Glucuronidation. *In*: Mulder, G.J. (Ed.), *Conjugation Reactions in Drug Metabolism; an Integrated Approach*. Taylor and Francis, London.

Muller, W., Muller, I. In *Microsclae Aquatic Toxicology: Advance, Techniques and Practise*; Wells, P. G., Lee, K. H., Blaise, C., Eds.; CRC Lewis: Boca Raton, FL, 1998; pp 97-112.

Narbonne, J.F., Daubeze, M., Clerandeau, C., Garrigues, P., 1989. Scale of classification based on biochemical markers in mussels: application to pollution monitoring in European coasts. *Biomarkers* **4**, 415-25.

NAS, 1980. The international Mussel Watch. Washington (DC): *National Academy of Sciences of the* United States *of America*.

Nasci, C., Nesto, N., Monteduro, R. A., Da Ros, L., 2002. Field application of biochemical markers and a physiological index in the mussel, *Mytilus galloprovincialis*: transplantation and biomonitoring studies in the lagoon of Venice (NE Italy). *Marine Environmental Research* **54**, 811-816.

Nesatyy, V.J. and Suter, M.J.F., 2007. Proteomics for the analysis of environmental stress responses in organisms. *Environmental Science and Technology* **41**, 6891– 6900.

Nesatyy, V.J. and Suter, M.J.F., 2008. Analysis of environmental stress response on the proteome level. *Mass Spectrometry Reviews* **27**, 556-574.

Nesto, N., Bertoldo, M., Nasci, C., Da Ros, L., 2004. Spatial and temporal variation of biomarkers in mussels (*Mytilus galloprovincialis*) from the Lagoon of Venice, Italy. *Marine Environmental Research* **58***,* 287-291.

Nice, H. E., Thorndyke, M. C., Morritt, D., Steele, S., Crane, M., 2000. Development of *Crassostrea gigas* larvae is affected by 4-nonylphenol. *Marine Pollution Bulletin* **40**, 491-496.

Nice, H.E., Thorndyke, M. C., Morritt, D., Steele, S., Crane, M., 2005. Sperm motility in the Pacific oyster (*Crassostrea gigas*) is affected by nonylphenol. *Marine Pollution Bulletin*, **50***,* 1668-1674.

Nimrod, A. C., Benson, W. H., 2004. Estrogenic responses to xenobiotics in channel catfish (*Ictalwus punctatus*). *Marine Environmental Research*, **42***,* 155-160.

NRC: Committee on Biological Markers of the National Research Council, 1987. Biological markers in environmental health research. *Environmental Health Perspectives* **74**, 3-9.

Nuwaysir, E.F., Bittner, M., Trent, J., Barrett, J.C., Afshari, C.A., 1999. Microarrays and toxicology: the advent of toxicogenomics. *Molecular Carcinogenesis* **24**, 153-9. Quality status report 2000: region IV—Bay of Biscay and Iberian coast. OSPAR Commission, London, 2000; 1–134.

Okai, Y., Sato, E., Higashi-Okai, K., Inoue, M., 2004. Effect of endocrine disruptor para-nonylphenol on the cell growth and oxygen radical generation in *Escherichia coli* mutant cells deficient in catalase and superoxide dismutase. *Free Radical Biology and Medicine* **37**, 1412-1418.

Olabarrieta, I., L'Azou, B., Yuric, S., Cambar, J., Cajaraville, M.P., 2001. In vitro effects of cadmium on two different animal cell models. *Toxicol. In Vitro* 15, 511–517.

Olsson, B., Bradley, B.P., Gilek, M., Reimer, O., Shepard, J.L., Tedengren, M., 2004. Physiological and proteomic responses in *Mytilus edulis* exposed to PCBs and PAHs extracted from Baltic Sea sediments. *Hydrobiologia* **514**, 15-27.

Pan, K., Wang, W., 2008. The subcellular fate of cadmium and zinc in the scallop *Chlamys nobilis* during waterborne and dietary metal exposure. *Aquat. Toxicol.*90, 253-260.

Park, J.D., Liu, Y., Klaassen, C.D., 2001. Protective effect of metallothionein against the toxicity of cadmium and other metals. *Toxicology*163, 93–100.

Pappin, D.J., Hojrup, P., Bleasby, A.J., 1993. Rapid identification of proteins by peptide-mass fingerprinting. *Current Biology* **3**, 327-332.

Patterson, S.D. and Aebersold, R.H., 2003. Proteomics: the first decade and beyond. *Nature Genetics* **33**, 311-323.

Peakall, D.B., 1994. The conceptual basis of the biomarker approach. *In*: Peakall DB, Shugart LR, editors. NATO Advanced Science Institutes Series. Berlin: Springer Verlag, 1993:15–29.

Pennie, W., Pettit, S.D., Lord, P.G., 2004. Toxicogenomics in risk assessment: an overview of an HESI collaborative research program. *Environmental Health Perspectives* **112**, 417–419.

Peters, L.D., O'Hara, S.C.M., Livingstone, D.R., 1996. Benzo[a]pyrene metabolism and xenobiotic stimulated reactive oxygen species generation by subcellular fraction of larvae of turbot (*Scophthalmus maximus* L.) *Comparative Biochemistry and Physiology* **114C**, 221-227.

Petrak, J., Ivanek, R., Toman, O., Cmejla, R., Cmejlova J., Vyoral, D., Zivny, J., Vulpe, C., 2008. Déjà vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. *Proteomics* **8**, 1744–1749.

Petricoin, E.E., Paweletz, C.P., Liotta, L.A., 2002. Clinical applications of proteomics: proteomic pattern diagnostics. *Journal of mammary gland biology and neoplasia* **7**, 433–40.

Pflieger, D., Le Caer, J.P., Lemaire, C., Bernard, B.A., Dujardin, G., 2000. Systematic identification of mitochondrial proteins by LCMS/ MS. *Analytical Chemistry* **74**, 2400-2406.

Phillips, D.J.H., 1986. The use of bio-indicators in monitoring conservative contaminants. Program design imperatives. *Marine Pollution Bulletin* **17**, 10-7.

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**, 27-33.

Porte, C., Janer, G., Lorusso, L.C., Ortiz-Zarragoitia, M., Cajaraville, M.P., Fossi, M.C., Canesi, L., 2006. Endocrine disruptors in marine organisms: approaches and perspectives. *Comparative Biochemistry and Physiology* **143C**, 303–315.

Prevodnik, A., Gardeström, J., Lilja, K., Elfwing, T., McDonagh, B., Petrović, N., Tedengren, M., Sheehan D., and Bollner T., 2007. Oxidative stress in response to xenobiotics in the blue mussel *Mytilus edulis* L.: Evidence for variation along a natural salinity gradient of the Baltic Sea. *Aquatic Toxicology* **82**, 63-71.

Prichard, A.K., Roby, D.D., Bowyer, R.T., Duffy, L.K., 1997. Pigeon guillemots as a sentinel species: a dose–response experiment with weathered oil in the field. *Chemosphere* **35(7)**, 1531–48.

Prusky, A.M. and Dixon, D.R., 2002. Effects of cadmium on nuclear integrity and DNA repair efficiency in the gill cells of *Mytilus edulis* L. *Aquatic Toxicology* **57**, 127-137.

Quinn, B, Gagnè, F., Costello, M., McKenzie, C., Wilson, J., Mothersill, C., 2006a. The endocrine disrupting effect of municipal effluent on the zebra mussel (*Dreissena polymorpha*). *Aquatic Toxicology* **66**, 279–292.

Quinn, B., Gagné, F., Blaise, C., Costello, Wilson, J.G., Mothersill, C, 2006b. Evaluation of the lethal and sub-lethal toxicity and potential endocrine disrupting effect of nonylphenol on the zebra mussel (*Dreissena polymorpha*). *Comparative Biochemistry and Physiology* **142C**, 118-127.

Rabilloud, T. (ed.), 2000. *Proteome research: Two-Dimensional Gel Electrophoresis and Identification Methods*. Springer Verlag, Heidelberg, 244 pp.

Regoli, F., Nigro, M., Orlando, E., 1998. Lysosomal and antioxidant responses to metals in the Antarctic scallop *Adamussium colbecki*. *Aquatic Toxicology* **40**, 375-392.

Remacle, J., Lambert, D., Raes, M., Pigeolet, E., Michiels, C., Toussaint, O., 1992. Importance of various antioxidant enzymes for cell stability Confrontation between theoretical and experimental data. Biochemical Journal **286**, 41-46.

Ricciardi, F., Matozzo, V., Marin, M.G., 2008. Effects of 4-nonylphenol exposure in mussels (*Mytilus galloprovincialis*) and crabs (*Carcinus aestuarii*) with particular emphasis on vitellogenin induction. *Marine Pollution Bulletin* **57**, 365-372.

Rodriguez-Ortega, M. J., Grosvik, B. E., Rodriguez-Ariza, A., Goksyr, A., López-Barea, J., 2003. Changes in protein expression profiles in bivalve molluscs (*Chamaelea gallina*) exposed to four model environmental pollutants. *Proteomics* **3**, 1535-1543.

Roméo, M. and Gnassia-Barelli, M., 1995. Metal distribution in different in tissues and in subcellular fractions of the Mediterranean clam *Ruditapes decussatus* treated with cadmium, copper or zinc. *Comparative Biochemistry Physiology* **111C**, 457-463.

Roméo, M., Gnassia-Barelli, M., 1997. Effect of Heavy Metals on Lipid Peroxidation in the Mediterranean Clam *Ruditapes decussatus*. *Comparative Biochemistry Physiology* **118C**, 33-37.

Romero-Ruiz, A., Carrascal, M., Alhama, J., Gómez-Ariza, J. L., Abian, J., López-Barea, J., 2006. Environmental proteomics studies in clams from the Donaña bank of Guadalquivir Estuary (SW Spain). *Proteomics* **6**, 245-255.

Romero-Ruiz, A., Alhama, J., Blasco, J., Gómez-Ariza, J.L., López-Barea, J., 2008. New metallothionein assay in *Scrobicularia plana*: Heating effect and correlation with other biomarkers. *Environmental Pollution* **156**, 1340-1347.

Ruokojärvi, P., Jaana, K., Sirpa, H., Terttu, V., 2004. Mussel incubation in the monitoring of PCDDs, PCDFs, PCBs, PBDEs and PCNs in two Finnish watercources. *Organohalogen Compounds* **66,** 1829-1833.

Sakakibara, Y., Hasegawa, K., Oda, T., Saito, H., Hirata, A., Matsuhashi, M., Sato, Y., 1990. Effects of synthetic estrogens, (*R*,*R*)-(+)-, (*S*,*S*)-(-)-, *dl*- and *meso*-hexestrol stereoisomers on microtubule assembly. *Biochemical Pharmacology* **39**, 167-172.

Samuelsson, M.L., Forlin, L., Karlsson, G., Adolfsson-Erici, M., Larsson, D.G.J., 2006. Using NMR metabolomics to identify responses of an environmental estrogen in blood plasma of fish. *Aquatic Toxicology* **78**,341-349.

Satarug, S., Haswell-Elkins, M.R., Moore, M.R., 2000. Safe levels of cadmium intake to prevent renal toxicity in human subjects. Review article. *The British Journal of Nutrition* **84**, 791-802.

Schiedek, D., Broeg, K., Barsiene, J., Lehtonen, K.K., Gercken, J., Pfeifer, S., Vuontisjarvi, H., Vuorinen, P.J., Dedonyte, V., Koehler, A., Balk, L., Schneider, R., 2006. Biomarker responses as indication of contaminant effects in blue mussel (*Mytilus edulis*) and female eelpout (*Zoarces viviparus*) from the southwestern Baltic Sea. *Marine Pollution Bulletin* **53**, 387–405.

Schirle, M., Heurtier, M.A., Kuster, B., 2003. Profiling core proteomes of human cell lines by one-dimensional PAGE and liquid chromatography-tandem mass spectrometry. *Molecular & Cellular Proteomics* **2**, 1297-1305.

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., Gunten, U.V., Wehrli, B., 2006. The challenge of micropollutants in aquatic systems. *Science* **313**, 1072-1077.

Serafim, A. and Bebianno, M.J., 2007a. Involvement of MT in Zn accumulation and elimination strategies in *Ruditapes decussatus*. *Archives of Environmental Contamination and Toxicology* **52**, 189-199.

Serafim, A. and Bebianno, M.J., 2007b. Kinetic model of cadmium accumulation and elimination and metallothionein response in *Ruditapes decussatus*. *Environmental Toxicology and Chemistry* **26**, 960-969.

Serafim, A. and Bebianno, M.J., 2009. Metallothionein role in the kinetic model of copper accumulation and elimination in the clam *Ruditapes decussatus*. *Environmental Research* **109(4)**, 390-9.

Servos, M.R., 1999. Review of the aquatic toxicity, estrogenic responses and bioaccumulation of alkylphenols and alkylphenol polyethoxylates. *Water Quality Research Journal of Canada* **34**, 123–177.

Sheehan, D. and Power, A., 1999. Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. *Comparative Biochemistry and Physiology* **123C**, 193–199.

Shepard, J.L. and Bradley, B.P., 2000. Protein expression signatures and lysosomal stability in *Mytilus edulis* exposed to graded copper concentrations. *Marine Environmental Research* **50**, 457–463.

Shepard, J.L. and Bradley, B.P., 2000. Protein expression signatures and lysosomal stability in *Mytilus edulis* exposed to graded copper concentrations. *Marine Environmental Research* **50**, 457-463.

Shepard, J.L., Olsson, B., Tedengren, M., Bradley, B.P., 2000. Protein expression signatures identified in *Mytilus edulis* exposed to PCBs, copper and salinity stress. *Marine Environmental Research* **50**, 337–340.

Sherman, M.Y. and Goldberg, A.L., 2001. Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron* **29**, 15-32.

Shi, D., Blackmore, G., Wang, W.-X., 2003. Effects of aqueous and dietary preexposure and resulting body burden on silver biokinetics in the green mussel *Perma viridis*. *Environmental Science and Technology* **37**, 936–943.

Shimada, Y., Okuno, S., Kawai, A., Shinomiya, H., Saito, A., Suzuki, M., Omori, Y., Nishino, N., Kanemoto, N., Fujiwara, T., Horie, M., Takahashi, E., 1998. Cloning and chromosomal mapping of a novel ABC transporter gene (hABC7), a candidate for X-linked sideroblastic anemia with spinocerebellar ataxia. *Journal of Human Genetics* **43,** 115-122.

Shin, P.K.S. and Lam, W.K.C., 2001. Development of a marine sediment pollution index. *Environmental Pollution* **113**, 281-291.

Simes, D.C., Bebianno, M. J., Moura, J. J.G., 2003, Isolation and characterisation of metallothionein from the clam *Ruditapes decussatus*. *Aquatic Toxicology* **63**, 307-318.

Simkiss, K., Taylor,M., Mason, A.Z., 1982. Metal detoxification and bioaccumulation in molluscs. *Marine Biology Letters* **3**, 187-201.

Skulachev, V.P., Chistyakov, V.V., Jasaitis, A.A., Smirnova, E.G., 1967. Inhibition of the respiratory chain by zinc ions. *Biochemical and Biophysical Research Communications* **26**, 1–6.

Slimane, T.A., Trugnan, G., Van IJzendoorn, S.C.D., Hoekstra, D., 2003. Raft-mediated trafficking of apical resident proteins occurs in both direct and transcytotic pathways in polarized hepatic cells: Role of distinct lipid microdomains. *Mol. Biol.Cell* 14 (2), 611-624.

Smaoui-Damak, W., Rebai, T., Berthet, B., Hamza-Chaffai, A., 2006. Does cadmium pollution affect reproduction in the clam *Ruditapes decussatus*? A one-year case study. *Comparative Biochemistry and Physiology* **143C**, 252-261.

Sokolova, I.M., 2004. Cadmium effects on mitochondrial function are enhanced by elevated temperatures in a marine poikilotherm, *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). *The Journal of Experimental Biology* **207**, 2639-2648.

Smital, T., Sauerborn, R., Hackenberger, B.K., 2003. Inducibility of the P-glycoprotein transport activity in the marine mussel *Mytilus galloprovincialis* and the freshwater mussel *Dreissena polymorpha*. *Aquatic Toxicology* **65**, 443-465.

Snape, J.R., Maund, S.J., Pickford, D.B., Hutchinson, T.H., 2004. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *Aquatic Toxicology* **67**, 143–154.

Sokolova, I.M., 2004. Cadmium effects on mitochondrial function are enhanced by elevated temperatures in a marine poikilotherm, *Crassostrea virginica* (Bivalvia: Ostreidae). *The Journal of Experimental Biology* **207**, 2639-48.

Sokolova, I.M., Evans, S., Hughes, F.M., 2004. Cadmium-induced apoptosis in oyster hemocytes involves disturbance of cellular energy balance but no mitochondrial permeability transition. *The Journal of Experimental Biology* **207**, 3369–3380.

Solé, M., 2000, Evidence of tributyltin on the MFO system of the clam *Ruditapes decussatus*: a laboratory and field approach. *Comparative Biochemistry and Physiology C* **125**, 93-101.

Spencer, J.P.E., Jenner, A., Aruoma, O.I., Cross, C.E., Wu, R., Halliwell, B., 1996. Oxidative DNA damage in human respiratory tract epithelial cells. Time of course in relation to DNA strand breakage. *Biochemical and Biophysical Research Communications* **224**, 17-22.

Stagg, R.M., 1998. The development of an international programme for monitoring the biological effects of contaminants in the OSPAR Convention area. *Marine Environmental Research* **46**, 307-313.

Staniek, K. and Nohl, H., 1999. H_2O_2 detection from intact mitochondria as a measure for one-electron reduction of dioxygen requires a non-invasive assay system. *Biochimica et Biophysica Acta* **1413**, 70-80.

Staniek, K. and Nohl H., 2000. Are mitochondria a permanent source of reactive oxygen species? *Biochimica et Biophysica Acta* **1460**, 268-75.

Staples, C., Mihaich, E., Carbone, J., Woodbrun, K., Klecka, G., 2004. A weight of evidence analysis of the chronic ecotoxicity of nonylphenol ethoxylates, nonylphenol ether carboxylates, and nonylphenol. *Human Ecology Risk Assessment* **10**, 999-1017.

Starita-Geribaldi, M., Roux, F., Garin, J., Chevallier, D., Fénichel, P., Pointis, G., 2003. Development of narrow immobilized pH gradients covering one pH unit for human seminal plasma proteomic analysis. *Proteomics* **3**, 1611–1619.

Stegeman, J.J., Brouwer, M., Richard, T.D.G., Förlin, L., Fowler, B.A., Sanders, B.M., van Veld, P.A., 1992. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. *In*: Huggett, R.J., Kimerly, R.A., Mehrle, P.M., Jr, Bergman, H.L. (Eds.), Biomarkers: Biochemical,

Physiological and Histological markers of Anthropogenic Stress. Lewis Publishers, Chelsea, MI, USA, pp. 235-335.

Stegeman, J.J., Hahn, M.E., 1994. Biochemistry and molecular biology of monooxygenase: current perspective on forms, functions, and regulation of cytochrome P450 in aquatic species. *In*: Malins, D.C., Ostrander, G.K. (Eds.), *Aquatic toxicology; Molecular, Biochemical and Cellular Perspectives*. Lewis Publishers, CRC press, Boca Raton, pp. 87-206.

Stenmark, H. and Olkkonen, V.M., 2001. The Rab GTPase family. *Genome Biology* **2 (5)**,3007.1–3007.7.

Stohs, S.J., Bagchi, D., Hassoun, E. and Bagchi, M., 2001. Oxidative mechanisms in the toxicity of chromium and cadmium ions. *Journal of Environmental Pathology, Toxicology and Oncology* **20**, 77-88.

Takata, T., Shimo-Oka, T., Miki, K., Fujii, N., 2005. Characterization of new D-β-aspartate-containing proteins in a lens-derived cell line. *Biochemical and Biophysical Research Communications* **334**, 1022-1031.

Tanguy, A., Bierne, N., Saavedra, C., Pina, B., Bachère, E., Kube, M., Bazin, E., Bonhomme, F., Boudry, P., Boulo, V., Boutet, I., Cancela, L., Dossat, C., Favrel, P., Huvet, A., Jarque, S., Jollivet, D., Klages, S., Lapègue, S., Leite, R., Moal, J., Moraga, D., Reinhardt, R., Samain, J.F., Zouros, E., Canário, A., 2008. Increasing genomic information in bivalves through new EST collections in four species: Development of new genetic markers for environmental studies and genome evolution. *Gene* **408(1-2)**, 27-36.

Taris, N., Lang, R.P., Camara, M.D., 2008. Sequence polymorphism can produce serious artefacts in real-time PCR assays: hard lessons from Pacific oysters. *BMC Genomics* **9**, 234.

Tedengren, M., Olsson, B., Bradley, B. P., and Zhou, L., 1999. Heavy metal uptake, physiological response and survival of the blue mussel (*Mytilus edulis*) from marine and brackish waters in relation to the induction of heat-shock protein 70. *Hydrobiologia* **393**, 261–269.

Tedesco, S., Doyle, H., Redmond, G., Sheehan, D., 2008. Gold nanoparticles and oxidative stress in *Mytilus edulis*. *Marine Environmental Research* **66**, 131-133.

Thuvander, A., 1989. Cadmium exposure of rainbow trout, *Salmo gairdneri* Richardson: effects on immune functions. *Journal of Fish Biology* **35**, 521-529

Tilton, S.C., Givan, S.A., Pereira, C.B., Bailey, G.S., Williams, D.E., 2006. Toxicogenomic profiling of the hepatic tumor promoters indole-3-carbinol, 17beta-estradiol and beta-naphthoflavone in rainbow trout. *Toxicological Sciences* **90**, 61–72.

Timbrell, J., 2001 (ed.). Principles of Biochemical Toxicology. Taylor and Francis, London.

Trethewey, R.N., Krotzky, A.J., Willmitzer, L., 1999. Metabolic profiling: a Rosetta Stone for genomics? *Current opinion in plant biology* **2**, 83–85.

Trethewey, R.N., 2004. Metabolite profiling as an aid to metabolic engineering in plants. *Current Opinion in Plant Biology* **7**, 196-201.

Tweeddale, H., Notley-McRobb, L., Ferenci, T., 1998. Effect of slow growth on metabolism of *Escherichia coli*, as revealed by global metabolite pool ("Metabolome") analysis. *Journal of Bacteriology* **180**, 5109–5116.

UNEP (United Nations Environmental Programme), 2003. Global report: regionally based assessment of persistent toxic substances. UNEP chemicals.

UNEP/RAMOGE, 1999. Manual on the Biomarkers Recommended for the MED POL Biomonitoring Programme. UNEP, Athens.

Unlu, M., Morgan, M.E., Minden, J.S., 1997. Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. *Electrophoresis* **18**, 2071-2077.

Van der Oost, R., Opperhuizen, A., Satumalay, K., Heida, H., Vermeulen, N.P.E., 1996. Biomonitoring aquatic pollution with feral eel (*Anguilla anguilla*): I. Bioaccumulation: biota-sedimentratios of PCBs, OCPs, PCDDs and PCDFs. *Aquatic Toxicology* **35**, 21-46.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk. *Environmental Toxicology and Pharmacology* **13**, 57-149.

Vazquez-Duhalt, R., Marquez-Rocha, F., Licea, A.F., Viana, M.T., 2005. Nonyphenol, an integrated vision of a pollutant. *Applied Ecology and Environmental Research* **4**, 1-25.

Venier, P., De Pitta, C., Pallavicini, A., Marsano, F., Varotto, L., Romuali, C., Dondero, F., Viarengo, A., and Lanfranchi, G., 2006. Development of mussel mRNA profiling: can gene expression trends reveal coastal water pollution? *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **602 (1-2)**, 121–134.

Vermeulen, N.P.E., 1996. Role of metabolism in chemical toxicity. *In*: *Ioannides, C. (Ed.), Cytochromes P450: Metabolic and Toxicological Aspects*. CRC Press, Boca Raton, FL, USA, pp. 29-53.

Viarengo, A., 1989. Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. *Critical Reviews in Aquatic Sciences* **1**, 295–317.

Viarengo, A., Nott, J.A., 1993. Mechanism of heavy metal cation homeostasis in marine invertebrates. *Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology and Endocrinology* **104**, 355-372.

Viarengo, A., Burlando, B., Cavaletto, M., Marchi, B., Ponzano, E., Blasco, J., 1999. Role of metallothionein against oxidative stress in the mussel *Mytilus galloprovincialis*. *The American Journal of Physiology* **277**, R1612-1619.

Viarengo, A., Burlando, B., Ceratto, N., Panfoli, I., 2000. Antioxidant role of metallothioneins: a comparative overview. *Cellular and Molecular Biology* **46**, 407-417.

Vioque-Fernández, A., Alves de Almeida, E., López-Barea, J., 2009. Assessment of Doñana National Park contamination in *Procambarus clarkii*: Integration of conventional biomarkers and proteomic approaches. *Science of the Total Environment* **407**, 1784-1797.

Waalkes, M.P. and R.R. Misra, 1996. Cadmium carcinogenicity and genotoxicity. *In Toxicology of Metals* (L. W. Chang, Ed.), pp. 231–244. CRC Press, Boca Raton, FL.

Wang, C.G., Zheng, R.H., Ding, X., Zuo, Z.H., Zhao, Y., Chen, Y.X., 2005. Effect of tributyltin, benzo[a]pyrene, and their mixture on the hepatic monooxygenase system in *Sebastiscus marmoratus*. *Bulletin of Environmental Contamination and Toxicology* **75**, 1214–9.

Wang, H. and Hanash, S., 2003. Multi-dimensional liquid phase based separations in proteomics. *Journal of Chromatography B* **787**, 11-18.

Washburn, M.P., Wolters, D., Yates, J.R. III, 2001. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nature Biotechnology* ***19***, 242-247.

Watanabe, M., Henmi, K., Ogawa, K. and Suzuki, T., 2003. Cadmium-dependent generation of reactive oxygen species and mitochondrial DNA breaks in photosynthetic and non-photosynthetic stains of *Euglena gracilis*. *Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology and Endocrinology* **134**, 227-234.

Weimin, F., Yilin, H., Changzhong, L., Liping, X., Rongqing Z., 2008. Cloning, Characterization, and Expression Analysis of Calreticulin from Pearl Oyster *Pinctada fucata*. Tsinghua Science and Technology **13**, 466-473.

WHO, 1993. Guidelines for Drinking-Water Quality. Vol. 1. Recommendations. 2nd ed. WHO, Geneva, pp. 30–44.

Wilkins, M.R., Sanchez, J.-C., Williams, K.L., Hochstrasser, D.F., 1996. Current challenges and future applications for protein maps and post-translational vector maps in proteome projects. *Electrophoresis* **17**, 830–838.

Williams, T.D., Diab, A.M., George, S.G., Godfrey, R.E., Sabine, V., Conesa, A., Minchin, S.D., Watts, P.C., Chipman, J.K., 2006. Development of the GENIPOL European Flounder (*Platichthys flesus*) microarray and determination of temporal transcriptional responses to cadmium at low dose. *Environmental Science and Technology* **40**, 6479–6488.

Williams, T.D., Diab, A., Ortega, F., Sabine, V.S., Godfrey, R.E., Falciani, F., Chipman, J.K., George, S.G., 2008. Transcriptomic responses of European flounder (*Platichthys flesus*) to model toxicants. *Aquatic Toxicology* **90(2)**, 83-91.

Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., Mann, M., 1996. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379**, 466-469.

Winzer, K., 2001. Oxidative stress in the marine environment prognostic tools for toxic injury in fish liver cells. Academic Thesis, University of Amsterdam.

Wittke, S., Kaiser, T., Mischak, H., 2004. Differential polypeptide display: the search for the elusive target. *Journal of Chromatography B* **803**, 17-26.

Wulfkuhle, J.D., McLean, K.C., Paweletz, C.P., Sgroi, D.C., Trock, B.J., Steeg, P.S., Petricoin, E.F., 2001. New approaches to proteomic analysis of breast cancer. *Proteomics* **1**, 1205–1215.

Xia, K. and Chang, Y.J., 2004. Potodegradation of the Endocrine-Disrupting Chemical 4-Nonylphenol in Biosolids Applied to Soil. *Journal of Environmental Quality* **33,** 1568-1574.

Yamada, H., Miyahara, T. and Sasaki, Y., 1993. Inorganic cadmium increases the frequency of chemically induced chromosome aberrations in cultured mammalian cells. *Mutation Research* **302(3)**, 137-145.

Ye, B., Maret,W., Vallee, B.L., 2001. Zinc metallothionein imported into liver mitochondria modulates respiration. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 2317–2322.

Yergey, A.L., Coorssen, J.R., Backlund, P.S. Jr., Blank, P.S., Humphrey, G.A., Zimmerberg, J., Campbell, J.M., Vestal, M.L., 2002. *De novo* sequencing of peptides using MALDI/TOF-TOF. *Journal of the American Society for Mass Spectrometry* **13**, 784-791.

Zangar, R.C., Varnum, S.M., Covington, C.Y. and Smith, R.D., 2004. *Disease Markers* **20**, 135–148. |

Zangar, R.C., Davydov, D.R., Verma, S., 2004. Mechanisms that regulate production of reactive oxygen species by cytochrome P-450. *Toxicology and Applied Pharmacology* **199**, 316–331.

Zapata, M., Tanguy, A., David, E., Moraga, D., Riquelme, C., 2009. Transcriptomic response of *Argopecten purpuratus* post-larvae to copper exposure under experimental conditions. *Gene* **442**, 37–46.

Zerial, M., and McBride, H., 2001. Rab proteins as membrane organizers. *Nat. Rev. Mol. Cell Biol.* 2, 107–117.

Zhong, Z., Troll, W., Koenig, K.L. and Frenkel, K., 1990. Carcinogenic sulfide salts of nickel and cadmium induce H_2O_2 formation by human polymorphonuclear leukocytes. *Cancer Research* **50**, 7570.

Zorita I, Apraiz I, Ortiz-Zarragoitia M, Orbea A, Cancio I, Soto M, Marigomez, I., Cajaraville, M.P., 2007 Assessment of biological effects of environmental pollution along the NW Mediterranean Sea using mussels as sentinel organisms. *Environmental Pollution* **148**, 236–50.

ERRATA

Page 144 the correct Figure 2 is presented below:

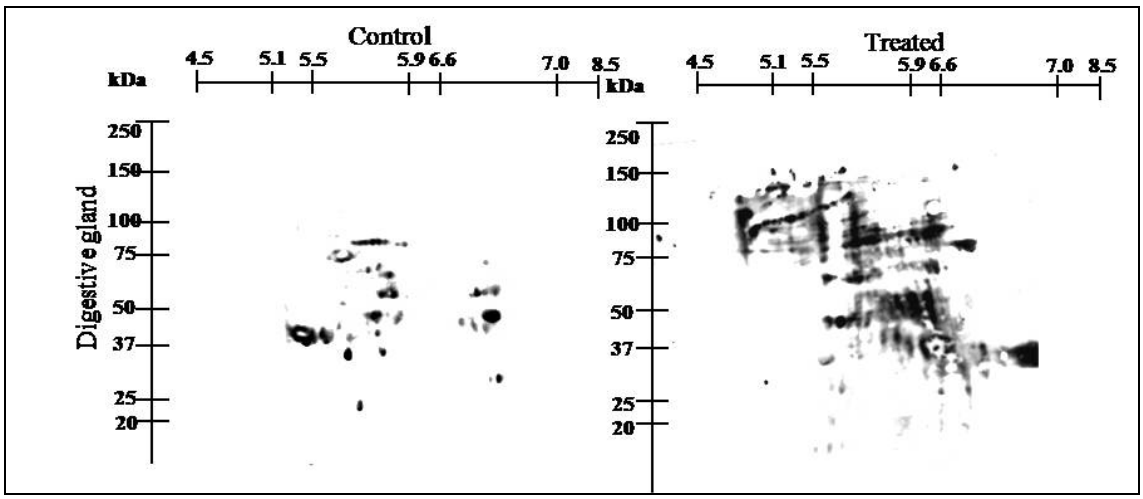

Figure 2 - Immunoblots of carbonylated proteins in 2D separations for digestive gland proteins from control and cadmium exposed clams.