UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

RESPOSTAS BIOQUÍMICAS E MOLECULARES EM OSTRAS DO MANGUE, *Crassostrea brasiliana*, EXPOSTAS A DIFERENTES CONTAMINANTES AMBIENTAIS

Tese submetida ao Programa de Pós-Graduação em Bioquímica da Universidade Federal de Santa Catarina para a obtenção do Título de Doutora em Bioquímica.

Orientador: Dr. Afonso Celso Dias Bainy

Karim Hahn Lüchmann

Florianópolis
2012

Catalogação na fonte pela Biblioteca Universitária
da
Universidade Federal de Santa Catarina

L936r Lüchmann, Karim Hahn
Respostas bioquímicas e moleculares em ostras do mangue, Crassostrea brasiliana, expostas a diferentes contaminantes ambientais [tese] / Karim Hahn Lüchmann ; orientador, Afonso Celso Dias Bainy. - Florianópolis, SC, 2012.
178 p.: il., grafs., tabs.

Tese (doutorado) - Universidade Federal de Santa Catarina, Centro de Ciências Biológicas. Programa de Pós-Graduação em Bioquímica.

Inclui referências

1. Bioquímica. 2. Ostra. 3. Marcadores biológicos. 4. Biotransformação (Metabolismo). 5. Antioxidantes. 6. Combustíveis diesel. 7. Esgotos. I. Bainy, Afonso Celso Dias. II. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Bioquímica. III. Título.

CDU 577

"Respostas bioquímicas e moleculares em ostras do mangue, *Crassostrea brasiliana*, expostas a diferentes contaminantes ambientais"

por

Karim Hahn Lüchmann

Tese julgada e aprovada em sua forma final pelos membros titulares da Banca Examinadora (Port. 10/PPGBQA/2012) do Programa de Pós-Graduação em Bioquímica - UFSC, composta pelos Professores Doutores:

Banca Examinadora:

Prof(a) Dr(a) Afonso Celso Dias Bainy (BQA/CCB/UFSC)

Prof(a) Dr(a) Eleine Francioni de Abreu Lima (CENPES/PETROBRAS)

Prof(a) Dr(a) Eduardo Alves de Almeida (UNESP/SJRP)

Prof(a) Dr(a) Edmundo C. Grisard (MIP/CCP/UFSC)

Prof(a) Dr(a) Alcir Luiz Dafre (BQA/CCB/UFSC)

Prof(a) Dr(a) Hernán Francisco Terenzi (BQA/CCB/UFSC)

Prof. Dr. Marcelo Farina
Coordenador do Programa de Pós-Graduação em Bioquímica

Florianópolis, 13 de março de 2012.

Dedico esta tese
Ao Fabão, minha outra metade...
Aos meus pais, Lígia e José Paulo, simplesmente por tudo!

AGRADECIMENTOS

Embora uma tese de doutorado seja, pela sua finalidade acadêmica, um trabalho individual, termino esta convicta de que sozinha não teria dado conta do recado. De fato, ao longo dos últimos quatro anos, muitas pessoas passaram pelo meu caminho e contribuíram, cada uma do seu jeito, para que este trabalho se tornasse realidade. Por essa razão, expresso meus sinceros agradecimentos:

Aos meus pais, José Paulo e Lígia, por todo o incentivo, carinho, paciência, apoio e amor incondicional. Tenho certeza que se não fosse por eles eu não teria chegado aqui, nem em lugar algum...

Ao meu marido e parceiro de vida e sonhos, Fabão. Agradeço por todo o amor, carinho, inspiração, ajuda, paciência, e inúmeras e lindas gargalhadas. I ♥ u

Ao meu orientador e amigo, Prof. Dr. Afonso Bainy. Agradeço pela confiança, liberdade, apoio e por toda a ajuda oferecida nesses últimos seis anos sob sua orientação. Sou muito grata por todo o aprendizado e pelas inúmeras oportunidades profissionais que me foram oferecidas. Muito obrigada!

Aos pesquisadores do exterior que contribuíram para o desenvolvimento de parte desta tese: Dr. Declan Schroeder, da *Marine Biological Association of the UK - MBA*; Dr. John Craft, da *Glasgow Caledonian University - GCU*; Dr. Jack Gilbert, do *Argonne National Laboratory*; e Dr. Kevin Chipman, da *University of Birmingham.* Em especial, agradeço ao Declan, pela ajuda, ensinamentos e por ter me acolhido em seu incrível grupo de pesquisa. Agradeço pela confiança depositada em mim, pelo tempo dedicado e pelo exemplo. Também sou grata ao John, que carinhosamente me recebeu em seu laboratório e muito me ensinou e apoiou; e ao Jack, que mesmo sem nunca ter me encontrado pessoalmente, acreditou no trabalho e financiou uma importante parte desta tese.

À Profa. Dra. Maria Risoleta F. Marques, que tem me acompanhado desde o meu início no LABCAI. Agradeço por todo o carinho, apoio, ensinamentos e confiança.

Aos queridos amigos e parceiros na tríade 'exposição-bancada-análise' Jacó e Lila que, sempre prontos para me ajudar no que fosse preciso, se transformaram no meu verdadeiro braço direito. Muito obrigada.

Ao Tarquin, que além de parceiro no laboratório, se tornou um grande amigo. Agradeço por toda a ajuda e pelo "empurrão" inicial para minha ida ao Reino Unido.

Aos demais amigos do LABCAI, fundamentais no desenvolvimento deste trabalho e nos diversos momentos de descontração: Ju, Fabrício, Pedro, Ana, Flávia, Maya, Naissa, Rafa, Mari, Clei, Elisa, Tomás, Gabriel, Cláudia, Laura, Rômi, Roberta, Tati, Shayla, Talita, Isis, Shantala, Arnaldo e Marcos. Agradeço ainda a Bel, Dani, Talita, Chris, Juliano e Eduardo, que embora não estejam mais no grupo, acompanharam e participaram de várias etapas desta tese. Ainda, sou especialmente grata ao Gui, pela constante ajuda com minhas infindáveis questões de bioinformática; e à Ninna, pelo apoio no desenvolvimento da primeira etapa da tese. Espero que o cansaço não tenha pregado uma peça à minha restrita e duvidosa memória...

À equipe LABCAI do projeto *Avaliação de biomarcadores bioquímicos, moleculares, histológicos e citogenéticos de contaminação aquática para a indústria do petróleo* (PETROBRAS), que pacientemente entendeu e respeitou minha ausência nos últimos meses para que eu terminasse a tese. Em especial, agradeço ao Prof. Afonso, à Lila e à Maya.

Aos pesquisadores/alunos do grupo de Biologia Molecular da *MBA*: Cecilia, Andrea, Luke, Kim, Matt, Rowena, Ian, Fred, Andrew, Grazyna, Manal, Yanan, Chloe, Colin, Michael e Glen,

que me acolheram com muito carinho e transformaram o doutorado sanduíche em uma experiência única.

Aos alunos do grupo do Prof. John Craft da *GCU*: Sandhya e Joey, pela ajuda e ótimos momentos de descontração dentro e fora do lab.

Ao Prof. Dr. Alcir L. Dafre e sua equipe do Laboratório de Defesas Celulares, em especial ao Trevisan, que sempre estiveram dispostos a contribuir para a realização deste trabalho.

À Dra. Márcia C. Bícego, Dra. Satie Taniguchi e Silvio T. Sasaki, do Laboratório de Química Orgânica Marinha do IO-USP, pelas análises de hidrocarbonetos nos tecidos das ostras.

Ao Prof. Dr. Edmundo Grisard e à Dra. Patrícia Stoco pelo sequenciamento de amostras de ostras.

Ao Prof. Cláudio Melo e equipe do Laboratório de Moluscos Marinhos (LMM - UFSC), que identificaram e forneceram as ostras e microalgas utilizadas nesta tese. Agradeço ao Carlos Henrique pelo auxílio na coleta e manutenção dos animais.

Aos pesquisadores Dr. Alistair Darby, Dra. Christiane Hertz-Fowler, Dr. Xuan Liu e Dra. Margaret Hughes, do *Centre for Genomic Research*, da *University of Liverpool*, pelo pirosequenciamento das amostras de ostras.

À Bela Tiwari, da *NERC Environmental Bioinformatics Center*, pelo auxílio na bioinformática.

Aos professores do Programa de Pós-Graduação em Bioquímica (PPGBQA) que colaboraram enormemente para minha formação. Em especial ao Prof. Dr. Marcelo Farina por todo o apoio.

Ao CNPq pelo financiamento através dos editais CT-Petro (Processo 550706/2005-4) e INCT-TA, e pela bolsa de Doutorado Sanduíche no Exterior.

À CAPES pela bolsa de doutorado.

Ao *Natural Environment Research Council* (*NERC*) pelo financiamento do transcriptoma da ostra.

À PETROBRAS e ao FINEP pelo financiamento indireto por meio do fornecimento de infra-estrutura ao LABCAI.

À Pró-Reitoria de Pós-Graduação (PRPG – UFSC) e ao PPGBQA que viabilizaram a continuidade de minha pesquisa, financiando meu retorno ao Reino Unido.

Aos secretários do PPGBQA Ronaldo e Roberto pela atenção e ajuda nas questões burocráticas.

Aos membros da banca examinadora pela disponibilidade em ceder tempo e esforço para contribuir com esta tese e com a minha formação.

Às minhas amigas-irmãs: Ju, Zah, Rê, Fefa, Dani, Cacá, Sandra, Nai e Tim (e seus respectivos), que pacientemente me aguentaram, me inspiraram, me alegraram, e me ajudaram, mesmo sem ter a menor ideia do que eu faço! Sei que até hoje minha profissão é um verdadeiro mistério para todas!

E finalmente, mas não menos importante, agradeço à minha família e à família Daura Jorge, que igualmente sem entender o que eu tanto faço no laboratório e no computador, respeitaram minhas ausências, e me deram todo o suporte e carinho. Um agradecimento especial para minha sogra, Teresa, e para minha querida e amada vó Mônica.

Muito obrigada!!

“All things are connected. Whatever befalls the earth befalls the sons of the earth. Man did not weave the web of life, he is merely a strand in it. Whatever he does to the web, he does to himself.”
Cacique Seattle

RESUMO

A contaminação dos ambientes costeiros apresenta-se como uma realidade na extensa costa brasileira. Dejetos e subprodutos das mais variadas atividades antrópicas, como os provenientes da atuação da indústria do petróleo e da ineficiência na coleta e tratamento do esgoto doméstico, provocam danos variados aos organismos expostos. Esses compostos químicos podem causar alterações biológicas de caráter molecular, celular, fisiológico ou ecológico, resultando em efeitos negativos não apenas para as comunidades naturais, como para os organismos destinados ao consumo humano. Desta forma, a identificação dessas alterações pode ser usada como biomarcador de contaminação aquática. O presente estudo contribui fornecendo informações para o estabelecimento de ferramentas sensíveis e viáveis para estudos ecotoxicológicos, através da avaliação de repostas de biomarcadores bioquímicos clássicos e da identificação de novos genes potenciais candidatos a biomarcadores. Para tal, ostras do mangue, *Crassostrea brasiliana*, foram expostas a diferentes contaminantes ambientais. São apresentados os resultados de biomarcadores bioquímicos em brânquia e glândula digestiva de ostras expostas por 96 horas a quatro concentrações da fração de óleo diesel acomodada em água (FAA). As respostas das enzimas antioxidantes e de fase II do sistema de biotransformação de xenobióticos, em conjunto com aspectos químicos, demonstraram que a ostra é capaz de bioacumular hidrocarbonetos alifáticos e aromáticos, além de responder a essa bioacumulação de forma concentração-dependente. Esses dados indicam um papel promissor dessa espécie como bioindicadora em programas de biomonitoramento ambiental. Entretanto, como muito pouco é conhecido sobre a biologia molecular de *C. brasiliana*, bibliotecas subtrativas de cDNA foram construídas. Esta metodologia possibilitou a identificação de 23 novos genes nessa espécie, comparando ostras expostas à FAA de óleo diesel por 24 horas e o grupo controle. Destes genes, três (*protease específica de ubiquitina 25 – USP25*, *precursor de dominina*, *nucleosídeo difosfato quinase B – NDPK-B*) foram validados por PCR

quantitativo em tempo real (qPCR), representando genes de interesse para estudos ecotoxicológicos em *C. brasiliana*. Ainda, com o intuito de aumentar as informações gênicas de *C. brasiliana*, a técnica de pirosequenciamento utilizando a plataforma 454 foi aplicada em ostras expostas à FAA de óleo diesel, ao fenantreno e ao esgoto doméstico. A partir desta abordagem metodológica de sequenciamento em grande escala, a montagem *de novo* das leituras geradas produziu o primeiro transcriptoma referência de *C. brasiliana*. Como resultado, 7.401 novos genes foram identificados, destacando-se os genes codificadores de proteínas possivelmente envolvidas no sistema de biotransformação de xenobióticos e no sistema de defesa antioxidante. Por fim, com o intuito de avaliar a utilidade dos dados gerados pelo banco de ESTs, o nível de transcrição de seis genes *CYP-like* e quatro *GST-like*, previamente identificados no transcriptoma referência, foram testados por qPCR, fornecendo subsídios para o entendimento dos mecanismos moleculares de toxicidade exercidos pelo fenantreno, um hidrocarboneto policíclico aromático (HPA) prioritário. Para tal, ostras foram expostas a duas concentrações de fenantreno (100 $\mu g.L^{-1}$ e 1000 $\mu g.L^{-1}$) por 24 horas. Os resultados obtidos indicam o papel de CYPs e GSTs no metabolismo de HPAs, uma vez que o tratamento induziu um aumento no nível dos transcritos. A maior resposta da brânquia, se comparada à glândula digestiva, sugere papéis diferenciados no metabolismo de xenobióticos destes tecidos, no qual o papel da brânquia é destacado. Os dados apresentados nessa tese demonstram a aplicabilidade do uso de biomarcadores bioquímicos e moleculares em estudos ecotoxicológicos utilizando a ostra *C. brasiliana* como bioindicador de contaminação aquática. Em última instância, os resultados constituem importantes fontes de informação para o biomonitoramento de águas contaminadas por derivados de petróleo e esgoto doméstico ao longo da costa brasileira.

Palavras-chave: *Crassostrea brasiliana*, biomarcadores, biotransformação de xenobióticos, defesa antioxidante, transcriptoma, óleo diesel, fenantreno, esgoto doméstico.

ABSTRACT

Contamination of the coastal marine environments has increased significantly along the Brazilian coast. Aquatic organisms are therefore continually exposed to by-products derived from a variety of human activities, such as petroleum industry and urban waste. Chemical contamination is considered to be one of the main causes of aquatic degradation, affecting different levels of biological organization including the subcellular. Thus, the biological responses, termed biomarkers, are used to assess the effects of chemical challenge on exposed organisms. The present study sheds light on biological information in order to improve the scientific basis for ecotoxicological studies. For that purpose, we measured the responses of 'classic' biochemical biomarkers and identified candidate genes for novel biomarker discovery in the mangrove oyster, *Crassostrea brasiliana*, exposed to environmental contaminants. The results are presented in four separate findings chapters. In Chapter 2, we show the biochemical responses and hydrocarbons bioaccumulation of the oyster exposed for 96 h to four sublethal concentrations of diesel fuel water accommodated fraction (WAF). The results of the antioxidant defenses, phase II biotransformation and chaperones parameters revealed clear biochemical responses to WAF challenge in the gill and digestive gland of oysters. Furthermore, the capacity of *C. brasiliana* to bioaccumulate aliphatic and aromatic hydrocarbons in a concentration-dependent manner is a strong indication of its suitability as a model in biomonitoring programs along the Brazilian coast. However, the genome resources for this species are limited. Thus, Chapter 3 shows the results and discussion on up- and down-regulation of identified genes in cDNA subtractive suppressive hybridization libraries from oysters exposed for 24 h to WAF. 23 novel genes were identified and three were validated by quantitative real-time PCR (qPCR), representing potential new biomarkers for ecotoxicological studies using *C. brasiliana* as bioindicator. Aiming to increase the genomic resources for *C. brasiliana* we pyrosequenced expressed genetic transcripts from the gill and

digestive gland of oysters exposed to 10% WAF, phenanthrene and domestic sewage, which are reported in Chapter 4. This methodological approach followed by *de novo* assembly has provided extensive genomic information to generate the first reference transcriptome for *C. brasiliana*. We identified 7,401 new genes, many of which related to the xenobiotics biotransformation system and the major antioxidant defence proteins. Finally, in Chapter 5 we evaluated the usefulness of the output derived from Chapter 4. We selected some putative *CYP*- and *GST*-related genes to evaluate the molecular mechanisms of phenanthrene toxicity in *C. brasiliana* by means of qPCR. We report, for the first time, a tissue-specific response of *CYP-like* and *GST-like* genes, and enzymatic and non-enzymatic antioxidant parameters in *C. brasiliana* exposed for 24 h to 100 $\mu g.L^{-1}$ and 1000 $\mu g.L^{-1}$ phenanthrene, a model PAH. The results indicated that 1000 $\mu g.L^{-1}$ phenanthrene can lead to the upregulation of both phase I and II genes and depletion of non-antioxidant parameters, with more pronounced effects in the gill. The biological responses in *C. brasiliana* support the use of this organism to investigate the molecular mechanism underlying PAH toxicity in bivalves. Altogether the results presented in this thesis provides insight into the applicability of different biochemical and molecular endpoints in oyster for ecotoxicological studies, thereby contributing to the definition of a better methodology for using the mangrove oyster *C. brasiliana* as a bioindicator organism to assess the potential toxic effects of petroleum by-products and domestic sewage along the Brazilian coast.

Keywords: *Crassostrea brasiliana*, biomarkers, xenobiotics biotransformation, antioxidant defence, transcriptome, pyrosequencing, diesel fuel, phenanthrene, domestic sewage.

LISTA DE FIGURAS

Capítulo 1

Figura 1. Mapa global do impacto antrópico em 20 tipos de ecossistemas costeiros e oceânicos. Em destaque as regiões fortemente impactadas no Caribe, no Mar do Norte, e em águas japonesas, e uma das menos impactadas (no quadrado à direita) na Austrália. Os números referem-se à escala de impacto acumulado definida pelos autores, variando do menos impactado (azul) ao mais impactado (vermelho). (Fonte: HALPERN et al., 2008; tradução nossa)..... 31

Capítulo 2

Figure 1. Superoxide dismutase (SOD) (A), catalase (CAT) (B) and glutathione peroxidase (GPx) (C) enzymes activity in the gill and digestive gland (dig gd) of the mangrove oyster *Crassostrea brasiliana* exposed for 96 h to four concentrations (2.5%, 5%, 10% and 20%) of diesel fuel water-accommodated fraction (diesel WAF), or seawater as the control group. Data are presented as mean + S.D. (n = 9 – 12 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc analysis. *p<0.05 when compared the diesel WAF exposed groups to the control for a given tissue. 61

Figure 2. Glutathione reductase (GR) (A), glucose-6-phosphate dehydrogenase (G6PDH) (B), γ-glutamyl transpeptidase (GGT) (C) and glutathione *S*-transferase (GST) (D) enzymes activity in the gill and digestive gland (dig gd) of the mangrove oyster *Crassostrea brasiliana* exposed for 96 h to four concentrations (2.5%, 5%, 10% and 20%) of diesel fuel water-accommodated fraction (diesel WAF), or seawater as the control group. Data are presented as mean + S.D. (n = 9 – 12 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc analysis. *p<0.05; **p<0.01; ***p<0.001 when compared the diesel WAF exposed groups to the control for a given tissue. 62

Figure 3. Immunocontent of heat-shock proteins Hsp60 (A) and Hsp90 (B) and lipid peroxidation levels (C) in the gill and digestive gland (dig gd) of the mangrove oyster *Crassostrea brasiliana* exposed for 96 h to four concentrations (2.5%, 5%, 10% and 20%)

of diesel fuel water-accommodated fraction (diesel WAF), or seawater as the control group. Data are presented as mean + S.D. (n = 9 – 12 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc analysis. *p<0.05; **p<0.01; ***p<0.001 when compared the diesel WAF exposed groups to the control for a given tissue. 63

Figure 4. Biplot from principal components analysis (PCA) of all biochemical parameters related to the diesel WAF exposure in both gill (A) and digestive gland (B). In gill, the first axis explained 66.7% of overall variation and the second 25.6%. In digestive gland, the first axis explained 78.2% of overall variation and the second 12.4%. 65

Capítulo 3

Figure 1. Relative gene transcription of putative up- and down-regulated genes identified in suppressive subtractive hybridization (SSH) libraries from the digestive gland of oysters *Crassostrea brasiliana* exposed for 24 h to 10% diesel fuel water-accommodated fraction (WAF). The gene transcript levels were assessed by quantitative real-time polymerase chain reaction (qPCR) and transcription of tester genes are relative to driver group in both SSH directions as follows: the forward library refers to expected upregulation genes in treated animals, where the diesel-challenged oysters are referred as "tester" and the control animals are called "driver"; the reverse library represents those genes in which downregulation by diesel WAF is expected, where the tester and driver designations for the groups are reversed. Note that the y axis is logarithmic to base 2. Results are expressed as mean ± standard error of the mean (n = 6 animals per group). Statistical analysis was performed by using a pairwise fixed reallocation randomization test. *Significant (p < 0.01) transcription change (REST 2009 software from Qiagen). USP25 = ubiquitin-specific peptidase 25; CFSP2 = Serine protease; USP = universal stress protein; NDPK-B = nucleoside diphosphate kinase B. 83

Capítulo 4

Figure 1. Overview of the *Crassostrea brasiliana* transcriptome assembly. (A) Frequency distribution of contig length. (B) Frequency distribution of the number of reads assembled into contigs. 100

Figure 2. Species distribution of the top blastx hit in NCBI nr protein database for each contig of the *Crassostrea brasiliana* transcriptome. Asterisks denote bivalve mollusc species..... 102

Figure 3. Gene Ontology GO terms for the transcriptome sequences of *Crassostrea brasiliana*. (A) GO Biological Process, (B) GO Molecular Function and (C) GO Cellular Component. 104

Figure 4. General enzyme classification based on enzyme commission (EC) numbers for the contigs of *Crassostrea brasiliana*. 106

Capítulo 5

Figure 1. Disposal of phenanthrene by mangrove oysters *Crassostrea brasiliana*. Phenanthrene at 100 (A) or 1000 (B) $\mu g.L^{-1}$ nominal concentration was followed in the seawater (salinity 25 ppt) over time in the absence (squares) or in the presence (circles) of oysters. 135

Figure 2. Relative gene transcription of putative phase I biotransformation of xenobiotics genes in the gill (A) and digestive gland (B) of the mangrove oyster *Crassostrea brasiliana* exposed to phenanthrene at 100 $\mu g.L^{-1}$ and 1000 $\mu g.L^{-1}$ for 24 h. The gene transcript levels were assessed by qPCR and transcription is relative to control group. Note the y axis is logarithmic to base 2. Statistical analysis was performed by using a pairwise fixed reallocation randomization test. *Represents significant transcription change (REST 2009 software Qiagen®, $p < 0.01$). 136

Figure 3. Relative gene transcription of putative phase II biotransformation of xenobiotics genes in the gill (A) and digestive gland (B) of the mangrove oyster *Crassostrea brasiliana* exposed to phenanthrene at 100 $\mu g.L^{-1}$ and 1000 $\mu g.L^{-1}$ for 24 h. The gene transcript levels were assessed by qPCR and transcription is relative to control group. Note the y axis is logarithmic to base 2. Statistical analysis was performed by using a pairwise fixed reallocation randomization test. *Represents significant transcription change (REST 2009 software Qiagen®, $p < 0.01$). GST classes/isoforms: *ΩGST* (omega), *MGST3* (microsomal GST isoform 3), *πGST* (pi). *ΩGST-like* could not be amplified in the digestive gland. 137

Figure 4. Thiol status measured as levels of (A) non-proteic thiols (NPSH), (B) total glutathione (GSH-t) and (C) proteic thiols (PSH)

in the gill and digestive gland (dig gd) of the mangrove oyster *Crassostrea brasiliana* exposed to phenanthrene at 100 μg.L^{-1} and 1000 μg.L^{-1} for 24 h, plus the seawater as the control group. Results are expressed as mean ± standard deviation (S.D.) (n = 7 - 8 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc analysis.*Represents significant differences, $p < 0.05$.....138

Figure 5. Graphs depict activity of the phase II biotransformation of xenobiotics, antioxidant and related enzymes in the gill and digestive gland (dig gd) of the mangrove oyster *Crassostrea brasiliana* exposed to phenanthrene at 100 μg.L^{-1} and 1000 μg.L^{-1} for 24 h: (A) glutathione *S*-transferase (GST), (B) catalase (CAT), (C) glutathione peroxidase (GPx), (D) glutathione reductase (GR) and (E) glucose-6-phosphate dehydrogenase (G6PDH). Results are expressed as mean ± standard deviation (S.D.) (n = 7 - 8 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc analysis.*Represents significant differences, $p < 0.05$.....139

LISTA DE TABELAS

Capítulo 1

Tabela 1: Plataformas de sequenciamento de próxima geração (NGS) atualmente disponíveis e suas principais características. 46

Tabela 2. Resumos dos principais biomarcadores e/ou técnicas utilizadas em cada capítulo desta tese. 48

Capítulo 2

Table 1. Aliphatic hydrocarbons levels ($ug.g^{-1}$) on a wet weight basis detected in whole soft tissues of oysters from both control and diesel WAF exposed groups. 59

Table 2. Aromatic hydrocarbons levels ($ng.g^{-1}$) on a wet weight basis detected in whole soft tissues of oysters from both control and diesel WAF exposed groups. 60

Capítulo 3

Table 1. Primer sequences used for qPCR for the amplification of genes of interest from both forward and reverse libraries with putative gene name and amplicon size (bp). 81

Table 2. General characteristics of suppressive subtractive libraries and expressed sequence tags obtained from digestive gland of *Crassostrea brasiliana*[a]. 82

Table 3. List of identified suppressive subtractive hybridization up-regulated genes in the digestive gland of *Crassostrea brasiliana* exposed to diesel fuel water-accommodated fraction. 86

Table 4. List of identified suppressive subtractive hybridization down-regulated genes in the digestive gland of *Crassostrea brasiliana* exposed to diesel fuel water-accommodated fraction. 87

Capítulo 4

Table 1. Summary statistics for 454 sequencing and assembly for *Crassostrea brasiliana*. 99

Table 2. Summary information for the identified *Crassostrea brasiliana* genes putatively involved in phase I of the metabolism of biotransformation of xenobiotics. 108

Table 3. Summary information for the identified *Crassostrea brasiliana* genes putatively involved in phases II and III of the metabolism of biotransformation of xenobiotics. 110

Table 4. Summary information for the identified *Crassostrea brasiliana* genes putatively involved in the antioxidant defence system. 113

Table 5. Contribution of each environmental contaminant to generate the contigs under the functional gene groups of interest for ecotoxicology. 116

Capítulo 5

Table 1. Primer sequences used for the qPCR for the amplification of each target and endogenous reference gene (ERG) with putative gene name and amplicon size (bp). 132

Table 2. Tissue levels of phase II biotransformation of xenobiotics, antioxidant and related parameters in the gill and digestive gland of the untreated (control group) mangrove oyster *Crassostrea brasiliana*. Results are expressed as mean ± standard error of the mean (S.E.M.). 140

LISTA DE ABREVIATURAS E SÍMBOLOS

Σ: total, soma
~: aproximadamente
µg: micrograma
$\mu g.g^{-1}$: micrograma por grama
$\mu g.L^{-1}$: micrograma por litro
µL: microlitro
$^1O_2$: oxigênio singlete
454 GS-FLX: plataforma 454 de pirosequenciamento, Roche
ABC: família de glicoproteínas transmembranas; do inglês *ATP binding cassete*
AH: hidrocarboneto alifático; do inglês *aliphatic hydrocarbon*
ATP: adenosina trifosfato
BEN: Balanço Energético Nacional
BLAST: algoritmo para comparação de sequências biológicas utilizando alinhamento local; do inglês *Basic Local Alignment Search Tool*
BLASTn: busca em um banco de sequências nucleotídicas a partir de uma sequência nucleotídica utilizando o algoritmo BLAST
BLASTx: busca em um banco de sequências de proteínas a partir de uma sequência de nucleotídeos traduzida, utilizando o algoritmo BLAST
BTEX: benzeno, tolueno, etilbenzeno e xilenos
CAT: catalase
cDNA: sequência nucleotídica complementar de DNA
CDNB: 2,4-dicloronitrobenzeno
$cels.mL^{-1}$: número de células por mililitro
CFSP2: serina protease; do inglês *Chlamys farreri serine protease*
Ct: limiar de detecção em uma reação de qPCR; do inglês *cycle threshold*
CuOOH: hidroperóxido de cumeno
CYP450 (ou CYP): citocromo P450 – número após a sigla representa família
ddNTP: dideoxinucleotídeo trifosfato
DMSO: dimetilsulfóxido
DNA: ácido desoxirribonucléico
DNase: desoxiribonuclease
DTNB: 5,5'-ditio-bis-2-nitrobenzoato
DTT: ditiotreitol
E: eficiência da reação de qPCR
EBI: Instituto Europeu de Bioinformática; do inglês *European Bioinformatics Institute*
EC: número de classificação de enzimas; do inglês *Enzyme Commission*

EDTA: ácido etilenodiamino tetra-acético
ERG: gene constitutivo; do inglês *endogenous reference gene*
EROs (*ROS*): espécies reativas de oxigênio; do inglês *reactive oxygen species*
EST: etiqueta de sequência expressa; do inglês *expressed sequence tags*
E-value (ou *e*): probabilidade de duas sequências alinharem ao acaso
F: valor da distribuição F de Fisher
FAA (*WAF*): fração acomodada em água; do inglês *water-accommodated fraction*
g: força da gravidade
g: grama
G6PDH: glicose-6-fosfato desidrogenase
GC/MS: técnica acoplada de cromatografia gasosa e espectrometria de massa; do inglês *gas chromatography-mass spectrometry*
GenBank: banco de dados do NCBI
GGT: γ-glutamil transpeptidase
GO: ontologia de genes; do inglês *Gene Ontology*
GPx: glutationa peroxidase
GR: glutationa redutase
Grx: glutaredoxina
GSH: glutationa reduzida
GSH-t: glutationa total
GSSG: glutationa oxidada
GST: glutationa *S*-transferase – letra após a sigla representa isoforma
h: horas
H_2O_2: peróxido de hidrogênio
HPA (*PAH*): hidrocarboneto policíclico aromático; do inglês *polycyclic aromatic hydrocarbon*
HPLC: cromatografia líquida de alta eficiência; do inglês *high-performance liquid chromatography*
Hsp: proteína de choque térmico; do inglês *heat shock protein* – número após a sigla representa família
IgG: imunoglobulina G ou anticorpo G
KAAS: servidor de anotação automática; do inglês *KEGG Automatic Annotation Server*
KCl: cloreto de potássio
kDa: quilodalton
KEGG: Enciclopédia de Genes e Genoma de Kioto; do inglês *Kyoto Encyclopedia of Genes and Genomes*
KO: números de ontologia KEGG; do inglês *KEGG ontology*
L: litro

LB: meio Luria
LPO: peroxidação lipídica ou lipoperoxidação; do inglês *lipid peroxidation*
M: molar
mA: milliampere
m^3: metro cúbico
Mb: mega pares de bases = 1.000.000 bp
mg: miligrama
min: minutos
mL: mililitro
mM: milimolar
mRNA: RNA mensageiro
MRP: proteína de resistência a múltiplas drogas *multidrug resistance proteins* – número após a sigla representa família
mtDNA: DNA mitocondrial
MXR: proteína de resistência a múltiplos xenobióticos; do inglês *multixenobiotic resistance*
n: número amostral
$NADP^+$: nicotinamida adenina dinucleotídeo fosfato oxidada
NADPH: nicotinamida adenina dinucleotídeo fosfato reduzida
NCBI: Centro Nacional de Informação Biotecnológica dos EUA; do inglês *National Center for Biotechnology Information*
ND: não detectado
NDPK-B: nucleosídeo-difosfato quinase B; do inglês *nucleoside diphosphate kinase B*
ng: nanograma
$ng.g^{-1}$: nanograma por grama
NGS: sequenciamento de próxima geração; do inglês *next-generation sequencing*
nm: nanômetro
NPSH: tióis não protéicos; do inglês *non-protein thiols*
nr: não redundante
NRTC: controle com amostra tratada com DNase mas não transcrita reversamente para reação de qPCR; do inglês *no reverse transcriptase control*
NS: não significativo
NTC: controle sem amostra para reação de qPCR; do inglês *no template control*
$O_2^{\cdot-}$: ânion superóxido
^oC: graus Celsius
OD: densidade ótica; do inglês *optical density*
p: significância estatística

pb: pares de bases
PCA: análise de componentes principais; do inglês *principal component analysis*
PCR: reação em cadeia da polimerase; do inglês *polymerase chain reaction*
Pfam: banco de dados para classificação de proteínas
pH: pontencial hidrogeniônico
PMSF: fenil-metil-sulfunil-fluoreto
PNSB: Pesquisa Nacional de Saneamento Básico
Prx: peroxiredoxina
PSH: tióis protéicos; do inglês *protein thiols*
PTP: placa para pirosequenciamento; do inglês *picotiter plate*
qPCR: PCR quantitativo em tempo real
r: coeficiente de correlação de Pearson
RNA: ácido ribonucléico
RNase: ribonuclease
RPM: rotações por minuto
rRNA: RNA ribossomal
RT-PCR: PCR via transcrição reversa; do inglês *reverse transcription PCR*
S.D.: desvio padrão; do inglês *standard deviation*
S.E.M.: erro padrão da média; do inglês *standard error of the mean*
s: segundos
SNPs: variantes em uma única posição nucleotídica; do inglês *single nucleotide polymorphisms*
SOD: superóxido dismutase
SSH: hibridação subtrativa supressiva; do inglês *suppressive subtractive hybridization*
SULT: sulfotransferase
Tris-HCl: tris(hidroximetil)aminometano-hidrocloreto
Trx: tioredoxina
UCM: mistura complexa não resolvida; do inglês *unresolved complex mixture*
UniProt: banco de dados de proteínas; do inglês *Universal Protein Resource*
US EPA: Agência de Proteção Ambiental dos Estados Unidos; do inglês *United States Environmental Protection Agency*
USP: proteína universal de stress; do inglês *universal stress protein*
USP25: protease específica para ubiquitina isoforma 25; do inglês *ubiquitin specific peptidase 25*
v/v: volume/volume
w/v: peso/volume; do inglês *weight/volume*
ΔΔCt: método comparativo de Ct para quantificação relativa da transcrição do gene de interesse

SUMÁRIO

CAPÍTULO 1. INTRODUÇÃO GERAL 29
Ecotoxicologia aquática: por que e para quê? 29
Contaminação aquática e monitoramento ambiental 30
Organismos aquáticos como bioindicadores de contaminação ambiental 36
Biomarcadores de contaminação aquática 37
Biomarcadores bioquímicos 38
Biomarcadores moleculares 41
Estrutura e objetivos da tese 47

CAPÍTULO 2. Biochemical biomarkers and hydrocarbons concentrations in the mangrove oyster *Crassostrea brasiliana* following exposure to diesel fuel water-accommodated fraction 51
Abstract 52
Introduction 52
Materials and methods 55
Results 58
Discussion 65
Conclusions 69
Acknowledgments 69
References 70

CAPÍTULO 3. Suppressive subtractive hybridization libraries prepared from the digestive gland of the oyster *Crassostrea brasiliana* exposed to diesel fuel water-accommodated fraction 77
Abstract 78
Introduction 78
Material and methods 79
Results and discussion 81
Acknowledgment 88
References 88

CAPÍTULO 4. Transcriptome pyrosequencing and *de novo* analysis for the mangrove oyster *Crassostrea brasiliana* exposed to environmental contaminants: application for discovering candidate genes for ecotoxicological studies 91
Abstract 92
Background 92
Materials and methods 94

Results and discussion 98
Conclusions 117
Acknowledgements 117
References 117

CAPÍTULO 5. Upregulation of new putative biotransformation genes and antioxidant responses in the mangrove oyster *Crassostrea brasiliana*: insights on molecular mechanisms of phenanthrene toxicity 125
Abstract 126
Introduction 126
Materials and methods 128
Results 134
Discussion 140
Acknowledgments 145
References 145

CAPÍTULO 6. 153
Considerações finais 153
Conclusões 157

REFERÊNCIAS BIBLIOGRÁFICAS CITADAS NO CAPÍTULO 1 .. 161

Capítulo 1
Introdução geral

Ecotoxicologia aquática: por que e para quê?

Desde o advento da Revolução Industrial, as sociedades vivenciam processos de mudanças cujo ritmo não encontra precedentes na história. A infindável gama de possibilidades proporcionada por essa nova realidade traz também efeitos negativos, senão devastadores, aos ambientes naturais. Para alguns, este novo período é conhecido como Antropoceno (ROCKSTRÖM et al., 2009[1]), quando atividades humanas passaram a ter um impacto significativo na Terra e no funcionamento de seus ecossistemas, culminando com o aparecimento de um inédito movimento ambiental global. Um registro importante para o avanço dessa nova consciência foi a publicação do livro "Primavera Silenciosa", de Rachel Carson, em 1962, despertando interesse para os efeitos tóxicos de compostos químicos em populações naturais, que passaram a ser frequentemente identificados em diferentes partes do mundo (COLBORN; DUMANOSKI; MYERS, 1996). Na medida em que as evidências da toxicidade de compostos químicos em ambientes naturais iam se tornando inquestionáveis, o processo de conhecimento foi avançando e se especializando. Assim, ocorreu a separação da toxicologia clássica em um novo ramo, a ecotoxicologia (WALKER et al., 1996). Com termo cunhado por René Truhaut, em 1969, e fazendo uma alusão às palavras "ecologia" e "toxicologia", a ecotoxicologia pode ser definida como o ramo da toxicologia cujo objetivo é entender e prever os efeitos causados por compostos químicos em comunidades naturais sob pressão antrópica (CHAPMAN, 2002).

De acordo com uma estimativa recente sobre o número de substâncias químicas existentes hoje no mundo, Binetti, Costamagna e Marcello (2008) chegaram à alarmante cifra de 60 milhões de químicos diferentes, sendo que 4.000 novos são produzidos todos os dias. Embora nem todas as substâncias tenham como destino final os ambientes naturais, muito pouco é conhecido sobre seus efeitos toxicológicos, sendo este um enorme desafio às agências ambientais em todo o mundo (JUDSON et al., 2009). De fato, Binetti, Costamagna e Marcello (2008) estimaram que apenas 10% dos químicos atualmente conhecidos foram avaliados - quanto a sua toxicidade - por órgãos competentes na Europa e Estados Unidos, indicando uma lacuna de informação que urge por respostas. Nesse contexto, os ecossistemas aquáticos apresentam-se

[1] As referências bibliográficas utilizadas neste capítulo estão listadas no final da tese.

como os mais vulneráveis, pois são considerados os receptores finais de substâncias químicas provenientes das mais variadas fontes (WINSTON; DI GIULIO, 1991).

Considerando essa notória suscetibilidade dos ecossistemas aquáticos, uma ênfase crescente vem sendo dada ao desenvolvimento de métodos para avaliar e compreender os efeitos causados por diferentes tipos de químicos sobre organismos aquáticos. Esse movimento teve início na década de 30, quando alguns pesquisadores realizaram uma série de observações empíricas para avaliar os efeitos tóxicos de substâncias químicas em peixes (MACEK, 1980). Sem regulamentação, as experimentações não apresentavam *status* de ciência e foram, por muitos anos, negligenciadas e até vistas com certa descrença nos corredores acadêmicos da época. Apenas recentemente, na década de 70, uma nova área da emergente ecotoxicologia foi proposta, dando origem à ecotoxicologia aquática (MACEK, 1980). Segundo Zagatto e Bertoletti (2006), este ramo foi criado com o intuito de identificar e compreender os mecanismos de toxicidade dos compostos químicos no meio aquático, bem como diagnosticar a qualidade ambiental, e, ainda, prever impactos nesses ambientes. Neste cenário, que combina alta exposição de ecossistemas vulneráveis a atividades humanas com um acelerado crescimento populacional - evidenciado pela extraordinária marca de sete bilhões de habitantes recentemente atingida (TOLLEFSON, 2011), a ecotoxicologia aquática caracteriza-se como uma área com desafios extremos e de elevada importância para a sociedade moderna.

Contaminação aquática e monitoramento ambiental

É evidente a dependência do homem por bens e serviços ambientais (HALPERN et al., 2008). Estima-se, por exemplo, que o valor monetário global dos serviços ambientais ultrapasse os 33 trilhões de dólares por ano, dos quais 21 relacionam-se a serviços provenientes de ecossistemas marinhos, o que é consideravelmente maior que os 18 trilhões de dólares gerados anualmente na economia mundial (estimativas para o ano de 1997; CONSTANZA et al., 1997). No entanto, ao explorar serviços ambientais, inevitavelmente deixa-se um rastro de interferências antrópicas nos ambientes naturais (DONEY, 2010). De fato, Halpern et al. (2008) mapearam todas as regiões costeiras e oceânicas do globo e, através de técnicas de modelagem utilizando diversos descritores ambientais e indicadores de impacto, identificaram que atualmente não há área livre de influência humana (Figura 1). Segundo os mesmos autores, aproximadamente 41% dos

ecossistemas aquáticos sofrem com impactos de média a alta magnitude, os quais se localizam, principalmente, em regiões densamente ocupadas e industrializadas.

De acordo com a Figura 1 (fonte: HALPERN et al., 2008), atualmente toda a costa brasileira pode ser classificada como média ou altamente impactada, e algumas regiões, como a Sudeste, apresentam níveis alarmantes de influência humana. Por este cenário, a costa brasileira merece especial atenção, tendo em vista que atuais tendências de crescimento econômico associadas à expectativa de intensa exploração de novas áreas petrolíferas expõem o país a uma situação de potencial risco ambiental. Adicionalmente, ainda que o crescimento observado nos últimos anos seja inquestionável, o Brasil continua sendo um país em desenvolvimento, cujas necessidades essenciais, como saneamento básico, por exemplo, estão muito aquém da demanda hoje existente (BAER, 2008). Diante dessa combinação de vetores de impacto, que vão da crescente exploração do petróleo à alarmante carência de infra-estrutura básica, medidas preventivas são capitais para avaliar, monitorar, e em muitos casos evitar atividades impactantes sobre nossos ecossistemas, especialmente os costeiros.

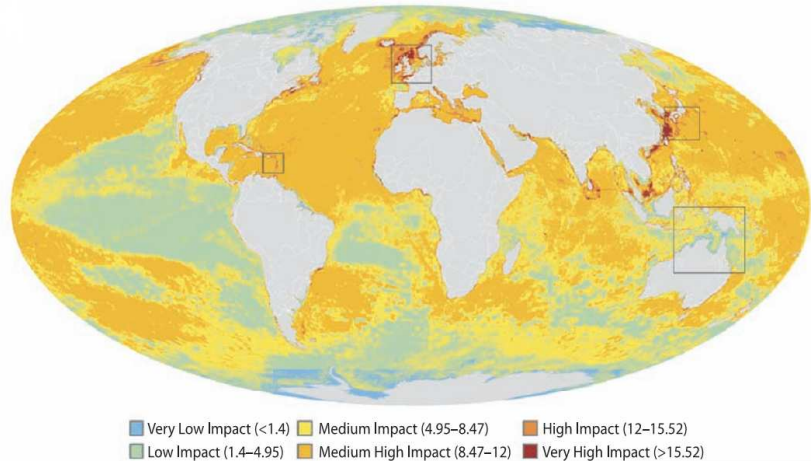


Figura 1. Mapa global do impacto antrópico em 20 tipos de ecossistemas costeiros e oceânicos. Em destaque as regiões fortemente impactadas no Caribe, no Mar do Norte, e em águas japonesas, e uma das menos impactadas (no quadrado à direita) na Austrália. Os números referem-se à escala de impacto acumulado definida pelos autores, variando do menos impactado (azul) ao mais impactado (vermelho). (Fonte: HALPERN et al., 2008; tradução nossa).

Dentre os impactos antropogênicos nas regiões costeiras, a contaminação aquática caracteriza-se como uma das mais complexas e, portanto, mais desafiadoras de se avaliar. Dejetos e subprodutos das mais variadas atividades, como os provenientes da atuação da indústria do petróleo e da ineficiência na coleta e tratamento do esgoto doméstico, compreendem misturas complexas, que quando não tratados e lançados diretamente no ambiente, podem exercer danos variados nos organismos expostos. Esses compostos químicos, tecnicamente chamados de xenobióticos, podem causar alterações biológicas em nível molecular, celular ou fisiológico, resultando em um efeito negativo não apenas nas comunidades animais e vegetais naturais, como nos organismos cultivados e destinados para o consumo humano (MOORE et al., 2004).

Os impactos da indústria petrolífera decorrem tanto de exposição aguda quanto crônica aos seus efluentes. Acidentes ambientais, como derrames de petróleo e seus derivados, constituem a principal fonte de contaminação aguda e estão relacionados a impactos na biota aquática e nos ecossistemas como um todo (KENNISH, 1992). Em escala mundial, destacam-se alguns acidentes: a colisão entre os petroleiros *Atlantic Empress* e *Aegean Captain* em julho de 1979, derramando 287.000 m^3 de óleo na costa de Trinidad e Tobago; o derramamento de cerca de 260.000 m^3 de petróleo na costa da Angola em maio de 1991; o rompimento do tanque *Prestige* na costa da Espanha em 2002 derramando mais de 60.000 m^3 de óleo combustível; e o acidente envolvendo o petroleiro *Exxon Valdez*, quando em 1989 derramou sua carga de aproximadamente 37.000 m^3 de petróleo na costa do Alasca (ITOPF, 2010). Entretanto, o caso mais dramático é o recente acidente no Golfo do México, Estados Unidos. Neste evento, uma plataforma da *British Petroleum* localizada na costa americana explodiu, resultando no derrame de cerca de 780.000 m^3 de petróleo entre os meses de abril e julho de 2010. Para alguns, este é considerado o maior desastre ambiental envolvendo a atuação da indústria do petróleo, tendo em vista a localização do acidente e a ampla dispersão do produto (ANTONIO; MENDES; THOMAZ, 2011; BARRON, 2011). No Brasil, destacam-se: o derrame de aproximadamente 1.300 m^3 de óleo combustível marítimo na Baía da Guanabara, Rio de Janeiro, em janeiro de 2000 (MENICOM et al., 2002); o derramamento de cerca de 7.200 m^3 de petróleo no Canal de São Sebastião, São Paulo, em março de 2000; o vazamento de 4.000 m^3 de petróleo devido ao rompimento de um oleoduto da Refinaria Presidente Vargas (REPAR), localizada no Paraná, em julho do mesmo ano; e em novembro de 2004, o navio-tanque *Vicuña*, de bandeira chilena, que sofreu uma explosão no porto de Paranaguá (Paraná)

resultando no derrame de mais de 6.500 m³ de litros de diferentes tipos de óleo e metanol. Segundo Filho (2006), impactos severos nos ecossistemas adjacentes foram registrados em todos os eventos.

Embora acidentes envolvendo a atuação da indústria do petróleo tenham grande destaque na mídia, a contaminação crônica corresponde à maior parcela de petróleo introduzida nos ecossistemas costeiros. De acordo com Gabardo (2007) e Tiburtius et al. (2005), o aporte crônico de petróleo e seus derivados pode ocorrer através do escoamento urbano, de efluentes industriais e domésticos, da navegação, do transporte e da produção em áreas *offshore*, além de pequenos e contínuos vazamentos de combustíveis em pontos de distribuição. Como a maior parte do consumo de petróleo ocorre em terra, os rios, sistemas de drenagem de águas pluviais e de esgotamento sanitário carreiam a maior parcela de óleo que chega aos ecossistemas costeiros (FILHO, 2006; LATIMER; ZHENG, 2003). Porém, vale ressaltar que muitos destes compostos também ocorrem naturalmente na região costeira e oceanos por exsudação natural de petróleo (BURGESS; AHRENS; HICKEY, 2003).

Dentre os derivados do petróleo, o óleo diesel apresenta-se como um potencial contaminante de ecossistemas aquáticos pela sua utilização como combustível marítimo de pequenas e médias embarcações, e navios. Além disso, efluentes contendo óleo diesel constantemente atingem as regiões costeiras como consequência de sua utilização como combustível de veículos rodoviários e máquinas em geral (KENNISH, 1992). Vale salientar que hoje, no Brasil, aproximadamente 40% da energia consumida é proveniente do petróleo e derivados, dos quais o óleo diesel apresenta elevada representatividade. Segundo recente relatório sobre o balanço energético no país desenvolvido pelo Ministério de Minas e Energia, o óleo diesel é o principal combustível utilizado tanto no setor de transportes (48,65%), como no agropecuário (58,2%) (BEN, 2011). A demanda por óleo diesel é crescente tendo em vista o aumento da frota rodoviária nacional em 41,2% para veículos pesados, e 10,6% para veículos leves em relação ao ano de 2009 (BEN, 2011), o que igualmente representa um potencial aumento no aporte de contaminantes nos ambientes costeiros.

De acordo com Neff (2002), o óleo diesel, também chamado de óleo n° 2, é considerado um óleo de densidade média, composto basicamente por hidrocarbonetos alifáticos (HAs) e aromáticos. Dentre os aromáticos, os monocíclicos ou BTEX (benzeno, tolueno, etilbenzeno e xilenos), que apresentam apenas um anel aromático, são os mais abundantes, seguidos pelos policíclicos (HPAs). Destes, os mais

representativos são aqueles que contêm de dois a quatro anéis (NEFF; STOUT; GUNSTER, 2005). Dentre os HPAs, o naftaleno e fenantreno, e seus derivados metilados e/ou alquilados, são os mais frequentemente encontrados no óleo diesel (IRWIN et al., 1997a; VANZELLA; MARTINEZ; CÓLUS, 2007).

No ambiente aquático, o óleo diesel pode promover sérios distúrbios nos organismos expostos, tanto por meio da fração insolúvel em água, que pode, por exemplo, comprometer as trocas gasosas quando em contato com as brânquias (PACHECO; SANTOS, 2001), como pela fração hidrossolúvel, através da biodisponibilização de HAs e HPAs (BARRON et al., 1999; MEADOR, 2003; SIMONATO; GUEDES; MARTINEZ, 2008). Segundo Neff (2002), embora os HAs exerçam efeitos narcóticos nos organismos, são os BTEX que merecem atenção em termos de risco ambiental. Ainda que voláteis, estes compostos apresentam moderada solubilidade em água e são rapidamente bioacumulados e eliminados, podendo exercer efeitos tóxicos nos organismos expostos (NEFF, 2002). Por outro lado, mesmo que pouco representativos quando comparados aos HAs e BTEX, os HPAs são considerados os componentes de maior toxicidade aos organismos aquáticos (FRANCIONI et al., 2007a, 2007b; MEADOR, 2003; NEFF, 2002). Dentre seus efeitos tóxicos, os relacionados a processos genotóxicos (AKCHA et al., 2000; MACHELLA; REGOLI; SANTELLA, 2005), carcinogênicos (AAS et al., 2000; AKCHA et al., 2003; DI et al., 2011), e no estabelecimento de estresse oxidativo (BARŠIENĖ et al., 2006; CAJARAVILLE et al., 2006; FROUIN et al., 2007; LIVINGSTONE, 2001, 2003; VAN DER OOST; BEYER; VERMEULEN, 2003) são comumente relatados.

Componentes químicos do petróleo e seus derivados também merecem atenção sob a ótica da ecotoxicologia aquática. Dentre eles, destaca-se o fenantreno, um hidrocarboneto aromático tricíclico, semivolátil, abundante e amplamente distribuído no meio aquático, além de ser considerado um composto tóxico para diversos organismos aquáticos (IRWIN et al., 1997b; MIRANDA, 2008). De fato, estudos recentes demonstraram seus efeitos no sistema imunológico de moluscos bivalves (HANNAM et al., 2010), e no estabelecimento de estresse oxidativo em peixes (JEE; KANG, 2005; OLIVEIRA; PACHECO; SANTOS, 2008; SUN et al., 2006; YIN et al., 2007) e microcrustáceos (FELDMANNOVÁ et al., 2006; NEWSTED; GIESY, 1987). Alterações comportamentais também foram recentemente relatadas em peixes (CARVALHO et al., 2008; CORREIA et al., 2007), fornecendo importantes evidências experimentais sobre a toxicidade do

fenantreno, indicando seu potencial para danos ecológicos. Outra importante característica deste composto refere-se à sua estrutura química. Apesar de não apresentar propriedade carcinogênica, o fenantreno possui a região de baía, característica também presente em outros HPAs considerados carcinogênicos, como o benzo[a]pireno (BaP) (ZHONG, 2011). É, portanto, considerado um bom modelo para estudos do metabolismo de HPAs (SCHOBER et al., 2010), sendo classificado como um poluente prioritário pela *US EPA* (2009).

Além de derivados de petróleo, ambientes aquáticos são constantemente impactados pelo esgoto doméstico. Segundo Chaler e colaboradores (2004), estes efluentes compreendem misturas complexas de diferentes classes de químicos, os quais incluem HPAs, organoclorados, bifenilas policloradas, metais traço, detergentes, pesticidas, além de fármacos e produtos de higiene pessoal. Ainda, potenciais desreguladores endócrinos, ou seja, compostos sintéticos ou naturais com capacidade de alterar o sistema endócrino dos animais podem ser encontrados no esgoto doméstico (BOGERS et al., 2007), além da contaminação biológica por patógenos virais e bacterianos (PARASHAR et al., 2003). Neste sentido, a avaliação dos efeitos tóxicos em organismos expostos ao esgoto constitui um dos maiores desafios para a ecotoxicologia aquática atual. No Brasil, este desafio é ainda mais evidente, tendo em vista a insuficiência - como já citado - na oferta de serviços de saneamento básico. Uma pesquisa recentemente realizada pelo Ministério das Cidades (PNSB, 2010) reforçou a problemática no país, demonstrando que pouco mais da metade dos municípios brasileiros (55,2%) apresentam serviço de esgotamento sanitário. É importante salientar que a pesquisa refere-se apenas à existência do serviço no município, sem considerar a extensão da rede, a qualidade do atendimento, ou se o esgoto, depois de recolhido, é tratado (PNSB, 2010). Neste sentido, o cenário apresenta-se ainda mais crítico.

Tendo em vista as complexas fontes de contaminação do meio aquático, há, atualmente, crescente demanda por ferramentas sensíveis para avaliação dos impactos antrópicos nas populações naturais ameaçadas. Neste contexto, programas de biomonitoramento ambiental são propostos com o intuito de alertar e prevenir danos nos ecossistemas costeiros, recomendando: 1) o uso de organismos bioindicadores de contaminação; 2) a realização de análises químicas, que determinam analiticamente a concentração dos contaminantes; 3) e a investigação de parâmetros biológicos, como a utilização de biomarcadores de contaminação aquática, que reflitam os impactos dos xenobióticos nos

organismos expostos (ICES, 2004; LAW et al., 2011; MARTÍN-DÍAZ et al., 2004; OLF, 2005).

Organismos aquáticos como bioindicadores de contaminação ambiental

Em um cenário ideal, o monitoramento ambiental avaliaria mudanças na estrutura e composição das comunidades biológicas frente a qualquer perturbação ambiental. Entretanto, em um cenário real, o tempo necessário para se conhecer as respostas dos vários grupos de organismos presentes em um determinado ambiente seria demasiado longo. Assim, múltiplos grupos taxonômicos têm sido utilizados com o intuito de aperfeiçoar a avaliação da qualidade ambiental (ROSENBERG; RESH, 1993). Segundo Beeby (2001), esses organismos apresentam diferentes denominações, as quais diferem conforme sua aplicação: monitores, quando organismos são utilizados para indicar algum efeito adverso nas suas funções, tais como em parâmetros fisiológicos e bioquímicos; sentinela, quando, através de análises químicas, os organismos podem refletir concentrações de contaminantes biodisponíveis no ambiente; e indicadores, que, com um cunho mais ecológico, acusam mudanças nas estruturas populacionais causadas pelo impacto antrópico. Entretanto, nesta tese escolhemos o termo comumente utilizado em estudos ambientais: bioindicadores (LAW et al., 2011). Segundo Holt e Miller (2011), bioindicador é qualquer espécie, comunidade ou processo biológico utilizado para avaliar a qualidade ambiental, identificando potenciais alterações ao longo do tempo.

No ambiente aquático, dois grupos de animais são predominantemente utilizados como bioindicadores de contaminação: peixes e moluscos bivalves. Isso porque ambos contam com mecanismos de bioacumulação e biomagnificação (FERNANDES et al., 2007; HEDOUIN et al., 2006; WEISBROD et al., 2007). Peixes estão presentes em praticamente qualquer corpo d'água, possuem uma vasta variedade de nichos ecológicos entre as espécies, além de transportarem os contaminantes do meio aquático para o terrestre através do processo de biomagnificação ao longo da cadeia trófica (PADMINI, 2010). Por outro lado, os bivalves, como ostras e mexilhões, são amplamente utilizados como bioindicadores por apresentarem ampla distribuição geográfica e hábito filtrador, o que acarreta em altas taxas de acumulação de contaminantes em seus tecidos (ICES, 2004; SHEEHAN; POWER, 1999). Além disso, são cosmopolitas, sésseis, eurialinos, facilmente amostrados e representam um importante recurso

nutricional e econômico para as populações humanas em regiões costeiras (BAINY et al., 2000; CHEUNG et al., 2001; ICES, 2004).

Na costa brasileira, esforços têm sido direcionados para a escolha de espécies que apresentem tanto interesse econômico como ecológico. Nesse contexto, a ostra do mangue, *Crassostrea brasiliana* (Lamarck, 1819), merece destaque. Segundo Ferreira e Neto (2006), além de cultivado com sucesso, este bivalve apresenta boa taxa de sobrevivência no estado de Santa Catarina, fato este corroborado por Pie et al. (2006) para as demais regiões do país. Ainda, esta espécie caracteriza-se por habitar regiões estuarinas e de manguezais, as quais, sabidamente, são expostas a aportes periódicos de contaminantes de variadas fontes. Ademais, apresenta ampla distribuição geográfica, sendo encontrada desde o sul do Brasil até o Pará (região norte), indicando sua potencialidade de uso em programas de biomonitoramento ambiental na costa brasileira (LAZOSKI, 2004). Considerando os aspectos supracitados, a ostra *C. brasiliana* foi escolhida como organismo modelo para os experimentos mostrados nesta tese (LÜCHMANN et al., 2011).

Biomarcadores de contaminação aquática

Em um contexto ecotoxicológico, biomarcadores são definidos como alterações biológicas de caráter molecular, celular ou fisiológico que expressam os efeitos tóxicos causados pelos contaminantes ambientais (WALKER et al., 1996). Tendo em vista a imediata resposta biológica no nível subindividual, Huggett et al. (1992) destacam o papel dos biomarcadores como sinalizadores da degradação ambiental, com potencial para prever danos em níveis maiores de organização ecológica, como populações e comunidades biológicas (RAND, 1995). Em termos práticos, os biomarcadores compreendem ferramentas com a função de refletir a interação entre o sistema biológico e os xenobióticos presentes no ambiente.

As vantagens no uso de biomarcadores em estudos ecotoxicológicos e programas de biomonitoramento ambiental referem-se, na maioria dos casos, a um menor custo associado à maior rapidez e facilidade das técnicas quando comparadas às tradicionais análises químicas, além de detectar mudanças antes do nível populacional (GALLOWAY et al., 2002). Ainda, Mayeux (2004) enfatiza que o uso de biomarcadores pode aumentar a sensibilidade de resposta como estimativa do grau de exposição ou análise de risco. Entretanto, algumas desvantagens podem ser identificadas. Por exemplo, as respostas de biomarcadores podem variar de acordo com alterações nas condições

ambientais, como variações naturais de salinidade, temperatura e pH, estação do ano, além da interferência de fatores biológicos, como tamanho, gênero e estágio reprodutivo do organismo bioindicador, gerando incertezas nos resultados obtidos (MARTÍN-DÍAZ et al., 2004). Ainda, há relatos de significativa variabilidade interindividual, especialmente quando se analisa populações provenientes de ambientes naturais (TALEB et al., 2009; WEBB; GAGNON, 2009). Assim, esforços devem ser direcionados no sentido de promover um maior conhecimento da variabilidade natural dos biomarcadores nas espécies bioindicadoras, bem como estabelecer métodos com alto grau de sensibilidade e reprodutibilidade.

Biomarcadores bioquímicos

Muito xenobióticos ambientais são lipofílicos, o que faz com que sejam rapidamente absorvidos pelos organismos aquáticos através das membranas lipídicas de diferentes tecidos. Assim, a presença de um elaborado sistema bioquímico de biotransformação parece ser o principal mecanismo de proteção celular dos organismos expostos a esses compostos. Neste sistema, cujas enzimas apresentam elaborado nível de organização nos compartimentos celulares, os contaminantes são metabolizados, facilitando assim o processo de eliminação (SACCO, 2006). Dentro das células, as enzimas localizam-se, principalmente, no citoplasma e membranas de organelas, como retículo endoplasmático e mitocôndria, além de membranas citoplasmáticas. Segundo Sacco (2006), o metabolismo de xenobióticos constitui um fator crucial no destino e toxicidade do composto químico em um organismo, determinando sua rota metabólica: a biotransformação, e potencial excreção, ou bioacumulação nas células adiposas. Nesse sentido, o entendimento do sistema de biotransformação em organismos aquáticos representa um dos principais alvos em estudos ecotoxicológicos.

O processo de biotransformação de xenobióticos é formado por enzimas de fase I, II e III. As de fase I, conhecidas como monooxigenases e constituídas, principalmente, pelo sistema citocromo P450 (CYP) e flavina-monoxigenases (FMOs) (NIYOGI et al., 2001), desempenham papel central na metabolização de xenobióticos lipofílicos, tornando-os hidrofílicos através de reações de detoxificação ou bioativação (OMIECINSKI et al., 2011). Após esta fase de ativação, os xenobióticos eletrofílicos podem ser conjugados a várias moléculas endógenas, como a glutationa (GSH), reduzindo sua toxicidade. Esta reação de conjugação pode ser catalisada por um conjunto de enzimas

de fase II, tais como a glutationa *S*-transferase (GST), resultando em produtos mais hidrossolúveis, permitindo que o sistema de transporte de fase III elimine estes conjugados para o meio extracelular (FITZPATRICK et al., 1997; HAYES; FLANAGAN; JOWSEY, 2005). Na fase III de biotransformação de xenobióticos, proteínas de membrana, componentes do sistema de resistência a múltiplos xenobióticos (*multixenobiotic resistance – MXR*), expulsam os xenobióticos da célula (LUCKENBACH; EPEL, 2008).

Dentre as enzimas supracitadas, CYPs e GSTs são largamente utilizadas como biomarcadores de contaminação (BEBIANNO; BARREIRA, 2009; VAN DER OOST; BEYER; VERMEULEN, 2003). No entanto, o mecanismo de ação e as potenciais diferenças quanto a especificidade e magnitude de resposta ainda não foram totalmente elucidadas em organismos aquáticos. Parte desta problemática refere-se à complexidade de ambas as famílias de enzimas. Por exemplo, componentes do sistema CYP compreendem uma complexa superfamília de enzimas, que em humanos é formada por 57 isoformas distribuídas em 18 famílias e 42 subfamílias (NEBERT; RUSSELL, 2002; OMIECINSKI, 2011), enquanto as GSTs são classificadas em sete classes de enzimas citosólicas, além de isoformas mitocondriais e microssomais (HAYES; FLANAGAN; JOWSEY, 2005). Entretanto, em comparação a modelos mamíferos, permanece em aberto o número total de CYPs e GSTS existentes em moluscos bivalves (MIAO et al, 2011; ZANETTE et al., 2010). Dessa maneira, ainda é difícil definir precisamente as funções catalíticas ou a importância fisiológica e toxicológica exercida por estas enzimas nestes organismos.

As defesas antioxidantes constituem sistemas bioquímicos que tendem a proteger as células contra os efeitos deletérios de espécies reativas de oxigênio (EROs). As EROs incluem radicais livres derivados do O_2, como o radical ânion superóxido ($O_2^{\cdot-}$), o oxigênio singlete ($^1O_2$) e o radical hidroxila ($^{\cdot}OH$), bem como os derivados não radicalares, como o peróxido de hidrogênio (H_2O_2). Uma vez formadas, as EROs podem reagir com diferentes alvos celulares levando à formação de lesões oxidativas em proteínas, DNA e lipídios (ALMEIDA et al., 2007; HALLIWELL; GUTTERIDGE, 2007). Assim, a célula possui um complexo sistema de detoxificação de EROs, formada tanto por moléculas não-enzimáticas, como a glutationa (GSH), como por enzimas com funções definidas neste processo. Especificamente, a enzima superóxido dismutase (SOD) remove o radical ânion superóxido, enquanto as enzimas catalase (CAT) e glutationa peroxidase (GPx) atuam na detoxificação do peróxido de hidrogênio (BRAY; LEVY,

2000). Adicionalmente, esse sistema conta com enzimas complementares, tais como a glutationa redutase (GR) que, como o próprio nome sugere, catalisa a redução da glutationa oxidada (GSSG) em sua forma reduzida (GSH), além da enzima glicose-6-fosfato desidrogenase (G6PDH), que fornece a nicotinamida-adenina dinucleotídeo fosfato reduzida (NADPH) como equivalente redutor para a conversão de GSSG em GSH. Ainda, a enzima γ-glutamil transpeptidase (GGT) é responsável pela transferência de grupos glutamil da GSH para inúmeros aminoácidos ou peptídeos, além de fornecer resíduos de cisteína para síntese protéica através da degradação da glutationa (LIU et al., 1998).

Na célula, as EROs podem ser naturalmente formadas durante o processo de fosforilação oxidativa para obtenção de energia, ação fagocitária de células de defesa e reações enzimáticas (STOREY, 1996). Entretanto, a produção dessas espécies tóxicas pode ser aumentada pela presença de certas classes de xenobióticos, como consequência das reações de biotransformação de compostos orgânicos, tais como quinonas e HPAs, e ainda, pela presença de íons metálicos livres gerando ciclos de reações oxidativas (AKCHA et al., 2003; PADMINI, 2010; REGOLI et al., 2002; WALKER et al., 2011). De fato, estudos realizados em organismos aquáticos demonstraram a indução de estresse oxidativo causada pela exposição a contaminantes ambientais (ANSALDO; NAJLE; LUQUET, 2005; BAINY et al., 1996; GALAR-MARTINEZ et al., 2010; NOGUEIRA et al., 2011). Segundo Halliwell e Gutteridge (2007), o estresse oxidativo é definido como um desequilíbrio entre a produção de EROs e as defesas antioxidantes, com consequente efeito em diferentes funções celulares. De acordo com Badmini (2010), alterações no sucesso reprodutivo, aumento na suscetibilidade a doenças e, em última instância, na sobrevivência dos organismos são alguns dos efeitos observados em animais sob condições de estresse oxidativo. Dessa forma, essa condição biológica resulta em uma série de implicações ecológicas para as comunidades naturais, o que reforça a importância de se avaliar parâmetros antioxidantes em animais expostos a contaminantes ambientais.

A indução da síntese de proteínas de estresse ou proteínas de choque térmico (*heat shock proteins* - *Hsp*) também está incluída entre os biomarcadores bioquímicos para avaliação da contaminação aquática. A estrutura altamente conservada dessas proteínas entre diferentes táxons sugere que elas desempenham um papel essencial na manutenção de processos celulares. Por exemplo, sob condições normais na célula, as Hsp podem atuar como chaperonas, estabilizando os intermediários

de polipeptídeos recém sintetizados. Essas proteínas atuam ainda, na transdução de sinais, no transporte de proteínas, na estabilização de fatores de transcrição e contra o efeito proteotóxico causado por uma grande variedade de agentes estressores físicos e químicos (IVANINA; SOKOLOVA; SUKHOTIN, 2008; PANTZARTZI et al., 2010; PRATT, 1997; SNYDER; GIRVETZ; MULDER, 2001). Conforme seu peso molecular (em kDa, quilodalton) as Hsp podem ser divididas em sete famílias: Hsp10, pequenas Hsp (com peso entre 15 e 35 kDa), Hsp40, Hsp60, Hsp70, Hsp90 e Hsp110 (PADMINI, 2010). Destas, as Hsp10 e Hsp60 são mitocondriais, enquanto as demais são essencialmente citoplasmáticas. Sob o ponto de vista ecotoxicológico, as famílias mais bem estudadas em animais aquáticos são: as Hsp60, que se mostraram fortemente induzíveis por metais no mexilhão *Perna perna* (FRANCO et al., 2006) e por derivados de petróleo no rotífero *Brachionus plicatilis* (WHEELOCK et al., 2002); as Hsp70, com menores níveis da proteína identificados em ostras *Crassostrea gigas* expostas a metais (BOUTET et al., 2003) e peixes *Oreochromis niloticus* mantidos em ambiente potencialmente contaminado (FRANCO et al., 2010); e Hsp90, que assim como as Hsp70, apresentaram níveis menores da proteína, em relação ao grupo controle, em ostras *Crassostrea virginica* tratadas com cádmio (IVANINA; TAYLOR; SOKOLOV, 2009) - embora Choi, Jo e Choi (2008) tenham observado um padrão oposto quando ostras *C. gigas* foram expostas ao mesmo metal.

Biomarcadores moleculares

Paralelamente à utilização de biomarcadores bioquímicos, técnicas disponibilizadas pela ciência genômica estão sendo padronizadas e aplicadas em estudos ecotoxicológicos, com o intuito de compreender os mecanismos moleculares envolvidos nas respostas tóxicas de organismos expostos a contaminantes ambientais (ÁLAMO et al., 2012; CHAPMAN, et al. 2011). A ecotoxicogenômica, área que compreende o emprego das ferramentas de genômica em estudos de ecotoxicologia, apresenta vantagens frente aos biomarcadores bioquímicos clássicos, uma vez que respostas bioquímicas podem ser inespecíficas ao contaminante avaliado (SNAPE et al., 2004). Historicamente, os biomarcadores normalmente utilizados em programas de biomonitoramento ambiental são provenientes da toxicologia clássica e são destinados, em última instância, à avaliação e previsão dos efeitos tóxicos de fármacos e químicos ambientais na saúde humana (FORBES; PALMQVIST; BACH, 2006). Nestes programas, potenciais problemas na interpretação de dados, e consequentemente na

avaliação de risco ambiental, podem ser enfrentados quando são avaliadas espécies cujas respostas e mecanismos moleculares de toxicidade são pouco conhecidos. Dessa forma, a ecotoxicogenômica apresenta-se como uma área promissora em estudos ambientais, uma vez que fornece subsídios tanto para a identificação da relação de dose-resposta para cada classe de contaminante, como para o reconhecimento da potencial variação de respostas entre espécies, e o entendimento das vias metabólicas envolvidas nos mecanismos de toxicidade (VAN AGGELEN et al., 2010). Ainda, novos e específicos biomarcadores podem ser identificados, contribuindo para o estabelecimento de diretrizes e prioridades junto a agências reguladoras ambientais (VAN AGGELEN et al., 2010).

Técnicas de “ômica” incluem uma ampla variedade de plataformas metodológicas visando análises de genomas, transcritos, metabólitos e proteínas, que, por sua vez, constituem as respectivas áreas de estudo atualmente denominadas genômica, transcriptômica, metabolômica e proteômica (NRC, 2007). Basicamente, essas áreas constituem a chamada “era pós-genômica”, cujo marco inicial é considerado o sequenciamento do primeiro genoma humano em 2001 (VENTER et al., 2001). Seus termos representam neologismos com o emprego do sufixo "oma" ou "ômica", que em grego significam "todo", "completo" ou "inteiro" (AMARAL et al., 2006). Dentro do enfoque da ecotoxicogenômica, e com o auxílio da bioinformática, essas abordagens “holísticas” focam principalmente na transcrição diferencial e na identificação de genes em organismos expostos a diferentes classes de contaminantes ambientais por ferramentas de transcriptoma (JAYAPAL et al., 2010; SNAPE et al., 2004).

Tradicionalmente, projetos de transcriptoma de organismos cujo genoma permanece desconhecido, ou pouco caracterizado, baseiam-se na geração e caracterização de etiquetas de sequências transcritas (*expressed sequence tags* - ESTs) (BOUCK; VISION, 2007; BURNETT et al., 2007; JOUBERT et al., 2010; ROBERTS et al., 2009; VENIER et al., 2006, 2009). Estas sequências parciais chamadas ESTs são resultado do sequenciamento de DNA complementar (cDNAs), e representam o conjunto de RNA mensageiros (mRNA) produzidos por um organismo em um determinado tempo, local ou condição ambiental. Nesse sentido, bibliotecas de ESTs fornecem um dos melhores custos efetivos para a descoberta de genes e padrões de transcrição gênica, uma vez que a maioria das sequências obtidas codifica proteínas, e a ausência de íntrons e regiões intergênicas facilita a análise e a consequente interpretação dos resultados (OHLROGGE; BENNING, 2000). Dados

de ESTs são gerados em grande escala, geralmente representando diversos tecidos e tratamentos e permitindo diferentes aplicações em estudos relacionados a respostas ao nível de transcrição. Com este propósito, diferentes bibliotecas podem ser comparadas, resultando na transcrição gênica diferencial, e técnicas de arranjos de cDNA (*cDNA arrays*) podem ser desenvolvidas para avaliar a transcrição de até dezenas de milhares de genes simultaneamente (BOUCK; VISION, 2007), como recentemente demonstrado em estudos de cunho ecotoxicológico com organismos aquáticos (CHANEY; GRACEY, 2011; DONDERO et al. 2006; VANDEGEHUCHTE et al., 2010; WILLIAMS et al., 2008). Adicionalmente, as bibliotecas de ESTs podem ser normalizadas por meio da utilização da técnica de hibridação subtrativa supressiva (*suppressive subtractive hybridization*, *SSH*), isolando genes diferencialmente transcritos por determinados tipos celulares sob condições fisiológicas ou experimentais específicas (DIACHENKO et al., 1996). A partir dessa abordagem, duas populações de cDNA são comparadas, isolando transcritos diferencialmente expressos (WINSTANLEY, 2008).

Apesar de inicialmente desenvolvida para identificar genes relacionados a doenças humanas, a SSH possui aplicações em diversos organismos, sendo também aplicada com êxito em estudos ecotoxicológicos com animais aquáticos (VENIER et al., 2009). Por exemplo, a SSH foi recentemente utilizada para identificar genes induzidos e reprimidos em resposta à exposição a hidrocarbonetos derivados de petróleo em ostras do Pacífico *C. gigas* (BOUTET; TANGUY; MORAGA, 2004) e em copépodos *Calanus finmarchicus* (HANSEN et al., 2007), e para identificar genes através de ESTs que tiveram sua transcrição alterada em *C. gigas* e no abalone *Haliotis rufescens* devido à exposição a pesticidas e cobre, respectivamente (COLLIN et al., 2010; SILVA-ACIARES et al., 2011). A versatilidade da técnica também é confirmada nos diversos estudos que a empregam para as mais variadas aplicações, como por exemplo, para o isolamento de genes diferencialmente transcritos de *C. gigas* e *Haliotis diversicolor* infectados com patógenos ambientais (RENAULT et al. 2011; WANG et al., 2008), e na identificação de novos biomarcadores ambientais em peixes *Dicentrarchus labrax* expostos *in situ* a diferentes fontes de contaminação costeira (NOGUEIRA et al., 2010). Neste contexto, nosso grupo de pesquisa também vem utilizando a mesma técnica a fim de identificar genes induzidos e/ou reprimidos por esgoto doméstico em duas espécies de ostras, *C. gigas* (MEDEIROS et al., 2008; TOLEDO-SILVA, 2009) e *Crassostrea rhizophorae* (ZANETTE, 2009), além de

avaliar o efeito de derivados de petróleo na abundância diferencial de transcritos em peixes *Poecilia vivipara* (MATTOS et al., 2010).

Tradicionalmente, as sequências de ESTs são determinadas pelo sequenciamento de Sanger, também chamado de método de terminação da cadeia por dideoxinucleotídeos (ddNTP) ou sequenciamento de primeira geração (*first-generation sequencing*) (SCHADT; TURNER; KASARSKIS, 2010). Entretanto, dependendo da aplicação, este método apresenta limitações relacionadas ao tempo de processamento e ao custo elevado, especialmente quando se trata de bibliotecas de cDNA não normalizadas. Neste sentido, novas tecnologias de sequenciamento - conhecidas como sequenciamento de próxima geração (*next-generation sequencing, NGS*) - estão em desenvolvimento e representam uma alternativa viável para projetos de transcriptoma, tanto para espécies modelo, como para aquelas cujo genoma ainda não foi sequenciado (EKBLOM; GALINDO, 2011; SCHIRMER et al., 2010; SHENDURE; JI, 2008). Estas novas técnicas de NGS criam uma mudança de paradigmas dentro da abordagem da ecotoxicogenômica aquática.

São quatro as principais plataformas de NGS atualmente disponíveis: 454/Roche; Illumina/Solexa; ABI SOLiD™, e HeliScope™. Destas, as três primeiras constituem a segunda geração da tecnologia de sequenciamento (*second-generation sequencing*), e a última, mesmo sendo a mais recente e ainda pouco estudada, promete o sequenciamento de molécula única com leituras mais extensas, e constitui a terceira geração da tecnologia de sequenciamento (*third-generation* sequencing) (SCHADT; TURNER; KASARSKIS, 2010). Embora os sistemas de NGS compreendam bioquímicas de sequenciamento diferentes (Tabela 1), todos promovem o sequenciamento de ácidos nucléicos em plataformas capazes de gerar informação sobre milhões de pares de bases em uma única corrida. Nesse sentido, os últimos avanços dessas tecnologias resultaram em: 1) um aumento dramático da velocidade e da facilidade de aquisição de grande quantidade de dados; 2) a redução espetacular dos custos do sequenciamento; 3) e a consequente democratização do sequenciamento de ácidos nucléicos (KAMB, 2011; SHENDURE; JI, 2008). Adicionalmente, técnicas de NGS dispensam métodos de clonagem tradicionalmente laboriosos (CARVALHO; SILVA, 2010), além da vantagem de não requerer conhecimentos prévios sobre tamanho e estrutura dos transcritos (SCHIRMER et al., 2010).

A tecnologia de sequenciamento de ESTs foi recentemente aplicada em estudos com organismos aquáticos, como por exemplo, com as ostras *C. virginica* (QUILANG et al., 2007; WANG; GUO, 2007) e

C. gigas (YU; LI, 2008), com os mexilhão *Mytilus galloprovincialis* (VENIER et al., 2009) e com o bivalve Antártico *Laternula elliptica* (PARK et al., 2008). No entanto, os trabalhos supracitados usaram o tradicional método de sequenciamento de Sanger, e apenas recentemente plataformas de NGS passaram a ser aplicadas em espécies aquáticas (SCHIRMER et al., 2010; VAN AGGELEN et al., 2010). Dentre os sistemas de NGS, o pirosequenciamento na plataforma 454 é a mais utilizada, como pode ser evidenciado nos estudos realizados com os moluscos *Bathymodiolus azoricus* (BETTENCOURT et al., 2010), *Laternulla elliptica* (CLARK et al., 2010), *M. galloprovincialis* (CRAFT et al., 2010), *Pactinopecten yessoensis* (HOU et al., 2011), *Pinctada margaritifera* (JOUBERT et al., 2010) e *Ruditapes philippinarum* (MILAN et al., 2011), com o coral *Acropora millipora* (MEYER et al., 2009), com o krill *Euphausia superba* (CLARK et al., 2011), e com os peixes *Oncorhynchus mykiss* (SALEM et al., 2010), *Poecilia reticulata* (FRASER et al., 2011), e *Salmo salar* (QUINN et al., 2008). A plataforma 454 produz as maiores leituras (*reads*) (aproximadamente 330 bp) (Tabela 1), tornando mais eficiente tanto o processo de montagem (*assembly*) em um projeto de sequenciamento *de novo* (sequenciamento de genomas desconhecidos), quanto a posterior anotação funcional de genes (KUMAR; BLAXTER, 2010; MARGULIES et al., 2005). Dessa forma, o pirosequenciamento é o método mais indicado quando pouco é conhecido sobre o genoma de uma espécie (EKBLOM; GALINDO, 2011), o que justifica a sua crescente utilização em projetos de transcriptoma de espécies de interesse ecotoxicológico.

Tabela 1: Plataformas de sequenciamento de próxima geração (NGS) atualmente disponíveis e suas principais características.

Plataforma	**Preparação da amostra**	**Bioquímica de sequenciamento**	**Comprimento das leituras[§]**	**Tempo e rendimento[‡] por corrida**
454/Roche	Fragmentação ou MP*; PCR em emulsão (*emPCR*)	Polimerase (pirosequenciamento)	330	7 horas; 0,45
Illumina/Solexa	Fragmentação ou MP; PCR em fase sólida	Polimerase (terminadores reversíveis)	75 ou 100	4 dias; 18
ABI SOLiD	Fragmentação ou MP; PCR em emulsão (*emPCR*)	Ligase (octâmeros com codificação de duas bases)	50	7 dias; 30
HeliScope	Molécula única	Polimerase (extensão assíncrona)	32	8 dias; 37

Fonte: Metzker (2010); Shendure e Ji (2008). *MP (*mate-paired*): corrida pareada; [§]Comprimento médio em pares de bases (pb); [‡]Rendimento em gigabases (Gb).

Estrutura e objetivos da tese

Após a contextualização apresentada nos parágrafos anteriores sobre o papel e a importância da ecotoxicologia aquática - suas ferramentas, limitações e desafios -, torna-se evidente a necessidade de novos estudos que vislumbrem o preenchimento das muitas lacunas ainda abertas. Neste cenário, o objetivo desta tese foi investigar a sensibilidade e aplicação de alguns biomarcadores bioquímicos e moleculares em ostras *Crassostrea brasiliana* tratadas com diferentes contaminantes ambientais, com o intuito de fornecer insights sobre respostas e mecanismos de toxicidade, bem como contribuir para o estabelecimento de ferramentas sensíveis para a avaliação de impactos no ambiente aquático. Com este propósito, três classes de contaminantes foram escolhidas: a fração de óleo diesel acomodada em água (FAA) e o esgoto doméstico, como representantes de misturas complexas de xenobióticos; e o fenantreno, um composto simples comumente utilizado como modelo em estudos de toxicidade de HPAs. Cabe ressaltar que os dados gerados nesta tese podem se tornar importantes fontes de informação para a utilização na avaliação de águas contaminadas por compostos derivados de petróleo e esgoto doméstico. Espera-se, em última instância, contribuir para o desenvolvimento de um efetivo programa de biomonitoramento ambiental na costa brasileira.

Os capítulos desta tese foram separados de acordo com o desenho experimental, métodos e parâmetros escolhidos com o intuito de atingir os objetivos propostos. Com exceção do Capítulo 1, que representa o prefácio, e o Capítulo 6, que apresenta as principais considerações e conclusões finais desta tese, cada capítulo representa um manuscrito preparado para publicação, além de um capítulo já publicado (LÜCHMANN et al., 2011 – Capítulo 2) e outro aceito para publicação (LÜCHMANN et al., 2012 – Capítulo 3). Como os capítulos foram trabalhados em colaboração com outros pesquisadores, os mesmos são apresentados como co-autores. Todos os manuscritos foram preparados em inglês, seguindo, parcialmente, a formatação das revistas nas quais os mesmos foram (ou serão) submetidos. Para facilitar a visualização da estrutura deste documento, a Tabela 2 mostra os biomarcadores e as abordagens metodológicas utilizadas em cada capítulo.

O estudo apresentado no Capítulo 2 mostra as respostas de biomarcadores bioquímicos considerados clássicos sob a ótica da ecotoxicologia aquática, como a atividade das enzimas SOD, CAT, GR, GPx, G6PDH, GGT e GST, e a expressão das proteínas Hsp60 e Hsp90. Essas análises foram conduzidas em brânquia e glândula digestiva de ostras expostas por 96 horas a quatro concentrações de FAA de óleo

diesel, cujos resultados permitiram, ainda, a comparação da sensibilidade de resposta entre os dois tecidos estudados. Dados de bioacumulação também são apresentados.

Tabela 2. Resumos dos principais biomarcadores e/ou técnicas utilizadas em cada capítulo desta tese.

Biomarcador e/ou técnica analisada	**Capítulo**			
	2	**3**	**4**	**5**
Atividade da enzima superóxido dismutase (SOD)	X			
Atividade da enzima catalase (CAT)	X			X
Atividade da enzima glutationa peroxidase (GPx)	X			X
Atividade da enzima glutationa redutase (GR)	X			X
Atividade da enzima glicose-6-fosfato desidrogenase (G6PDH)	X			X
Atividade da enzima glutationa *S*-transferase (GST)	X			X
Atividade da enzima γ-glutamil transpeptidase (GGT)	X			
Expressão da proteína de estresse Hsp60	X			
Expressão da proteína de estresse Hsp90	X			
Hibridação subtrativa supressiva (SSH)		X		
Sequenciamento de EST			X	
Níveis de mRNA dos genes (SSH) por PCR quantitativo em tempo real (qPCR)		X		
Níveis de mRNA dos genes *CYP-like* e *GST-like* por qPCR				X

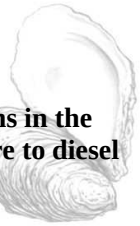
O Capítulo 3 aborda os primeiros dados moleculares obtidos nesta tese. Os resultados são provenientes da construção de bibliotecas subtrativas de cDNA de ostras, através da técnica de SSH, com o objetivo de identificar novos genes candidatos a biomarcadores de exposição a derivados de petróleo. Os resultados da validação dos genes identificados, pelo método de PCR quantitativo em tempo real (qPCR), também são evidenciados. Os parâmetros foram analisados em glândula digestiva de ostras expostas por 24 horas a uma única concentração de FAA de óleo diesel.

O Capítulo 4 compreende os resultados obtidos do primeiro projeto transcriptoma em grande escala utilizando sequenciamento de próxima geração (plataforma 454) em um organismo de interesse ecotoxicológico no Brasil. Para esta abordagem, foram utilizadas ostras expostas à FAA de óleo diesel, fenantreno e esgoto doméstico, cujos resultados obtidos representam uma importante contribuição para a

ecotoxicologia nacional. Mais especificamente, o trabalho faz uma descrição geral do transcriptoma da *C. brasiliana*, comparando os resultados com dados disponíveis para outras espécies cujos genomas ainda não foram sequenciados. Ainda que de forma preliminar, este estudo também identifica genes de importância ecotoxicológica.

O estudo descrito no Capítulo 5 resulta da aplicação de métodos bioquímicos e moleculares em brânquia e glândula digestiva de ostras expostas por 24 horas a duas concentrações de fenantreno. Nele, resultados de biomarcadores antioxidantes não-enzimáticos, como o estado tiólico celular, bem como biomarcadores enzimáticos, como CAT, GPx, GR, G6PDH e GST, são abordados de forma comparativa entre os dois tecidos estudados. Adicionalmente, sequências gênicas similares a *CYP* e *GST* identificadas no Capítulo 4 foram utilizadas para avaliação da transcrição destes genes, por qPCR, em resposta à exposição ao fenantreno. Dados referentes ao monitoramento dos níveis de fenantreno na água durante a exposição também são apresentados.

Finalmente, o Capítulo 6 sumariza os principais achados dos Capítulos 2, 3, 4 e 5, bem como destaca as considerações finais e principais conclusões alcançadas ao final dos quatro anos deste estudo.



Capítulo 2
Biochemical biomarkers and hydrocarbons concentrations in the mangrove oyster *Crassostrea brasiliana* following exposure to diesel fuel water-accommodated fraction

Karim H. Lüchmann[a], Jacó J. Mattos[a], Marília N. Siebert[a], Ninna Granucci[a], Tarquin S. Dorrington[a], Márcia C. Bícego[b], Satie Taniguchi[b], Silvio T. Sasaki[b], Fábio G. Daura-Jorge[c], Afonso C.D. Bainy[a]

Publicado na Aquatic Toxicology Volume 105, Números 3-4: 652-660, 2011

[a]Laboratório de Biomarcadores de Contaminação Aquática e Imunoquímica, Departamento de Bioquímica, Universidade Federal de Santa Catarina, Florianópolis, Brazil; [b]Laboratório de Química Orgânica Marinha, Instituto Oceanográfico, Universidade de São Paulo, São Paulo, Brazil; [C]Laboratório de Mamíferos Aquáticos, Departamento de Ecologia e Zoologia, Universidade Federal de Santa Catarina, Florianópolis, Brazil.

Abstract
Understanding the toxic mechanisms by which organisms cope to environmental stressful conditions is a fundamental question for ecotoxicology. In this study, we evaluated biochemical responses and hydrocarbons bioaccumulation of the mangrove oyster *Crassostrea brasiliana* exposed for 96 h to four sublethal concentrations of diesel fuel water accommodated fraction (WAF). For that purpose, enzymatic activities (SOD, CAT, GPx, GR, G6PDH, GST and GGT), Hsp60 and Hsp90 immunocontent and LPO levels were determined in the gill and digestive gland of oysters and related to the hydrocarbons accumulated in the whole soft tissues. The results of this study revealed clear biochemical responses to diesel fuel WAF exposure in both tissues of the oyster. The capacity of *C. brasiliana* to bioaccumulate aliphatic and aromatic hydrocarbons in a concentration-dependent manner is a strong indication of its suitability as a model in biomonitoring programs along the Brazilian coast, which was also validated by the response of the antioxidant defenses, phase II biotransformation and chaperones. Hsp60 levels and GGT activity were the most promising biomarkers in the gill, while GST and GR activities stood out as suitable biomarkers for the detection of diesel toxicity in the digestive gland. The decrease of SOD activity and Hsp90 levels may also reflect a negative effect of diesel exposure regardless the tissue. The present results provide a sound preliminary report on the biochemical responses of *C. brasiliana* challenged with a petroleum by-product and should be carefully considered for use in the monitoring of oil and gas activities in Brazil.
Keywords: Diesel; Biomarkers; Pollution; Oyster; *Crassostrea brasiliana*

Introduction

The recent discovery of potentially massive oil reserves in Brazilian offshore waters has projected the country into becoming one of the world's largest producers of crude oil (Wertheim, 2009). There is, therefore, a need for monitoring of petroleum industry activities and the possibility of marine contamination. Oil exploration, production and transport potentially expose marine organisms to petrochemical compounds; hence, a pollution-monitoring program is a priority to assess the effects of petroleum by-products on such organisms. It may also enhance any decision-making in the public and private sectors on the environmental effects of accidents, such as the recent BP oil spill in the Gulf of Mexico.

Filter-feeding mollusks, such as oysters, play a significant role as sentinel organisms in monitoring programs due to their capacity to bioaccumulate environmental contaminants as well as to respond to their presence (Bebianno and Barreira, 2009; Solé et al., 2007). Moreover, they are sessile, globally distributed and economically important. Chemical analysis of contaminants in bivalve tissues has been recommended for biomonitoring (Solé et al., 2007), which gives an indication of the bioavailable fraction of environmental contamination and of direct exposure to chemicals. However, they do not necessarily reveal potential biological effects of the contaminants (Baumard et al., 1999) and biomarkers have been developed to detect and evaluate the effects of exposure to contaminants in the aquatic environment (Richardson et al., 2008).

Brazilian oyster production has expanded considerably over the past years, and the native mangrove oyster *Crassostrea brasiliana* has become prevalent in oyster farms in Brazil (Pie et al., 2006). In addition, this species naturally occurs throughout Brazilian coast in environments known to be exposed to petroleum by-products, such as diesel fuel (oil N°2), the most common fuel used by boats in the world (Kennish, 1992).

Petroleum by-products and their metabolic products are able to cause a range of biochemical responses involved in the biotransformation of xenobiotics, the antioxidant defense system and general cellular metabolism in marine bivalves (Altenburger et al., 2003; Banni et al., 2010; Bebbiano and Barreira, 2009; Lima et al., 2007). Under these conditions, a metabolic impairment might result in the formation of excessive amounts of ROS (reactive oxygen species) that can lead to oxidative stress. As a result, the normal intracellular reducing environment is compromised, damaging proteins, nucleic acids and lipids. Lipid peroxidation is considered a major mechanism by which oxyradicals can cause injury, impairing cellular function and ultimately resulting in the failure of normal cell function (Livingstone, 2001). Links between hydrocarbon exposure and increased levels of lipid peroxidation in bivalves have been shown by Bebianno and Barreira (2009) and Lima et al. (2007).

However, to protect against the deleterious effects of ROS, cells contain a complex network of antioxidant defenses, composed of both enzymatic and nonenzymatic antioxidants (Halliwell and Gutteridge, 2007). The antioxidant system involves enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). SOD dismutates the superoxide anion radical ($O_2^{\cdot-}$) into hydrogen

peroxide (H_2O_2), which is degraded by CAT and GPx (Halliwell and Gutteridge, 2007). Ancillary enzymes, such as glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH), recycle GSH and NADPH, respectively, contributing to the maintenance of an optimal intracellular redox environment for proper function of cellular proteins (Circu and Aw, 2010). Among the nonenzymatic defenses, glutathione (GSH), the most abundant intracellular nonprotein thiol, participates in many important biological processes including protection against toxic compounds. Different enzymes mediate the metabolism of glutathione and γ-glutamyl transpeptidase (GGT) is involved in glutathione synthesis, exerting an essential role in oxidant-challenged cells (Liu et al., 1998).

Enzymes implicated in the elimination of by-products of ROS play an important role as indirect antioxidants (Boutet et al., 2004), such as glutathione *S*-transferase (GST). GSTs are a group of multifunctional enzymes catalyzing the conjugation of a broad range of electrophilic substrates, generally produced during phase I of biotransformation of organic compounds, to endogenous glutathione (GSH) (Storey, 1996). These enzymes are involved in the cellular detoxification and excretion of many xenobiotics, being ideal as biomarkers of polycyclic aromatic hydrocarbon (PAH) exposure in marine bivalves (Bebianno and Barreira, 2009). Moreover, the heat shock proteins (Hsps) have also been considered a potential petroleum hydrocarbon target (Downs et al., 2001; Wolfe et al., 1999). Ubiquitous in nature, Hsps play a role as molecular chaperones, being able to protect cells against the toxic effects of xenobiotics (Kalmar and Greensmith, 2009). Hsps also play a role in a number of other cellular processes that occur during and after exposure to oxidative stress (Kalmar and Greensmith, 2009), although their exact role in protection from the effects of petroleum hydrocarbons is not fully understood.

Despite the existence of several studies that evaluate the impact of petroleum by-products in the field using oysters, relatively few have focused on the effects of complex mixtures of water-accommodated hydrocarbons from the petroleum by-products under laboratory exposure. Furthermore, the combination of chemical analysis with biochemical biomarker assessment has not been applied along the Brazilian coast. In line with this approach, the present work integrates for the first time the assessment of a set of putative biochemical biomarkers of hydrocarbons exposure in the mangrove oyster *Crassostrea brasiliana* with the characterization of the water accommodated hydrocarbons derived from diesel fuel.

Materials and methods

Experimental design

Adult mangrove oysters, *C. brasiliana*, of similar shell length (4 – 5.5 cm) were obtained from an oyster farming area at the Laboratório de Moluscos Marinhos (UFSC) in Florianópolis, southern Brazil, and immediately transported to the laboratory for an acclimation period of 7 days. Under laboratory conditions, the oysters were held in aerated 0.45 μm-filtered seawater at a constant temperature (21 °C) and salinity (25 ppt), and fed on microalgae (*Chaetoceros muelleri* and *Isochrysis* sp.) twice a day at a density of 3.3 x 10^6 cels.mL^{-1} and 2.2 x 10^6 cels.mL^{-1}, respectively.

Groups of 10 oysters were exposed for 96 hours to four concentrations (2.5%, 5.0%, 10.0% and 20.0%) of diesel fuel water-accommodated fraction (diesel WAF), with seawater used as the control group. Diesel fuel was purchased at a Petrobras petrol station and WAF was obtained according to Singer et al. (2000) with minor modifications. Briefly, one part (1 L) of fresh diesel fuel was diluted with nine parts (9 L) of the 0.45 μm-filtered seawater (salinity 25 ppt) in a sealed 14 L glass flask protected from light, in order to minimize evaporation and degradation of the fuel components. The diesel-water mixture was stirred for 23 hours with the tissue homogenizer Glas-Col at 1600 rpm at a constant temperature of 21 °C. The mixture was then made to stand for 1 hour before the lower layer of water (diesel WAF) was transferred into the aquaria. The four concentrations of diesel WAF were prepared through dilutions of the WAF with the control seawater. The animals were not fed during the exposure period and the experiment was carried out in duplicate. No mortality was observed in both the control and treated groups.

After the 96 h experimental period, three oysters from each aquarium, totalizing six animals from each experimental group, were killed, pooled, wrapped in aluminum foil and immediately frozen at -80 °C for further chemical analysis. For biochemical parameters, six oysters from each aquarium, totalizing 12 animals from each experimental group, were killed and the gill and digestive gland were immediately excised, frozen in liquid nitrogen and stored at -80 °C until preparation for analysis.

Chemical analysis

The procedure for the analysis of aliphatic hydrocarbons (AH) and polycyclic aromatic hydrocarbons (PAH) followed that described in MacLeod et al. (1985) with minor modifications. Briefly, five grams of

wet tissue was extracted, after the addition of anhydrous Na_2SO_4, with hexane/dichloromethane 50% (v/v) using Soxhlet apparatus for 8 h. Before extraction, n-hexadecene, n-eicosene, d_8-naphthalene, d_{10}-acenaphthene, d_{10}-phenanthrene, d_{12}-chrysene, and d_{12}-perylene were added to all samples, blanks and reference material as surrogates. Aliphatic hydrocarbons were eluted in a partially deactivated (5%) silica:alumina column chromatography with 40 mL of n-hexane (F1) and PAHs with 45 mL of a 1:1 mixture of n-hexane and methylene chloride (F2). The PAH fraction was further purified by high-performance liquid chromatography (HPLC) to remove lipids and finally concentrated to a volume of 1 mL in hexane and internal standards tetradecene and d_{12}-benzo(b)fluoranthene were, respectively, added to F1 and F2 before gas chromatographic analysis. Aliphatic hydrocarbons were analyzed by gas chromatography using a flame ionization detector (FID). PAHs were quantitatively analyzed by gas chromatograph coupled to a mass spectrometer (GC/MS) in a selected ion mode (SIM).

Tissue preparation for biochemical analyses

Gill and digestive gland of each oyster were individually weighed and homogenized in 1:4 w/v chilled buffer (20 mM Tris-HCl buffer, pH 7.6, containing 0.5 M sucrose, 1 mM DTT, 1 mM EDTA, 0.15 M KCl and 0.1 mM PMSF) using the tissue homogenizer *Tissue-Tearor*™. The homogenates were centrifuged at 9000 *g* for 30 min at 4 °C, followed by a second centrifugation of the supernatants at 37000 *g* for 74 min at 4 °C to obtain the cytosolic fraction. The resulting supernatants were used for measurements of antioxidant enzyme activity and Hsp90 immunocontent. The pellets from the first centrifugation were used for measurement of γ-glutamyl transpeptidase activity, Hsp60 immunocontent and lipid peroxides contents. Total protein levels were quantified in both supernatant and pellet according to Peterson (1977) using bovine serum albumin as standard.

Enzyme assays

The cytosolic copper/zinc superoxide dismutase (SOD) activity was determined by an indirect method through the inhibition of cytochrome *c* reduction in the presence of hypoxanthine/xantine oxidase $O_2^{\cdot-}$ generator system at 550 nm (McCord and Fridovich, 1969). Catalase (CAT) activity was measured by the decrease in absorbance at 240 nm by H_2O_2 decomposition, according to Beutler (1975). Glutathione peroxidase (GPx) activity was measured indirectly by

monitoring the NADPH oxidation rate at 340 nm according to Wendel (1981) using cumene hydroperoxide (CuOOH) as substrate. Glutathione reductase (GR) activity was quantified by the NADPH oxidation rate at 340 nm (Sies et al., 1979). Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined following the method of Glock and McLean (1953), which evaluates the increase in absorbance at 340 nm, caused by the reduction of $NADP^+$ to NADPH. Glutathione *S*-transferase (GST) activity was assayed by increasing absorbance at 340 nm, using 1-chloro-2,4 dinitrobenzene (CDNB) as substrate (Keen et al., 1976). γ-glutamyl transpeptidase (GGT) activity was determined using commercially available kit (Biotecnica Ltda). All enzyme assays using visible wavelengths were carried out by use of 96 wells plates reader (Spectramax 250, Molecular Devices, Sunnyvale, CA), while CAT activity was assayed in a Perkin Elmer Lambda Bio20 UV/visible spectrophotometer (Perkin Elmer, Cambridge, UK).

Hsp immunodetection

Samples of equal total protein content (30 µg of protein) were separated by SDS-PAGE using 10% gels under denaturant conditions and transferred to nitrocellulose membrane for 90 min using 400 mA current. The membranes were blocked for 1 h with 5% (w/v) nonfat powered milk in TBS-T (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5). Membranes were then washed three times with TBS (10 mM Tris, 150 mM NaCl, pH7.5) and incubated with primary antibody. For Hsp detection, rabbit polyclonal antibody SPA-805 (1:10000) (StressGen), anti-insect Hsp60, and mouse monoclonal antibody AC88 (1:2000) (Calbiochem), anti-mouse Hsp90, were used as primary antibodies. NA934 (1:1000) donkey anti-rabbit IgG peroxidase-linked (Amersham) and NA931 (1:1000) sheep anti-mouse IgG peroxidase-linked (Amersham) were used as the secondary antibody for detecting both isoforms. Because only one immunoreactive band was observed on the western blots for both antibodies, a dot-blot procedure was used for quantification of samples. A Bio-Dot® Microfiltration Apparatus (Bio-Rad) provided a reproducible method for binding Hsp proteins onto the nitrocellulose membranes. Immunodetection of Hsp60 and Hsp90 were completed on the membrane in an identical manner to the western blots. Immunoblotting was developed using the enhanced chemiluminescence (ECL) system (Amersham, São Paulo) and Hsp60 and Hsp90 expression was quantified by densitometric analysis of the immunoreactive bands using the Scion Image® software.

Lipid peroxidation (LPO)

Oxidative stress damage was measured in terms of lipid peroxidation determination (LPO), according to Hermes-Lima et al. (1995) with minor modifications. Cumene hydroperoxide was used as a standard.

Statistical analysis

Differences in mean values were analyzed by one-way ANOVA followed by complementary Tukey test with significance level established at $p<0.05$. Normality (Shapiro-Wilks test) and homogeneity of variances assumptions were previously checked (Bartlett's test) and logarithmic transformation was applied when necessary (Zar, 1999). Outliers were excluded according to the Grubbs test. Pearson Correlation matrix was also calculated to study the relationships between the biochemical biomarkers measured. Statistical analyses were performed with the software GraphPad 5.0.

A principal component analysis (PCA) on correlation matrix was used to explore and describe the relationship between the biochemical biomarkers and the concentration of WAF for each tissue separately. PCA was applied on the media of all samples for each biochemical response and treatment. The data was previously log-transformed ($\log_{[x+1]}$) and the analyses were performed with the software CANOCO 4.5.

Results

Aliphatic and polycyclic aromatic hydrocarbon concentrations

Aliphatic and polycyclic aromatic hydrocarbon concentrations were determined in the whole soft tissues of oysters from both control and diesel WAF exposed groups. The concentrations of different types of aliphatic hydrocarbons, total *n*-alkanes and isoprenoids (pristane and phytane), and total aliphatic hydrocarbons (ΣAH) are shown in Table 1. The ΣAH levels varied among experimental groups, ranging from 43.8 $ug.g^{-1}$ in the control group to 292 $ug.g^{-1}$ in the 20% diesel WAF group. Oysters from the control group accumulated only biogenic *n*-alkanes, characteristic of both phytoplankton (C_{15}, C_{16} and C_{17}) and superior plants (C_{23}-C_{33}) (data not shown). In oysters exposed to diesel WAF, *n*-alkanes from both biogenic and petrogenic sources were detected (data not shown). Oysters exposed to diesel presented a ratio of pristane/phytane ranged from 1.5 to 2.5 (Table 1), values similar to the diesel WAF itself used in the exposures (data not shown). The unresolved complex mixture (UCM) levels were also determined in the oysters (Table 1). The UCM is an indicator of petrogenic sources and is composed by both ramified and cyclic aliphatic hydrocarbons (Readman

et al., 2002). UCM levels were above the detection limits only in the exposed groups, where oysters from 2.5%, 5%, 10% and 20% diesel WAF accumulated 56.2 ug.g^{-1}, 98.3 ug.g^{-1}, 64.5 ug.g^{-1} and 201 ug.g^{-1}, respectively (Table 1).

The levels of individual and total polycyclic aromatic hydrocarbons (ΣPAH) accumulated in the oysters are summarized in Table 2. The ΣPAH concentrations increased in a concentration-dependent manner along the experimental range, from 2.6 µg.g^{-1} in the control group to 115 µg.g^{-1} in the 20% diesel WAF group (Table 2). Oysters exposed to diesel WAF accumulated only low molecular weight PAHs, with 2 or 3 aromatic rings, the most water soluble PAHs. Individual parental PAH determination showed that biphenyl, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene were detected in all the exposed groups (Table 2), with phenanthrene being the most abundant, accounting for 3.7% of total PAHs. Among the non-parental PAHs, the sum of trimethylnaphthalenes (52.5%), followed by the sum of dimethylnaphthalenes (26.7%) were the most abundant of all the accumulated PAHs.

Table 1. Aliphatic hydrocarbons levels (ug.g^{-1}) on a wet weight basis detected in whole soft tissues of oysters from both control and diesel WAF exposed groups.

	Control	2.5% WAF	5% WAF	10% WAF	20% WAF
Pristane	0.174	0.320	0.374	0.725	1.84
Phytane	<0.085	0.139	0.255	0.400	1.15
Pristane/phytane	ND	2.3	1.5	1.8	1.6
Total *n*-alkanes	1.5	2.2	2.9	3.4	9.9
UCM	<5.34	56.2	98.3	64.5	201
ΣAH	43.8	95.9	154	119	292

UCM, unresolved complex mixture; ΣAH, total aliphatic hydrocarbons; ND, not detected.

Table 2. Aromatic hydrocarbons levels ($ng.g^{-1}$) on a wet weight basis detected in whole soft tissues of oysters from both control and diesel WAF exposed groups.

	Control	2.5% WAF	5% WAF	10% WAF	20% WAF
Σmethylnaphthalene	62.9	1053	279	1164	4446
Biphenyl	39.8	152	145	373	1273
Σethylnaphthalene	<4.71	213	391	1108	3494
Σdimethylnaphthalene	92.4	1738	3441	10,862	33,364
Acenaphtene	21.9	53.4	109	320	862
Σtrimethylnaphthalene	1404	2164	10,386	27,098	57,751
Fluorene	147	114	303	872	2005
Dibenzothiophene	54.0	21.6	68.0	167	444
Phenanthrene	14.4	226	598	1673	4350
Anthracene	<3.46	8.30	31.4	73.8	150
Σmethyldibenzothiophene	4.56	29.7	162	339	663
Σdimethylphenantrene	<5.85	228	1253	2841	5553
Fluoranthene	<2.45	9.40	41.1	77.0	148
Pyrene	<5.08	19.0	92.5	186	369
Benz[a]anthracene	<3.52	<3.52	<3.52	<3.52	<3.52
Chrysene	<7.04	<7.04	<7.04	<7.04	<7.04
Benzo[b]fluoranthene	<2.53	<2.53	<2.53	<2.53	<2.53
Benzo[k]fluorantene	<2.40	<2.40	<2.40	<2.40	<2.40
Benzo[e]pyrene	<2.56	<2.56	<2.56	<2.56	<2.56
Benzo[a]pyrene	<2.03	<2.03	<2.03	<2.03	<2.03
Perylene	<1.21	<1.21	<1.21	<1.21	<1.21
Indeno [1,2,3-cd]pyrene	<1.41	<1.41	<1.41	<1.41	<1.41
Dibenzo[ah]anthracene	<2.56	<2.56	<2.56	<2.56	<2.56
Benzo(ghi)perylene	<10.3	<10.3	<10.3	<10.3	<10.3
ΣPAH	2596	6029	17,301	47,153	114,872

ΣPAH, total polycyclic aliphatic hydrocarbons.

Biochemical biomarkers

Fig. 1 presents the activity of antioxidant enzymes in the gill and digestive gland of oysters exposed to different diesel WAF concentrations. Antioxidant enzymes SOD, CAT and GPx showed the

same pattern of response in both tissues. SOD activity showed a decreasing trend response with a significant difference in oysters exposed to 20% WAF compared to control [$F_{(4,53)}$ = 3.171; p<0.05 for gill and $F_{(4,51)}$ = 2.941; p<0.05 for digestive gland] (Fig. 1A). Unlike the SOD activity, the levels of CAT and GPx activity did not differ between the experimental and control groups (p>0.05) (Figs. 1B and 1C, respectively).

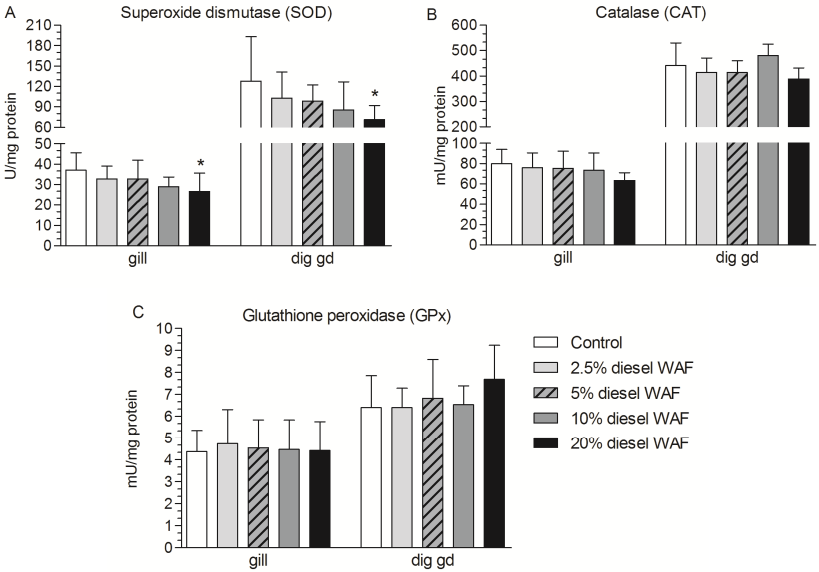


Figure 1. Superoxide dismutase (SOD) (A), catalase (CAT) (B) and glutathione peroxidase (GPx) (C) enzymes activity in the gill and digestive gland (dig gd) of the mangrove oyster *Crassostrea brasiliana* exposed for 96 h to four concentrations (2.5%, 5%, 10% and 20%) of diesel fuel water-accommodated fraction (diesel WAF), or seawater as the control group. Data are presented as mean + S.D. (n = 9 – 12 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc analysis. *p<0.05 when compared the diesel WAF exposed groups to the control for a given tissue.

Ancillary enzyme GR presented a distinct pattern between tissues. GR activity differed among experimental groups only in digestive gland with significantly higher activities in oysters exposed to 5%, 10% and 20% diesel WAF when compared to the control [$F_{(4,51)}$ = 11.52; p<0.001] (Fig. 2A). G6PDH however, did not differ among the experiment groups in both tissues (p>0.05) (Fig. 2B).

GGT activity also presented a different response pattern regarding the tissues. In gills, GGT activity was significantly induced in oysters exposed to 2.5% and 20% WAF. In digestive gland, no significant differences were observed (p>0.05) (Fig. 2C). GST activity was significantly higher in the digestive glands of oysters exposed to 10% and 20% diesel WAF than the control [$F_{(4,50)}$ = 4.532; p<0.001] (Fig. 2D). In gills, GST activity remained unchanged in the exposed oysters compared to unexposed ones (p>0.05) (Fig. 2D).

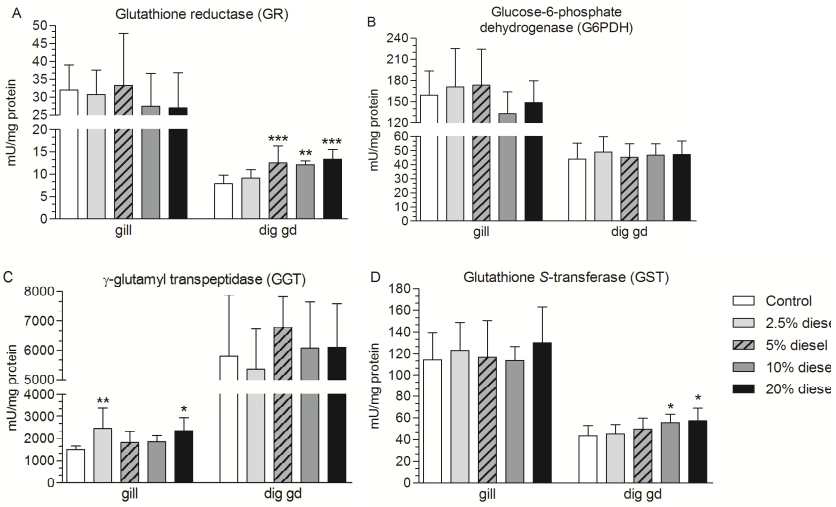


Figure 2. Glutathione reductase (GR) (A), glucose-6-phosphate dehydrogenase (G6PDH) (B), γ-glutamyl transpeptidase (GGT) (C) and glutathione *S*-transferase (GST) (D) enzymes activity in the gill and digestive gland (dig gd) of the mangrove oyster *Crassostrea brasiliana* exposed for 96 h to four concentrations (2.5%, 5%, 10% and 20%) of diesel fuel water-accommodated fraction (diesel WAF), or seawater as the control group. Data are presented as mean + S.D. (n = 9 – 12 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc analysis. *p<0.05; **p<0.01; ***p<0.001 when compared the diesel WAF exposed groups to the control for a given tissue.

The immunocontent of Hsp60, Hsp90 and the levels of lipid peroxidation in both gill and digestive gland are shown in Figs. 3A, 3B and 3C, respectively. Hsp60 levels of gill were significantly higher in the 2.5% and 20% groups, when compared to control [$F_{(4,48)}$ = 6.098; p<0.001]. In the digestive gland however, the Hsp60 levels were similar

among all the experimental groups (p>0.05) (Fig. 3A). Opposite to Hsp60, the levels of Hsp90 exhibited similar trends between tissues and were significantly lower in exposed groups compared to control [$F_{(4,51)}$ = 7.733; p<0.001 for gill and $F_{(4,52)}$ = 5.979; p<0.001 for digestive gland] (Fig. 3B).

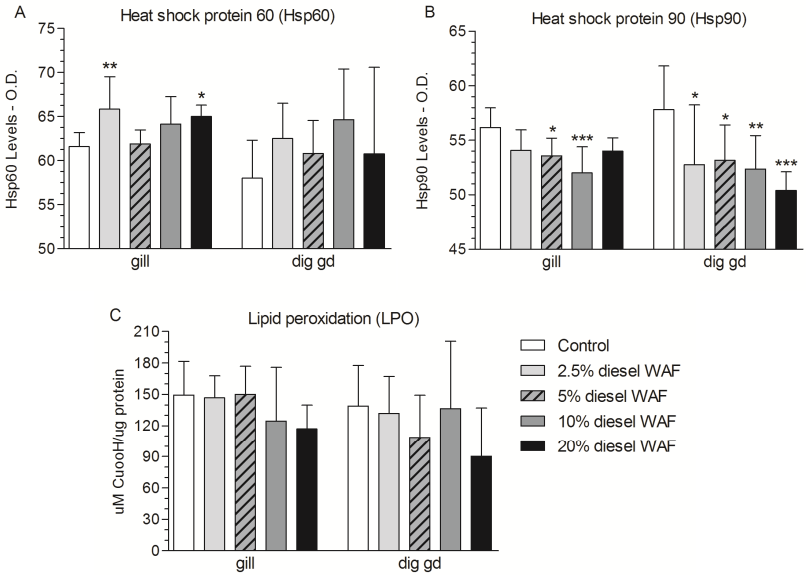


Figure 3. Immunocontent of heat-shock proteins Hsp60 (A) and Hsp90 (B) and lipid peroxidation levels (C) in the gill and digestive gland (dig gd) of the mangrove oyster *Crassostrea brasiliana* exposed for 96 h to four concentrations (2.5%, 5%, 10% and 20%) of diesel fuel water-accommodated fraction (diesel WAF), or seawater as the control group. Data are presented as mean + S.D. (n = 9 – 12 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc analysis. *p<0.05; **p<0.01; ***p<0.001 when compared the diesel WAF exposed groups to the control for a given tissue.

Significant correlations between the investigated biomarkers were found in both tissues. In gill, CAT activity was positively correlated with SOD (r = 0.45; p<0.001), GPx (r = 0.40, p<0.01), GR (r = 0.60; p<0.01) and G6PDH (r = 0.68, p<0.001) activities. In the same tissue, GST was positively correlated with GR (r = 0.43; p<0.01) and GPx activities (r = 0.61; p<0.01). Significant negative correlations were

found among LPO levels and GPx (r = -0.35, $p<0.05$) and GST activity (r = -0.28, $p<0.05$) and Hsp60 levels (r = -0.30, $p<0.05$) in the gill.
In digestive gland a positive correlation was found between GST and GR activities (r = 0.76; $p<0.001$), which was also correlated with GPx (r = 0.62; $p<0.001$). Significant negative correlations were identified among Hsp90 and GR (r = -0.47; $p<0.01$), GST (r = -0.48; $p<0.01$) and G6PDH (r = -0.32; $p<0.05$) activities. LPO levels were negatively correlated with GR activity (r = -0.42; $p<0.01$) and positively correlated with Hsp90 (r = 0.40; $p<0.01$).

Relationship between aliphatic and polycyclic aromatic hydrocarbons concentrations and biochemical parameters

The relationship between aliphatic and polycyclic aromatic hydrocarbons accumulated in oyster whole soft tissue and the biochemical parameters either in the gill or in the digestive gland of *C. brasiliana* was assessed by principal components analysis (PCA). Fig. 4 presents the PCA ordination output for both tissues (Fig. 4A for gill and Fig. 4B for digestive gland).

From the gill plot, the two main components explained 92.3% of the total variation. Component 1 explained 66.7%, while component 2 explained 25.6%. The ordination diagram presented a clear difference between treatments along component 1. The biochemical parameters Hsp60 levels, GST and GGT activities showed a positive correlation with component 1, which were higher in the highest diesel WAF concentration (20%) (Fig. 4A). On the other hand the same figure shows that the contents of Hsp90 and LPO together with the activities of SOD and CAT presented a negative correlation in component 1, being higher in control and lower in 20% diesel WAF concentration. Component 2 was positively correlated with G6PDH and GPx activities, which were higher in the 2.5% diesel WAF concentration (Fig. 4A).

From the digestive gland plot (Fig. 4B), the two main components explained 90.6% of total variation, where component 1 explained 78.2% and component 2 explained 12.4%. A similar pattern to the gill plot was noted in the digestive gland plot with a clear relationship between treatments and component 1. The biochemical parameters that showed a positive correlation with component 1 were mainly GR, GST and GPx activities, which were higher in the 20% diesel WAF concentration (Fig. 4B). A negative correlation with component 1 was observed mainly to SOD activity and LPO and Hsp90 levels that were higher in the control treatment. Component 2 showed a

positive correlation with CAT activity and Hsp60 levels, which were higher in the 10% diesel WAF concentration (Fig. 4B).

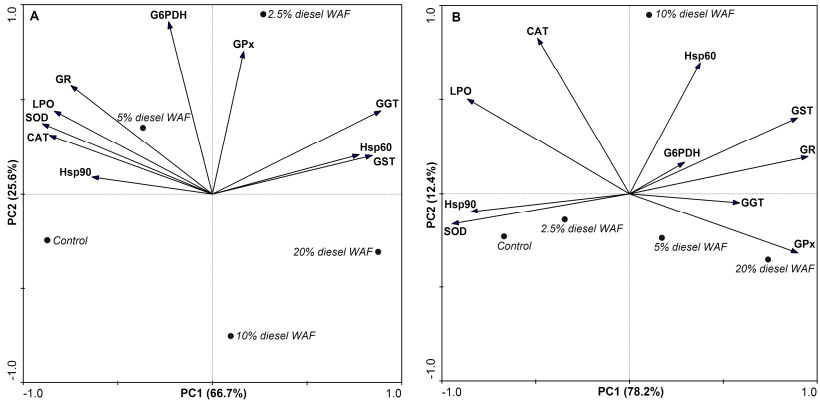


Figure 4. Biplot from principal components analysis (PCA) of all biochemical parameters related to the diesel WAF exposure in both gill (A) and digestive gland (B). In gill, the first axis explained 66.7% of overall variation and the second 25.6%. In digestive gland, the first axis explained 78.2% of overall variation and the second 12.4%.

Discussion

Oysters accumulated both aliphatic and aromatic hydrocarbons in a concentration-dependent manner reaching large values in the highest diesel WAF concentration (20%). Such an increase reflects their ability to bioaccumulate petrogenic hydrocarbons as previously reported in *Crassostrea virginica* (Noreña-Barroso et al., 1999; Sanders, 1995) and *Crassostrea gigas* (Bado-Nilles et al., 2010). Bivalves accumulate PAHs directly from the water-phase and food, and the organisms readily accumulate the more-soluble PAHs than the less-soluble and heavier PAHs (Neff, 2002). In fact, the total PAHs accumulated in oyster tissues were mostly due to the uptake of the 2 ring PAH non-parental naphthalene and the 3 ring PAHs phenanthrene. This can be explained by the increase of K_{ow} of the alkyl PAHs, which is also often associated with an increased trend to bioaccumulate when compared to parent compound PAHs (Irwin et al., 1997). Thus, the accumulation of low molecular weights PAHs reflects not only the chemical nature of diesel fuel itself, but also the uptake pathway for hydrocarbon accumulation by *C. brasiliana*.

The production of contaminant-stimulated reactive oxygen species (ROS) and their resulting oxidative damage seems to be the main mechanism of petroleum PAHs toxicity in marine bivalves (Banni et al., 2010; Bebbiano and Barreira, 2009; Frouin et al., 2007). To cope with this pro-oxidative challenge, bivalves generally produce increased SOD activity as the first line of enzymatic antioxidant defense (Ansaldo et al., 2005; Cheung et al., 2004; Lima et al., 2007). However, this inducibility should not be considered as a general rule since results have not always been consistent and no-effect or decreased SOD activity has been reported in mollusks exposed to environmental stressors (Cossu et al., 1997; Livingstone, 2001; Verlecar et al., 2008). Indeed, in the present study SOD activity of gill and digestive gland decreased with a marked difference in the highest diesel WAF concentration. According to Escobar et al. (1996), SOD activity can be inhibited by ROS, which could be a part of the diesel toxicity response in *C. brasiliana*.

CAT and GPx are known to serve as protective responses to scavenge the generation of ROS and enhanced activities represent an important adaptation to pollutant-induced stress (Cheung et al., 2001; Cossu et al., 1997; Richardson et al., 2008). However, the results obtained here show that diesel WAF did not elicit the expected effects on these enzymes in both tissues. Although not statistically significant, CAT activity in the gill followed the same decreased SOD activity trend for the highest diesel WAF concentration. This result offers evidence toward the functional linkage between SOD and CAT, which is further supported by the positive correlation found between their activities in the gill. On the other hand, digestive gland CAT and GPx activities of both tissues remained unchanged regardless the diesel concentration. Absence of induction of CAT and GPx activities has previously been reported in the mussel *Mytilus galloprovincialis* exposed to B[a]P for 72 hours (Banni et al., 2010) and *Perna viridis* treated with mercury chloride for 15 d (Verlecar et al., 2008). On the other hand, GPx had already been found to be a susceptible antioxidant in the freshwater bivalve *Unio tumidus* exposed to environmental contaminants (Cossu et al., 1997) and is considered an efficient protective enzyme against lipid peroxidation in aquatic organisms (Winston and Di Giulio, 1991). Together these results suggest that (i) despite the decrease in SOD and CAT activities together with the unchanged activity of GPx, antioxidant systems of *C. brasiliana* remained active enough to prevent lipid peroxidation and (ii) the oysters also used other approaches than

enzymatic antioxidants to cope with the toxic challenge in consequence to accumulated hydrocarbons.

GST activity was significantly increased in digestive gland of oysters exposed to diesel WAF. GST is involved in phase II of the biotransformation process by conjugating reduced glutathione (GSH) to different electrophilic compounds leading to their detoxification (Storey, 1996). GST is also thought to play a peroxidase activity, exerting a significant supplementary antioxidant role in the cell (Barata et al., 2005; Bebianno and Barreira, 2009), and previous studies have associated its induction to petroleum exposure in bivalves (Banni et al., 2010; Boutet et al., 2004; Lima et al., 2007; Silva et al., 2005; Solé et al., 2007). From the data obtained in this study, it appears clear that GST activity of the digestive gland was effective in preventing damage on lipid membranes of exposed oysters, verified as such by the unchanged LPO levels. A significant negative correlation between GST and LPO was also found in the gill, reinforcing the protective role of GST in *C. brasiliana* following diesel exposure.

This putative GST protection could be mediated by the increased GR activity seen in the digestive gland of exposed oysters. GR is a NADPH-dependent enzyme that plays an essential role in the maintenance of GST activity through the regeneration of GSH from GSSG (Verlecar et al., 2008). Hence, GR induction becomes important during stress conditions, which is seen here through the significant positive correlation between GR and GST activities in both the gill and digestive gland. This finding is consistent with recent reports carried out with mussels challenged with PAHs and other pollutants (Akcha et al., 2000; Gamble et al., 1995; Porte et al., 1991; Sáenz et al., 2010), indicating that GR activity, along with GST, could be sensitive indicators of hydrocarbon exposure in *C. brasiliana*. However, to keep GR activity, a constant NADPH-generating capacity is required within the cell. Such a feature can be achieved by the activity of G6PDH, an ancillary enzyme of the antioxidant defense system (Bainy et al., 1996). Although G6PDH activity did not differ among the experimental groups, a significant positive correlation was found between G6PDH and GST and GR activities in both tissues, suggesting that G6PDH, together with GR, is involved in the maintenance of reduced GSH levels, which in turn is used as substrate by GST (Sáenz et al., 2010).

GGT activity was the only enzyme of the glutathione pathway induced in the gills following the diesel WAF exposure. GGT is important for oxidant-challenged cells to maintain the intracellular GSH concentration, and an increase in such activity may represent a higher

availability of cysteine residues to GSH formation (Liu et al., 1998). GSH comprises the main non-protein antioxidant defense against oxidative injuries (Farid et al., 2009; Halliwell and Gutteridge, 2007; Hannam et al., 2010), and from the data obtained in this study we can hypothesize that the enhanced GGT activity kept the cellular redox balance in the gill of oysters exposed to diesel WAF. The enhanced gill GGT activity together with the unchanged GR and GST activities suggests that oyster tissues differ in the maintenance of intracellular GSH levels.

Diesel fuel WAF was also able to induce a significant increase of Hsp60 immunocontent in the gill. Although poorly understood in marine invertebrates in terms of pollutant response, Hsp60 is thought to be strongly induced by petroleum hydrocarbons (Snyder et al., 2001; Wheelock et al., 2002), as previously observed by Downs et al. (2001), Oberdörster et al. (1999) and Wolfe et al. (1999). Differential expression of Hsp60 in response to diesel WAF indicates a particular physiological condition for the mitochondria. According to Downs et al. (2001), Hsp60 is a biomarker for the rate of protein mitochondrial turnover, which is expected to be higher under cellular stress, especially in the presence of ROS. Increased levels of Hsp60 suggest that protein denaturation, protein import and synthesis increased in the gill cells of exposed oysters. Moreover, these data indicate that the stress response of oysters in terms of Hsp60 induction in gill is a rapid reaction to short term events whereas hepatic tissues require the accumulation of proteotoxic chemicals following chronic exposure, such as observed in fish (Triebskorn et al., 2002; Webb and Gagnon, 2009). Hence, Hsp60 induction seems to be an adaptive mechanism of this tissue against diesel generated toxicity, and is suggested as a sensitive biomarker for mitochondrial protein turnover in *C. brasiliana* when the stressor is diesel fuel.

Inversely, diesel fuel lead to a significant concentration-dependent inhibition of Hsp90 levels in both tissues, with a marked response in the digestive gland. This was a surprising result in our work, since Hsp90 is a ubiquitous multifunctional chaperone that is involved in protein folding, cytoprotection, as well as in number of cellular regulatory pathways following a stressor challenge (Li et al., 2009). Based on our data, we can speculate that diesel WAF inhibited the synthesis of Hsp90 by some alteration on a transcriptional or translational level, or by the inhibition of the signal-transduction cascade for induction of Hsp90 (Downs et al., 2001). Moreover, we cannot discount the idea that the cells from both gill and digestive gland

are protected by the action of the remaining Hsp90 or other proteins, precluding the need for protein chaperoning in the cytoplasm.

Our results also corroborate the different mechanisms in mollusk tissues that cope with the stress from hydrocarbons exposure (Banni et al., 2010). According to the PCA results, the gill and digestive gland of *C. brasiliana* show some differences in terms of biochemical response when exposed to diesel fuel WAF. The differences relate to the putative regulation of the glutathione pathway, which in gill it is achieved through an induced GGT activity, and in digestive gland through enhanced GR activity. Moreover, gill and digestive gland also differ regarding the chaperone role and the phase II biotransformation process, shown here as Hsp60 levels and GST activity. Regardless the tissue, PCA also showed that 20% diesel WAF was indeed the concentration with more influence on the biochemical responses elicited in *C. brasiliana* after 96 h of exposure.

Conclusions

In conclusion, the mangrove oyster *C. brasiliana* exposed to four sublethal concentrations of diesel fuel water accommodated fraction (WAF) is able to bioaccumulate both aliphatic and aromatic hydrocarbons in a concentration-dependent manner in a short period of time. In this process, toxic effects were identified in terms of biochemical biomarkers responses, which lead to an induction of protective phase II and ancillary enzymes GST and GR in digestive gland, and an elevation of Hsp60 levels and GGT activity in gill tissue. The results indicate that oyster's tissues handle differently during a chemical exposure, which might reflect the differences in the mechanism in which the tissues cope to the stress. The gill seemed to be a target tissue for short-term events while the digestive gland may be of greater value to determine the oyster's stress response under chronic exposure to stressors in its environment. Moreover, this study provided evidence towards the suitability of using a set of putative biochemical biomarkers in the oyster *C. brasiliana* for the monitoring of oil and gas activities in Brazil.

Acknowledgments

This work was supported by a grant (550706/2005-4) from CNPq-CTPetro to ACDB. The Brazilian agencies Capes (KHL; JJM and MNS) and CNPq (ACDB; TSD and F.G.D.J.) fellowships are acknowledged. We are grateful to Dr. Cláudio M.R. Melo and to M.Sc. Carlos H.A.M. Gomes for supplying the oysters used in this study.

References

Akcha, F., Izuel, C., Venier, P., Budzinski, H., Burgeot, T., Narbonne, J.-F., 2000. Enzymatic biomarker measurement and study of DNA adduct formation in benzo[a]pyrene-contaminated mussels, *Mytilus galloprovincialis.* Aquat. Toxicol. 49, 269–287.

Altenburger, R., Segner, H., Van dar Oost, R., 2003. Biomarkers and PAHs prospects for the assessment of exposure and effects in aquatic systems. In: Douben, P.E.T. (Ed.), PAHs: An Ecotoxicological Perspective. Wiley, Chichester, England, pp. 147–171.

Ansaldo, M., Najle, R., Luquet, C.M., 2005. Oxidative stress generated by diesel seawater contamination in the digestive gland of the Antarctic limpet *Nacella concinna*. Mar. Environ. Res. 59, 381–390.

Bado-Nilles, A., Renault, T., Faury, N., Le Floch, S., Quentel, C., Auffret, M., Thomas-Guyon, H., 2010. *In vivo* effects of LCO soluble fraction on immunerelated functions and gene transcription in the Pacific oyster, *Crassostrea gigas* (Thunberg). Aquat. Toxicol. 97, 196–203.

Bainy, A.C.D., Saito, E., Carvalho, P.S.M., Junqueira, V.B.C., 1996. Oxidative stress in gill, erythrocytes, liver and kidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. Aquat. Toxicol. 34, 151–162.

Banni, M., Negri, A., Dagnino, A., Jebali, J., Ameur, S., Boussetta, H., 2010. Acute effects of benzo[a]pyrene on digestive gland enzymatic biomarkers and DNA damage on mussel *Mytilus galloprovincialis*. Ecotoxicol. Environ. Saf. 73, 842–848.

Barata, C., Lekumberri, I., Vila-Escalé, M., Prat, N., Porte, C., 2005. Trace metal concentration, antioxidant enzyme activities and susceptibility to oxidative stress in the tricoptera larvae *Hydropsyche exocellata* from the Llobregat river basin (NE Spain). Aquat. Toxicol. 74, 3–19.

Baumard, P., Budzinski, H., Garrigues, P., Narbonne, J.F., Burgeot, T., Michel, X., Bellocq, J., 1999. Polycyclic aromatic hydrocarbon (PAH) burden of mussels (*Mytilus* sp.) in different marine environments in relation with sediment PAH contamination, and bioavailability. Mar. Environ. Res. 47, 415–439.

Bebianno, M.J., Barreira, L.A., 2009. Polycyclic aromatic hydrocarbons concentrations and biomarker responses in the clam *Ruditapes decussates* transplanted in the Ria Formosa lagoon. Ecotoxicol. Environ. Saf. 72, 1849–1860.

Beutler, E., 1975. Red cell metabolism: a manual of biochemical methods, Grune & Stratton, New York.

Boutet, I., Tanguy, A., Moraga, D., 2004. Response of the Pacific oyster *Crassostrea gigas* to hydrocarbon contamination under experimental conditions. Gene. 329, 147–157.

Carlberg, I., Mannervik, B., 1985. Glutathione reductase. Meth. Enzymol. 113, 484–490.

Cheung, C.C.C., Zheng, G.J., Li, A.M.Y., Richardson, B.J., Lam, P.K.S., 2001. Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*. Aquat. Toxicol. 52, 189–203.

Cheung, C.C.C., Siu, W.H.L., Richardson, B.J., De Luca-Abbott, S.B., Lam, P.K.S., 2004. Antioxidant responses to benzo[a]pyrene and Aroclor 1254 exposure in the green-lipped mussel, *Perna viridis*. Environ. Pollut. 128, 393–403.

Circu, M.L., Aw, T.Y., 2010. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic. Biol. Med. 15, 749–62.

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

Downs, C.A., Dillon Jr, R.T., Fauth, J.E., Woodley, C.M., 2001. A molecular biomarker system for assessing the health of gastropods (*Ilyanassa obsoleta*) exposed to natural and anthropogenic stressors. J. Exp. Mar. Biol. Ecol. 259, 189–214.

Escobar, J.A., Rubio, M.A., Lissi, E.A., 1996. SOD and catalase inactivation by singlet oxygen and peroxyl radical. Free Radic. Biol. Med. 20, 285–290.

Farid, N.M., Hamed, R.R., Shokeer, A.G., 2009. Glutathione and its Related Enzymes in Fasciola Snails (*Lymnaea natalensis*): Purification and Characterization of Glutathione Transferase. Res. J. Agric. Biol. Sci. 5, 317–325.

Frouin, H., Pellerin, J., Fournier, M., Pelletier, E., Richard, P., Pichaud, N., Rouleau, C., Garnerot, F., 2007. Physiological effects of polycyclic aromatic hydrocarbons on soft-shell clam *Mya arenaria*. Aquat.Toxicol. 82, 120–134.

Gamble, S.C., Goldfarb, P.S., Porte, C., Livingstone, D.R., 1995. Glutathione Peroxidase and other antioxidant enzyme function in marine invertebrates (*Mytilus edulis*, *Pecten maximus*, *Carcinus maenas* and *Asterias rubens*). Mar. Environ. Res. 39, 191–195.

Glock, G.E., McLean, P., 1953. Further studies on the properties and assay of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. Biochem. J. 55, 400–408.

Halliwell, B., Gutteridge, J., 2007. Free Radicals in Biology and Medicine, Oxford University Press, Nova York.

Hannam, M.L., Bamber, S.D., Galloway, T.S., Moody, A.J., Jones, M.B., 2010. Effects of the model PAH phenanthrene on immune function and oxidative stress in the haemolymph of the temperate scallop *Pecten maximus*. Chemosphere. 78, 779–784.

Hermes-Lima, M., Willmore, W.G., Storey, K., 1995. Quantification of lipid peroxidation in tissue extracts based on Fe(III)xilenol orange complex formation. Free Radic. Biol. Med. 19, 271–280.

Irwin, R.J., Van Mouwerik, M., Stevens, L., Seese, M.D., Basham, W., 1997. Environmental Contaminants Encyclopedia. National Park Service, Water Resources Division, Fort Collins, Colorado. Distributed within the Federal Government as an Electronic Document (Projected public availability on the internet or NTIS: 1998).

Kalmar, B., Greensmith, L., 2009. Induction of heat shock proteins for protection against oxidative stress. Adv. Drug. Deliv. Rev. 28, 310–318.

Keen, J.H., Habig, W.H., Jakoby, W.B., 1976. Mechanism for several activities of the gluthatione *S*-transferases. J. Biol. Chem. 251, 6183–6188.

Kennish, M.J., 1992. Ecology of Estuaries: Anthropogenic Effects. Londres: CRC Press, p. 494.

Li, F., Luan, W., Zhang, C., Zhang, J., Wang, B., Xie, Y., Li, S., Xiang, J., 2009. Cloning of cytoplasmic heat shock protein 90 (FcHSP90) from *Fenneropenaeus chinensis* and its expression response to heat shock and hypoxia. Cell Stress Chaperones. 14, 161–172.

Lima, I., Moreira, S.M., Rendón-Von Osten, J., Soares, A.M.V.M., Guilhermino, L., 2007. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. Chemosphere. 66, 1230–1242.

Liu, R.-M., Shi, M.M., Giulivi, C., Forman, H.J., 1998. Quinones increase g-glutamyl transpeptidase expression by multiple mechanisms in rat lung epithelial cells. Am. J. Physiol. Lung Cell. Mol. Physiol. 274, 330–336.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.

MacLeod, W.D., Brown, D.W., Friedman, A.J., Burrows, D.G., Maynes, O., Pearce, R.W., Wigren, C.A., Bogar, R.G., 1985. Standard Analytical Procedures of the NOAA National Analytical Facility, 1985–1986. Extractable Toxic Organic Components, 2nd ed. U.S. Department of Commerce, NOAA Technical Memo, NMFS-F/NWC-9. NOAA Northwest Fisheries Science Center, Seattle,WA, p. 121.

Neff, J.M., 2002. Polycyclic aromatic hydrocarbons in the ocean. In: Neff, J.M. (Ed.), Bioaccumulation in Marine Organisms: Effect of Contaminants from Oil Well Produced Water. Elsevier, Amsterdam, pp. 241–318.

Noreña-Barroso, E., Gold-Bouchot, G., Zapata-Perez, O., Sericano, J.L., 1999. Polynuclear Aromatic Hydrocarbons in American Oysters *Crassostrea virginica* from the Terminos Lagoon, Campeche, Mexico. Mar. Pollut. Bull. 38, 637–645.

Oberdörster, E., Martin, M., Ide, C.F., McLachlan, J.A., 1999. Benthic community structure and biomarker induction in grass shrimp in an estuarine system. Arch. Environ. Contam. Toxicol. 37, 512–518.

Peterson, G.L., 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 83, 346–356.

Pie, M.R., Ribeiro, R.O., Boeger, W.A., Ostrensky, A., Falleiros, R.N., Angelo, L., 2006. A simple PCR-RFLP method for the discrimination of native and introduced oyster species (*Crassostrea brasiliana*, *C. rhizophorae* and *C. gigas*; Bivalvia: Ostreidae) cultured in Southern Brazil. Aquacult. Res. 37, 1598–1600.

Porte, C., Sole, M., Albaiges, J., Livingstone, D.R., 1991. Responses of mixed-function oxygenase and antioxidase enzyme system of *Mytilu*s sp. to organic pollutants. Comp. Biochem. Physiol. 100 C, 183–186.

Readman, J.W., Fillmann, G., Tolosa, I., Bartocci, J., Villeneuve, J.-P., Catinni, C., Mee, L.D., 2002. Petroleum and PAH contamination of the Black Sea. Mar. Pollut. Bull. 44, 48–62.

Richardson, B.J., Mak, E., Luca-Abbott, S.B.D., Martin, M., McClellan, K., Lam, P.K.S., 2008. Antioxidant responses to polycyclic aromatic hydrocarbons and organochlorine pesticides in green-lipped mussels (*Perna viridis*): Do mussels ‘‘integrate” biomarker responses? Mar. Pollut. Bull. 57, 503–514.

Sáenz, L.A., Seibert, E.L., Zanette, J., Fiedler, H.D., Curtius, A.J., Ferreira, J.F., Almeida, E.A., Marques, M.R.F., Bainy, A.C.D., 2010. Biochemical biomarkers and metals in *Perna perna* mussels from mariculture zones of Santa Catarina, Brazil. Ecotoxicol. Environ. Saf. 73, 796–804.

Sanders, M., 1995. Distribution of polycyclic aromatic hydrocarbons in oyster (*Crassostrea virginica*) and surface sediment from two estuaries in South Carolina. Arch. Environ. Contam. Toxicol. 28, 397–405.

Sies, H., Koch, O.R., Martino, E., Boveris, A., 1979. Increased biliary glutathione disulfide release in chronically ethanol treated rats. FEBS Lett. 103, 287–290.

Silva, A.Z., Zanette, J., Ferreira, J.F., Guzenski, J. Marques, M.R.F., Bainy, A.C.D., 2005. Effects of salinity on biomarker responses in

Crassostrea rhizophorae (Mollusca, Bivalvia) exposed to diesel oil. Ecotoxicol. Environ. Saf. 62, 376–382.

Singer, M.M., Aurand, D., Bragin, G.E., Clark, J.R., Coelho, G.M., Sowby, M.L., Tjeerdema, R.S., 2000. Standardization of the preparation and quantitation of water-accommodated fractions of petroleum for toxicity testing. Mar. Pollut. Bull. 40, 1007–1016.

Snyder, M.J., Girvetz, E., Mulder, E.P., 2001. Induction of marine mollusc stress proteins by chemical or physical stress. Environ. Contam. Toxicol. 41, 22–29.

Solé, M., Buet, A., Ortiz, L., Maynou, F., Bayona, J.M., Albaigés, J., 2007. Bioaccumulation and biochemical responses in mussels exposed to the water-accommodated fraction of the Prestige fuel oil. Sci. Mar. 71, 373–382.

Storey, K.B., 1996. Oxidative stress: animal adaptations in nature. Braz. J. Med. Biol. Res. 29, 1715–1733.

Triebskorn, R., Adam, S., Casper, H., Honnen, W., Pawert, M., Schramm, M., Schwaiger, J., Köhler, H.-R., 2002. Biomarkers as diagnostic tools for evaluating effects of unknown past water quality conditions on stream organisms. Ecotoxicology. 11, 451–465.

Verlecar, X.N., Jena, K.B., Chainy, G.B.N., 2008. Modulation of antioxidant defences in digestive gland of *Perna viridis* (L.), on mercury exposures. Chemosphere. 71, 1977–1985.

Webb, D., Gagnon, M.M., 2009. The value of stress protein 70 as an environmental biomarker of fish health under field conditions. Environ. Toxicol. 24, 287–295.

Wendel, A., 1981. Glutathione peroxidase. Meth. Enzymol. 77, 325–333.

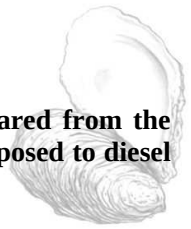
Wertheim, P.H., 2009. Brazilian pre-salt reserves bring role of oil industry into question. Oil&Gas Journal.

Wheelock, C.E., Baumgartner, T.A., Newman, J.W., Wolfe, M.F., Tjeerdema, R.S., 2002. Effect of nutritional state on HSP60 levels in the rotifer *Brachionus plicatilis* following toxicant exposure. Aquat. Toxicol. 61, 89–93.

Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat. Toxicol. 19, 137–161.

Wolfe, M.F., Olsen, H.E., Gasuad, K.A., Tjeerdema, R.S., Sowby, M.L., 1999. Induction of heat-shock protein 60 in *Isochrysis galbana* exposed to sublethal preparation of dispersant and Prudhoe Bay crude oil. Mar. Environ. Res. 7, 473–489.

Zar, J.H., 1999. Biostatistical analysis, Prentice-Hall, Englewood Cliffs.



Capítulo 3

Suppressive subtractive hybridization libraries prepared from the digestive gland of the oyster *Crassostrea brasiliana* exposed to diesel fuel water-accommodated fraction

Karim H. Lüchmann[†]; Jacó J. Mattos[†]; Marília N. Siebert[†]; Tarquin S. Dorrington[†]; Guilherme Toledo-Silva[†,‡]; Patricia H. Stoco[§]; Edmundo C. Grisard[§]; Afonso C.D. Bainy[†]

Aceito para publicação na Environmental Toxicology and Chemistry

[†]*Laboratório de Biomarcadores de Contaminação Aquática e Imunoquímica, Departamento de Bioquímica, Núcleo de Estudos em Patologia Aquícola, Universidade Federal de Santa Catarina, Florianópolis, SC, 88034-257, Brazil;* [‡]*Laboratório de Análise Genética e Molecular, Centro de Biologia Molecular e Engenharia Genética, Instituto de Biologia, Departamento de Genética e Evolução, Universidade Estadual de Campinas, Campinas, SP, 13083-875, Brazil;* [§]*Laboratório de Protozoologia, CCB, Universidade Federal de Santa Catarina, Florianópolis, SC, 88049-900, Brazil*

Abstract

Diesel fuel can cause adverse effects in marine invertebrates by mechanisms that are not clearly understood. The authors used suppressive subtractive hybridization (SSH) to identify genes up- and down-regulated in *Crassostrea brasiliana* exposed to diesel fuel. Genes putatively involved in protein regulation, innate immune, and stress response, were altered by diesel challenge. Three genes regulated by diesel were validated by quantitative real-time polymerase chain reaction (qPCR). This study sheds light on transcriptomic responses of oysters to diesel pollution.

Keywords: Oyster, *Crassostrea brasiliana*, Gene transcription, SSH, Diesel fuel

Capítulo publicado no jornal Environmental Toxicology and Chemistry, **Wiley/Blackwell Publisher DOI: 10.1002/etc.1837**
Disponível em:
http://onlinelibrary.wiley.com/doi/10.1002/etc.1837/abstract.

Capítulo 4

Transcriptome pyrosequencing and *de novo* analysis for the mangrove oyster *Crassostrea brasiliana* exposed to environmental contaminants: application for discovering candidate genes for ecotoxicological studies

Karim H. Lüchmann[1], Jack A. Gilbert[2], John A. Craft[3], J. Kevin Chipman[4], Tarquin S. Dorrington[1], Jacó J. Mattos[1], Marília N. Siebert[1], Afonso C.D. Bainy[1], Declan C. Schroeder[5*]

Preparado para submissão na BMC Genomics

[1]Biochemistry Department, Federal University of Santa Catarina, Florianópolis 88040-900, SC, Brazil; [2]Argonne National Laboratory, Chicago, United States of America; [3]Biological and Biomedical Sciences, Glasgow Caledonian University, Glasgow, United Kingdom; [4]School of Biological Sciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom; [5]Marine Biological Association of the United Kingdom (MBA), Citadel Hill, Plymouth PL1 2PB, United Kingdom.

Abstract

***Background*:** Although bivalves are among the most studied aquatic organisms due to their ecological role, economic importance and use as bioindicators in pollution biomonitoring, very little information is available on their genome sequences. This study reports the *de novo* transcriptome sequencing using 454 GS-FLX from the gill and digestive gland of the tropical oyster *Crassostrea brasiliana* challenged with environmental contaminants. The transcriptomic sequences should be useful for gene discovery, and to help to elucidate the mechanism of toxicity of petroleum by-products and domestic sewage.

***Results*:** Using 454 pyrosequencing, we obtained a total of 399,291 reads consisting in 96,582,549 bp, which were *de novo* assembled into 20,938 contigs, with an average size of 575 bp, representing a dramatic expansion of existing cDNA sequences available for *C. brasiliana*. Blast searching of non-redundant public databases returned 7,401 significant matches to Eukaryotic proteins. In depth analysis of the data revealed an extensive catalogue of the xenobiotics biotransformation system and the major antioxidant proteins. An initial assessment of putative genes of ecotoxicology interest allowed us to identify 41 CYP450s, 26 GSTs, 8 SULTs, 9 MRPs and 28 genes putatively involved in the antioxidant defence.

***Conclusions*:** We show that second-generation sequencing provided an inedited and reliable reference transcriptome of a non-model species. This resource provides clues to the identification of potential biotransformation and antioxidant genes involved in the detoxification of environmental contaminants of *C. brasiliana*, and lays the foundation for future functional genomics studies.

Background

The mangrove oysters, *Crassostrea brasiliana*, are commonly distributed along the Brazilian coast where they play a relevant economic and ecological role. Oysters, as other bivalve molluscs, are sessile, filter feeders, accumulate various water contaminants in their tissues and may be considered as an ideal bioindicator for pollution monitoring in coastal waters in many areas of the world (Bado-Nilles et al., 2010). However, such as for other Ostreidae species, there are many aspects of their biology which require elucidation. For instance, the endpoints used for monitoring effects are based on a small number of

biomarkers which have their origins in human toxicology and are not bivalve-specific (Forbes et al., 2006).

In view of the ecological and economical importance of oysters to the coastal areas, understanding their biology, susceptibility to pollutants and differential stress resistance has become an important issue for modern ecotoxicology. In particular, genomic resources such as genome or transcriptome sequences would make possible studies to address these processes. However, genomic data available for oysters are limited (Tanguy et al., 2008), and recently sequence-based strategies have been developed for transcriptome studies. Among them, Expressed Sequence Tags (ESTs) sequencing programs have proven to be an effective method for gene discovery and have been widely used for initiating genomic research in non-model and un-or under- annotated organisms such as oysters (Fleury et al., 2009; Tanguy et al., 2008; Wang and Guo, 2007). ESTs collections provide information on the part of the genome that is expressed and can be valuable for genome annotation and analysis, discovery of single nucleotide polymorphisms (SNPs), and expression studies (Joubert et al., 2010). However, genomic sequences resources available for *C. brasiliana* are extremely limited.

Traditionally, ESTs projects of *Crassostrea* sp. have been based on classical cloning and Sanger sequencing strategies, but because next-generation sequencing technologies provide much higher throughput than Sanger sequencing at a lower cost (Ekblom and Galindo, 2011), these new technologies have been showed great potential for expanding sequence database of bivalves and other non-model species (*i.e.* Clark et al., 2010; Craft et al., 2010; Hou et al., 2011; Joubert et al., 2010; Meyer et al., 2009). For those organisms where the genome data is limited, the 454 pyrosequencing platform is most frequently used, as longer reads are generated and then are more amenable to further annotation and analysis (Kumar and Blaxter, 2010).

With the aim of increasing the genomic resources for the mangrove oyster *C. brasiliana*, we performed the first *de novo* transcriptome pyrosequencing for this species. More specifically, the objectives of this study are to discover genes that encode enzymes putatively involved in the biotransformation of xenobiotics and stress response in the oyster *C. brasiliana* exposed to three different environmental contaminants: phenanthrene, diesel fuel water-accommodated fraction (WAF) and domestic sewage. The rational for selecting those contaminants in this study lies in their prevalence in aquatic environments and importance as

chemical models for ecotoxicological studies. Phenanthrene, a 3-ring compound included in the US-EPA priority pollutant list, is one of the most abundant PAHs in the aquatic environment as a result of human activities (US EPA, 2009). This lipophilic and low molecular weight PAH can be easily taken up by aquatic organisms (Oliveira et al., 2007), with a greater bioaccumulation rate in bivalve molluscs (Hannam et al., 2010). In contrast, diesel WAF comprises a model for complex mixtures derived from petroleum industry activities. Diesel fuel is one of the most common aquatic contaminants which has recently been showed to exert biochemical effects and bioaccumulation trends in *C. brasiliana* (Lüchmann et al., 2011). Furthermore, domestic sewage was chosen based on the high inputs of untreated sewage discharges in coastal ecosystems around the world and its effects on transcriptional levels of oysters (Medeiros et al., 2008).

C. brasiliana was challenged to each contaminant separately and total RNA was extracted from the gill and digestive gland. Libraries of cDNA were constructed from total RNA and non-normalized libraries were sequenced using the 454 GS-FLX platform. The transcriptome was assembled using the pool of sequencing data from all cDNA libraries and resulting contigs were annotated given raise to the first *de novo* transcriptome for the mangrove oyster *C. brasiliana*. Sequences were screened to identify relevant genes involved in the biotransformation of xenobiotics and associated antioxidant defence. Results demonstrated the capability of using 454 sequencing data to identify genes of interest on a non-model species with promising relevance for ecotoxicology studies and aquatic monitoring programs.

Materials and methods

Oyster collection and chemical exposures

Specimens of the mangrove oyster, *C. brasiliana*, of similar shell length (5-8 cm), were obtained from an oyster farming area at the Marine Mollusks Laboratory, Federal University of Santa Catarina, Florianópolis, southern Brazil, and were immediately transported to the University laboratory where they were maintained in the aerated tanks with 0.45 μm filtered seawater at 21 °C and 25 ppt. Oysters were fed on microalgae (*Chaetoceros muelleri* and *Isochrysis* sp.) twice a day at a density of 3.3×10^6 cels.mL^{-1} and 2.2×10^6 cels.mL^{-1}, respectively, and water was changed daily for at least 7 days for acclimatization prior to the exposure experiments. Oysters were then randomly divided into

glass exposure tanks and held fasting for 24 hours prior to the chemical exposures at the same previous seawater conditions. The exposure experiments to diesel fuel water-accommodated fraction (WAF), phenanthrene (PHE) and domestic sewage were carried out in different occasions but the oysters were supplied from the same brood stock of the same mollusc culture facility, and were submitted to the same acclimatization process as described above.

Groups of 10 oysters were exposed for 24 and 72 h to 10% of diesel fuel diesel WAF (v/v), with seawater used as the control group. Diesel fuel was purchased at a Petrobras petrol station and WAF was obtained according to Singer et al. (2000) with minor modifications. Briefly, one part (1 L) of fresh diesel fuel was diluted with nine parts (9 L) of the 0.45 μm filtered seawater (salinity 25 ppt) in a sealed 14 L glass flask protected from light, in order to minimize evaporation and degradation of the fuel components. The diesel–water mixture was stirred for 23 h with the homogenizer Glas-Col (LLC) using a steel modified pestle at 1600 rpm at a constant temperature of 21 °C. The mixture was then made to stand for 1 h before the lower layer of water (diesel WAF) was transferred into the aquaria. The 10% diesel WAF was prepared through dilution of the WAF with the control seawater. The animals were not fed during the whole exposure period and the experiment was carried out in duplicate. No mortality was observed in both the control and treated groups. The diesel WAF concentration was chosen based on previous results of biochemical biomarkers measured in *C. brasiliana* (Lüchmann et al., 2011).

Phenanthrene (PHE) (Sigma-Aldrich, P1140-9) was first dissolved in dimethyl sulfoxide (DMSO) and then added 0.45 μm filtered seawater (salinity 25 ppt) to achieve a final nominal PHE concentration of 1000 $\mu g.L^{-1}$, equivalent to 5.6 μM, and a final DMSO concentration of 0.01% (v/v). Oysters were transferred to glass tanks which were covered to avoid evaporation of PHE. Oysters were exposed for 24 h and were not fed during the exposure period. Control was carried out at the same condition except for introducing 0.01% (v/v) DMSO only. No mortality was observed in both the control and treated groups. The sublethal PHE concentration was chosen based on realistic environmental concentrations found nearby petroleum exploration area (Anyakora et al., 2005).

Sewage exposure was performed according to Medeiros et al. (2008) with minor modifications. Briefly, domestic sewage was collected at the influent duct of the downtown wastewater treatment

plant (Florianópolis, southern Brazil) after solid material grid removal, and diluted to 33% (v/v) using 0.45 μm filtered seawater (salinity 25 ppt). Oysters were placed in glass tanks, where the chemical challenge took place. Exposure was carried out for 24 h and oysters were not fed during the exposure period. Control was carried out at the same condition. No mortality was observed in both the control and treated groups.

After chemical exposures, twelve oysters from diesel fuel WAF and phenanthrene experiments, and seven from the domestic sewage exposure were sacrificed and the gill and digestive gland were immediately excised, flash frozen in liquid nitrogen and stored at -80 °C until further analysis. Total RNA used for the control group was extracted from three oysters of each control group used in the experiments cited before totalizing twelve animals.

Total RNA isolation and preparation of cDNA libraries

Total RNA from gill and digestive gland of each oyster was individually isolated using TRIzol reagent (Invitrogen, UK) and purified with the Nucleospin RNA II Total RNA Isolation Kit (AbGene, UK) following the supplier's protocol with minor modifications. Briefly, 50 – 100 mg of each tissue was mechanically disrupted in the presence of 1 mL TRIzol using a homogenizer (Tissue-Tearor, BioSpec Products). TRIzol protocol was strictly followed until achieve the upper aqueous phase, where 200 μL was transferred to a new tube for on-column precipitation using the Nucleospin RNA II Total RNA Isolation Kit (AbGene, UK). RNA was then eluted in 60 μL of RNase free water. The residual genomic DNA contamination was removed during the RNA cleanup using the DNase I digestion as instructed by the manufacturer (AbGene, UK). The integrity of the purified total RNA was assessed using formaldehyde agarose gel electrophoresis, and RNA quantity was determined by NanoDrop ND-1000 spectrophotometer (Thermo Scientific, UK).

Equal quantities of purified total RNA were pooled into 10 samples: gill control, digestive gland control, gill diesel WAF 24 h, digestive gland diesel WAF 24 h, gill diesel WAF 72 h, digestive gland diesel WAF 72 h, gill phenanthrene, digestive gland phenanthrene, gill sewage, and digestive gland sewage, and the pools were used for synthesis of non-normalized full-length double-stranded cDNA (ds-cDNA). cDNA libraries were constructed for each sample using the

SMARTer PCR cDNA Synthesis Kit (Clontech, Paris) according to the manufacturer's instructions. Full-length ds-cDNA templates were then amplified by long-distance PCR using the Advantage 2 PCR Kit (Clontech, Paris). To ensure that the PCR products were not over amplified, the optimal number of PCR cycles was determined according to the manufacturer's guidelines, which was verified by agarose gel electrophoresis. The products were purified with DNA Clean & Concentrator™-5 Kit (Zymo Research, USA). The amplified cDNA libraries were verified for quality by microcapillary electrophoresis (Agilent Bioanalyzer 2100, Agilent Technologies) and quantified using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, UK).

Transcriptome pyrosequencing

cDNA libraries obtained from the gill and digestive gland of each treatment were pooled into four samples based on the tissue and treatment: (1) gill and digestive gland control, (2) gill and digestive gland sewage, (3) gill and digestive gland phenanthrene, (4) gill and digestive gland 24 h and 72 h diesel WAF. The libraries were then submitted to size-selection in a gel and cDNA fragments larger than 700 bp were sheared by nebulisation into smaller pieces (500 – 700 bp) to produce short, random fragments appropriate for 454 sequencing. Each library was sequenced twice (large and small fragments) and MID tags were used to enable subsequent identification of size-selected portions from each library dataset. The eight tagged cDNAs libraries (two for each cDNA library) were then combined and pyrosequenced on the half of a PicoTiter Plate using the 454 Genome Sequencer FLX System according to the manufacturers' instructions (454/Roche, http://www.454.com). Pyrosequencing was performed by the Center for Genomic Research at the University of Liverpool.

Sequence data analysis and assembly

The raw sequence data obtained from the four cDNA libraries were pooled and subject to filtering and trimming of SMARTer™ adaptors for cDNA synthesis, primers, poly (A/T) tails and potential contaminating vector sequences. Following the sequence trimming and size selection (>150 bp), the reads were assembled using SeqMan NGen v 3.0.4 (DNASTAR). Default parameters for *de novo* assembly of '454' reads were used, except for two settings: the 'Min Match Percentage'

was set to 80 (instead of 85) and minimum number of reads to form a consensus sequences, which was set to a minimum of 2 (instead of 10). The first parameter represents the minimum percentage of identity required to join two sequences in the same contig and the second was chosen in order to assemble low frequent reads, which can represent weakly transcribed genes.

Functional annotation

Following the *de novo* assembly, contigs and singletons were compared to NCBI's non-redundant (nr) nucleotide database using blastn (*E* value threshold of 10^{-6}) to identify putative contamination by ribosomal RNA, genomic DNA and mitochondrial DNA. Sequences that did not have blastn matches to the sequences cited above were aligned against the NCBI non-redundant (nr) protein database using blastx algorithm, with a cutoff *E* value of $< 10^{-6}$. Both blastn and blastx similarity searches were performed using Blast2GO PRO v.2.5.0 software (Conessa et al., 2005). Resulting top 10 blastx hits were fed into Blast2GO in order to retrieve associated Gene Ontology (GO) terms describing biological processes, molecular functions, and cellular components. By using specific gene identifiers and accession numbers, Blast2GO produces all GO annotations as well as corresponding enzyme commission numbers (EC) for sequences with an *E* value cut off set at 10^{-6}. InterPro terms were also obtained from InterProScan at EBI, converted and merged to GOs using Blast2GO software. Finally, the KEGG (Kyoto Encyclopedia of Genes and Genome) orthology (KO) identifiers, or the K numbers, were generated using the web-based server KAAS (KEGG Automatic Annotation Server) (Moriya et al., 2007), resulting in the mapping of putative KEGG metabolic pathways of *C. brasiliana*.

Results and discussion

454 sequencing and de novo assembly of the oyster transcriptome

We created 10 non-normalized cDNA libraries based on RNA extracted from the gill and digestive gland of *C. brasiliana* exposed to three environmental contaminants: domestic sewage, phenanthrene and diesel fuel water-accommodated fraction (WAF), plus a control group. From these, four cDNA libraries were pooled based on the tissue and treatment, and were subjected to a single 454 GS-FLX run. This sequencing run yielded a total of 96,582,549 bp of sequence data, in

the form of 399,291 raw reads with an average length of 260 bp. After cleaning the data and removing small reads (<150 bp), a total of 246,514 reads were assembled into contiguous sequences (contigs), and 152,777 reads were identified as singletons (reads not assembled into contigs), which were excluded from further analysis. An overview of the sequencing and assembly process is presented in Table 1.

Table 1. Summary statistics for 454 sequencing and assembly for *Crassostrea brasiliana*.

Total number of reads	399,291
Total bases	96,582,549
Total bases for assembly	4,205,453
Total number of assembled reads	246,514
Total number of contigs	20,938
Number of contigs with 2 reads	8,010
Number of contigs with > 2 reads	12,928
Total number of singletons	152,777

De novo assembly of the clean reads produced 20,938 contigs, ranging in size from 144 to 4,662 bp, with an average length of 575 bp. 10,260 contigs were greater than 500 bp (49.0%), of which 1,556 (15.2%) were longer than 1 Kb, and 50% of the assembled bases were incorporated into contigs greater than 911 bp (N50 = 911 bp). These results resembled the size distribution of contigs generated from 454 reads reported in previous studies (Bettencourt et al., 2011; Fraser et al. 2011; Hou et al., 2011). The size distribution of the contigs is shown in Figure 1A. The average number of reads per contig was 12 (range 2-38,260 number of reads). 247 contigs comprised more than 100 reads, with the largest contig of 4,462 bp comprising 504 sequences. Only one contig (860 bp in size) comprised more than 7,000 reads, containing the most reads with 38,260 sequences. Nonetheless, the length of contigs was significantly correlated with the number of sequences assembled into them (Figure 1B) (Pearson r = 0.53; n = 20,938; $p < 0.0001$), as expected for 454 reads (Parchman et al., 2010).

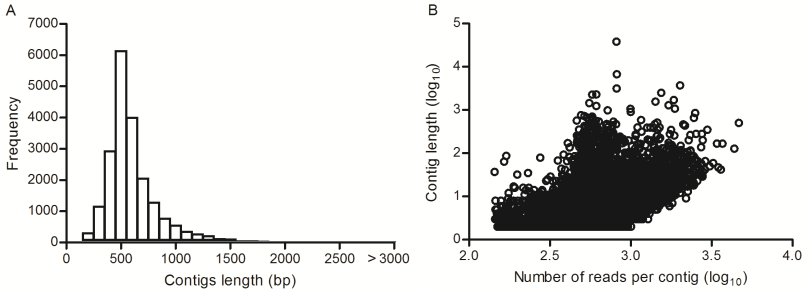


Figure 1. Overview of the *Crassostrea brasiliana* transcriptome assembly. (A) Frequency distribution of contig length. (B) Frequency distribution of the number of reads assembled into contigs.

Functional annotation

One objective of this study was to assign hypothetical protein sequence and function to each EST. All contigs were used as queries to search annotated protein databases and were assigned a gene description and/or GO term using Blast2GO platform (Götz et al., 2008). However, we annotated our dataset by first searching the NCBI non-redundant (nr) nucleotide databases using blastn (*E* value threshold of 10^{-6}) to identify rRNA genes and mitochondrion sequences (search in hit description for the exact word 'mitochondrion', referred here as mtDNA). The rational for selecting blastn algorithm for initial search lies in the absence of blastx hits of most contigs with matches to rRNA genes and mtDNA (data not shown), which could lead to biases in the types of genes during the annotation of the *C. brasiliana* transcriptome. Of 20,938 contigs, 127 had matches to rRNA genes and 90 to mtDNA sequences, representing 1.04% the entire contig dataset. The majority of the 217 contigs annotated using blastn corresponded to oyster rRNA and mtDNA, with 88.19% of rRNA genes and 98.89% of mitochondrion sequences matching *Crassostrea* sp. sequences. Of the 127 contigs that had matches to rRNA genes, 57 hit to *C. virginica*, 20 to *C. gigas* and 12 to *C. brasiliana*. This result seems to represent the ESTs availability for the *Crassostrea* genus at the NCBI database, which is dominated by *C. gigas* sequences, followed by *C. virginica*, representing 93.36% and 6.59%, respectively, of all ESTs for this genus. Likely, of 90 contigs with hit to mitochondrion sequences, 58 matched to *C. virginica*, 26 to *C. hongkongensis*, 2 to *C. gigas*, 1 to *C. iredalei* and 1 to *C. nippona*. Besides representing the major availability of genomic sequences for

these species, the results also show the relative close relationship between *C. brasiliana* to *C. virginica*, as previously reported by Melo et al. (2010).

Excluding sequences with best blastn hits to rRNA genes and mitochondrion sequences, 20,721 (98.96%) sequences were annotated by searching the NCBI non-redundant (nr) protein database using blastx (*E* value < 10^{-6}). A total of 7,466 contigs (36.04%) had significant blastx matches. Of these, 7,401 were aligned to Eukaryotic proteins, and 65 sequences hit to Prokaryotic and viruses proteins (0.31%), which were excluded from the *C. brasiliana* transcriptome dataset. Contigs with significant blastx hits corresponded to 6,015 unique accession numbers, of which 542 were matched by multiple queries. These 542 subject sequences were matched by 1,386 query sequences (~2.56 matched queries per subject, on average). The average length of annotated contigs was 691 bp, while non-annotated contigs averaged 509 bp. Nonetheless, longer contigs were more likely to have blastx matches to the NCBI annotated protein databases; logistic regression indicated that contigs length was a significant explanatory variable of the presence of blastx match to the annotated protein database (slope = 0.0024; intercept = -1,997, p = <0.0001). 71.4% of our contigs over 1 kb in length had blastx matches, whereas only 12.1% of contigs shorter than 300 bp did.

The percentage of sequences of *C. brasiliana* with functional annotation is consistent with the 30 to 40% values previously reported for *de novo* transcriptome assemblies of non-model eukaryotes (Fraser et al., 2011; Parchman et al., 2010; Vera et al., 2008). Interestingly, the frequency of annotated contigs of the present study (36%) is higher when compared to other non-model marine invertebrates which EST collections have been recently sequenced (c.f. 12% in the blue mussel *Mytilus galloprovincialis* (Craft et al., 2010), 17% in the Antarctic bivalve *Laternulla elliptica* (Clark et al., 2010), 24% in the Manila clam, *Ruditapes philippinarum* (Milan et al., 2011), 25% in the krill *Euphausia superba* (Clark et al., 2011) and 28% in the Yesso scallop *Pactinopecten yessoensis* (Hou et al., 2011). This can be due to the increased number of large scale transcriptome projects of non-model organisms, which has resulted in higher availability of sequences in public databases for phylogenetically closely related species (Ekblom; Galindo, 2011). In fact, taxa with the most matches were amphioxus (*Branchiostoma floridae*; 959 matches, 13%), acorn worm (*Saccoglossus kowalevskii*; 785 matches, 11.1%), purple sea urchin (*Strongylocentrotus purpuratus*; 317 matches, 4.5%), and Pacific oyster

(*Crassostrea gigas*; 316 matches, 4.5%) (Figure 2). Nonetheless, among the matches with *B. floridae* and *S. kowalevskii*, only 21 and 41 contigs, respectively, corresponded to known proteins, and the remaining sequences were related to hypothetical or predicted proteins. The similarity of blastx results with those species further supports their close evolutionary relationship with molluscs (Eirín-López et al., 2008; Putnam et al., 2008).

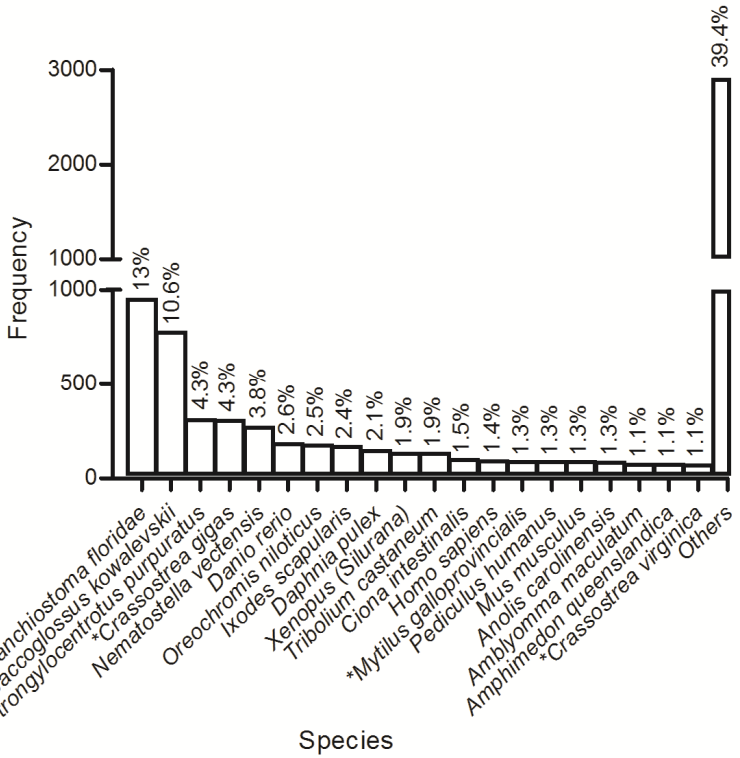
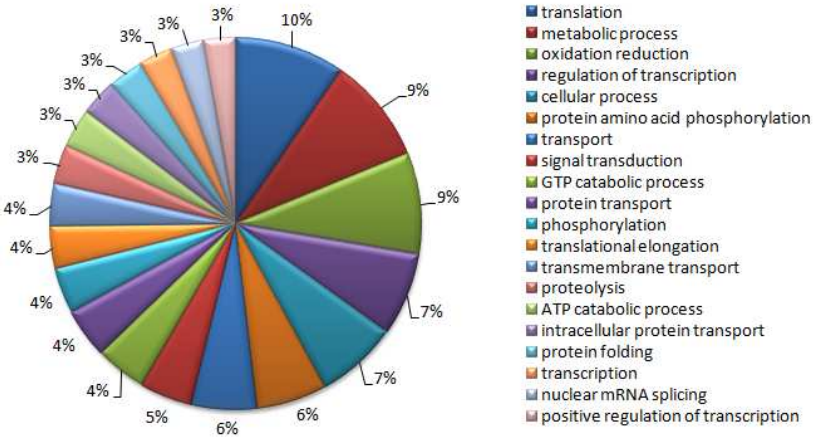


Figure 2. Species distribution of the top blastx hit in NCBI nr protein database for each contig of the *Crassostrea brasiliana* transcriptome. Asterisks denote bivalve mollusc species.

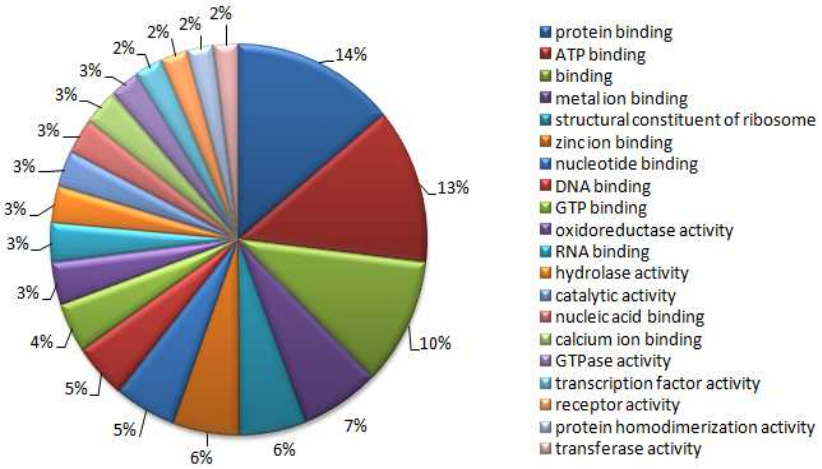
Oyster sequences that had matches in NCBI nr protein database were subject to Gene Ontology (GO) analysis in Blast2GO in order to assign putative functions for the *C. brasiliana* ESTs. GO terms allow a coherent annotation of genes products based on a controlled vocabulary and hierarchical relationships for molecular function,

cellular component and biological processes (Ashburner et al., 2000). Of 7,401 annotated sequences, 6,379 (86.2%) were assigned with one or more GO terms, which were further validated resulting in 4,381 annotated sequences (59.2%). Due to the “true path rule”, this validation step removes parent terms when a child term is present, assuring more specific functional annotations for a given sequence (Götz; Conesa, 2011). Following this approach, a total of 25,046 GO assignments were obtained, with 44.5% for Biological Process, 30.5% for Molecular Function, and 24.1% for Cellular Component. Among the 4,381 transcripts for which we obtained GO terms, we observed a wide diversity of functional categories represented on the GO database. Figure 3 shows a total of 20 GO categories for all three major GO functional domains into which the transcripts were classified. The most prominent GO Biological Process categories were “translation” (GO:0006412), “metabolic process” (GO:0008152) and “oxidation reduction” (GO:0055114) (each with 8-10% of the total). Within the GO Molecular Function, “protein binding” (GO:0005515) was most prevalent term, followed by “ATP binding” (GO:0005524) and “binding” (GO:0005488) (each with about 10-14%). Regarding the GO Cellular Component, the most represented categories were “cytoplasm” (GO:0005737), “nucleus” (GO:0005634), followed by “cytosol” (GO:0005829) (each with 11-13% of total hits). Together, the overall distribution of GO categories suggests that the 454 sequencing provided a comprehensive representation of the *C. brasiliana* transcriptome.

A



B



C

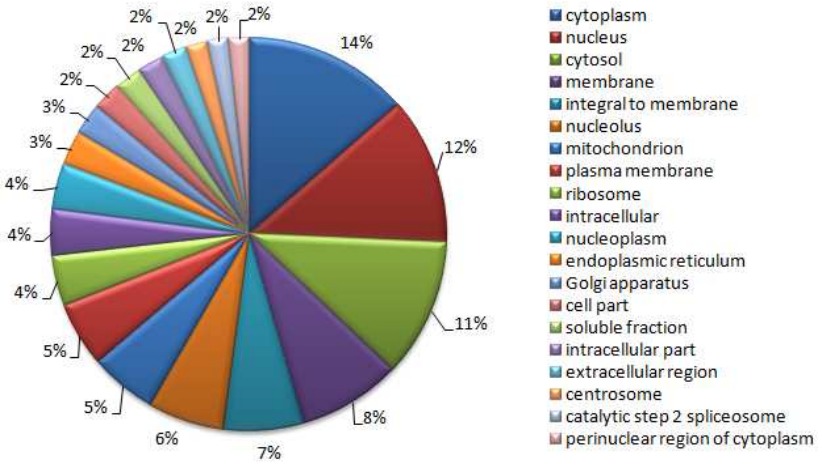


Figure 3. Gene Ontology GO terms for the transcriptome sequences of *Crassostrea brasiliana*. (A) GO Biological Process, (B) GO Molecular Function and (C) GO Cellular Component.

Despite GO analysis, we searched the annotated sequences for the enzyme commission (EC) numbers using Blast2Go. EC numbers were assigned to 1,341 sequences, with 31.1% and 30.4% corresponding to transferases and hydrolases, respectively, followed by oxidoreductases

(16.7%) and ligases (13.2%) (Figure 4). To further evaluate the effectiveness of our annotation process, we assigned the annotated sequences for the KEGG orthology (KO) identifiers, or the K numbers, using the web-based server KAAS (KEGG Automatic Annotation Server) (Moriya et al., 2007). In total, out of 7,401 annotated sequences, 2,673 sequences had K numbers. To identify the biological pathways that are active in *C. brasiliana*, the 2,673 KO identifiers were integrated into the KEGG resource by the KAAS service, resulting in the assignment to 276 different KEGG pathways. The number of pathways identified in this study is superior to other previous studies (*i.e.* Hao et al., 2011; Hou et al., 2011) and might suggest that the *C. brasiliana* sequence data contain a large diversity of genes involved in a variety of biological processes, and do not contain notable biases towards particular categories of genes. The pathways with most representation by the unique sequences were “ribosome” and “spliceosome”, followed by “oxidative phosphorylation”, “protein processing in endoplasmic reticulum” and “RNA transport”. Interestingly, “Huntington's disease” and “Alzheimer's disease” were amongst the well-represented metabolic pathways which might suggest a bias in favour to well annotated human genes available in public databases. Moreover, pathways commonly related to xenobiotic biotransformation and response to stress in aquatic organisms were represented by sequences classified into “metabolism of xenobiotics by cytochrome P450”, “drug metabolism - cytochrome P450”, “drug metabolism - other enzymes”, “glutathione metabolism”, “and “pentose phosphate pathway”. Taken together, the putative KEGG pathways identified in the current study shed light on specific responses and functions involved in the molecular processes of *C. brasiliana*. Additionally, these annotations provide a valuable resource for the identification of novel genes involved in the pathways of xenobiotic biotransformation and stress responses.

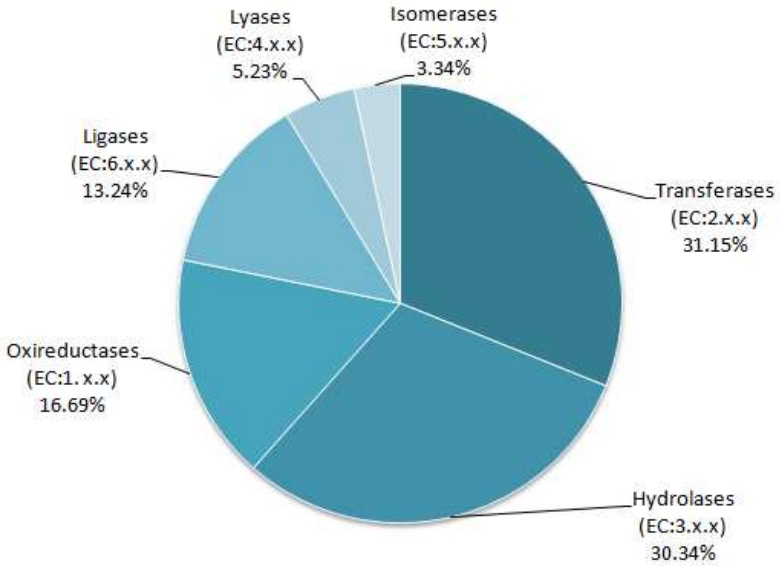


Figure 4. General enzyme classification based on enzyme commission (EC) numbers for the contigs of *Crassostrea brasiliana*.

Detection of transcripts encoding proteins involved in xenobiotic biotransformation

In Eukaryotes, the biotransformation of most lipophilic xenobiotics can be divided in three phases. During phase I, polar groups are introduced into the xenobiotic molecule, which is usually catalysed by the cytochrome P450 enzymes, making it a suitable substrate for phase II reactions. The metabolites formed by phase I reactions may undergo further metabolism by conjugation to polar endogenous substrates which is mainly catalyzed by enzymes like glutathione *S*-transferases (GSTs) and sulfotransferases (SULT) whereby lipophilic compounds are transformed into hydrophilic conjugates (Walker et al., 1996). In phase III specialized transporters recognize the conjugates, and expel them out of the cell (Homolya et al., 2003). Thus, the response of enzymes belonging to those phases represents a defence mechanism developed by an organism exposed to contaminants and have been used as biomarkers to evaluate both exposure to, and effects of, environmental pollutants. Based on this assumption and considering the importance of oysters for aquatic biomonitoring programs, the most interest of our research was to identify the presence of transcripts involved in the metabolism of xenobiotic biotransformation and associated antioxidant defence,

providing a baseline for future studies on the environmental stress response of *C. brasiliana*. Therefore, the selection of candidate genes involved in phases I, II and III presented in Tables 2 and 3 was based on queries matching xenobiotics detoxification-related criteria allowing the identification of relevant sequences from the oyster transcriptome.

Transcripts encoding putative CYP450s

A total of 41 CYP450-related contigs were identified in the reference transcriptome. The transcripts were first identified based on the closest blastx hits in the NCBI nr database, followed by the identification of at least one of the descriptors provided by the InterPro Scan: IPR001128 (Cytochrome P450 Family); IPR002401 (Cytochrome P450, E-class, group I); Pfam domain PF00067 (Cytochrome P450); and/or SSF48264 (Cytochrome P450 superfamily). Of 41 contigs with matches to CYP450s, 11 did not have an InterPro ID, but were manually blasted against the protein NCBI nr database, confirming our provisional annotation as putative CYP450s using an *E* value cut off of 10^{-6} and a minimum of 40% identity with the subject sequence. CYP450s were assigned to nine CYP families, and the representatives of all major CYP clans in protostomes (2-4 and mitochondrial; Karatolos et al., 2011) were found in this dataset (Table 2). Of 41 identified CYP450s, 11 belonged to the CYP2 family, nine to the CYP4 family, eight to the CYP17 family, seven to the CYP3 family and the rest to the CYP1, CYP20, CYP24, CYP27 and mitochondrial families (Table 2).

Among the putative CYP450 families with ecotoxicology interest identified in this study, CYP1 and CYP2 are known to be implicated in the metabolism of environmental contaminants of deuterostomes (Nebert; Russel, 2002; Kubota et al., 2011). The involvement of CYP families in protostomes is weakly known, but in insects, CYP3 and CYP4 seem to be involved in the metabolism of insecticides (Feyereisen, 2006). Likely, CYP2 and CYP4 families were recently identified and suggested to be involved in defences against toxic chemicals in bivalve molluscs (Zanette et al., 2010; Miao et al., 2011, respectively). In this study, transcripts encoding putative CYP2 family were most representative followed by CYP4 and CYP17. The CYP2 gene family is the largest and most diverse CYP family, which has evolved complex roles in physiology and toxicology, and diverse regulatory mechanisms (Goldstone et al., 2006). Interestingly,

cormorants exposed to environmental pollutants showed differential regulation in the transcription level which was dependent on the chemical class, with chlorinated compounds exerting no effects on these genes whilst perfluorinated compounds were negatively correlated to CYP2 levels (Kubota et al., 2011). However, in a recent study carried out in our laboratory, oyster *C. brasiliana* showed an up-regulation of CYP2-like genes following short-term exposure to phenanthrene[2].

Table 2. Summary information for the identified *Crassostrea brasiliana* genes putatively involved in phase I of the metabolism of biotransformation of xenobiotics.

Candidate genes	Occurrence*	Average contig size	Average reads per contigs
Cytochrome P450			
Clan 2[§]			
CYP450, family 1	2	1,173 bp	14
CYP450, family 2	11	843 bp	14
CYP450, family 17	8	1,094 bp	11
Clan3			
CYP450, family 3	7	769 bp	5
Clan 4			
CYP450, family 4	9	506 bp	2
Clan mitochondrial			
CYP450, family 20	1	1,779 bp	20
CYP450, family 24	1	916 bp	3
CYP450, family 27	1	708 bp	3
CYP450 mitochondrial	1	804 bp	3

*Number of contigs obtained in this study that had a hit with the corresponding proteins in the NCBI nr database. [§]Clan classification according to Nelson (1998).

Despite CYP2, transcripts encoding putative CYP17 proteins were well represented in *C. brasiliana* transcriptome (Table 2). Based on the closest blastx hits in the NCBI nr database these transcripts had a high similarity to CYP356A1 of *C. gigas*, which is closely related to members of the CYP17 and CYP1 families (Toledo-Silva et al., 2008). Although CYP17 is thought to be related to the steroid metabolism, the role of homologous sequences of the xenobiotic detoxification is also suggested (Toledo-Silva et al., 2008). In fact, *CYP356A1-like* was over-

[2] See Chapter 5.

expressed in the gill of *C. brasiliana* exposed to phenanthrene[1] and together with CYP2 are therefore candidate biomarkers for PAH biotransformation in bivalve molluscs. Furthermore, two sequences were aligned to CYP1 family, which is reported to be the main phase I enzyme family involved in PAHs metabolism. However, the role and even the existence of CYP1 in molluscs are poorly known, requiring more studies to address this issue.

The CYP4 gene family encodes a diverse number of enzymes, with functions related to the hydroxylation of fatty acids and eicosanoids in vertebrates (Kikuta et al., 2002), and xenobiotic biotransformation in polychaetes and insects (Rewitz et al., 2006). Likely, a wide variety of factors have been showed to regulate CYP4 family members, ranging from hormones in vertebrates (Simpson, 1997) to dissolved oxygen in bivalves (Snyder et al., 2001). There is also recent evidence of the regulation of environmental pollutants on CYP4 related genes in bivalves, in which benzo[*a*]pyrene (BaP) decreased the transcription levels of CYP4 in scallops (Miao et al., 2011). In the present study, the number of genes encoding putative CYP4 genes is close to that in the whitefly *Trialeurodes vaporarium* and in the bed bug *Cimex lectularius*, where 13 and 17 sequences were identified, respectively (Karatolos et al., 2011; Bai et al., 2011). Finally, less abundant transcripts encoding other CYP450 families were identified in *C. brasiliana* reference transcriptome and fell in Clans 3 and Mitochondrial (Table 2).

Transcripts encoding putative glutathione S*-transferases (GSTs) and sulfotransferases (SULTs)*

In the *C. brasiliana* dataset 25 contigs were identified with high sequence similarity to GSTs. The transcripts were first identified based on the closest blastx hits in the NCBI nr database, followed by the identification of at least one of the descriptors provided by the InterPro Scan: IPR004045 (Glutathione *S*-transferase, N-terminal); IPR010987 (Glutathione *S*-transferase_C-like); Pfam domain PF00043 (Glutathione *S*-transferase, C-terminal domain); and/or Pfam domain PF02798 (Glutathione *S*-transferase, N-terminal domain) for the identification of both cytosolic or microsomal GSTs. Furthermore, a search for microsomal GSTs was carried out by the identification of the MAPEG (Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism) descriptors derived from InterPro Scan: IPR023352 (MAPEG domain); IPR001129 (MAPEG protein); and Pfam domain PF01124 (MAPEG family). Ten sequences did not have InterPro IDs

but were manually aligned against the protein NCBI nr database, confirming our provisional annotation as putative GSTs using an *E* value cut off of 10^{-6} and a minimum of 40% identity with the subject sequence. Of the 26 GST-related contig sequences, 20 were assigned to seven cytosolic classes and six to three microsomal GSTs (Table 3). Interestingly, independent on the GST class the percentage of similarity between *C. brasiliana* putative GSTs and those of other species was higher (> 40%) than in GSTs sequences of other mollusc species, such as *C. gigas* (Boutet et al., 2004) and *Mytilus edulis* (Lüdeking and Kohler, 2002).

Table 3. Summary information for the identified *Crassostrea brasiliana* genes putatively involved in phases II and III of the metabolism of biotransformation of xenobiotics.

Candidate genes	Occurrence*	Average contig size	Average reads per contigs
Phase II - Glutathione *S*-transferases			
GST alpha	1	512 bp	3
GST omega	4	646 bp	7
GST pi	2	819 bp	11
GST theta	2	678 bp	3
GST sigma	6	710 bp	7
GST mu	1	1,048 bp	14
GST rho	3	798 bp	41
GST microsomal 1	2	541 bp	11
GST microsomal 2	1	464 bp	12
GST microsomal 3	3	821 bp	14
Phase II - Sulfotransferases			
Sulfotransferases	8	572 bp	4
Phase III - Multidrug resistance proteins			
MRP1	8	514 bp	4
MRP4	1	489 bp	3

*Number of contigs obtained in this study that had a hit with the corresponding proteins in the NCBI nr database.

Most of the identified GSTs were assigned to the sigma (6 sequences), followed by the omega class (4 sequences), members of which are known to play a role in the xenobiotic detoxification and in the protection against oxidative stress (Board et al. 2000; Fonseca et al, 2010; Milan et al., 2011). Indeed, omega GST has recently been

reported to be useful as a biomarker for hydrocarbon[3] and domestic sewage exposure in oysters (Boutet et al., 2004). The remaining contigs identified here with matches to cytosolic forms were further designed into putative pi, mu, theta, rho and theta classes. Of these, pi GST is known to inactivate products of oxidative damage, such as lipoperoxidation products, lipid hydroperoxides and their derivatives (Doyen et al., 2008) which has been implicated to play an important role in the detoxification of BaP in scallops (Miao et al., 2011). Mu class has also been proposed as biomarkers for hydrocarbons exposure in oysters, since it was over-expressed in *C. gigas* under chemical stress conditions (Boutet et al., 2004). Additionally, class theta, which has recently been identified as most responsive cytosolic GST in fish challenged with organic compounds (Williams et al., 2008), was well represented in this study although very little is known of this class in molluscs (Whalen et al., 2008). Interestingly, the contig assembled by the most reads (contig 504) aligned to rho class (103 reads), which appears to match peptides to theta GST sequences (Whalen et al., 2008). In fact, one contig provisionally assigned to the rho class also presented high identity (>60%) to the theta family of fish (data not shown). This is not surprising considering that rho and theta classes were similarly responsive to organic compounds in the flounder *Platichthys flesus* (Williams et al., 2008).

With regards to microsomal GSTs, six contigs were likely to be assigned to MAPEG family, with subgroups 1 (MGST1) and 3 (MGST3) most represented (Table 3). MAPEG members constitute a unique branch where most of the proteins are involved in the production of eicosanoids (Hayes et al., 2005), although evidences show that microsomal GSTS are capable of detoxifying organic xenobiotics in fish and human (Hayes et al., 2005; Williams et al., 2008). Finally, and not less important, transcripts encoding sulfotransferases (SULTs) were also identified in the *C. brasiliana* transcriptome (Table 3). These genes were assigned to putative SULTs following the identification of one of the descriptors derived from InterPro Scan: Pfam domain PF00685 (Sulfotransferase domain) and/or IPR000863 (Sulfotransferase domain). Two contigs did not have InterPro IDs but were assigned by the same criteria described above for CYPs and GSTs. The presence of putative SULTs, a key component of phase II metabolism of endogenous and exogenous compounds, suggests a role in the detoxification or endocrine

[3] See Chapter 5.

metabolism of oysters, as previously suggested for mussel (Janer et al., 2005; Lavado et al., 2006). Furthermore, expression of SULTs enzymes has recently been associated to PAHs in the channel catfish *Ictalurus punctatus* (Gaworecki et al., 2004), although their inducibility is yet to be confirmed for several species, including molluscs (Janer et al., 2005; Milan et al., 2011; Roméo and Wirgin, 2011).

Transcripts encoding putative multidrug resistance proteins (MRPs)

Conjugates formed by phase II reactions are eliminated from the cells by the transport across the plasma membrane into the extracellular space, which is mediated by the multidrug resistance proteins (MRPs) (Homolya et al., 2003). Nine MRPs exist and all belong to the superfamily of ATP-Binding Cassette (ABC) transporters (Hayes et al., 2005). In the *C. brasiliana* dataset nine contigs were identified as putative MRPs based on the closest blastx hits in the NCBI nr database, followed by the identification of at least one of the descriptors provided by the InterPro Scan: IPR003439 (ABC transporter-like); IPR001140 (ABC transporter); IPR011527 (ABC transporter, transmembrane domain, type 1); IPR017940 (ABC transporter, integral membrane, type 1); Pfam domain PF00005 (ABC transporter); and Pfam domain PF00664 (ABC transporter transmembrane region). Two contigs did not have InterPro IDs but were manually annotated by the same criteria described before. Of the nine contigs with MRPs matches, 8 were most likely to be related to MRP1 and only one to MRP4 (Table 3). In mammals, MRP1 is thought to export phase II-by products and compounds complexed with endogenous glutathione (GSH), and plays, therefore, an essential role in detoxification and defence against oxidative stress (Homolya et al., 2003). In aquatic invertebrates, MRPs are called as multixenobiotic resistance (MXR), and even without a clear classification as for mammals, are known to provide protection against toxicants (Luckenbach and Epel, 2008). In fact, previous examination has shown an over-expression of MXR-like genes in *C. gigas* exposed to domestic sewage (Medeiros et al., 2008). Taken together our findings suggest the importance of MRPs genes in providing protection against environmental pollutants of *C. brasiliana*.

Detection of antioxidant genes of interest for ecotoxicology

Given that the exposure to pollutants and further biotransformation may generate reactive oxygen species (ROS) that have the potential to cause damage to DNA, proteins and lipids, we are also interested in genes that participate in general antioxidant defence. Cellular protection

against the deleterious effects of ROS cells has been attributed to a complex network composed by both enzymatic and nonenzymatic antioxidants (Halliwell and Gutteridge, 2007). A number of contigs encoding genes putatively involved in the antioxidant defence were identified in the *C. brasiliana* transcriptome (Table 4). The transcripts were identified based on the closest blastx hits in the NCBI nr database and included those encoding the following enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), thioredoxins (Trx) (also the thioredoxin reductase - TrxR and thioredoxin peroxidase - TPx), peroxiredoxin (Prx), glutaredoxin (Grx), glutathione reductase (GR), quinone oxidoreductase (also called DT-diaphorase), and the ancillary enzyme glucose-6-phosphate dehydrogenase (G6PDH). Of those, Trx-related contigs were the most represented in our dataset (11 contigs with 7 reads in average). Trx-related enzymes are involved in thiol-based redox regulation and belong to a thioredoxin (Trx) superfamily, which also consists of GPx, Prx and Grx (Shchedrina et al., 2007). Such activities have been proposed to protect against metal exposure and virus-induced oxidative stress in molluscs (Nikapitiya et al., 2009; Trevisan et al., 2011), and thus may represent interesting antioxidant biomarkers with promising potential for ecotoxicological studies in *C. brasiliana*.

Table 4. Summary information for the identified *Crassostrea brasiliana* genes putatively involved in the antioxidant defence system.

Candidate genes	**Occurrence***	**Average contig size**	**Average reads per contigs**
Superoxide dismutase	3	894 bp	45
Catalase	2	1325 bp	22
Glutathione peroxidase	2	577 bp	7
Glutathione reductase	1	536 bp	5
Peroxiredoxin	3	829 bp	11
Glutaredoxin	4	435 bp	6
Thioredoxin	8	657 bp	7
Thioredoxin reductase	2	564 bp	4
Thioredoxin peroxidase	1	989 bp	10
Quinone oxidoreductase	2	529 bp	2
G6PDH	2	888 bp	5

*Number of contigs obtained in this study that had a hit with the corresponding proteins in the NCBI nr database.

Contigs with similarity to SOD and CAT enzymes were assembled by the most reads, in average 45 and 22 respectively, suggesting their involvement in the cellular protection against ROS of *C. brasiliana* challenged with environmental pollutants as previously reported for mussel and limpet (Ansaldo et al., 2005; Cheung et al., 2004; Lima et al., 2007). Furthermore, two contigs were identified with high sequence similarity to quinone oxidoreductase, or DT-diaphorase. The latter enzyme is generally considered as a detoxification enzyme because of its ability to reduce reactive quinones to less reactive and less toxic hydroquinones (Siegel et al., 2004). Knowledge about DT-diaphorase in invertebrates is still limited, but a few reports have demonstrated its potential function as antioxidant in molluscs (*i.e.* reviewed by Manduzio et al., 2005). Altogether these findings show promising insight for understanding antioxidant metabolism in oysters, however further functional studies (such as the analysis of transcript levels) are required to elucidate the role and regulation of such genes in *C. brasiliana.*

Contribution of the environmental contaminants to the genes of interest for ecotoxicology

To reveal if the environmental contaminants contributed to the overall *C. brasiliana* transcriptome, we analysed the reads within the 113 contigs under the 33 functional gene groups of interest for ecotoxicology presented in Tables 2, 3 and 4. Among the 9 CYP-related gene groups, the families 2, 3, 4 and 17 were mostly originated from the environmental contaminants, whereas CYP1-like and CYP20-like genes were equally represented by reads originated from control and exposed groups. Among these, CYP family 2 was the most represented by reads from the diesel and domestic sewage treatments, indicating its role in detoxification of organic compounds such as PAHs, which has been previously reported in cormorant and mouse (Kubota et al., 2011; Schober et al., 2010). Similarly, contigs of family 17 were generated by reads from all the exposed groups, with higher contribution of diesel exposure (Table 5). On the other hand, the families 24, 27 and CYP mitochondrial were generated by reads from stressed groups only; although those contigs were produced by three sequences each (Table 5).

Concerning contigs of the functional gene groups of phase II biotransformation of xenobiotics, GST omega, followed by GST theta, GST sigma, sulfotransferase, GST rho and GST microsomal 2 were

most generated by reads from the diesel experiment. In addition, GST sigma was well represented by reads from the sewage group. Interestingly, phenanthrene exposure contributed mainly to contigs of the functional groups GST omega, GST mu, GST alpha and GST microsomal 3 (Table 5). This finding is consistent with previously documented report that PAHs induced GST omega and GST mu in different tissues of *C. gigas* (Boutet et al., 2004). With regards to phase III genes, the environmental contaminants with most contribution were diesel for both MRP1 and MRP4, followed by phenanthrene for MRP1, and sewage for MRP4 (Table 5). Those preliminary analyses showed that the presence of contigs of phase I, phase II and phase III of xenobiotic metabolism in *C. brasiliana* are mainly due to the chemical exposures.

Among the functional gene groups related to antioxidant defence system, glutathione reductase, quinone oxidoreductase and G6PDH were generated mainly by reads from phenanthrene exposure. Likely, catalase was originated by sequences from phenanthrene, although reads from further treatments contributed to the generation of this gene group. In addition, diesel contributed mainly for the generation of contigs of peroxiredoxin, thioredoxin peroxidase, G6PDH and thioredoxin functional groups (Table 5). Interestingly, the environmental contaminants seemed to contribute in a lesser extent to the contigs of SOD, GPx and glutaredoxin functional groups, which might suggest a general response in oysters challenged under controlled laboratory conditions.

Combining detoxification of xenobiotics and antioxidant results, we can suggest that all the environmental contaminants applied in this study contributed to the *C. brasiliana* transcriptome. The results also suggest that some classes of genes are more influenced by a particular contaminant, indicating its potential application as molecular biomarker in biomonitoring programs. However, additional experiments using approaches such as quantitative real-time PCR and/or global mapping sequences to the reference transcriptome are required to validate the results from this preliminary analysis.

Table 5. Contribution of each environmental contaminant to generate the contigs under the functional gene groups of interest for ecotoxicology.

Candidate genes	Control	Sewage	Phenanthrene	Diesel WAF
Phase I				
CYP450, family 1	8	2	10	8
CYP450, family 2	13	51	14	79
CYP450, family 17	2	24	21	45
CYP450, family 3	2	7	7	19
CYP450, family 4	1	1	4	14
CYP450, family 20	6	1	5	8
CYP450, family 24	0	1	1	1
CYP450, family 27	0	1	1	1
CYP450 mitochondrial	0	0	2	1
Phase II				
GST alpha	0	0	1	1
GST omega	0	3	8	17
GST pi	6	4	8	4
GST theta	0	1	0	5
GST sigma	5	18	2	20
GST mu	2	1	4	7
GST rho	24	8	21	70
GST microsomal 1	5	1	5	10
GST microsomal 2	2	0	1	9
GST microsomal 3	7	6	16	12
Sulfotransferase	5	0	5	14
Phase III				
MRP1	4	2	8	17
MRP4	0	1	0	2
Antioxidants				
Superoxide dismutase	56	20	20	39
Catalase	5	9	15	14
Glutathione peroxidase	4	0	4	5
Glutathione reductase	0	1	4	0
Peroxiredoxin	5	3	7	18
Glutaredoxin	11	2	0	11
Thioredoxin	15	3	12	26
Thioredoxin reductase	2	0	3	2
Thioredoxin peroxidase	2	0	3	5
Quinone oxidoreductase	0	1	2	1
G6PDH	0	1	5	4

Conclusions

This study is the first to describe a *de novo* transcriptome for the mangrove oyster *Crassostrea brasiliana* using 454 pyrosequencing technology with emphasis to pathways related to detoxification of environmental contaminants. To date, the lack of genomic data available for this species has hampered characterization of the molecular mechanisms underlying resistance to aquatic contaminants. The ~20,000 genes described here represent a dramatic expansion of existing cDNA sequence available for *C. brasiliana*. We have identified genes that are potential candidates as biomarkers for ecotoxicological studies including those encoding enzymes putatively involved in the biotransformation of xenobiotics and those encoding enzymes of antioxidant defence system. This is in agreement with previous reports on enzymatic activities on bivalves exposed to environmental contaminants, and adds to the current knowledge on the molecular biology and biochemistry of stress response in oysters. Furthermore, the EST libraries developed in this study can be used as reference transcriptome for further studies on gene expression using *C. brasiliana* and other bivalve species, addressing in particular the molecular mechanisms underlying the susceptibility to pollutants and differential stress resistance. In long term this research should be helpful in management of biomonitoring programs using oysters as bioindicator species.

Acknowledgements

This research was supported by grants from NERC, UK to JAG and CNPq to ACDB (CT-Petro 550706/2005-4 and CNPq INCT-TA). KHL was a Guest Student at the Marine Biological Association of the United Kingdom and Glasgow Caledonian University and was supported by a CAPES PhD Fellowship and CNPq PhD Sandwich Fellowship, Brazil. ACDB was recipient of the CNPq Productivity Fellowship, Brazil. We would like to thank Fabrício Flores Nunes and Christielly Rodrigues da Silva for their assistance during the sewage experiment.

References

Ansaldo M, Najle R, Luquet CM: **Oxidative stress generated by diesel seawater contamination in the digestive gland of the Antarctic limpet *Nacella concinna*.** *Mar Environ Res* 2005, **59:**381-390.

Anyakora C, Ogbeche A, Palmer P, Coker, H: **Determination of polynuclear aromatic hydrocarbons in marine samples of Siokolo Fishing Settlement.** *J Chromatogr* 2005, **1073:**323-330.

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, et al.: **Gene Ontology: tool for the unification of biology.** *Nature Genet* 2000, **25**:25-29.

Bado-Nilles A, Renault T, Faury N, Le Floch S, Quentel C, Auffret M, Thomas-Guyon H: ***In vivo* effects of LCO soluble fraction on immunerelated functions and gene transcription in the Pacific oyster, *Crassostrea gigas* (Thunberg).** *Aquat Toxicol* 2010, **97:**196-203.

Bai X, Mamidala P, Rajarapu SP, Jones SC, Mittapalli O: **Transcriptomics of the Bed Bug (*Cimex lectularius*).** PLoS ONE 2011, **6:**e16336.

Bebianno MJ, Barreira LA: **Polycyclic aromatic hydrocarbons concentrations and biomarker responses in the clam *Ruditapes decussates* transplanted in the Ria Formosa lagoon**. *Ecotoxicol Environ Saf* 2009, **72:**1849-1860.

Bettencourt R, Pinheiro M, Egas C, Gomes P, Afonso M, Shank T, Santos RS: **High-throughput sequencing and analysis of the gill tissue transcriptome from the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus*.** *BMC Genomics* 2010, **11:**559.

Board PG, Coggan M, Chelvanayagam G, Easteal S, Jermiin LS, Schulte GK, Danley DE, Hoth LR, Griffor MC, Kamath AV, Rosner MH, Chrunyk BA, Perregaux DE, Gabel CA, Geoghegan KF, Pandit J: **Identification, characterization, and crystal structure of the omega class glutathione transferases.** *J Biol Chem* 2000, **275:**24798-24806.

Boutet I, Tanguy A, Moraga D: **Characterisation and expression of four mRNA sequences encoding glutathione S-transferases pi, mu, omega and sigma classes in the Pacific oyster *Crassostrea gigas* exposed to hydrocarbons and pesticides.** *Mar Biol* 2004, **146:**53-64.

Cheung CCC, Siu WHL, Richardson BJ, De Luca-Abbott SB, Lam PKS: **Antioxidant responses to benzo[a]pyrene and Aroclor 1254 exposure in the green-lipped mussel, *Perna viridis*.** *Environ Pollut* 2004, **128:**393-403.

Clark MS, Thorne MA, Vieira FA, Cardoso JC, Power DM, Peck LS: **Insights into shell deposition in the Antarctic bivalve *Laternula elliptica*: gene discovery in the mantle transcriptome using 454 pyrosequencing.** *BMC Genomics* 2010, **11:**362.

Clark MS, Thorne MAS, Toullec J-Y, Meng Y, Guan LL, Peck LS, Moore S: **Antarctic Krill 454 Pyrosequencing Reveals Chaperone and Stress Transcriptome.** *PLoS ONE* 2011, **6:**e15919.

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M: **Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research.** *Bioinformatics* 2005, **21:**3674-3676.

Craft JA, Gilbert JA, Temperton B, Dempsey KE, Ashelford K, Tiwari B, Hutchinson TH, Chipman JK: **Pyrosequencing of *Mytilus galloprovincialis* cDNAs: tissue-specific expression patterns.** *PLoS ONE* 2010, **5:**e8875.

Doyen P, Bigot A, Vasseur P, Rodius F**: Molecular cloning and expression study of pi-class glutathione S-transferase (pi-GST) and selenium-dependent glutathione peroxidase (Se-GPx) transcripts in the freshwater bivalve *Dreissena polymorpha*.** *Comp Biochem Physiol C* 2008, **147:**69-77.

Eirín-López JM, Frehlick LJ, Chiva M, Saperas N, Ausió J: **The sperm proteins from amphioxus mirror its basal position among chordates and redefine the origin of vertebrate protamines.** *Mol Biol Evol* 2008, **25:**1705-1713.

Ekblom R, Galindo J: **Applications of next generation sequencing in molecular ecology of non-model organisms.** *Heredity* 2011, **107:**1-15.

Feyereisen R: **Evolution of insect P450.** *Biochem Soc Trans* 2006, **34:**1252-1255.

Fleury E, Huvet A, Lelong C, Lorgeril J, Boulo V, Gueguen Y, Bachère E, *et al.*: **Generation and analysis of a 29,745 unique Expressed Sequence Tags from the Pacific oyster (*Crassostrea gigas*) assembled into a publicly accessible database: the GigasDatabase.** *BMC Genomics* 2009, **10:**341.

Fonseca RR, Johnson WE, O'Brien SJ, Vasconcelos V, Antunes A: **Molecular evolution and the role of oxidative stress in the expansion and functional diversification of cytosolic glutathione transferases.** *BMC Evol Biol* 2010, **10:**281.

Forbes VE, Palmqvist A, Bach L: **The use and misuse of biomarkers in ecotoxicology.** *Environ Toxicol Chem* 2006, **25:**272-280.

Fraser BA, Weadick CJ, Janowitz I, Rodd H, Hughes KA: **Sequencing and characterization of the guppy (*Poecilia reticulata*) transcriptome.** *BMC Genomics* 2011, **12:**202.

Gaworecki KM, Rice CD, van den Hurk P: **Induction of phenol-type sulfotransferase and glucuronosyltransferase in channel catfish and mummichog.** *Mar Environ Res* 2004, **58:**525-528.

Goldstone HM, Stegeman JJ: **A revised evolutionary history of the CYP1A subfamily: gene duplication, gene conversion, and positive selection.** *J Mol Evol* 2006, **62:**708-717.

Götz S, Garcia-Gomez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, Robles M, Talon M, Dopazo J, Conesa A: **High-throughput functional annotation and data mining with the blast2go suite.** *Nucleic Acids Res* 2008, **36**:3420-3435.

Götz S, Conesa A: **Visual gene ontology based knowledge discovery in functional genomics.** In *Knowledge oriented applications in data mining.* Edited by Funatsu K, Hasegawa K. India: InTech; 2011, 245-260.

Halliwell B, Gutteridge J: **Free Radicals in Biology and Medicine.** Nova York: Oxford University Press; 2007.

Hannam ML, Bamber SD, Galloway TS, Moody AJ, Jones MB: **Effects of the model PAH phenanthrene on immune function and oxidative stress in the haemolymph of the temperate scallop *Pecten maximus*.** *Chemosphere* 2010, **78:**779-784.

Hao DC, Ge G, Xiao P, Zhang Y, Yang L: **The first insight into the tissue specific *Taxus* transcriptome via Illumina second generation sequencing.** *PLoS ONE* 2011, **6:**e21220.

Hayes JD, Flanagan JU, Jowsey IR: **Glutathione transferases.** *Annu Rev Pharmacol Toxicol* 2005, **45:**51-88.

Homolya L, Varadi A, Sarkadi B: **Multidrug resistance-associated proteins: Export pumps for conjugates with glutathione, glucuronate or sulfate.** *Biofactors* 2003, **17:**103-114.

Hou R, Bao Z, Wang S, Su H, Li Y, Du H, Hu J, Wang S, Hu X: **Transcriptome Sequencing and *De Novo* Analysis for Yesso Scallop (*Patinopecten yessoensis*) Using 454 GS FLX.** *PLoS ONE* 2011, **6:**e21560.

Janer G, Mesia-Vela S, Kauffman FC, Porte C: **Sulfatase activity in the oyster *Crassostrea virginica*: Its potential interference with sulfotransferase determination.** *Aquat Toxicol* 2005, **15:**92-95.

Joubert C, Piquemal D, Marie B, Manchon L, Pierrat F, Zanella-Cleon I, Cochennec-Laureau N, Gueguen Y, Montagnani C: **Transcriptome and**

proteome analysis of *Pinctada margaritifera* calcifying mantle and shell: focus on biomineralization. *BMC Genomics* 2010, **11:**613.

Karatolos N, Pauchet Y, Wilkinson P, Chauhan R, Denholm I, Gorman K, Nelson DR, Bass C, Ffrench-Constant RH, Williamson MS: **Pyrosequencing the transcriptome of the greenhouse whitefly, *Trialeurodes vaporariorum* reveals multiple transcripts encoding insecticide targets and detoxifying enzymes.** *BMC Genomics* 2011, **12:**56.

Kikuta Y, Kusunose E, Kusunose M: **Prostaglandin and leukotriene omega-hydroxylases.** *Prostaglandins Other Lipid Mediat* 2002, **68-69:**345-362.

Kubota A, Stegeman JJ, Goldstone JV, Nelson DR, Kim EY, Tanabe S, Iwata H: **Cytochrome P450 CYP2 genes in the common cormorant: Evolutionary relationships with 130 diapsid CYP2 clan sequences and chemical effects on their expression.** *Comp Biochem Physiol C Toxicol Pharmacol* 2011, **153:**280-289.

Kumar S, Blaxter ML: **Comparing *de novo* assemblers for 454 transcriptome data.** *BMC Genomics* 2010, **11:**571.

Lavado R, Janer G, Porte C: **Steroid levels and steroid metabolism in the mussel *Mytilus edulis*: the modulating effect of dispersed crude oil and alkylphenols.** *Aquat Toxicol* 2006, **78:**65-72.

Lima I, Moreira SM, Rendón-Von Osten J, Soares AMVM, Guilhermino L: **Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal.** *Chemosphere* 2007, **66:**1230-1242.

Lüchmann KH, Mattos JJ, Siebert MN, Granucci N, Dorrington TS, Bicego MC, Taniguchi S, Sasaki ST, Daura-Jorge FG, Bainy ACD: **Biochemical biomarkers and hydrocarbons concentrations in the mangrove oyster *Crassostrea brasiliana* following exposure to diesel fuel water-accommodated fraction.** *Aquat Toxicol* 2011, **105:**652-660.

Luckenbach T, Epel D: **ABCB- and ABCC-type transporters confer multixenobiotic resistance and form an environment-tissue barrier in bivalve gills.** *Am J Physiol Regul Integr Comp Physiol* 2008, **294:**R1919-R1929.

Lüdeking A, Kohler A: **Identification of six mRNA sequences of genes related to multixenobiotic resistance (MXR) and biotransformation in *Mytilus edulis*.** *Mar Ecol Prog Ser* 2002, **238:**115-124

Manduzio H, Rocher B, Durand F, Galap C, Leboulenger F: **The point about oxidative stress in molluscs.** *ISJ* 2005, **2:**91-104.

Medeiros ID, Siebert MN, Toledo-Silva G, Rodrigues TB, Marques MRF, Bainy ACD: **Induced gene expression in oyster *Crassostrea gigas* exposed to sewage.** *Environ Toxicol Pharmacol* 2008, **26:**362-365.

Melo AGC, Varela ES, Beasley CR, Schneider H, Sampaio I, Gaffney PM, Reece KS, Tagliaro CH: **Molecular identification, phylogeny and geographic distribution of Brazilian mangrove oysters (*Crassostrea*).** *Genet Mol Biol* 2010, **33:**564-572.

Meyer E, Aglyamova GV, Wang S, Buchanan-Carter J, Abrego D, Colbourne JK, Willis BL, Matz MV: **Sequencing and de novo analysis of a coral larval transcriptome using 454 GSFlx.** *BMC Genomics* 2009, **10:**219.

Miao J, Pan L, Liu N, Xu C, Zhang L: **Molecular cloning of CYP4 and GSTpi homologues in the scallop *Chlamys farreri* and its expression in response to Benzo[a]pyrene exposure.** *Mar Genomics* 2011, **4:**99-108.

Milan M, Coppe A, Reinhardt R, Cancela LM, Leite RB, Saavedra C, Ciofi C, Chelazzi G, Patarnello T, Bortoluzzi S, Bargelloni L: **Transcriptome sequencing and microarray development for the Manila clam, *Ruditapes philippinarum*: genomic tools for environmental monitoring.** *BMC Genomics* 2011, **12:**234.

Moriya Y, Itoh M, Okuda S, Yoshizawa AC, Kanehisa M: **KAAS: an automatic genome annotation and pathway reconstruction server.** *Nucleic Acids Res* 2007, **35:**182-185.

Nebert DW, Russell DW: **Clinical importance of the cytochromes P450.** *Lancet* 2002, **360:**1155-1162.

Nelson DR: **Metazoan cytochrome P450 evolution.** *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1998, **121:**15-22.

Nikapitiya C, De Zoysa M, Whang I, Kim CG, Lee YH, Kim SJ, Lee J: **Molecular cloning, characterization and expression analysis of peroxiredoxin 6 from disk abalone *Haliotis discus discus* and the antioxidant activity of its recombinant protein.** *Fish Shellfish Immunol* 2009, **27:**239-249.

Oliveira M, Pacheco M, Santos MA: **Cytochrome P4501A, genotoxic and stress responses in golden grey mullet (*Liza aurata*) following short-term exposure to phenanthrene.** *Chemosphere* 2007, **66:**1284-1291.

Parchman TL, Geist KS, Grahnen JA, Benkman CW, Buerkle CA: **Transcriptome sequencing in an ecologically important tree species: assembly, annotation, and marker discovery.** *BMC Genomics* 2010, **11:**180.

Putnam NH, Blow MJ, Butts T, Ferrier DEK, Furlong RF, Hellsten U, Kawashima T, Robinson-Rechavi M, Shoguchi D, Terry A, Yu JK, Benito-Gutiérrez EL, et al.: **The amphioxus genome and the evolution of the chordate karyotype.** *Nature* 2008, **453:**1064-1071.

Rewitz KF, Styrishave B, Lϕbner-Olesen A, Anderson O: **Marine invertebrate cytochrome P450: emerging insights from vertebrate and insect analogies.** *Comp Biochem Physiol C* 2006, **143:**363-381.

Roméo M, Wirgin I: **Biotransformation of organic contaminants and the acquisition of resistence.** In *Tolerance to Environmental Contaminants*. Edited by Amiard-Triquet C, Rainbow PS, Romeo M. Florida: CRC Press; 2011, 175-208.

Schober W, Pusch G, Oeder S, Reindl H, Behrendt H, et al.: **Metabolic activation of phenanthrene by human and mouse cytochromes P450 and pharmacokinetics in CYP1A2 knockout mice.** *Chemico-Biol Inter* 2010, **183:**57-66.

Shchedrina VA, Novoselov SV, Malinouski MY, Gladyshev VN: **Identification and characterization of a selenoprote in family containing a diselenide bond in a redox motif.** *Proc Natl Acad Sci USA* 2007, **104:**13919-13924.

Siegel D, Gustafson DL, Dehn DL, Han JY, Boonchoong P, Berliner LJ, Ross D: **NAD(P)H:quinone oxidoreductase 1: role as a superoxide scavenger.** *Mol Pharmacol* 2004, **65:**1238-1247.

Simpson AECM: **The cytochrome P450 4 (CYP4) family.** *Gen Pharmac* 1997, **28:**351-359.

Singer MM, Aurand D, Bragin GE, Clark JR, Coelho GM, Sowby ML, Tjeerdema RS: **Standardization of the preparation and quantitation of water-accommodated fractions of petroleum for toxicity testing.** *Mar Pollut Bull* 2009, **40:**1007-1016.

Snyder M, Girvetz E, Mulder E: **Induction of marine mollusc stress proteins by chemical or physical stress.** *Arch Environ Con Tox* 2001, **44:**22-29.

Tanguy A, Bierne N, Saavedra C, Pina B, Bachere E, Kube M, Bazin E, Bonhomme F, Boudry P, Boulo V, et al.: **Increasing genomic**

information in bivalves through new EST collections in four species: development of new genetic markers for environmental studies and genome evolution. *Gene* 2008, **408:**27-36.

Toledo-Silva G, Siebert MN, Medeiros I, Sincero T, Moraes M, Goldstone, Bainy, ACD: **Cloning a new cytochrome P450 isoform (CYP356A1) from oyster *Crassostrea gigas*.** *Mar Environ Res* 2008, **66:**15-18.

Trevisan R, Mello DF, Fisher AS, Schuwerack PM, Dafre AL, Moody AJ: **Selenium in water enhances antioxidant defenses and protects against copper-induced DNA damage in the blue mussel *Mytilus edulis*.** *Aquat Toxicol* 2011, **101:**64-71.

US EPA: United States Environmental Protection Agency Priority Pollutants [http://www.epa.gov/waterscience/methods/pollutants.htm]

Vera JC, Wheat CW, Fescemyer HW, Frilander MJ, Crawford DL, Hanski I, Marden JH: **Rapid transcriptome characterization for a nonmodel organism using 454 pyrosequencing.** *Mol Ecol* 2008, **17:**1636-47.

Walker CH, Sibly RM, Hopkin SP, Peakall DB: **Principles of Ecotoxicology.** Londres: Taylor & Francis; 1996.

Wang Y, Guo X: **Development and Characterization of EST-SSR Markers in the Eastern Oyster *Crassostrea virginica.*** *Mar Biotechnol* 2007, **9:**500-511.

Whalen KE, Morin D, Lin CY, Tjeerdema RS, Goldstone JV, Hahn ME: **Proteomic identification, cDNA cloning and enzymatic activity of glutathione *S*-transferases from the generalist marine gastropod, *Cyphoma gibbosum*.** *Arch Biochem Biophys* 2008, **478:**7-17.

Williams TD, Diab A, Ortega F, Sabine VS, Godfrey RE, et al.: **Transcriptomic responses of European flounder (*Platichthys flesus*) to model toxicants.** *Aquat Toxicol* 2008, **90:**83-91.

Zanette J, Goldstone JV, Bainy ACD, Stegeman JJ: **New cytochrome P450 genes in bivalves -Identification of CYPs from mussel and oyster ESTs.** *Mar Environ Res* 2010, **69:**S1.

Capítulo 5

Upregulation of new putative biotransformation genes and antioxidant responses in the mangrove oyster *Crassostrea brasiliana*: insights on molecular mechanisms of phenanthrene toxicity

Karim H. Lüchmann[a,*]; Tarquin S. Dorrington[a]; Rafael Trevisan[a]; Alcir L. Dafre[a]; Flávia L. Zacchi[a]; Mariana C. Naldi[a]; Declan C. Schroeder[b]; Afonso C.D. Bainy[a]

Preparado para submissão na Environmental Science & Technology

[a]Biochemistry Department, Federal University of Santa Catarina, Florianópolis 88040-900, SC, Brazil; [b]Marine Biological Association of the United Kingdom (MBA), Citadel Hill, Plymouth PL1 2PB, United Kingdom.

Abstract

Phenanthrene, a major component of crude oil, is one of the most abundant Polycyclic Aromatic Hydrocarbons (PAHs) in aquatic ecosystems, and is readily bioavailable to marine organisms. Understanding the fate and toxicity of PAHs in animals require knowledge on the xenobiotics biotransformation and antioxidant defence systems, which are poorly known in bivalves. In this study, we report, for the first time, tissue-specific transcription analysis of *CYP-like* and *GST-like* genes, by means of quantitative real-time PCR, and enzymatic and non-enzymatic antioxidant parameters in the oyster *Crassostrea brasiliana* following 24 h exposure to 100 and 1000 $\mu g.L^{-1}$ phenanthrene, a model PAH. The results indicated that 1000 $\mu g.L^{-1}$ phenanthrene can lead to the upregulation of both phase I and II genes, with more pronounced effects in the gill. Both *CYP-like* and *GST-like* are transcribed in a tissue-specific manner, reflecting the importance of gill in the detoxification of PAHs. The biological responses in *C. brasiliana* support the use of this organism to investigate the molecular mechanism underlying PAH toxicity in bivalves.

Keywords: *Crassostrea brasiliana*; phenanthrene; quantitative real-time PCR; CYP; GST; glutathione; antioxidant enzymes

Introduction

Aquatic biomonitoring programs represent a feasible tool to assess the health and provide early warnings of coastal and marine environments. However, the success of these programs relies on the assertive choice of parameters to be employed, which may represent a challenge by itself. Recent efforts have highlighted the use of a multiple approaches combining the assessment of biochemical, molecular and physiological endpoints, known as biomarkers, emphasising the use of mollusc species as bioindicator organisms (Galloway et al., 2002). Due to their worldwide distribution, ecological habitat and significant ability to bioaccumulate pollutants, bivalves, such as oysters, play a significant role in environmental studies (Bebianno and Barreira, 2009; Frouin et al., 2007; Lüchmann et al., 2011; Solé et al., 2007).

In biomonitoring programs, differences in the response of biomarkers are expected to be attributed exclusively to the chemical exposure. Nevertheless, recent studies have reported that biomarkers appear to have variable responses to factors other than the contaminant

challenge, such as the biological rhythms, reproductive cycles and environmental conditions (*i.e.* Fernandéz et al., 2010; Forbes et al., 2006; Zanette et al., 2011). To address this issue it is imperative renewed efforts to understand the biology of target species and to comprehend their responses to chemical exposure. This study, therefore, aims to gain insights regarding the acute effects of phenanthrene, a model polycyclic aromatic hydrocarbon (PAH).

Phenanthrene, a 3-ring compound included in the US Environmental Protection Agency (EPA) priority pollutant list, is one of the most abundant PAHs in the aquatic environment resulting from human activities (US EPA, 2009). Also, its low molecular weight makes it more water soluble, contributing to its abundance in oil polluted waters where it is readily bioavailable, exhibiting a large bioaccumulation factor in aquatic organisms, which further potentiate toxic effects (Hannam et al., 2010; Oliveira et al., 2007; Yin et al., 2007). While various mechanisms of phenanthrene toxicity have been proposed (Hecht et al., 2008), the most convincing evidences involve metabolic activation by members of the cytochrome P450 (CYP) superfamily (Shou et al., 1994). CYPs consist of several multigenic families of structurally and functionally related heme-proteins, playing an important role in phase I biotransformation of xenobiotics (Omiecinski et al., 2011). The metabolites formed by phase I reactions may undergo further metabolism by conjugation with an endogenous substrates catalyzed by "transferases" enzymes, such as glutathione *S*-transferases (GSTs), resulting in more hydrophilic conjugates that are usually readily excreted from the organism (Omiecinski et al., 2011). However, while numerous studies in bivalves have shown that GST activity is inducible by PAHs (Banni et al., 2010; Boutet et al., 2004; Le Pennec and Le Pennec, 2003; Lima et al., 2007; Lüchmann et al. 2011; Solé et al., 2007; Zanette et al., 2011), almost nothing is known about the phase I biotransformation in bivalves. The first basic question "*are specific CYPs modulated in bivalves challenged by environmental contaminants?*" remains to be determined. Along this lack of knowledge about the role of CYPs, the molecular mechanisms of phenanthrene detoxification remain to be elucidated.

Another feasible mechanism of phenanthrene toxicity has been proposed to involve generation of reactive oxygen species (ROS), as by-products of biotransformation reactions (Reis-Henriques et al., 2009), which may cause damage to proteins, DNA and lipids, leading to their functional impairment (Livingstone, 2001; Yin et al., 2007).

Thus, the coordinated response of different cellular defences, such as the antioxidant system, the biotransformation and the detoxification systems are important determinants in maintaining cellular function (Winston and Di Giulio, 1991). ROS are counteracted by an intricate antioxidant defence system that includes the enzymatic scavengers such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). As many biochemical pathways, the ancillary enzymes glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH) are required to maintain glutathione (GSH) in the reduced state ant thus maintain the turnover rate of GSH-dependent peroxidases and transferases. Due to its abundance, protein thiols (PSH) have been taken as an important antioxidant system (Reischl et al., 2007), that along with non-proteic thiols groups (NPSH), basically GSH, are the main reducing buffers of the cell (Sies, 1999).

Based on our previous study on the biochemical responses of *C. brasiliana* to complex mixtures of water-accommodated hydrocarbons from diesel fuel (Lüchmann et al., 2011), the present study is the first attempt to elucidate the early molecular and biochemical responses to phenanthrene searching for its toxic mechanisms in *C. brasiliana* under controlled laboratory conditions. Two sublethal concentrations of phenanthrene were chosen based on previous reports carried out with bivalves (Hannam et al., 2010; Wootton et al., 2003) and on realistic environmental concentrations found nearby petroleum exploration area (Anyakora et al., 2005). Therefore, responses to phenanthrene were studied at the biochemical and transcriptional level of genes belonging to phase I (CYPs) and phase II (GSTs) metabolism of xenobiotics and on biochemical parameters related to antioxidant defences. The results obtained in this study may also provide background information on putative biomarkers which may contribute to enhance any decision-making in the public and/or private sectors on the environmental effects to petroleum industry-related accidents.

Materials and methods

Chemicals

Phenanthrene (P1140-9; 98% purity) and all other chemicals used in this work were purchased from Sigma-Aldrich (São Paulo) and were from the highest commercial grade available. Reagents used for total RNA isolation were provided by Sigma-Aldrich, Invitrogen (São

Paulo) and Macherey-Nagel (Alvorada); kits used for cDNA synthesis and quantitative real-time PCR (qPCR) reactions were purchased from Qiagen (São Paulo).

Animals and exposure conditions

Mangrove oysters (*Crassostrea brasiliana*) of similar shell length (6.0 – 8.0 cm) were collected at an oyster farm at Sambaqui beach (Marine Mollusks Laboratory, UFSC) in Florianópolis, southern Brazil. After collection, the animals were immediately transported to the laboratory and placed in aerated tanks with 0.45 µm-filtered seawater (1 L of seawater per animal), at 21 °C and salinity 25 ppt. Oysters were fed twice a day on microalgae (*Chaetoceros muelleri* and *Isochrysis* sp.) at a density of 3.3 x 10^6 cels.mL^{-1} and 2.2 x 10^6 cels.mL^{-1}, respectively, and water was changed daily. Animals were maintained for at least seven days for acclimatization prior to the chemical exposure. Phenanthrene was first dissolved in dimethyl sulfoxide (DMSO) and this was then added to filtered seawater to achieve a final nominal phenanthrene concentration of 100 and 1000 µg.L^{-1}, equivalent to 0.56 and 5.6 µM, and a final DMSO concentration of 0.01% (v/v). Oysters were then randomly divided into three glass exposure tanks which were individually aerated and covered with glass to avoid evaporation of phenanthrene, and held fasting for 24 h prior to the exposure. Control group was carried out under similar conditions except for the introduction of 0.01% (v/v) DMSO only. During the 24 h exposure period control and exposed organisms were not fed to prevent potential bioaccumulation of phenanthrene by food. No mortality was observed in the control and treated groups.

Seawater phenanthrene monitoring

To monitor the experimental amounts of phenanthrene, phenanthrene concentration was followed over a 24 h period. At indicated intervals, a sample was collected from the DMSO-containing seawater and from the phenanthrene-containing seawater in the presence and in the absence of animals. After sample collection, fluorimetric readings (240 nm excitation/360 nm emission) were immediately taken. Samples were collected in autoclaved 5 mL amber bottles and fluorescent readings were made in triplicate using a serial dilution of 2 mg.L^{-1} phenanthrene as

reference standard curve. Seawater phenanthrene analyses were conducted using spectrofluorimeter (Spectramax 250, Molecular Devices, Sunnyvale, CA). In order to determine the initial rate and half-life of phenanthrene disappearance due to oysters, the decrease in fluorescence in tanks devoid of animals was subtracted from the actual readings and presented as *C. brasiliana*-dependent consumption of phenanthrene.

Treatment and sample preparation

Eight oysters at each exposure concentration were sacrificed after 24 h exposure, and tissue samples of gill and digestive gland were immediately excised and processed for thiol measurements. Partial gill and digestive gland samples were also dissected and preserved in RNA*later* (Sigma-Aldrich) and kept on ice until frozen at -20 °C for further gene transcription analysis or samples were directly frozen in liquid nitrogen and stored at -80 °C for enzymatic assays.

Gene selection

Partial sequences for six CYP-related genes (*CYP2AU1* which was assigned by David Nelson, two *CYP2-like*, *CYP3A2-like*, *CYP356A1-like* and *CYP4-like*) and four GST-related genes (*GST-like*, *GST omega-like*, *GST pi-like*, *GST microsomal 3-like*) were selected from the *C. brasiliana* transcriptome database[4]. The genes were annotated based on the closest blastx hits in the NCBI nr database (*E* value threshold of 10^{-6}). The *alpha tubuline-like* gene was used as endogenous reference gene (ERG). Primers were designed using Primer3 software, following the requirements for qPCR assays.

Isolation of total RNA and procedure of reverse transcription (RT)

Total RNA from gill and digestive gland of each oyster was individually isolated using TRIzol reagent (Invitrogen) and further purified with the Nucleospin RNA II Total RNA Isolation kit (Macherey-Nagel) following the supplier's protocol with minor modifications. Briefly, 50 – 100 mg of each tissue was mechanically disrupted in the presence of 1 mL TRIzol using a homogenizer

[4] See Chapter 4.

(Tissue-Tearor, BioSpec Products). TRIzol protocol was strictly followed until the upper aqueous phase was achieved, where 200 µL was transferred to a new tube for on-column precipitation using the Nucleospin RNA II Total RNA Isolation kit. RNA was then eluted in 60 µL of RNase free water. The residual genomic DNA contamination was removed during the RNA cleanup using the RNase-free DNase I digestion as instructed by the manufacturer (Macherey-Nagel). RNA concentration and purity were then measured using a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific). Only high purity samples (OD 260/280 > 1.8; OD 260/230 > 1.8) were further processed. 1 µg of total RNA per sample was DNase treated then reverse transcribed using a QuantiTect Reverse Transcription kit (Qiagen) with a mixture of oligo-dT and random primers. A pool of samples from all treatments was used for no reverse transcription control (NRTC) which was removed post DNase treatment but before the RT step. Aliquots of the RT mixture were diluted at 1/10 with nuclease-free water before use. The resulting neat and diluted cDNA and NRTC were stored at -20 °C.

qPCR analysis

The relative levels of gene transcripts in the gill and digestive gland from control and exposed oysters were investigated by qPCR using the Rotor-Gene SYBR Green PCR kit (Qiagen) and Rotor-Gene 6000 real-time qPCR system (Qiagen) with primers specific to *C. brasiliana* (Table 1). qPCR was performed in technical duplicates for each sample in a 20 µL reaction volume containing 10 µL Rotor-Gene SYBR Green PCR kit, 1 µL of each primer (1 µM), 2 µL of diluted cDNA sample, NRTC or no template control (NTC) and 6 µL of nuclease-free water. PCR amplification was performed using the fast two-step cycling program as follows: 5 min at 95 °C, and 40 cycles of 5 s at 95 °C and 10 s at 60 °C as instructed by the manufacturer. PCR products were subjected to melt curve analysis (ramp from 72 °C to 95 °C, rising by 1 °C each step, acquire to green fluorescence) to ensure that non-specific priming was absent in samples, and selected samples were gel verified by electrophoresis to check for single amplicons and primer dimers. Each plate included negative control (NRTC to ensure no background DNA contamination after DNase treatment) and NTC (sterile water).

Table 1. Primer sequences used for the qPCR for the amplification of each target and endogenous reference gene (ERG) with putative gene name and amplicon size (bp).

Gene name	Primer sequence 5'-3'	Amplicon size (bp)
ERG		
Alpha tubuline-like	F - TGA GGC CCG TGA AGA TCT TGC TGC R - ACC ACC CTC CTC TTC AGC TTC ACC T	145
Phase I biotransformation cytochrome P450 genes		
CYP2AU1	F - AAC GGC AAG AGG TGT AAG GTT TGC R - TAA TCC ATC ACC CGG ATT GGC AGA	158
CYP2-like 1	F - TCG TGC TCC TTT ACG AGT TGA CGA R - ATA TGC CGG GAG ATC CAT GTC GAA	91
CYP2-like 2	F - CGC TTC GCA GTC CAA GTT GAC AAA R - ATC GTG TTT GGG TTC AGG TAT GCG	136
CYP3A2-like	F - AGT GGA CGT CAA CAA CTG GAT CGT R - TGG AAC ACC ATA CCT CCG GAA CAA	103
CYP4-like	F - TTA ATG GCC AGA ACC TTT GCT GCC R - GAC GTC ATT GCC TCA ACT GCC TTT	98
CYP356A1-like	F - TGT TCA GGC CCA ACA ACT CTG TCA R - GGG AGT GGA CTC AAC CAG ATT CAC AA	114
Phase II biotransformation glutathione S-transferase genes		
GST-like	F - ACT CAT ACC ATC CGA CAA AGC CCA R - TGG CAT CCT CTG CCT TCT TCT TGA	167
GST omega-like	F - ATT GGC ACA CGT ACC TCG TCT GAT R - TTA ATG GGA CCG CCA GAA GGT CAT	175
GST microsomal 3-like	F - GCA TTG TCT GGT GTG GTT TGG TGT R - CCT GAG AGT ATG ATG CAG CTT GCA GA	153
GST pi-like	F - ATG GCG TTG GAT TGC ACT AAC TGG R - ACG GAC GCT ACT GGT GGA CAA TAA	100

PCR efficiency (*E*) was determined for each primer pair by constructing a standard curve from serial dilutions as follows: equal amounts of cDNA from all samples were pooled and serially diluted to generate efficiency curves from four cDNA concentrations. All efficiency curves had a R^2 greater than 0.99 and efficiencies between 95–105%. Cycle threshold (Ct) values corresponded to the number of cycle at which the fluorescence emission monitored in real-time exceeded the threshold limit. Ct and *E* were obtained using the Rotor-Gene 6000 real-time qPCR system.

Protein and non-protein thiols

After dissection, tissue samples of gill and digestive gland (approximately 100 mg per animal) were immediately homogenized in 1

mL of 0.5 M perchloric acid, and centrifuged at 15,000 *g* for 2 min at 4 °C. The supernatant was assayed for total glutathione (GSH-t) and for non-protein thiols (NPSH), whereas the pellet was carefully solubilised in 500 µL of 0.5 M Tris-HCl buffer pH 8.0, containing 1% SDS, and assayed for protein thiols (PSH).

Total glutathione (GSH-t), comprising both reduced (GSH) and oxidized (as disulphide, GSSG) glutathione forms, was determined by the GR-DTNB recycling assay, as previously described by Akerboom and Sies (1981), using a Cary 50 UV/Vis Spectrophotometer (Varian Inc., Palo Alto). A standard curve using known GSH amounts was used to obtain actual values.

NPSH, representing the low-molecular weight thiols such as glutathione and cysteine, and PSH, comprising the reduced thiols present in proteins, were measured colorimetrically using the Ellman's reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (Ellman, 1959). For NPSH, the acid extract was added to 0.5 M Tris-HCl buffer pH 8.0 and 0.25 mM DTNB. For PSH, the resuspended pellet was added to 0.5 M Tris-HCl buffer pH 8.0, containing 1% SDS and 0.25 mM DTNB, and let react for 30 minutes before readings. The thiol levels were estimated at 412 nm using the DTNB molar extinction coefficient of 13,600 using a Cary 50 UV/Vis Spectrophotometer (Varian Inc., Palo Alto).

Enzyme assays

Gill and digestive gland of each oyster were individually weighed and homogenized in 1:4 w/v chilled buffer (20 mM Tris-HCl buffer pH 7.6, containing 0.5 M sucrose, 1 mM DTT, 1 mM EDTA, 0.15M KCl and 0.1 mM PMSF) using the tissue homogenizer *Tissue-Tearor*™. The homogenates were centrifuged at 9000 *g* for 30 min at 4 °C, followed by a second centrifugation of the supernatants at 37,000 *g* for 74 min at 4 °C to obtain the cytosolic fraction. Total protein levels were quantified in the supernatant according to Peterson (1977) using bovine serum albumin as standard.

Catalase (CAT) turnover rate was determined by the decrease in absorbance at 240 nm by H_2O_2 decomposition, according to Beutler (1975). Glutathione peroxidase (GPx) activity was measured indirectly by monitoring the NADPH oxidation rate at 340 nm according to Sies et al. (1979) using cumene hydroperoxide (CuOOH) as substrate. Glutathione reductase (GR) activity was quantified by the NADPH oxidation rate at 340 nm (Sies et al., 1979). Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined following the method of Glock and McLean (1953), which evaluates the increase in

absorbance at 340 nm caused by the reduction of $NADP^+$ to NADPH. Glutathione *S*-transferase (GST) activity was assayed by increasing absorbance at 340 nm, using 1-chloro-2,4 dinitrobenzene (CDNB) as GST universal substrate (Keen et al., 1976). All enzyme assays using visible wavelengths were carried out using 96 wells plates reader (Spectramax 250, Molecular Devices, Sunnyvale, CA), while CAT activity was assayed in a Perkin Elmer Lambda Bio20 UV/visible spectrophotometer (Perkin Elmer, Cambridge).

Statistical analysis

Biochemical parameters (thiols and enzyme activities) were measured in 8 individuals from each treatment. Differences in mean values were analyzed by one-way ANOVA followed by complementary Tukey test when convenient. Student's *t*-test was also performed to compare the basal levels (control groups) of thiols and enzymatic activities between tissues. Normality (Shapiro-Wilks test) and homogeneity of variances assumptions were previously checked (Bartlett's test) (Zar, 1999) and outliers were excluded according to the Grubbs test. Differences were considered statistically significant when $p < 0.05$ and analyses were performed with the software GraphPad 5.0.

Gene transcription levels were assessed in 7 individuals from each treatment. The relative mRNA expression ratio for a considered gene was analyzed using an efficiency corrected ΔΔCt method, normalizing to the endogenous reference gene (ERG) (Pfaffl et al., 2002). *p*-values were obtained using a pair wise fixed reallocation randomization test (2,000 iterations) using the relative expression software tool (REST 2009) (Pfaffl et al., 2002). Differences were considered statistically significant when $p < 0.01$.

Results

Seawater phenanthrene monitoring

To assess phenanthrene stability during the oyster exposure, experimental amounts of phenanthrene (0.01% DMSO final concentration) were added into the seawater to provide nominal concentrations of 100 or 1000 $\mu g.L^{-1}$ (Figure 1). Phenanthrene concentrations decreased over time in the water at an initial rate of 15.8 + 0.6 or 130.5 ± 12.8 $\mu g.L^{-1}.h^{-1}$ in the 100 or 1000 $\mu g.L^{-1}$ tanks, an 8.3 fold increase in the initial rate of phenanthrene consumption by the oyster. The spontaneous decrease in phenanthrene was subtracted from rates obtained in the presence of animals. As can be seen, oysters readily remove phenanthrene from the water, supposedly by detoxification

reactions, presenting a half life of 2.85 or 7.75 h at 100 or 1000 μg.L^{-1}. These data undoubtedly demonstrate a high phenanthrene consumption rate by *C. brasiliana*, probably due to detoxification mechanisms, which remains to be proven. The 8.3 fold increase in initial decay indicates that the absorbing/degrading system is not yet saturated, even at the highest concentration studied.

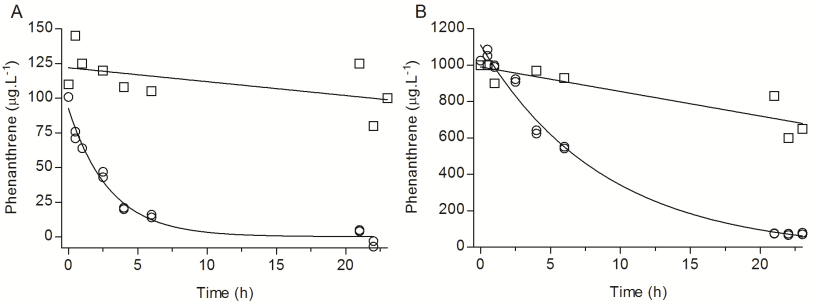


Figure 1. Disposal of phenanthrene by mangrove oysters *Crassostrea brasiliana*. Phenanthrene at 100 (A) or 1000 (B) μg.L^{-1} nominal concentration was followed in the seawater (salinity 25 ppt) over time in the absence (squares) or in the presence (circles) of oysters.

Phenanthrene effects on oyster gene transcription

Four genes, out of six, putatively involved in phase I biotransformation of xenobiotics showed a marked upregulation in the gill of oysters exposed to 1000 μg.L^{-1} phenanthrene (Figure 2A). All studied genes belonging to the *CYP2* family were upregulated between 9 and 16 fold, of which *CYP2-like 2* showed the strongest upregulation (~16-fold), followed by *CYP2AU1* (~12-fold), and *CYP2-like 1* (~9-fold) as compared to control group. *CYP356A1-like* was upregulated nearly 9-fold relative to the control group, while transcripts for *CYP3A2-like* and *CYP4-like* remained unchanged. Interestingly, only *CYP2-like 2* gene was upregulated approximately 8-fold after exposure to 100 μg.L^{-1} phenanthrene as compared to untreated control animals (Figure 2A).

Transcript levels for putative CYP-related genes in digestive gland (Figure 2B) exhibited a distinct pattern of response to phenanthrene exposure as compared to gill, with upregulation only for *CYP2AU1* (~3-fold) after exposure to 1000 μg.L^{-1} and none at 100 μg.L^{-1} concentration.

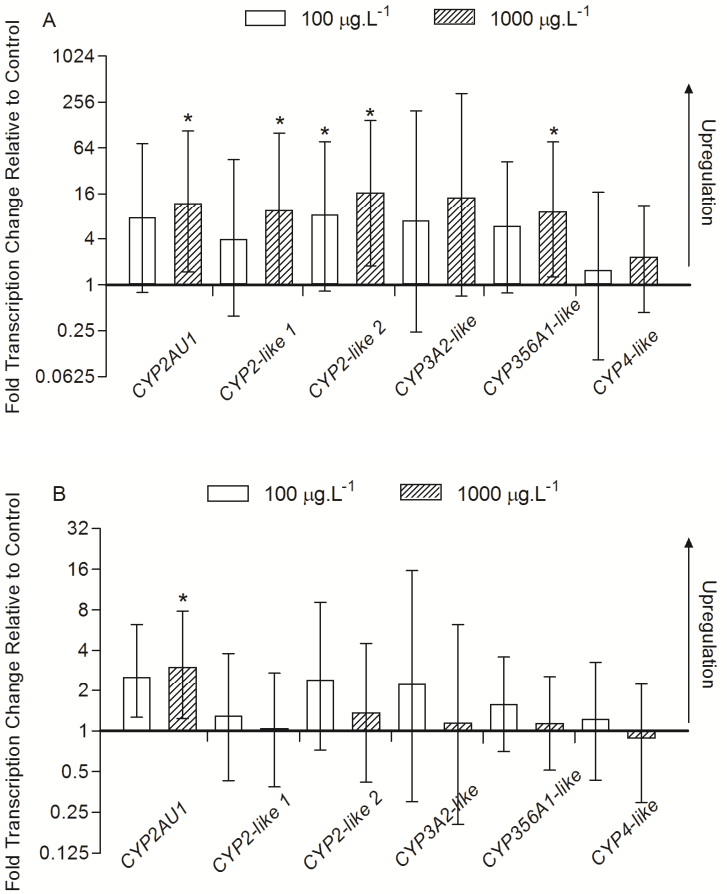


Figure 2. Relative gene transcription of putative phase I biotransformation of xenobiotics genes in the gill (A) and digestive gland (B) of the mangrove oyster *Crassostrea brasiliana* exposed to phenanthrene at 100 μg.L^{-1} and 1000 μg.L^{-1} for 24 h. The gene transcript levels were assessed by qPCR and transcription is relative to control group. Note the y axis is logarithmic to base 2. Statistical analysis was performed by using a pairwise fixed reallocation randomization test. *Represents significant transcription change (REST 2009 software Qiagen®, $p < 0.01$).

Concerning genes related to GSTs putatively involved in phase II xenobiotics detoxification, of the four GST-related genes assayed in this study, two showed significant upregulation relative to the control group,

but only for the gill at the highest concentration of phenanthrene (1000 μg.L^{-1}). *GST omega-like* and *GST microsomal 3-like* were upregulated approximately 9-fold and 6-fold, respectively (Figure 3A). No significant differences were observed in GST-related genes in the digestive gland for both 100 and 1000 μg.L^{-1} groups (Figure 3B).

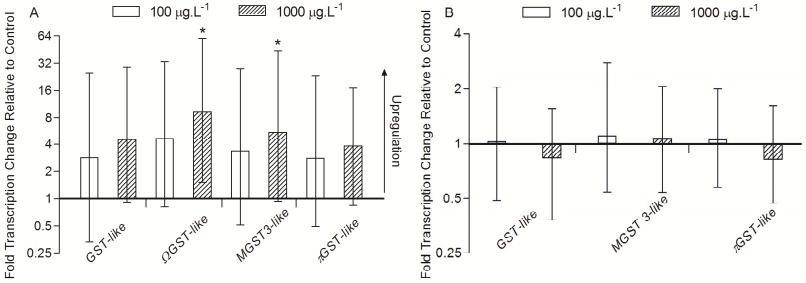


Figure 3. Relative gene transcription of putative phase II biotransformation of xenobiotics genes in the gill (A) and digestive gland (B) of the mangrove oyster *Crassostrea brasiliana* exposed to phenanthrene at 100 μg.L^{-1} and 1000 μg.L^{-1} for 24 h. The gene transcript levels were assessed by qPCR and transcription is relative to control group. Note the y axis is logarithmic to base 2. Statistical analysis was performed by using a pairwise fixed reallocation randomization test. *Represents significant transcription change (REST 2009 software Qiagen®, $p < 0.01$). GST classes/isoforms: *ΩGST* (omega), *MGST3* (microsomal GST isoform 3), *πGST* (pi). *ΩGST-like* could not be amplified in the digestive gland.

Biochemical parameters

Figure 4 shows the thiol status measured in the gill and digestive gland of *C. brasiliana* exposed to phenanthrene for 24 h. NPSH levels decreased in the gill and digestive gland of animals exposed to 1000 μg.L^{-1} phenanthrene [$F_{(2,19)} = 4.131$; $p < 0.05$ for gill and $F_{(2,21)} = 3.830$; $p < 0.05$ for digestive gland], as shown in Figure 4A. The levels of GSH-t were significantly decreased in the gill following exposure to phenanthrene [$F_{(2,19)} = 3.662$; $p < 0.05$]. In digestive gland, no significant differences in the levels of GSH-t were observed ($p > 0.05$) (Figure 4B). The levels of PSH did not differ among experimental groups for both tissues ($p > 0.05$) (Figure 4C).

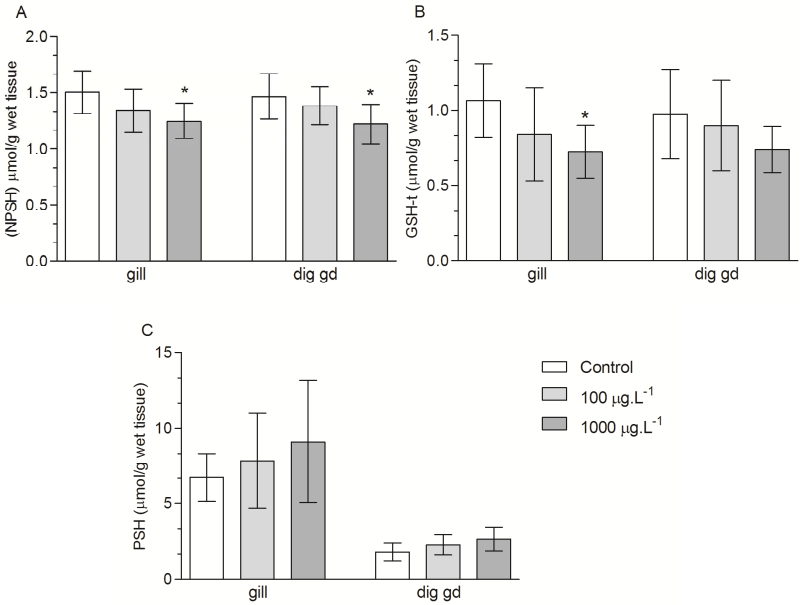


Figure 4. Thiol status measured as levels of (A) non-proteic thiols (NPSH), (B) total glutathione (GSH-t) and (C) proteic thiols (PSH) in the gill and digestive gland (dig gd) of the mangrove oyster *Crassostrea brasiliana* exposed to phenanthrene at 100 µg.L^{-1} and 1000 µg.L^{-1} for 24 h, plus the seawater as the control group. Results are expressed as mean ± standard deviation (S.D.) (n = 7 - 8 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc analysis.*Represents significant differences, p < 0.05.

As an index of phase II biotransformation of xenobiotics, GST activity decreased in the gill of animals exposed to 100 µg.L^{-1} phenanthrene [$F_{(2,21)}$ = 3.405; p < 0.05], but activity remained unchanged at 1000 µg.L^{-1} reaching similar values to the control group (p > 0.05) (Figure 5A), while GST activity did not change in digestive gland (p > 0.05) (Figure 5A). Phenanthrene treatment did not produce any significant alteration in antioxidant (CAT and GPx) and related enzymes (GR and G6PDH) in the gill or digestive gland of *C. brasiliana* (Figures 5B - E).

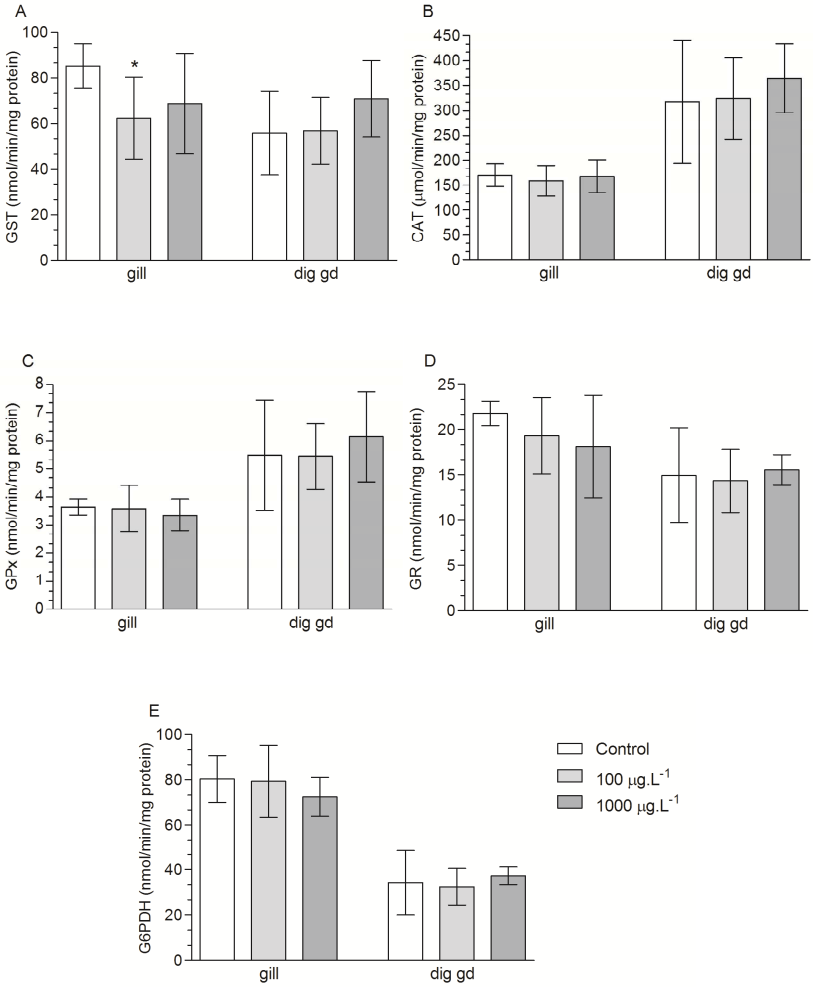


Figure 5. Graphs depict activity of the phase II biotransformation of xenobiotics, antioxidant and related enzymes in the gill and digestive gland (dig gd) of the mangrove oyster *Crassostrea brasiliana* exposed to phenanthrene at 100 µg.L^{-1} and 1000 µg.L^{-1} for 24 h: (A) glutathione *S*-transferase (GST), (B) catalase (CAT), (C) glutathione peroxidase (GPx), (D) glutathione reductase (GR) and (E) glucose-6-phosphate dehydrogenase (G6PDH). Results are expressed as mean ± standard deviation (S.D.) (n = 7 - 8 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc analysis.*Represents significant differences, $p < 0.05$.

Tissues differences in the levels of phase II biotransformation, antioxidant and related parameters of oysters from the control group are shown in Table 2. Oysters showed higher PSH levels and GST, GR and G6PDH activities in the gill, whereas CAT and GPx activities were significantly higher in the digestive gland. However, NPSH and GSH-t were not altered between tissues ($p > 0.05$).

Table 2. Tissue levels of phase II biotransformation of xenobiotics, antioxidant and related parameters in the gill and digestive gland of the untreated (control group) mangrove oyster *Crassostrea brasiliana*. Results are expressed as mean ± standard error of the mean (S.E.M.).

Parameters	**Gill**	**Digestive gland**	**Fold variation to gill**	***p***
Non-proteic thiols (NPSH)	1.50 ± 0.07	1.47 ± 0.07	1.0	NS
Total glutathione (GSH-t)	1.07 ± 0.09	0.97 ± 0.10	1.1	NS
Proteic thiols (PSH)	6.72 ± 0.57	1.80 ± 0.64	3.7	***
Glutathione *S*-transferase (GST)	85.30 ± 3.45	55.87 ± 6.50	1.5	**
Catalase (CAT)	170.0 ± 7.93	317.3 ± 43.78	-1.9	***
Glutathione peroxidase (GPx)	3.54 ± 0.13	5.70 ± 0.32	-1.6	***
Glutathione reductase (GR)	21.80 ± 0.51	14.96 ± 1.85	1.5	**
Glucose-6-phosphate dehydrogenase (G6PDH)	88.38 ± 3.66	34.25 ± 5.02	2.6	***

Significant differences are as follow: $**p < 0.01$ and $***p < 0.001$; $n = 7 – 8$ animals per group; NS: non-significant.

Discussion

We were able to show a rapid uptake of phenanthrene by the oysters (Figure 1), despite the presence of a small spontaneous decay, which provides evidence of the ability of *C. brasiliana* to dispose low molecular PAHs, confirming our previous study with this species (Lüchmann et al., 2011). Bioaccumulation of PAHs has also been reported for other bivalves, such as *Crassostrea virginica* (Elder and Dresler, 1988), *Mytilus galloprovincialis* (Valavanidis et al., 2008), *Perna perna* (Francioni et al., 2007) and *Pecten maximus* (Hannam et al., 2010).

Once phenanthrene has been taken up by an organism it may be subjected to biotransformation reactions to further detoxification and excretion (Hannam et al., 2010). In mammals, these reactions are known to be mediated by the cytochrome P450 (CYP) enzymes during phase I biotransformation, producing reactive intermediates, such as diol

epoxides (Pushparajah et al., 2008; Shou et al., 1994). According to Hecht et al. (2008, 2009), PAH bay region diol epoxides are suitable substrates for phase II reactions. However, very little is known on the molecular mechanisms of phenanthrene metabolism in bivalves. With that in mind we selected six putative CYP-related genes from families 2, 3, 4 and 17, and four GST-related genes putatively belonging to class omega, pi and microsomal 3 to investigate their modulation at the transcriptional level in the gill and digestive gland of *C. brasiliana* exposed to phenanthrene for 24 h.

We found that the CYP2 gene family was the most induced by phenanthrene in oysters, which is in line with previous findings for *CYP2* transcript levels in rat and mouse challenged with the same PAH (Schober et al., 2010; Shou et al., 1994). Interestingly, mRNA expression levels for the three CYP2 genes in the gill of *C. brasiliana* presented similar responses to phenanthrene treatment, with increased transcription found for *CYP2-like 2* (~16-fold), followed by *CYP2AU1* (~12-fold) at the highest concentration of 1000 $\mu g.L^{-1}$. Similarly, *CYP2-like 2* transcript levels were induced nearly 3 fold in the gill of oysters treated by 100 $\mu g.L^{-1}$ phenanthrene. These data indicates the role of the CYP2 enzymes in the detoxification of environmental contaminants as previously reported by Kubota et al. (2011). Moreover, *CYP356A1-like*, which is closely related to members of the CYP1 and CYP17 gene families, was strongly upregulated (~9-fold) in the gill of oysters exposed to 1000 $\mu g.L^{-1}$ phenanthrene. In a previous study carried out by our group, *CYP356A1* were induced in oyster *Crassostrea gigas* after exposure to domestic sewage (Toledo-Silva et al., 2008), which gives support to its role in xenobiotic biotransformation. It is also interesting to note that in our study the gill presented a significant upregulation in their CYP-related genes, in contrast to the weak effect of phenanthrene to the digestive gland, considered the main detoxifying organ of bivalves (Verlecar et al., 2007). Our results point the gill as an important tissue for the phase I biotransformation of PAHs.

The absence of changes in transcript abundance for *CYP3A2-like* and *CYP4-like* in exposed oysters suggests that these CYP families do not present short term activation in phenanthrene metabolism of *C. brasiliana*. Compared to previous studies on aquatic organisms, our results showed different trends which are somehow unexpected as CYP3 and CYP4 enzymes are likely to be involved in cell detoxification. For instance, previous studies have shown a moderate upregulation of *CYP3A* in red mullet captured in the north-eastern Adriatic Sea, close to an oil refinery (Torre et al., 2010), and an induction of *CYP3-like* in

mussel treated by beta-naphtoflavone (Zanette, 2009). Furthermore, Sabourault et al. (1999) found a decrease in *CYP4* mRNA levels sea bass exposed to benzo[a]pyrene, which has recently been supported by Miao et al. (2011), who showed similar findings in scallops. A possible explanation for this discrepancy could be due to different intrinsic features of CYP subfamilies regarding different expression patterns and distributions, as observed for CYP4 genes in the polychaetes *Nereis virens* and *Capitella capitata* sp. following exposure to PAHs (Li et al., 2004; Rewitz et al., 2004).

Concerning phase II biotransformation genes, in our study we investigated four *GST-like* genes but not all of the GST classes and tissues showed transcriptional changes after exposure to phenanthrene. Only the gill *GST omega-like* and *GST microsomal 3-like* showed obvious increased mRNA levels (~9-fold and ~6-fold, respectively) for 1000 $\mu g.L^{-1}$ concentration. In line with this, a significant upregulation for *GST omega* was reported by Boutet et al. (2004) in *C. gigas* treated by hydrocarbons. The authors also found induction of *GST mu*, indicating that the extent of *GST* induction towards PAHs in mollusc is species-specific, which is further supported by the elevated transcript levels of *GST pi* found in scallop *Chlamys farreri* exposed to benzo[a]pyrene (Miao et al., 2011). This discrepancy in the extent of induction has been previously reported for mammalian cells, which supports the idea that phenanthrene detoxification is a gene class-, dose- and cell-specific process (Hecht et al., 2008; Sundberg et al., 2002).

Unlike gene transcription, GST catalytic activity showed a significant decrease in the gill after exposure to 100 $\mu g.L^{-1}$ phenanthrene. This is consistent with previous findings obtained for fish exposed to the same PAH (Oliveira et al., 2008). Therefore, combining no upregulation of GST-related genes and significant decrease in GST activity results, we can suggest that the absence of transcriptional GST activity did not produce enough of the enzyme to cope with phenanthrene detoxification in the cells. Furthermore, different sensitivity towards inducers at transcript level, mRNA processing and protein stability may have led to discrepancies between induction of gene transcription and enzyme activity, as reported by Trisciani et al. (2011). Curiously, the elevated transcript levels for *GST omega-like* and *GST microsomal 3-like* in the gill after exposure to 1000 $\mu g.L^{-1}$ phenanthrene seemed to maintain GST enzyme production similar to basal levels identified in the control group. This is further supported by the marked reduction seen in GSH-t levels in the gill. According to Hecht et al. (2008) and Pushparajah et al. (2008), GST are considered

highly effective enzymes in conjugating metabolically activated PAHs to glutathione, leading to depletion of GSH content (Dickinson and Forman, 2002). In line with this, in a recent work carried out with *C. gigas*, Trevisan et al. (2012) observed that oysters exposed for 18 h to the eletrophilic compound CDNB had depleted levels of GSH-t and PSH in the gill. The authors also observed a 2.7 times increase in the gill GST activity, followed by the appearance of the CDNB-glutathione conjugate in seawater, which clearly reinforce the idea of thiol consumption during metabolism of eletrophilic compounds and the induction of phase II enzymes in such situations. However, the reduction in GSH-t and NPSH levels following phenanthrene exposure is likely to indicate enhanced conjugation allied to the formation of ROS, which in turn, can overwhelm this antioxidant defence system, as previously reported in scallops by Hannam et al. (2010). Whether GSH-t was depleted by GST conjugation activity or ROS formation is unknown, with further investigation required to determine if phenanthrene exposure plays a pro-oxidative role in *C. brasiliana*. Moreover, it has to be emphasized that the GST activity was measured utilizing the universal GST substrate CDNB, and previous report have evidenced that many GST classes are devoid or present only very low activity toward CDNB (*i.e.* Dixon and Edwards, 2010). Thus, one must be aware that the quantification of GST-related genes levels and GST activity in this study might not be measurements of the same biological response.

Because of numerous studies have reported changes in the activity of antioxidant enzymes in aquatic organisms following exposure to phenanthrene (*i.e.* Correia et al., 2007; Hannam et al., 2010; Oliveira et al., 2008), in this study, we also assessed the effects of this PAH on the activity of CAT, GPx, GR and G6PDH in the gill and in digestive gland of *C. brasiliana*. Our results showed that phenanthrene did not elicit any significant response of antioxidant and related enzymes in oysters after 24 h exposure. This is consistent with previously documented report that enzymatic changes related to PAHs exposure tends to be time-dependent with most pronounced differences seen after 48 h exposure (Ansaldo et al., 2005; Banni et al., 2010; Solé et al., 2007). In fact, we have recently showed that *C. brasiliana* revealed clear biochemical responses to diesel fuel water-accommodated fraction after 96 h of exposure (Lüchmann et al., 2011), indicating that 24 h might not have been long enough to activate antioxidant defence system at protein level following phenanthrene exposure. This would also be the case for GST-protein synthesis. However, Bebianno and Barreira (2009) found a significant

increase of antioxidant enzymes in the clam *Ruditapes decussatus* at the first day following transplant to PAHs polluted areas. Thus, further studies are required to understand the temporal modulation of antioxidant defences in *C. brasiliana* following exposure to PAHs.

Based on the differences between tissues seen in this study for both molecular and biochemical parameters we can postulate that *C. brasiliana* shows tissue-specific responses following phenanthrene challenge. It is supported by the lesser responsiveness of CYP- and GST-related genes seen in digestive gland, which is consistent to the idea that gill is the target organ for rapid reaction to short-term events whereas hepatic tissues require the accumulation of chemicals following chronic exposure, such as previously observed for biochemical parameters in fish (Webb and Gagnon, 2009). This is further supported by the lower antioxidant levels in the digestive gland, as compared to the gill, which includes lower PSH levels and GR, G6PDH and GST activities. It is interesting to note that the gill of some bivalve species also present higher activity of GST, G6PDH and GR as compared to the digestive gland (Almeida et al., 2005; McDonagh and Sheehan, 2008; Lopez-Galindo et al., 2010). Altogether our findings indicate that gill seems to present a more potent repertoire related to thiol and biotransformation metabolism: a) higher thiol pool in proteins; b) better capacity to regenerate oxidized GSH through elevated GR and G6PDH; c) higher capacity of conjugation through GST; d) prompt responses at the transcriptional level amplifying phase I (CYPs) and phase II (GSTs) genes. These data reinforces the idea that gill is a very important detoxification organ, in line with Luckenbach and Epel (2008) proposal.

In conclusion, while phenanthrene induced transcription of CYP- and GST-related genes, it also reduced both NPSH and GSH-t levels and GST activity following 24 h exposure. Transcription upregulation of CYP2 gene family, *CYP356A1-like*, *GST omega-like* and *GST microsomal 3-like* occurred in a tissue-specific manner, reflecting the importance of the gill in the mechanism of PAH detoxification. Further investigations into the substrate selectivity of catalytic activity of GSTs are essential for completely understanding their role in xenobiotic biotransformation in bivalves, as well as a time course study to unravel long-term responses. Finally, our study goes some way toward achieving these goals and sheds light on tissue-specific transcription of biotransformation-related genes and catalytic activity of antioxidant parameters which should also increase the set of potential biomarkers of aquatic contamination in bivalves.

Acknowledgments

This work was supported by CNPq (National Council for Research Development), INCT–TA (National Institute of Science and Technology – Aquatic Toxicology) and FAPESC (Foundation for the Support of Scientific and Technological Research in the State of Santa Catarina). The provided scholarships are sincerely appreciated (KHL; TSD; RT from Capes and MCN from CNPq). ACDB and ALD are CNPq research fellows. We would like to thank Jacó Joaquim Mattos and Marília Nardelli Siebert for their assistance in dissecting the oysters.

References

Akerboom, T. P.; Sies, H. Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. *Methods Enzymol.* **1981**, *77*, 373–382.

Almeida, E. A.; Bainy, A. C. D.; Dafre, A. L.; Gomes, O. F.; Medeiros, M. H. G.; Mascio, P. D. Oxidative stress in digestive gland and gill of the brown mussel *Perna perna* exposed to air and re-submersed. *J. Exp. Mar. Biol. Ecol.* **2005**, *318*, 21–30.

Ansaldo, M.; Najle, R.; Luquet, C. M. Oxidative stress generated by diesel seawater contamination in the digestive gland of the Antarctic limpet *Nacella concinna*. *Mar. Environ. Res.* **2005**, *59*, 381–390.

Anyakora, C.; Ogbeche, A.; Palmer, P.; Coker, H. Determination of polynuclear aromatic hydrocarbons in marine samples of Siokolo Fishing Settlement. *J. Chromatogr.* **2005**, *1073*, 323–330.

Banni, M.; Negri, A.; Dagnino, A.; Jebali, J.; Ameur, S.; Boussetta, H. Acute effects of benzo[a]pyrene on digestive gland enzymatic biomarkers and DNA damage on mussel *Mytilus galloprovincialis*. *Ecotoxicol. Environ. Saf.* **2010**, *73*, 842–848.

Bebianno, M. J.; Barreira, L. A. Polycyclic aromatic hydrocarbons concentrations and biomarker responses in the clam *Ruditapes decussates* transplanted in the Ria Formosa lagoon. *Ecotoxicol. Environ. Saf.* **2009**, *72*, 1849–1860.

Beutler, E. *Red cell metabolism: a manual of biochemical methods*; Grune & Stratton: New York, **1975**.

Boutet, I.; Tanguy, A.; Moraga, D. Characterisation and expression of four mRNA sequences encoding glutathione S-transferases pi, mu, omega and sigma classes in the Pacific oyster *Crassostrea gigas* exposed to hydrocarbons and pesticides. *Mar. Biol.* **2004**, *146*, 53–64.

Correia, A. D.; Goncalves, R.; Scholze, M.; Ferreira, M.; Henriques, M. A. R. Biochemical and behavioral responses in gilthead seabream (*Sparus aurata*) to phenanthrene. *J. Exp. Mar. Biol. Ecol.* **2007**, *347*, 109–122.

Dickinson, D. A.; Forman, H. J. Cellular glutathione and thiols metabolism. *Biochem. Pharmacol.* **2002**, *64*, 1019–1026.

Dixon, D. P.; Edwards, R. Glutathione S-transferases. In *The Arabidopsis Book*. American Society of Plant Biologists: Rockville, MD, **2010**.

Elder, J. F.; Dresler, P. V. Accumulation and bioconcentration of polycyclic aromatic hydrocarbons in a nearshore estuarine environment near a Pensacola (Florida) creosote contamination site. *Environ. Pollut.* **1988**, *49*, 117–132.

Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.

Fernández, B.; Campillo, J. A.; Martínez-Gómez, C.; Benedicto, J. Antioxidant responses in gills of mussel (*Mytilus galloprovincialis*) as biomarkers of environmental stress along the Spanish Mediterranean coast. *Aquat. Toxicol.* **2010**, *99*, 186–197.

Forbes, V. E.; Palmqvist, A.; Bach, L. The use and misuse of biomarkers in ecotoxicology. *Environ. Toxicol. Chem.* **2006**, *25*, 272–280.

Francioni, E.; Wagener, A. L. R.; Scofield, A. L.; Depledge, M. H.; Cavalier, B. Evaluation of the mussel *Perna perna* as a biomonitor of polycyclic aromatic hydrocarbon (PAH) exposure and effects. *Mar. Pollut. Bull.* **2007**, *54*, 329–338.

Frouin, H.; Pellerin, J.; Fournier, M.; Pelletier, E.; Richard, P.; Pichaud, N.; Rouleau, C.; Garnerot, F. Physiological effects of polycyclic aromatic hydrocarbons on soft-shell clam *Mya arenaria*. *Aquat.Toxicol.* **2007**, *82*, 120–134.

Galloway, T. S.; Sanger, R. C.; Smith, K. L.; Fillmann, G.; Readman, J. W.; Ford, T. E.; Depledge, M. H. Rapid assessment of marine pollution using multiple biomarkers and chemical immunoassays. *Environ. Sci. Technol.* **2002**, *36*, 2219–2226.

Glock, G. E.; McLean, P. Further studies on the properties and assay of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. *Biochem. J.* **1953**, *55*, 400–408.

Hannam, M. L.; Bamber, S. D.; Galloway, T. S.; Moody, A. J.; Jones, M. B. Effects of the model PAH phenanthrene on immune function and oxidative stress in the haemolymph of the temperate scallop *Pecten maximus*. *Chemosphere* **2010**, *78*, 779–784.

Hecht, S. S.; Villalta, P. W.; Hochalter, J. B. Analysis of phenanthrene diol epoxide mercapturic acid detoxification products in human urine: relevance to molecular epidemiology studies of glutathione-S-transferase polymorphisms. *Carcinogenesis* **2008**, *29*, 937–943.

Hecht, S. S.; Berg, J. Z.; Hochalter, J. B. Preferential Glutathione Conjugation of a Reverse Diol Epoxide Compared to a Bay Region Diol Epoxide of Phenanthrene in Human Hepatocytes: Relevance to Molecular Epidemiology Studies of Glutathione-S-Transferase Polymorphisms and *Cancer. Chem. Res. Toxicol.* **2009**, *22*, 426–432.

Keen, J. H.; Habig, W. H.; Jakoby, W. B. Mechanism for several activities of the gluthatione *S*-transferases. *J. Biol. Chem.* **1976**, *251*, 6183–6188.

Kubota, A.; Stegeman, J. J.; Goldstone, J. V.; Nelson, D. R.; Kim, E. Y.; Tanabe, S.; Iwata, H. Cytochrome P450 CYP2 genes in the common cormorant: Evolutionary relationships with 130 diapsid CYP2 clan sequences and chemical effects on their expression. Comp. Biochem. Physiol. C Toxicol. Pharmacol. **2011**, *153*, 280–289.

Le Pennec, G.; Le Pennec, M. Induction of glutathione-*S*-transferases in primary cultured digestive gland acini from the mollusk bivalve *Pecten maximus* (L.): application of a new cellular model in biomonitoring studies. *Aquat. Toxicol.* **2003**, *64*, 131–142.

Li, B.; Bisgaard, H. C.; Forbes, V. Identification and expression of two novel cytochrome P450 genes, belonging to CYP4 and a new CYP331

family, in the polychaete *Capitella capitata* sp.I. *Biochem. Biophys. Res. Commun.* **2004**, *325*, 510–517.

Lima, I.; Moreira, S. M.; Rendón-Von O. J.; Soares, A. M. V. M.; Guilhermino, L. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. *Chemosphere* **2007**, *66*, 1230–1242.

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

Lopez-Galindo, C.; Vargas-Chacoff, L.; Nebot, E.; Casanueva, J.; Rubio, D.; Mancera, J.; Sole, M. Sublethal responses of the common mussel (*Mytilus galloprovincialis*) exposed to sodium hypochlorite and Mexel (R) 432 used as antifoulants. *Ecotoxicol. Environ. Saf.* **2010**, *73*, 825-834.

Lüchmann, K. H.; Mattos, J. J.; Siebert, M. N.; Granucci, N.; Dorrington, T. S.; Bicego, M. C.; Taniguchi, S.; Sasaki, S. T.; Daura-Jorge, F. G.; Bainy, A. C. D. Biochemical biomarkers and hydrocarbons concentrations in the mangrove oyster *Crassostrea brasiliana* following exposure to diesel fuel water-accommodated fraction. *Aquat. Toxicol.* **2011**, *105*, 652–660.

Luckenbach, T.; Epel, D. ABCB and ABCC type transporters confer multixenobiotic resistance and form an environment-tissue barrier in bivalve gills. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2008**, *294*, R1919-29.

McDonagh, B.; Sheehan, D. Effects of oxidative stress on protein thiols and disulphides in *Mytilus edulis* revealed by proteomics: actin and protein disulphide isomerase are redox targets. *Mar. Environ. Res.* **2008**, *66*, 193-195.

Miao, J.; Pan, L.; Liu, N.; Xu, C.; Zhang, L. Molecular cloning of CYP4 and GSTpi homologues in the scallop *Chlamys farreri* and its expression in response to Benzo[a]pyrene exposure. *Mar. Genomics* **2011**, *4*, 99–108.

Oliveira, M.; Pacheco, M.; Santos, M. A. Cytochrome P4501A, genotoxic and stress responses in golden grey mullet (*Liza aurata*)

following short-term exposure to phenanthrene. *Chemosphere* **2007**, *66*, 1284–1291.

Oliveira, M.; Pacheco, M.; Santos, M. A. Organ specific antioxidant responses in golden grey mullet (*Liza aurata*) following a short-term exposure to phenanthrene. *Sci. Total Environ.* **2008**, *396*, 70–78.

Omiecinski, C. J.; Heuvel, J. P. V.; Perdew, G. H.; Peters, J. M. Xenobiotic metabolism, disposition, and regulation by receptors: From biochemical phenomenon to predictors of major toxicities. *Toxicol. Sci.* **2011**, *120*, S49–S75.

Peterson, G. L. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **1977**, *83*, 346–356.

Pfaffl, M. W.; Horgan, G. W.; Dempfle, L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucl. Acids. Res.* **2002**, *30*, e36.

Pushparajah, D. S.; Umachandran, M.; Plant, K. E.; Plant, N.; Ioannides, C. Up-regulation of the glutathione S-transferase system in human liver by polycyclic aromatic hydrocarbons; comparison with rat liver and lung. *Mutagenesis* **2008**, *23*, 299–308.

Reischl, E.; Dafre, A. L.; Franco, J. L.; Wilhelm Filho, D. Distribution, adaptation and physiological meaning of thiols from vertebrate hemoglobins. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **2007**, *146*, 22–53.

Reis-Henriques, M. A.; Ferreira, M.; Coimbra, A. M.; D'Silva, C.; Costa, J.; Shailaja, M. S. Phenanthrene and nitrite effects on juvenile sea bass, *Dicentrarchus labrax*, using hepatic biotransformation enzymes, biliary fluorescence, and micronuclei as biomarkers. *Cienc. Mar.* **2009**, *35*, 29–40.

Rewitz, K.; Kjellerup, C.; Jørgensen, A.; Petersen, C.; Andersen, O. Identification of two *Nereis virens* (Annelida: Polychaeta) cytochromes P450 and induction by xenobiotics. *Comp. Biochem. Physiol. Part C* **2004**, *138*, 89–96.

Sabourault, C.; Amichot, M.; Pauron, D.; Bergé, J. B.; Lafaurie, M.; Girard, J. P. Cloning of CYP4F7, a kidney-specific P450 in the sea bass *Dicentrarchus labrax*. *Biochem. Bioph. Res. Commun.* **1999,** *258*, 155–161.

Schober, W.; Pusch, G.; Oeder, S.; Reindl, H.; Behrendt, H.; Buters, J. T. M. Metabolic activation of phenanthrene by human and mouse cytochromes P450 and pharmacokinetics in CYP1A2 knockout mice. *Chemico-Biol. Inter.* **2010**, *183*, 57–66.

Shou, M.; Korzekwa, K. R.; Krausz, K. W.; Crespi, C. L.; Gonzalez, F. J.; Gelboin, H. V. Regio-and stereo-selective metabolism of phenanthrene by twelve cDNA expressed human, rodent, and rabbit cytochromes P450. *Cancer Letters* **1994**, *83*, 305–313.

Sies, H.; Koch, O. R.; Martino, E.; Boveris, A. Increased biliary glutathione disulfide release in chronically ethanol treated rats. *FEBS Lett*. **1979**, *103*, 287–290.

Sies, H. Glutathione and its role in cellular functions. *Free Radic. Biol. Med.* **1999**, *27*, 916–921.

Solé, M.; Buet, A.; Ortiz, L.; Maynou, F.; Bayona, J. M.; Albaigés, J. Bioaccumulation and biochemical responses in mussels exposed to the water-accommodated fraction of the Prestige fuel oil. *Sci. Mar.* **2007**, *71*, 373–382.

Sundberg, K.; Dreij, K.; Seidel, A.; Jernstrom, B. Glutathione conjugation and DNA adduct formation of dibenzo[a, l]pyrene and benzo[a]pyrene diol epoxides in V79 cells stably expressing different human glutathione transferases. *Chem. Res. Toxicol.* **2002**, *15*, 170–179.

Toledo-Silva, G.; Siebert, M. N.; Medeiros, I.; Sincero, T.; Moraes, M.; Goldstone, J. V.; Bainy, A. C. D. Cloning a new cytochrome P450 isoform (CYP356A1) from oyster *Crassostrea gigas*. *Mar. Environ. Res.* **2008**, *66*, 15–18.

Torre, D. C.; Corsi, I.; Nardi, F.; Perra, G.; Tomasino, M. P.; Focardi, S. Transcriptional and post-transcriptional response of drug-metabolizing enzymes to PAHs contamination in red mullet (Mullus barbatus, Linnaeus, 1758): a field study. *Mar. Environ. Res.* **2010**, *70*, 95–101.

Trevisan, R.; Arl, M.; Sacchet, C. L.; Engel, C. S.; Danielli, N. M.; Mello, D. F.; Brocardo, C.; Maris, A. F.; Dafre, A. L. Antioxidant deficit in gills of Pacific oyster (*Crassostrea gigas*) exposed to chlorodinitrobenzene increases menadione toxicity. *Aquat. Toxicol.* **2012**, *108*, 85–93.

Trisciani, A.; Corsi, I.; Torre, C. D.; Perra, G.; Focardi, S. Hepatic biotransformation genes and enzymes and PAH metabolites in bile of common sole (*Solea solea*, Linnaeus, 1758) from an oil-contaminated site in the Mediterranean Sea: a field study. *Mar. Pollut. Bull.* **2011**, *62*, 806–814.

US EPA. *United States Environmental Protection Agency Priority Pollutants* Website; http://www.epa.gov/waterscience/methods/pollutants.html, **2009**.

Valavanidis, A.; Vlachogianni, T.; Triantafillaki, S.; Dassenakis, M.; Androutsos, F.; Scoullos, M. Polycyclic aromatic hydrocarbons in surface seawater and in indigenous mussels (*Mytilus galloprovincialis*) from coastal areas of the Saronikos Gulf (Greece). *Estuar. Coast. Shelf Sci.* **2008**, *79*, 733–739.

Verlecar, X. N.; Jena, K. B.; Chainy, G. B. N. Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperature. *Chem. Biol. Interact.* **2007**, *167*, 219–226.

Webb, D.; Gagnon, M. M. The value of stress protein 70 as an environmental biomarker of fish health under field conditions. *Environ. Toxicol.* **2009**, *24*, 287–295.

Winston, G.W.; Di Giulio, R. T. Prooxidant and Antioxidant Mechanisms in Aquatic Organisms. *Aquat. Toxicol.* **1991**, *19*, 137–161.

Wootton, E. C.; Dyrynda, E. A.; Pipe, R. K.; Ratcliffe, N. A. Comparisons of PAH-induced immunomodulation in three bivalve molluscs. *Aquat. Toxicol.* **2003**, *65*, 13–25.

Yin, Y.; Jia, H.; Sun, Y.; Yu, H.; Wang, X.; Wu, J.; Xue, Y. Bioaccumulation and ROS generation in liver of *Carassius auratus*, exposed to phenanthrene. *Comp. Biochem. Physiol. Part C* **2007**, *145*, 288–293.

Zanette, J. Identificação e caracterização de marcadores moleculares para estudos ecotoxicológicos em moluscos bivalves e peixes. PhD Dissertation, Federal University of Santa Catarina, Florianópolis, SC, **2009**.

Zanette, J.; Almeida, E. A.; Silva, A. Z.; Guzenski, J.; Ferreira, J. F.; Di Mascio, P.; Marques, M. R. F.; Bainy, A. C. D. Salinity influences glutathione **S**-transferase activity and lipid peroxidation responses in the **Crassostrea gigas** oyster exposed to diesel oil. *Sci. Total Environ.* **2011**, *409*, 1976–1983.

Zar, J. H. *Biostatistical analysis;* Prentice-Hall: Englewood Cliffs, **1999**.

Capítulo 6

Considerações finais

Como abordado no Capítulo 1, os ecossistemas aquáticos são continuamente expostos a uma quantidade significativa de contaminantes, muitos dos quais derivados de atividades da indústria do petróleo e da ineficiência na coleta e tratamento do esgoto doméstico. Independente da origem, os contaminantes podem causar efeitos tóxicos nos organismos atingidos, cujas respostas biológicas podem ser utilizadas como uma ferramenta viável em programas de biomonitoramento ambiental. No entanto, para se determinar a ferramenta ideal a ser empregada nestes programas, estudos devem ser conduzidos, especialmente quando se trata de misturas complexas de xenobióticos. Paralelamente, a escolha do organismo bioindicador constitui um importante ponto a ser considerado. A utilização de moluscos bivalves em programas de biomonitoramento e estudos ecotoxicológicos vem sendo considerada como uma ferramenta promissora, sendo que no Brasil, a ostra do mangue, *Crassostrea brasiliana*, ocupa uma posição de destaque, uma vez que apresenta ampla distribuição em regiões costeiras potencialmente contaminadas, e representa um importante recurso nutricional e econômico para as populações destas áreas. Nesse sentido, esta tese teve como principal objetivo avaliar a sensibilidade e aplicação de alguns biomarcadores bioquímicos e moleculares em ostras, *C. brasiliana*, expostas a contaminantes ambientais. Respostas de biomarcadores bioquímicos clássicos foram avaliadas em ostras expostas à fração de óleo diesel acomodada em água (FAA) e ao fenantreno. Adicionalmente, técnicas de biologia molecular foram aplicadas em animais tratados com FAA, fenantreno e esgoto doméstico visando a identificação de novos genes candidatos a biomarcadores de contaminação aquática.

O estudo desenvolvido e demonstrado no Capítulo 2 incorporou o uso de biomarcadores bioquímicos clássicos, além de análise química, para avaliar o efeito toxicológico de quatro concentrações de FAA em brânquia e glândula digestiva de ostras expostas por 96 horas sob condições controladas de laboratório. Os resultados demonstraram que a ostra apresenta elevada capacidade de bioacumulação de hidrocarbonetos alifáticos e aromáticos de forma concentração-dependente. A resposta de alguns biomarcadores seguiu esta mesma tendência, identificada pelo aumento da atividade das enzimas GST e GR na glândula digestiva, bem como níveis elevados da proteína Hsp60,

e maior atividade da GGT em brânquias. Esses resultados sugerem que os tecidos das ostras apresentam mecanismos diferenciados de resposta frente à exposição química. Além disso, constituem as primeiras informações ecotoxicológicas para esta espécie, fornecendo subsídios para o estabelecimento de ferramenta de biomonitoramento com potencial aplicabilidade tanto por órgãos ambientais, como por segmentos relacionados à indústria de petróleo.

Adicionalmente ao uso de biomarcadores bioquímicos clássicos, como abordado no Capítulo 2, estudos ecotoxicológicos com organismos cuja biologia é pouco conhecida requerem a identificação de biomarcadores específicos para refinar e legitimar a avaliação da qualidade ambiental. Com este propósito, no Capítulo 3, a técnica de hibridação subtrativa supressiva (SSH) foi aplicada em glândula digestiva de ostras expostas por 24 horas a FAA 10%. A técnica foi aplicada em dois momentos diferentes, e resultou na identificação de novos genes de interesse ecotoxicológico classificados em diferentes categorias. Dentre eles estão os genes de resposta ao estresse (*USP*), genes envolvidos na regulação da síntese protéica (*CFSP2* e *USP25*), no metabolismo de nucleotídeos (*NDPK-B*), e sistema imunológico (*Precursor de dominina*). A partir das sequências obtidas nas SSHs, iniciadores específicos foram desenhados para alguns genes, com o objetivo de validar a transcrição diferencial dos mesmos através da técnica de PCR quantitativo em tempo real (qPCR). Dentre os resultados obtidos, vale destacar a validação de alguns genes identificados, como *Precursor de dominina*, *USP* e *NDPK-B*. Embora as propriedades bioquímicas e a importância fisiológica das proteínas codificadas por esses genes ainda permanecem para serem elucidadas em moluscos, os genes aqui identificados demonstraram elevado potencial como candidatos a biomarcadores de exposição a derivados de petróleo. Ainda, o perfil de transcrição diferencial identificado mostra que grande parte da maquinaria metabólica do organismo foi afetada pela exposição à FAA. Entretanto, vale ressaltar que nenhum dos biomarcadores empregados no Capítulo 2 foi identificado na SSH. Este resultado pode estar relacionado às condições de estringência do método, e/ou à baixa cobertura (*coverage*) no sequenciamento (pelo método de Sanger), tendo em vista que apenas uma pequena parcela das colônias de bactérias foram sequenciadas. Ainda, o caráter aleatório de escolha das colônias para sequenciamento pode ter desfavorecido transcritos pouco abundantes.

Desta maneira, com o intuito de aumentar a cobertura de sequenciamento e a consequente identificação de novos genes de interesse ecotoxicológico em ostras *C. brasiliana*, foi utilizada a técnica

de pirosequenciamento (plataforma 454, Roche), cujos resultados foram mostrados no Capítulo 4. Como essa técnica não necessita de uma etapa de clonagem, as sequências não tipicamente amostradas numa abordagem de sequenciamento de Sanger devido a problemas de clonagem, são mais representadas no conjunto de dados provenientes do pirosequenciamento, contribuindo, assim, para uma maior cobertura do transcriptoma. Neste contexto, foram construídas dez bibliotecas de cDNA não normalizadas de brânquia e glândula digestiva de ostras expostas à FAA de óleo diesel, ao fenantreno e ao esgoto doméstico, as quais foram agrupadas, resultando em quatro bibliotecas de cDNA flanqueadas por adaptadores, e pirosequenciadas. A partir desta abordagem metodológica de sequenciamento em massa, em uma corrida foram gerados 399.291 *reads*, em um total de 96.582.549 bp, dos quais 246.514 *reads* foram utilizados para montagem *de novo*. Vale ressaltar que o processo de montagem foi realizado em três momentos distintos, com softwares diferentes com o intuito de escolher a melhor estratégia de bioinformática para os dados gerados neste trabalho. Após montagem utilizando *Newbler v 3.4, CLC Genomics Workbench* e *SeqMan NGen v 3.0.4* (*DNASTAR*) seguida de análise dos *contigs* gerados, foi escolhido o *SeqMan* para a montagem *de novo* e análise do transcriptoma. Dessa forma, foi produzido o primeiro transcriptoma referência da ostra *C. brasiliana*. 20.938 *contigs* foram gerados, os quais foram anotados resultando na identificação de 7.401 genes com similaridade significativa a sequências traduzidas de eucariotos; 25.046 termos funcionais GO; e 276 diferentes vias metabólicas mapeadas (KEGG). A partir dos genes identificados, foi dada maior ênfase àquelas classes e/ou famílias de genes que codificam proteínas de interesse ecotoxicológico. Assim, uma ampla variedade de genes putativamente envolvidos no sistema de detoxificação de xenobióticos e no sistema de defesa antioxidante foi identificada. Os dados gerados neste capítulo possibilitaram, pela primeira vez na literatura, a verificação da existência de genes transcritos em *C. brasiliana* frente à exposição a estressores ambientais, os quais podem se tornar importantes fontes de informação para utilização no biomonitoramento de águas contaminadas por compostos derivados de petróleo e esgoto doméstico.

Vale ainda ressaltar que o trabalho apresentado no Capítulo 4 foi desenvolvido durante o estágio de doutorado Sanduíche no Reino Unido (entre os meses de maio e novembro de 2010) e envolveu a participação das seguintes instituições estrangeiras: *The Marine Biological Association of the United Kingdon* (Dr. Declan C. Schroeder), *Argonne National Laboratory* (Dr. Jack A. Gilbert), *Glasgow Caledonian*

University (Dr. John A. Craft) e *University of Birmingham* (Dr. Kevin Chipman) e *University of Liverpool* (equipe do *Centre for Genomic Research*), onde o pirosequenciamento foi realizado. Convém destacar que, os dados estão sendo trabalhados com o intuito de aperfeiçoar os resultados obtidos. Desta forma, espera-se, em um curto espaço de tempo, mapear os *reads* no transcriptoma referência visando a identificação do padrão de transcrição gênica global e, assim, entender a contribuição de cada contaminante para o transcriptoma. Com este intuito, alguns genes serão escolhidos e analisados por qPCR para validar os achados no transcriptoma. Ainda, análises filogenéticas serão realizadas para as sequências de interesse ecotoxicológico visando a confirmação das funções sugeridas, bem como a identificação da relação evolutiva entre diferentes espécies.

Em um estudo adicional, apresentado no Capítulo 5, a utilidade dos dados gerados no Capítulo 4 foram testados por qPCR, fornecendo subsídios para o entendimento dos mecanismos moleculares de toxicidade exercidos pelo fenantreno em ostras. Com base nas sequências gênicas geradas no transcriptoma da *C. brasiliana*, os níveis de mRNA de seis genes *CYP-like* e quatro *GST-like* foram analisados em brânquia e glândula digestiva de ostras expostas por 24 horas a 100 $\mu g.L^{-1}$ e 1000 $\mu g.L^{-1}$ de fenantreno. Os resultados obtidos mostram o papel das enzimas de fase I (CYP) e fase II (GST) na biotransformação de HPAs em ostras. Ainda, parâmetros antioxidantes enzimáticos e não-enzimáticos foram analisados, sendo observada uma redução nos níveis de NPSH e GSH-t nos animais expostos a 1000 $\mu g.L^{-1}$ de fenantreno, bem como uma diminuição da atividade da GST em brânquia na menor concentração analisada. Os resultados obtidos sugerem diferenças no mecanismo de resposta de toxicidade entre os tecidos, no qual o papel da brânquia é destacado. Juntos, esses dados fornecem as primeiras informações moleculares e bioquímicas dos mecanismos e respostas de toxicidade de ostras *C. brasiliana* expostas a um contaminante abundante e amplamente distribuído no meio aquático.

As informações geradas neste trabalho agora servem como base para futuros esforços ecotoxicológicos visando a elucidação do papel funcional de genes identificados, fornecendo subsídios para um melhor conhecimento científico dos mecanismos de toxicidade em moluscos bivalves. Dessa forma, espera-se contribuir para a compreensão de diferentes aspectos da bioquímica, biologia molecular e da fisiologia da ostra *C. brasiliana*, bem como de espécies relacionadas. O presente estudo sugere ainda a utilização da ostra *C. brasiliana* para o biomonitoramento ambiental, utilizando-se tanto biomarcadores

bioquímicos clássicos como os genes candidatos a biomarcadores de contaminação aquática.

Por fim, cabe ressaltar que embora esta tese forneça informações inéditas e contribua para a ecotoxicologia aquática, ela é finalizada abrindo uma ampla gama de possibilidades para novos estudos, cumprindo o ciclo da ciência, no qual novas perguntas são formuladas e novos horizontes apresentados.

Conclusões

Capítulo 2

- Ostras, *Crassostrea brasiliana*, expostas a quatro concentrações de fração de óleo diesel acomodada em água (FAA) por 96 horas bioacumularam hidrocarbonetos alifáticos e aromáticos de forma concentração-dependente;
- Biomarcadores bioquímicos analisados em brânquia e glândula digestiva de ostras seguiram a mesma tendência de resposta concentração-dependente, com um aumento nas atividades das enzimas GST, GR e GGT, e na expressão de Hsp60, além de uma diminuição da SOD e dos níveis de Hsp90;
- Em brânquia, GGT e Hsp60 foram os biomarcadores mais sensíveis, enquanto que na glândula digestiva, GST e GR mostraram-se mais responsivos, demonstrando que respostas variam entre tecidos;
- Os dados de análise química em conjunto com os resultados dos biomarcadores bioquímicos demonstraram a potencialidade de uso da ostra *C. brasiliana* como espécie bioindicadora de contaminação aquática.

Capítulo 3

- A utilização da técnica de SSH em glândula digestiva de ostras *C. brasiliana* expostas à FAA 10% por 24 horas gerou bibliotecas subtrativas contendo fragmentos de genes induzidos ou reprimidos pela exposição;
- Sequências anotadas sugerem que o óleo diesel modula transcrição de genes envolvidos na síntese de proteínas, manutenção do citoesqueleto e sistema imune;
- Oito genes foram escolhidos para validação por PCR quantitativo em tempo real (qPCR), dos quais quatro apresentaram transcrição diferencial estatisticamente significativa. Destes, o gene de resposta ao estresse (*USP*), e os genes envolvidos na regulação da síntese protéica

(*CFSP2* e *USP25*), no metabolismo de nucleotídeos (*NDPK-B*), e sistema imunológico (*Precursor de dominina*) são candidatos a biomarcadores de exposição a óleo diesel;
- Estudos futuros envolvendo a clonagem completa dos genes identificados e validados por qPCR são necessários para a classificação final dos genes.

Capítulo 4
- 399.391 *reads*, em um total de 96.582.549 bp, foram gerados por pirosequenciamento. Após seleção por qualidade e tamanho dos *reads*, 246.514 foram utilizados para montagem *de novo*, resultando em 20.938 *contigs* e 152.777 *singletons*;
- Cerca de 36% dos *contigs* gerados puderam ser anotados através de buscas por similaridade no banco de dados do *NCBI*, sendo que 7.401 genes apresentaram similaridade significativa a proteínas de eucariotos;
- Inferências funcionais foram feitas a aproximadamente 86% dos *contigs*, com 25.046 termos de ontologia dos genes (GO) identificados e 276 diferentes vias metabólicas mapeadas (KEGG);
- Análise funcional dos *contigs* resultou na identificação preliminar de 41 *CYP*s, 26 *GST*s, 8 *SULT*s e 9 *MRP*s classificados em genes codificadores de proteínas do sistema de biotransformação de xenobióticos, além de 28 proteínas do sistema de defesa antioxidante;
- Os dados fornecidos neste capítulo resultaram no primeiro transcriptoma referência da ostra *C. brasiliana*, aumentando consideravelmente o número de sequências disponíveis nos bancos de dados para o gênero *Crassostrea;*
- Espera-se, em um curto espaço de tempo, mapear os *reads* no transcriptoma referência visando a identificação do padrão de transcrição gênica global e, assim, entender a contribuição de cada contaminante para o transcriptoma.

Capítulo 5
- Seis novos genes de CYPs e quatro de GSTs identificados no transcriptoma de *C. brasiliana* foram utilizados para avaliar o mecanismo molecular de toxicidade de HPAs em brânquia e glândula digestiva de ostras expostas a duas concentrações (100 e 1000 $\mu g.L^{-1}$) de fenantreno por 24 horas;
- Os níveis de mRNA dos genes *CYP-like* e *GST-like*, analisados por qPCR, mostraram um padrão de transcrição dependente do tecido;
- Genes classificados como CYP das famílias 2 e 17, e GSTs das classes ômega e microssomal 3 foram os mais responsivos ao fenantreno, com

forte indução na brânquia dos animais expostos à concentração de 1000 $\mu g.L^{-1}$;

- Parâmetros antioxidantes enzimáticos e não-enzimáticos foram analisados, sendo observada uma redução nos níveis de NPSH e GSH-t em ostras expostas a 1000 $\mu g.L^{-1}$, bem como uma diminuição da GST em brânquias na menor concentração analisada;
- A brânquia mostrou ser o tecido mais responsivo ao fenantreno, indicando maiores esforços de análise neste tecido;
- Os resultados gerados neste capítulo são inéditos e representam os primeiros indícios do papel de enzimas de fase I e fase II do sistema de biotransformação de xenobióticos no metabolismo de HPAs em ostras *C. brasiliana*.

Referências bibliográficas citadas no Capítulo 1

AAS, E. et al. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. **Aquatic Toxicology**, v. 51, p. 241-258, 2000.

AKCHA, F. et al. Enzymatic biomarker measurement and study of DNA adduct formation in benzo[a]pyrene-contaminated mussels, *Mytilus galloprovincialis.* **Aquatic Toxicology**, v. 49, p. 269-287, 2000.

AKCHA, F. et al. **Role of DNA adduct formation in induced carcinogenesis**. In: DOUBEN, P.E.T. (Ed.). PAHs: An ecotoxicological perspective. West Sussex: John Wiley & Sons Ltd, 2003. p. 65-79.

ÁLAMO, M.J.P. et al. **Omics methodologies:** New tools in aquaculture studies. In: MUCHLISIN, Z.A. (Ed.). Aquaculture. InTech, 2012. Disponível em: <http://www.intechopen.com/articles/show/title/omics-methodologies-new-tools-in-aquaculture-studies2012>. Acesso em: 02 fev. 2012.

ALMEIDA, E.A. et al. Oxidative stress in *Perna perna* and other bivalves as indicators of environmental stress in the Brazilian marine environment: antioxidants, lipid peroxidation and DNA damage. **Comparative Biochemistry and Physiology A**, v. 146, p. 588-600, 2007.

AMARAL, A.M. et al. Plataformas tecnológicas no estudo da bactéria causadora do cancro cítrico: genômica, transcriptômica e proteômica. **Laranja**, v. 27, p. 355-377, 2006.

ANSALDO, M.; NAJLE, R.; LUQUET, C.M. Oxidative stress generated by diesel seawater contamination in the digestive gland of the Antarctic limpet *Nacella concinna*. **Marine Environmental Research**, v. 59, p. 381-390, 2005.

ANTONIO, F.J.; MENDES, R.S.; THOMAZ, S.M. Identifying and modeling patterns of tetrapod vertebrate mortality rates in the Gulf of Mexico oil spill. **Aquatic Toxicology**, v. 105, p. 177-179, 2011.

BAER, W. **The Brazilian economy:** Growth and development. Boulder: Lynne Rienner Publishers, 2008. 443 p.

BAINY, A.C.D. et al. Oxidative stress in gill, erythrocytes, liver and kidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. **Aquatic Toxicology**, v. 34, p. 151-162, 1996.

BAINY, A.C.D. et al. Biochemical responses in farmed mussel *Perna perna* transplanted to contaminated sites on Santa Catarina Island, SC, Brazil. **Marine Environmental Research**, v. 50, p. 411-416, 2000.

BARRON, M.G. et al. Are aromatic hydrocarbons the primary determinant of petroleum toxicity to aquatic organisms? **Aquatic Toxicology**, v. 46, p. 253-268, 1999.

BARRON, M.G. Ecological impacts of the deepwater horizon oil spill: implications for immunotoxicity. **Toxicologic Pathology**, 2011.

BARŠIENĖ, J. et al. Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish treated with crude oil. **Aquatic Toxicology**, v. 78, p. 99-104, 2006.

BEBIANNO, M.J.; BARREIRA, L.A. Polycyclic aromatic hydrocarbons concentrations and biomarker responses in the clam *Ruditapes decussates* transplanted in the Ria Formosa lagoon. **Ecotoxicology and Environmental Safety**, v. 72, p. 1849-1860, 2009.

BEEBY, A. What do sentinels stand for? **Environmental Pollution**, v. 112, p. 285-298. 2001.

BEN. **BALANÇO ENERGÉTICO NACIONAL 2011:** Ano base 2010. Empresa de Pesquisa Energética – Rio de Janeiro: EPE, 2011. 266 p.

BETTENCOURT, R. et al. High-throughput sequencing and analysis of the gill tissue transcriptome from the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus*. **BMC Genomics**, v. 11, p. 559, 2010.

BINETTI, R.; COSTAMAGNA, F.M.; MARCELLO, I. Exponential growth of new chemicals and evolution of information relevant to risk control. **Annali dell'Istituto Superiore di Sanità**, v. 44, p. 13-15, 2008.

BOGERS, R. et al. An in vitro / in vivo screening assay as a sensitive tool to assess endocrine disruptive activity in surface water. **Environment International**, v. 33, p. 292-301, 2007.

BOUCK, A.; VISION, T. The molecular ecologist's guide to expressed sequence tags. **Molecular Ecology**, v. 16, p. 907-924, 2007.

BOUTET, I. et al. Molecular identification and expression of heat shock cognate 70 (hsc70) and heat shock protein 70 (hsp70) genes in the Pacific oyster *Crassostrea gigas*. **Cell Stress Chaperones**, v. 8, p. 76-85, 2003.

BOUTET, I.; TANGUY, A.; MORAGA, D. Response of the Pacific oyster *Crassostrea gigas* to hydrocarbon contamination under experimental conditions. **Gene**, v. 329, p. 147-157, 2004.

BRAY, T.M.; LEVY, M.A. **Assessment of oxidative stress:** Concepts and approaches. In: BRAY, T.M.; SCHOENE, N.W. (Eds.). Models and methods in cell signaling and gene expression: Applications to oxidative stress research. London: OICA International, 2000. p. 1-14.

BURGESS, R.M.; AHRENS, M.J.; HICKEY, C.W. **Geochemistry of PAHs in aquatic environments:** Source, persistence and distribution. In: DOUBEN, P.E.T. (Ed.). PAHs: An ecotoxicological perspective. West Sussex: John Wiley & Sons Ltd, 2003. p. 35-45.

BURNETT, K.G. et al. *Fundulus* as the premier teleost model in environmental biology: Opportunities for new insights using genomics. **Comparative Biochemistry and Physiology D Genomics & Proteomics**, v. 2, p. 257-286, 2007.

CAJARAVILLE, M.P. et al. Signs of recovery of mussels health two years after the Prestige oil spill. **Marine Environmental Research**, v. 62, p. 337-341, 2006.

CARVALHO, P.S. et al. Effects of naphthalene and phenanthrene on visual and prey capture endpoints during early stages of the dourado *Salminus brasiliensis*. **Marine Environmental Research**, v. 66, p. 205-207, 2008.

CARVALHO, M.C.C.G.; SILVA, D.C.G. Sequenciamento de DNA de nova geração e suas aplicações na genômica de plantas. **Ciência Rural**, v. 40, p. 735-744, 2010.

CHALER, R. et al. Identification and quantification of n-octyl esters of alkanoic and hexanedioic acids and phthalates as urban wastewater

markers in biota and sediments from estuarine areas. **Journal of Chromatography A**, v. 1046, p. 203-210, 2004.

CHANEY, M.L.; GRACEY, A.Y. Mass mortality in Pacific oysters is associated with a specific gene expression signature. **Molecular Ecology**, v. 20, p. 2942-2954, 2011.

CHAPMAN, P.M. Integrating toxicology and ecology: putting the "eco" into ecotoxicology. **Marine Pollution Bulletin**, v. 44, p. 7-15, 2002.

CHAPMAN, R.W. et al. The transcriptomic responses of the eastern oyster, *Crassostrea virginica*, to environmental conditions. **Molecular Ecology**, v. 20, p. 1431-1449, 2011.

CHEUNG, C.C.C. et al. Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*. **Aquatic Toxicology**, v. 52, p. 189-203, 2001.

CHOI, Y.K.; JO, P.G.; CHOI, C.Y. Cadmium affects the expression of heat shock protein 90 and metallothionein mRNA in the Pacific oyster, *Crassostrea gigas*. **Comparative Biochemistry and Physiology Part C**, v. 147, p. 286-292, 2008.

CLARK, M.S. et al. Insights into shell deposition in the Antarctic bivalve *Laternula elliptica*: gene discovery in the mantle transcriptome using 454 pyrosequencing. **BMC Genomics**, v. 11, p. 362, 2010.

CLARK, M.S. et al. Antarctic krill 454 pyrosequencing reveals chaperone and stress transcriptome. **PLoS ONE**, v. 6, p. e15919, 2011.

COLBORN, T.; DUMANOSKI, D.; MYERS, J.P. **Our stolen future**. New York: Plume/Penguin Books, 1996. 306 p.

COLLIN, H. et al. Response of the Pacific oyster *Crassostrea gigas*, Thunberg 1793, to pesticide exposure under experimental conditions. **The Journal of Experimental Biology**, v. 213, p. 4010-4017, 2010.

CONSTANZA, R. et al. The value of the world's ecosystem services and natural capital. **Nature**, v. 387, p. 253-260, 1997.

CORREIA, A.D. et al. Biochemical and behavioral responses in gilthead seabream (*Sparus aurata*) to phenanthrene. **Journal of Experimental Marine Biology and Ecology**, v. 347, p. 109-122, 2007.

CRAFT, J.A. et al. Pyrosequencing of *Mytilus galloprovincialis* cDNAs: tissue-specific expression patterns. **PLoS ONE**, v. 5, p. e8875, 2010.

DI, Y. et al. Tissue-specific expression of *p53* and *ras* genes in response to the environmental genotoxicant benzo(a)pyrene in marine mussels. **Environmental Science & Technology**, v. 45, p. 8974-8981, 2011.

DIACHENKO, L. et al. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. **Proceedings of the National Academy of Sciences of the United States of America**, v. 93, p. 6025-6030, 1996.

DONDERO, F. et al. Gene transcription profiling in pollutant exposed mussels (*Mytilus* spp.) using a new low-density oligonucleotide microarray. **Gene**, v. 376, p. 24-36, 2006.

DONEY, S.C. The growing human footprint on coastal and open-ocean biogeochemistry. **Science**, v. 328, p. 1512-1516, 2010.

EKBLOM, R.; GALINDO J. Applications of next generation sequencing in molecular ecology of non-model organisms. **Heredity**, v. 107, p. 1-15, 2011.

FELDMANNOVÁ, M. et al. Effects of N-heterocyclic polyaromatic hydrocarbons on survival, reproduction, and biochemical parameters in *Daphnia magna*. **Environmental Toxicology**, v. 21, p. 425-431, 2006.

FERNANDES, C. et al. Bioaccumulation of heavy metals in *Liza saliens* from the Esmoriz-Paramos coastal lagoon, Portugal. **Ecotoxicology and Environmental Safety**, v. 66, p. 426-431, 2007.

FERREIRA, J.F.; NETO, F.M.O. Cultivo de moluscos em Santa Catarina. **INFOFISH**, v. 4, 2006.

FILHO, A.M.S. **Planos nacionais de contingência para atendimento a derramamento de óleo: análise de países representativos das Américas para implantação no caso do Brasil**. 2006. 217 f. Dissertação (Mestrado em Ciências em Planejamento Ambiental) - Programa de Pós-Graduação em Engenharia, Universidade Federal do Rio de Janeiro, Rio de Janeiro.

FITZPATRICK, P.J. et al. Assessment of a glutathione *S*-transferase and related protein in the gill and digestive gland of *Mytilus edulis* (L.), as a potential organic pollution biomarkers. **Biomarkers**, v. 2, p. 51-56, 1997.

FORBES, V.E.; PALMQVIST, A.; BACH, L. The use and misuse of biomarkers in ecotoxicology. **Environmental Toxicology & Chemistry**, v. 25, p. 272-280, 2006.

FRANCIONI, E. et al. Evaluation of the mussel *Perna perna* as a biomonitor of polycyclic aromatic hydrocarbon (PAH) exposure and effects. **Marine Pollution Bulletin**, v. 54, p. 329-338, 2007a.

FRANCIONI, E. et al. Polycyclic aromatic hydrocarbon in inter-tidal mussel *Perna perna*: Space-time observations, source investigation and genotoxicity. **Science of the Total Environment**, v. 372, p. 515-531, 2007b.

FRANCO, J.L. et al. Antioxidant status and stress proteins in the gills of the brown mussel *Perna perna* exposed to zinc. **Chemico-Biological Interactions**, v. 160, p. 232-240, 2006.

FRANCO, J.L. et al. Biochemical alterations in caged Nile tilapia *Oreochromis niloticus*. **Ecotoxicology and Environmental Safety**, v. 73, p. 864-872, 2010.

FRASER, B.A. et al. Sequencing and characterization of the guppy (*Poecilia reticulata*) transcriptome. **BMC Genomics**, v. 12, p. 202, 2011.

FROUIN, H. et al. Physiological effects of polycyclic aromatic hydrocarbons on soft-shell clam *Mya arenaria*. **Aquatic Toxicology**, v. 82, p. 120-134, 2007.

GABARDO, I.T. **Caracterização química e toxicológica da água produzida descartada em plataformas de óleo e gás na costa brasileira e seu comportamento dispersivo no mar**. 2007. 235 f. Tese (Doutorado em Química) - Programa de Pós-Graduação em Química, Universidade Federal do Rio Grande do Norte, Natal.

GALAR-MARTINEZ, M. et al. Oxidative stress induced on *Cyprinus carpio* by contaminants present in the water and sediment of Madin

Reservoir. **Journal of environmental science and health Part A**, v. 45, p. 155-160, 2010.

GALLOWAY, T.S. et al. Rapid assessment of marine pollution using multiple biomarkers and chemical immunoassays. **Environmental Science & Technology**, v. 36, p. 2219-2226, 2002.

HALLIWELL, B.; GUTTERIDGE, J. **Free radicals in biology and medicine**. Nova York: Oxford University Press, 2007. 851 p.

HALPERN, B.S. et al. A global map of human impact on marine ecosystems. **Science**, v. 319, p. 948-952, 2008.

HANNAM, M.L. et al. Effects of the model PAH phenanthrene on immune function and oxidative stress in the haemolymph of the temperate scallop *Pecten maximus*. **Chemosphere**, v. 78, p. 779-784, 2010.

HANSEN, B.J. et al. Suppression subtractive hybridization library prepared from the copepod *Calanus finmarchicus* exposed to a sublethal mixture of environmental stressors. **Comparative Biochemistry and Physiology Part D**, v. 2, p. 250-256, 2007.

HAYES, J.D.; FLANAGAN, J.U.; JOWSEY, I.R. Glutathione transferases. **Annual Review of Pharmacology and Toxicology**, v. 45, p. 51-88, 2005.

HEDOUIN, L. et al. Allometric relationships in the bioconcentration of heavy metals by the edible tropical clam *Gafrarium tumidum*. **Science of the Total Environment**, v. 366, p. 154-163, 2006.

HOLT, E.A.; MILLER, S.W. Bioindicators: using organisms to measure environmental impacts. **Nature Education Knowledge**, v. 2, p. 8, 2011.

HOU, R. et al. Transcriptome sequencing and *de novo* analysis for Yesso scallop (*Patinopecten yessoensis*) using 454 GS FLX. **PLoS ONE**, v. 6, p. e21560, 2011.

HUGGETT, R.J. et al. **Biomarkers:** Biochemical, physiological and histological markers of anthropogenic stress. Boca Raton: Lewis Publishers, 1992. 347 p.

ICES. **Report of the working group on biological effects of contaminants (WGBEC) 22-26 March 2004, Ostend, Belgium**. Copenhagen, Denmark. The International Council for the Exploration of the Sea (ICES), Marine Habitat Committee, 2004. 86 p.

IRWIN, R.J. et al. **Environmental contaminants encyclopedia PAHs entry**. Colorado: National Park Service, Water Resources Division, Fort Collins, 1997a. 176 p.

IRWIN, R.J. et al. **Environmental contaminants encyclopedia phenanthrene entry**. Colorado: National Park Service, Water Resources Division, Fort Collins, 1997b. 57 p.

ITOPF - INTERNATIONAL TANKER OWNERS POLLUTION FEDERATION LIMITED. **Oil tanker spill statistics:** 2010. Disponível em: <http://www.itopf.com/information-services/data-and-statistics/statistics/>. Acesso em: 01 fev. 2012.

IVANINA, A.V.; CHERKASOV, A.S.; SOKOLOVA, I.M. Effects of cadmium on cellular protein and glutathione synthesis and expression of stress proteins in eastern oysters, *Crassostrea virginica* Gmelin. **The Journal of Experimental Biology**, v. 211, p. 577-586, 2008.

IVANINA, A.V.; TAYLOR, C.; SOKOLOV, I.M. Effects of elevated temperature and cadmium exposure on stress protein response in eastern oysters *Crassostrea virginica* (Gmelin). **Aquatic Toxicology**, v. 91, p. 245-254, 2009.

JAYAPAL, M. et al. Environmental toxicogenomics: a post-genomic approach to analysing biological responses to environmental toxins. **The International Journal of Biochemistry & Cell Biology**, v. 42, p. 230-240, 2010.

JEE, J.-H; KANG, J.-C. Biochemical changes of enzymatic defense system after phenanthrene exposure in olive flounder, *Paralichthys olivaceus*. **Physiological Research**, v. 54, p. 585-591, 2005.

JOUBERT, C. et al. Transcriptome and proteome analysis of *Pinctada margaritifera* calcifying mantle and shell: focus on biomineralization. **BMC Genomics**, v. 11, p. 613, 2010.

JUDSON, R. et al. The toxicity data landscape for environmental chemicals. **Environmental Health Perspectives**, v. 117, p. 685-695, 2009.

KAMB, A. Next-generation sequencing and its potential impact. **Chemical Research in Toxicology**, v. 24, p. 1163-1168, 2011.

KENNISH, M.J. **Ecology of estuaries**: Anthropogenic effects. Londres: CRC Press, 1992. 494 p.

KUMAR, S.; BLAXTER, M.L. Comparing *de novo* assemblers for 454 transcriptome data. **BMC Genomics**, v. 11, p. 571, 2010.

LATIMER, J.S.; ZHENG, J. **The sources, transport, and fate of PAHs in the marine environment**. In: DOUBEN, P.E.T. (Ed.). PAHs: An ecotoxicological perspective. West Sussex: John Wiley & Sons Ltd, 2003. p. 9-33.

LAW, R.J. et al. **PREMIAM – Pollution Response in Emergencies Marine Impact Assessment and Monitoring: Post-incident monitoring guidelines**. Science Series Technical Report, Lowestoft, Cefas. Norwich: Crown, 2011. 164 p.

LAZOSKI, C. **Sistemática molecular e genética populacional de ostras brasileiras (*Crassostrea* spp.)**. 2004. 150 f. Tese (Doutorado em Genética) - Curso de Pós-Graduação em Genética, Universidade Federal do Rio de Janeiro, Rio de Janeiro.

LIU, R.-M. et al. Quinones increase γ-glutamyl transpeptidase expression by multiple mechanisms in rat lung epithelial cells. **American Journal of Physiology - Lung Cellular and Molecular Physiology**, v. 274, p. 330-336, 1998.

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

LIVINGSTONE, D.R. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. **Revue de Médecine Vétérinaire**, v. 154, p. 427-430, 2003.

LÜCHMANN, K.H. et al. Biochemical biomarkers and hydrocarbons concentrations in the mangrove oyster *Crassostrea brasiliana* following exposure to diesel fuel water-accommodated fraction. **Aquatic Toxicology**, v. 105, p. 652-660, 2011.

LÜCHMANN, K.H. et al. Suppressive subtractive hybridization libraries prepared from the digestive gland of the oyster *Crassostrea brasiliana* exposed to diesel fuel water-accommodated fraction. **Environmental Toxicology and Chemistry**, DOI 10.1002/etc.1837, 2012.

LUCKENBACH, T.; EPEL, D. ABCB and ABCC type transporters confer multixenobiotic resistance and form an environment-tissue barrier in bivalve gills. **American Journal of Physiology - Regulatory, Integrative and Comparative Physiology**, v. 294, p. R1919-1929, 2008.

MACEK, K.J. Aquatic toxicology: fact or fiction? **Environmental Health Perspectives**, v. 34, p. 159-162, 1980.

MACHELLA, N.; REGOLI, F.; SANTELLA, R.M. Immunofluorescent detection of 8-oxo-dG and PAH bulky adducts in fish liver and mussel digestive gland. **Aquatic Toxicology**, v. 71, p. 335-343, 2005.

MARGULIES, M. et al. Sequencing in microfabricated high-density picolitre reactors. **Nature**, v. 437, p. 376-380, 2005.

MARTÍN-DÍAZ, M.L. et al. Biomarkers as tools to assess sediment quality. Laboratory and field surveys. **Trends in Analytical Chemistry**, v. 23, p. 807-818, 2004.

MATTOS, J.J. et al. Differential gene expression in *Poecilia vivipara* exposed to diesel oil water accommodated fraction. **Marine Environmental Research**, v. 69, p. S31-33, 2010.

MAYEUX, R. Biomarkers: potential use and limitations. **Journal of the American Society for Experimental Neuro Therapies**, v. 1, p. 182-188, 2004.

MEADOR, J.P. **Bioaccumulation of PAHs in marine invertebrates**. In: DOUBEN, P.E.T. (Ed.). PAHs: An ecotoxicological perspective. West Sussex: John Wiley & Sons Ltd, 2003. p. 147-171.

MEDEIROS, I.D. et al. Differential gene expression in oyster exposed to sewage. **Marine Environmental Research**, v. 66, p. 156-157, 2008.

MENICOM, M.F.G. et al. Brazilian oil spills chemical characterization - case studies. **Environmental Forensics**, v. 3, p. 303-321, 2002.

METZKER, M.L. Sequencing technologies - the next generation. **Nature Reviews Genetics**, v. 11, p. 31-46, 2010.

MEYER, E. et al. Sequencing and de novo analysis of a coral larval transcriptome using 454 GSFlx. **BMC Genomics**, v. 10, p. 219, 2009.

MIAO, J. et al. Molecular cloning of CYP4 and GSTpi homologues in the scallop *Chlamys farreri* and its expression in response to benzo[a]pyrene exposure. **Marine Genomics**, v. 4, p. 99-108, 2011.

MILAN, M. et al. Transcriptome sequencing and microarray development for the Manila clam, *Ruditapes philippinarum*: genomic tools for environmental monitoring. **BMC Genomics**, v. 12, p. 234, 2011.

MIRANDA, V.J.M. **Degradação de naftaleno, fenantreno e benzo(a)pireno em solos e sedimentos de ambientes costeiros, oceânicos e antárticos**. 2008. 63 f. Dissertação (Mestrado em *Magister Scientiae*) - Programa de Pós-Graduação em Solos e Nutrição de Plantas, Universidade Federal de Viçosa, Viçosa.

MOORE, M.N. et al. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. **Mutation Research**, v. 552, p. 247-268, 2004.

NEBERT, D.W.; RUSSELL, D.W. Clinical importance of the cytochromes P450. **Lancet**, v. 360, p. 1155-1162, 2002.

NEFF, J.M. **Bioaccumulation in marine organisms**: Effect of contaminants from oil well produced water. Amsterdam: Elsevier Science Publishers, 2002. 452 p.

NEFF, J.M.; STOUT, S.A.; GUNSTER, D.G. Ecological risk assessment of PAH in sediments: identifying sources and ecological

hazard. **Integrated Environmental Assessment and Management**, v. 1, p. 22-33, 2005.

NEWSTED, J.L.; GIESY, J.P. Predictive models for photoinduced acute toxicity of polycyclic aromatic hydrocarbons to *Daphnia magna* Strauss (Cladocera, Crustacea). **Environmental Toxicology & Chemistry**, v. 6, p. 445-461, 1987.

NIYOGI, S. et al. Seasonal variation of antioxidant and biotransformation enzymes in barnacle, *Balanus balanoides*, and their relation with polyaromatic hydrocarbons. **Marine Environmental Research**, v. 52, p. 13-26, 2001.

NOGUEIRA, P. et al. **Novel potential molecular biomarkers of aquatic contamination in *Dicentrarchus labrax* and *Liza aurata***. In: HAMAMURA, N. et al. (Eds.). Interdisciplinary studies on environmental chemistry - biological responses to contaminants. Aveiro: Terrapub, 2010. p. 127-138.

NOGUEIRA, L. et al. Oxidative stress in Nile tilapia (*Oreochromis niloticus*) and armored catfish (*Pterygoplichthys anisitsi*) exposed to diesel oil. **Environmental Monitoring and Assessment**, v. 180, p. 243-255, 2011.

NRC - National Research Council. **Applications of toxicogenomic technologies to predictive toxicology and risk assessment**. Washington, DC: National Academy Press, 2007. Disponível em: <http://dels.nas.edu/resources/static-assets/materials-based-on-reports/reports-in-brief/toxicogenomic_technologies_final.pdf>.

OHLROGGE, J.; BENNING, C. Unraveling plant metabolism by EST analysis. **Current Opinion of Plant Biology**, v. 3, p. 224-228, 2000.

OLF. **The Norwegian Oil Industry Association Water Column monitoring:** Discharges risk assessment monitoring. Summary Report 2005. Stavanger, Noruega, 2005. 25 p.

OLIVEIRA, M.; PACHECO, M.; SANTOS, M.A. Organ specific antioxidant responses in golden grey mullet (*Liza aurata*) following a short-term exposure to phenanthrene. **Science of the Total Environment**, v. 396, p. 70-78, 2008.

OMIECINSKI, C.J. et al. Xenobiotic metabolism, disposition, and regulation by receptors: from biochemical phenomenon to predictors of major toxicities. **Toxicological Sciences**, v. 120, p. S49-S75, 2011.

PACHECO, M.; SANTOS, M.A. Biotransformation, endocrine, and genetic responses of *Anguilla Anguilla* L. to petroleum distillate products and environmentally contaminated waters. **Ecotoxicology and Environmental Safety**, v. 49, p. 64-75, 2001.

PADMINI, E. **Physiological adaptations of stressed fish to polluted environments:** Role of heat shock proteins. In: WHITACRE, D.M. (Ed.). Reviews of environmental contamination and toxicology. London: Springer, 2010. p. 1-28.

PANTZARTZI, C. et al. Promoter complexity and tissue-specific expression of stress response components in *Mytilus galloprovincialis*, a sessile marine invertebrate species. **PLoS Computational Biology**, v. 6, p. e1000847, 2010.

PARASHAR, U.D. et al. Global illness and deaths caused by rotavirus disease in children. **Emerging infectious diseases**, v. 9, p. 565-572, 2003.

PARK, H. et al. Analysis of ESTs and expression of two peroxiredoxins in the thermally stressed Antarctic bivalve *Laternula elliptica*. **Fish & Shellfish Immunology**, v. 25, p. 550-559, 2008.

PIE, M.R. et al. A simple PCR-RFLP method for the discrimination of native and introduced oyster species (*Crassostrea brasiliana*, *C. rhizophorae* and *C. gigas*; Bivalvia: Ostreidae) cultured in Southern Brazil. **Aquaculture Research**, v. 37, p. 1598-1600, 2006.

PNSB - **Pesquisa Nacional de Saneamento Básico 2008**. Rio de Janeiro: IBGE, 2010. 222 p.

PRATT, W.B. The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via MAP

kinase. **Annual Review of Pharmacology and Toxicology**, v. 37, p. 297-326, 1997.

QUILANG, J. et al. Generation and analysis of ESTs from the eastern oyster, *Crassostrea virginica* Gmelin and identification of microsatellite and SNP markers. **BMC Genomics**, v. 8, p. 157-168, 2007.

QUINN, N.L. et al. Assessing the feasibility of GS FLX pyrosequencing for sequencing the Atlantic salmon genome. **BMC Genomics**, v. 9, p. 404, 2008.

RAND, G.M. **Effects, environmental fate, and risk assessment**. Washington: Taylor & Francis, 1995. 1125 p.

REGOLI, F. et al. Application of biomarkers for assessing the biological impact of dredged materials in the Mediterranean: the relationship between antioxidant responses and susceptibility to oxidative stress in the red mullet (*Mullus barbatus*). **Marine Pollution Bulletin**, v. 44, p. 912-922, 2002.

RENAULT, T. et al. Suppression substractive hybridisation (SSH) and real time PCR reveal differential gene expression in the Pacific cupped oyster, *Crassostrea gigas*, challenged with Ostreid herpesvirus 1. **Developmental & Comparative Immunology**, v. 35, p. 725-735, 2011.

ROBERTS, S. et al. Analysis of genes isolated from plated hemocytes of the Pacific oyster, *Crassostrea gigas*. **Marine Biotechnology**, v. 11, p. 24-44, 2009.

ROCKSTRÖM, J. et al. A safe operating space for humanity. **Nature**, v. 461, p. 472-475, 2009.

ROSENBERG, D.M.; RESH, V.H. **Introduction to freshwater biomonitoring and benthic macroinvertebrates**. In: ROSENBERG, D.M.; RESH, V.H. (Eds.). Freshwater biomonitoring and benthic macroinvertebrates. New York: Chapman and Hall, 1993. p. 1-9.

SACCO, J.C. **Phase II biotransformation of xenobiotics in polar bear (*Ursus maritimus*) and channel catfish (*Ictalurus punctatus*)**. 2006. 170 f. Tese (Doctor of Philosophy) - University of Florida, Flórida.

SALEM, M. et al. Characterization of the rainbow trout transcriptome using Sanger and 454-pyrosequencing approaches. **BMC Genomics**, v. 11, p. 564, 2010.

SCHADT, E.E.; TURNER, S.; KASARSKIS, A. A window into third-generation sequencing. **Human Molecular Genetics**, v. 19, p. R227-240, 2010.

SCHIRMER, K.S. et al. Transcriptomics in ecotoxicology. **Analytical and Bioanalytical Chemistry**, v. 397, p. 917-923, 2010.

SCHOBER, W. et al. Metabolic activation of phenanthrene by human and mouse cytochromes P450 and pharmacokinetics in CYP1A2 knockout mice. **Chemico-Biological Interactions**, v. 183, p. 57-66, 2010.

SHEEHAN, D.; POWER, A. Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. **Comparative Biochemistry and Physiology**, v. 123, p. 193-199, 1999.

SHENDURE, J.; LI, H. Next-generation DNA sequencing. **Nature Biotechnology**, v. 26, p. 1135-1145, 2008.

SILVA-ACIARES, F. et al. Identification of genes expressed in juvenile *Haliotis rufescens* in response to different copper concentrations in the north of Chile under controlled conditions. **Marine Pollution Bulletin**, v. 62, p. 671-680, 2011.

SIMONATO, J.D.; GUEDES, C.L.B.; MARTINEZ, C.B.R. Biochemical, physiological, and histological changes in the neotropical fish *Prochilodus lineatus* exposed to diesel oil. **Ecotoxicology and Environmental Safety**, v. 69, p. 112-120, 2008.

SNAPE, J.R. et al. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. **Aquatic Toxicology**, v. 67, p. 143-154, 2004.

SNYDER, M.J.; GIRVETZ, E.; MULDER, E.P. Induction of marine mollusc stress proteins by chemical or physical stress. **Environmental Contamination and Toxicology**, v. 41, p. 22-29, 2001.

STOREY, K.B. Oxidative stress: animal adaptations in nature. **Brazilian Journal of Medical and Biological Research**, v. 29, p. 1715-1733, 1996.

SUN, Y. et al. Bioaccumulation, depuration and oxidative stress in fish *Carassius auratus* under phenanthrene exposure. **Chemosphere**, v. 63, p. 1319-1327, 2006.

TALEB, Z.M. et al. Biomonitoring of environmental pollution on the Algerian west coast using caged mussels *Mytilus galloprovincialis*. **Oceanologia**, v. 51, p. 63-84, 2009.

TIBURTIUS, E.R.L. et al. Degradação de BTXs via processos oxidativos avançados. **Química Nova**, v. 28, p. 61-64, 2005.

TOLEDO-SILVA, G. **Análise da expressão gênica diferencial em ostras-do-pacífico *Crassostrea gigas* expostas a esgoto doméstico *in situ***. 2009. 98 f. Dissertação (Mestrado em Biotecnologia), Programa de Pós-Graduação em Biotecnologia, Universidade Federal de Santa Catarina, Florianópolis.

TOLLEFSON, J. Seven billion and counting. **Nature**, v. 478, p. 300, 2011.

US EPA - United States Environmental Protection Agency. **Priority pollutants**, 2009. Disponível em: <http://www.epa.gov/waterscience /methods/pollutants.html>. Acesso em: 10 dez. 2010.

VAN AGGELEN, G. et al. Integrating omic technologies into aquatic ecological risk assessment and environmental monitoring: hurdles, achievements, and future outlook. **Environmental Health Perspectives**, v. 118, p. 1-5, 2010.

VAN DER OOST, R.; BEYER, J.; VERMEULEN, N.P.E. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. **Environmental Toxicology and Pharmacology**, v. 13, p. 57-149, 2003.

VANDEGEHUCHTE, M.B. et al. Gene transcription profiles, global DNA methylation and potential transgenerational epigenetic effects related to Zn exposure history in *Daphnia magna*. **Environmental Pollution**, v. 158, p. 3323-3329, 2010.

VANZELLA, T.P.; MARTINEZ, C.B.R.; CÓLUS, I.M.S. Genotoxic and mutagenic effect of diesel oil water-soluble fraction on an neotropical fish species. **Mutation Research**, v. 631, p. 36-43, 2007.

VENIER, P. et al. Development of mussel mRNA profiling: can gene expression trends reveal coastal water pollution? **Mutation Research**, v. 602, p. 121-134, 2006.

VENIER, P. et al. MytiBase: a knowledgebase of mussel (*M. galloprovincialis*) transcribed sequences. **BMC Genomics**, v. 10, p. 72-88, 2009.

VENTER, J.C. et al. The sequence of the human genome. **Science**, v. 291, p. 1304-1351, 2001.

WALKER, C.H. et al. **Principles of ecotoxicology**. Londres: Taylor & Francis, 1996. 321 p.

WALKER, C.W. et al. **p53 superfamily proteins in marine bivalve cancer and stress biology**. In: LESSER, M. (Ed.). Advances in marine biology. Oxford: Academic Press, 2011. v. 59, p. 1-36.

WANG, Y.; GUO, X. Development and characterization of EST-SSR markers in the eastern oyster *Crassostrea virginica*. **Marine Biotechnology**, v. 9, p. 500-511, 2007.

WANG, K.-J. et al. Identification of the up-regulated expression genes in hemocytes of variously colored abalone (*Haliotis diversicolor* Reeve, 1846) challenged with bacteria. **Developmental and Comparative Immunology**, v. 32, p. 1326-1347, 2008.

WEBB, D.; GAGNON, M.M. The value of stress protein 70 as an environmental biomarker of fish health under field conditions. **Environmental Toxicology**, v. 24, p. 287-295, 2009.

WEISBROD, A.V. et al. Workgroup report: review of fish bioaccumulation databases used to identify persistent, bioaccumulative, toxic substances. **Environmental Health Perspectives**, v. 115, p. 255-261, 2007.

WHEELOCK, C.E. et al. Effect of nutritional state on HSP60 levels in the rotifer *Brachionus plicatilis* following toxicant exposure. **Aquatic Toxicology**, v. 61, p. 89-93, 2002.

WILLIAMS, T.D. et al. Transcriptomic responses of European flounder (*Platichthys flesus*) to model toxicants. **Aquatic Toxicology**, v. 90, p. 83-91, 2008.

WINSTANLEY, C. **Molecular biomethods handbook**. Humana Press: Springer, 2008.

WINSTON, G.W.; DI GIULIO, R.T. Prooxidant and antioxidant mechanisms in aquatic organisms. **Aquatic Toxicology**, v. 19, p. 137-161, 1991.

YIN, Y. et al. Bioaccumulation and ROS generation in liver of *Carassius auratus*, exposed to phenanthrene. **Comparative Biochemistry and Physiology Part C**, v. 145, p. 288-293, 2007.

YU, H.; LI, Q. Exploiting EST databases for the development and characterization of EST–SSRs in the Pacific oyster (*Crassostrea gigas*). **Journal of Heredity**, v. 99, p. 208-214, 2008.

ZAGATTO, P.A.; BERTOLETTI, E. **Ecotoxicologia aquática:** Princípios e aplicações. São Paulo: Editora Rima, 2006. 464 p.

ZANETTE, J. **Identificação e caracterização de marcadores moleculares para estudos ecotoxicológicos em moluscos bivalves e peixes**. 2009. 180 f. Tese (Doutorado em Biotecnologia), Programa de Pós-Graduação em Biotecnologia, Universidade Federal de Santa Catarina, Florianópolis.

ZANETTE, J. et al. Identification of CYP genes in *Mytilus* (mussel) and *Crassostrea* (oyster) species: First approach to the full complement of cytochrome P450 genes in bivalves. **Marine Environmental Research**, v. 69, p. S1-S3, 2010.

ZHONG, Y. **Phenanthrene tetraols as probes for the metabolic activation of carcinogenic polycyclic aromatic hydrocarbons in humans**. 2011. 161 f. Tese (Doctor of Philosophy) - Faculty of the Graduate School, University of Minnesota, Minnesota.