store

Proteomics and ecotoxicology : marine invertebrates and endocrine disrupting chemicals

90 0858155 4

Reference Only

LIBRARY STORE

Copyright Statement

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no formation derived from it may be published without the author's prior consent.

Proteomics and ecotoxicology: marine invertebrates and endocrine disrupting chemicals

by

ANNE BJØRNSTAD

A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences
University of Plymouth
Drake Circus
Plymouth PL4 8AA
Devon, UK

In collaboration with
International Research Institute of Stavanger (IRIS-BIOMILJØ)

May 2008

University of Plymouth
Library
Item No.
900 858 1554
Shelfmark
THESIS 574.5833 BJO

Anne Bjørnstad

Proteomics and ecotoxicology: marine invertebrates and endocrine disrupting chemicals

Abstract

The key problem faced by environmental scientists is to predict and recognize the damaging effects of chemical pollutants on natural biota. The aim of this thesis was to evaluate the potential for proteomics in ecotoxicology and environmental risk assessment (ERA), with the hypothesis that proteomic technologies (i.e. ProteinChip technology in combination with SELDI TOF MS) could be a useful supplement to existing methods of environmental assessment, by providing a sensitive, non-invasive, rapid multi-endpoint assessment of effects of anthropogenic chemicals on organism *in vivo*. Three invertebrate species, *Mytilus edulis, Hyas araneus* and *Strongylocentrotus droebachiensis* was exposed to natural and anthropogenic chemicals in laboratory and field studies. Results revealed that proteomics was a sensitive endpoint, as all exposure regimes significantly affected protein expression. It was shown that plasma protein expression profiles contained information that was compound, dose, site, species and gender-specific. Regarding the latter; male and female organisms responded differently to all exposures both quantitatively (e.g. in terms of number of affected protein species) and qualitatively (e.g. in terms of type of affected protein species). Furthermore, genders have shown opposite responses following the same exposure regime. Equally, species-specific responses were observed. Moreover, exposing organisms to graded levels of contamination under controlled laboratory conditions and in the field revealed that different subsets of proteomes were affected at different levels of exposure. This finding represents an opportunity for applying proteomics for both prognostic (e.g. early warning of potential adverse effects or assessment of recovery) and diagnostic purposes. Moreover, those protein features that were changed by all exposure concentrations showed complex dose-response relationships, including both linear and various types of biphasic response-curves.

In summary, results from the present study indicate that proteomics have the potential to be a useful tool in ERA. For example, identification of key molecules could elucidate mechanism of action related to mixture effects, gender and species-specific susceptibility to environmental pollutants as well as dose-response relationships at low doses. Furthermore, key proteins (i.e. putative biomarkers) could be purified and coupled to e.g. a biosensor for automated monitoring.

List of contents

COPYRIGHT STATEMENT 1

ABSTRACT 3

LIST OF CONTENTS 4

LIST OF TABLES AND FIGURES 8

- TABLES 8
- FIGURES 11

ACKNOWLEDGEMENTS 20

PRESENTATIONS AND PUBLICATIONS 22

AUTHOR'S DECLARATION 23

ABBREVIATIONS 24

CHAPTER 1 29

GENERAL INTRODUCTION 29

1.1 THE CHALLENGE – POTENTIAL EDCS 30

- 1.1.1 CHEMICAL MODES OF ACTION ON (ENDOCRINE) SYSTEMS 33
  - *Endocrine systems* *33*
  - *How do hormones act?* *34*
  - *How do EDCs act?* *37*
- 1.1.2 EVIDENCE OF (ECOLOGICAL) EFFECTS OF EDCS 38
- 1.1.3 RESEARCH NEEDS AND CURRENT CONCERNS REGARDING EDCS 40

1.2 CURRENT ENVIRONMENTAL MONITORING 41

1.3 THE NEW METHODS – 'OMICS' TECHNOLOGIES 46

- 1.3.1 GENOMICS 50
- 1.3.2 TRANSCRIPTOMICS 51
- 1.3.3 PROTEOMICS 53
- 1.3.4 METABOLOMICS/METABONOMICS 54
- 1.3.5 WHICH IS THE PREFERRED 'OMIC' TECHNOLOGY? 55

1.4 THE CURRENT STATE OF 'EXPRESSION' PROTEOMICS 57

- 1.4.1 PROTEIN SEPARATION/ QUANTITATION – THE DISCOVERY PHASE 58
- 1.4.2 DATAMINING/BIOINFORMATICS 64
- 1.4.3 PROTEIN PURIFICATION AND IDENTIFICATION 65
- 1.4.4 PROMISES OF A PROTEOMIC APPROACH IN ECOTOXICOLOGY 66

1.5 STUDY ORGANISMS 67

- 1.5.1 MYTILUS EDULIS 69
- 1.5.2 HYAS ARANEUS 70
- 1.5.3 STRONGYLOCENTROTUS DROEBACHIENSIS 70

1.6 AIM OF THE STUDY 72

CHAPTER 2 73

MATERIAL AND METHODS 73

2.1 METHOD DEVELOPMENT - PROTEOMICS 74

- 2.1.1 STUDY DESIGN AND SAMPLING 75
- 2.1.2 SAMPLE PREPARATIONS WITH PROTEINCHIP ARRAY TECHNOLOGY 76
  - *A. ProteinChip arrays* *78*

B. Sample dilution ... 81
C. Buffer conditions for sample binding ... 84
D. Sample incubation ... 86
E. Wash conditions ... 88
F, G & H. Energy absorbing molecule (matrix) ... 88
2.1.3 DATA COLLECTION WITH SELDI TOF MS ... 91
2.1.4 DATA HANDLING AND STATISTICS ... 94

2.2 COLLECTION AND MAINTENANCE OF ANIMALS ... 99

2.3 EXPOSURE SYSTEM (USED IN LABORATORY STUDIES) ... 101

2.4 LABORATORY AND FIELD EXPERIMENTS ... 103
2.4.1 LABORATORY STUDY 1 (MIXTURE EFFECTS) ... 103
2.4.2 LABORATORY STUDY 2 (SPECIES AND GENDER-RELATED EFFECTS) ... 103
2.4.3 LABORATORY STUDY 3 (DOSE-RESPONSE RELATIONSHIPS) ... 104
2.4.4 FIELD VALIDATION STUDY (COPPER GRADIENT) ... 104

2.5 EXPOSURE MONITORING ... 105

2.6 CHEMICALS ... 105

CHAPTER 3 ... 106

EFFECTS OF OIL AND PRODUCED-WATER COMPOUNDS ON PROTEIN EXPRESSION IN THE MUSSEL *MYTILUS EDULIS* ... 106

***3.1* INTRODUCTION** ... 107

***3.2* MATERIAL AND METHODS** ... 110
3.2.1 EXPOSURE MONITORING ... 112
3.2.2 SAMPLE COLLECTION ... 113
3.2.3 SAMPLE PREPARATION ON PROTEINCHIP ARRAYS ... 113
3.2.4 SELDI TOF MS ANALYSIS ... 113
3.2.5 DATA HANDLING AND STATISTICS ... 114

***3.3* RESULTS AND DISCUSSIONS** ... 114
3.3.1 CHEMISTRY ... 114
3.3.2 PROTEOMIC RESPONSE ... 115
3.3.3 TREATMENT-SPECIFIC RESPONSE ... 121
3.3.4 GENDER-SPECIFIC RESPONSES ... 124
3.3.5 GENDER-SPECIFIC PROTEIN PATTERNS ... 126
3.3.6 REPRODUCIBILITY ... 130
3.3.7 USE OF (PROTE)OMIC DATA IN RISK ASSESSMENT ... 132

3.4 CONCLUSIONS ... 133

CHAPTER 4 ... 135

SPECIES AND GENDER-SPECIFIC RESPONSES TO ENDOCRINE DISRUPTING CHEMICALS ... 135

***4.1* INTRODUCTION** ... 136

***4.2* MATERIAL AND METHODS** ... 141
4.2.1 MONITORING OF ECDS DURING EXPOSURE ... 142
4.2.2 SAMPLE COLLECTION ... 143
4.2.3 SAMPLE PREPARATION ON PROTEINCHIP ARRAYS ... 143
4.2.4 SELDI TOF MS ANALYSIS ... 143
4.2.5 DATA HANDLING AND STATISTICS ... 144

***4.3* RESULTS AND DISCUSSIONS** ... 145
4.3.1 GENERAL RESPONSE ... 146

4.3.2 TREATMENT-SPECIFIC RESPONSE 148
4.3.3 SPECIES SIMILARITIES AND DIFFERENCES 152
4.3.4 GENDER-SPECIFIC RESPONSES 157

4.4 CONCLUSIONS 162

CHAPTER 5 164

DOSE-RESPONSE RELATIONSHIPS OF OIL ON PROTEIN EXPRESSION IN MOLLUSCS AND ECHINODERMS 164

***5.1* INTRODUCTION** 165

***5.2* MATERIAL AND METHODS** 168

5.2.1 EXPOSURE MONITORING 170
5.2.2 SAMPLE COLLECTION 171
5.2.3 SAMPLE PREPARATION ON PROTEINCHIP ARRAYS 171
5.2.4 SELDI TOF MS ANALYSIS 171
5.2.5 DATA HANDLING AND STATISTICS 172

***5.3* RESULTS** 172

5.3.1 CHEMISTRY 172
5.3.2 PROTEOMIC RESPONSE 174
5.3.3 DOSE-RESPONSE RELATIONSHIPS 175
5.3.4 MULTIVARIATE RESPONSE PATTERNS 178

5.4 DISCUSSION 183

5.5 CONCLUSIONS 189

CHAPTER 6 190

FIELD VALIDATION: PROTEIN EXPRESSION IN *MYTILUS EDULIS* DEPLOYED ALONG A FIELDGRADIENT IN THE VICINITY OF A COPPER MINE 190

***6.1* INTRODUCTION** 191

***6.2* MATERIAL AND METHODS** 194

6.2.1 MONITORING OF THE METAL GRADIENT 196
6.2.2 SAMPLE COLLECTION 196
6.2.3 SAMPLE PREPARATION ON PROTEINCHIP ARRAYS 196
6.2.4 SELDI TOF MS ANALYSIS 197
6.2.5 DATA HANDLING AND STATISTICS 197

***6.3* RESULTS** 197

6.3.1 CHEMISTRY 198
6.3.2 GENERAL EFFECT ON PROTEIN EXPRESSION 200
6.3.3 SITE/DOSE-RESPONSE RELATIONSHIPS 202
6.3.4 COMPARISON TO SEASONAL VARIATION IN REFERENCE MUSSELS 208
6.3.5 EFFECT OF THE POLLUTANT GRADIENT ON GENDER DIFFERENCES 209

6.4 DISCUSSION 211

6.5 CONCLUSIONS 218

CHAPTER 7 220

GENERAL DISCUSSION AND FUTURE PERSPECTIVES 220

7.1 GENERAL DISCUSSION 220

7.1.1 REQUIREMENTS/RESEARCH NEEDS OF ECOTOXICOLOGY/ERA 221
7.1.2 THE POTENTIAL FOR PROTEOMIC IN ECOTOXICOLOGY/ERA 224
7.1.3 THE CHALLENGES AND PITFALLS FOR PROTEOMICS IN ERA 227

7.1.4 OVERALL SUMMARY 229

7.2 FUTURE PERSPECTIVES 231

7.2.1 GATHER BASELINE INFORMATION 232
7.2.2 VALIDATION OF THE EXPERIMENTS AND RESULTS FROM THIS THESIS 232
7.2.3 PROTEIN IDENTIFICATION AND MECHANISTIC STUDIES 233
7.2.4 MAKING THE INFORMATION/KNOWLEDGE ACCESSIBLE AND USER-FRIENDLY FOR THE 'USERS' 234

BIBLIOGRAPHY 236

URL's: 295

PUBLICATION 1& 2 297

PUBLICATIONS IN PREPARATION 298

List of Tables and Figures

Tables

Table 1-1. The two categories of hormone receptors, classes of hormones that bind to them, and principal mechanisms of action. Adapted from http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/index.html........................29

Table 2-1. Factors addressed in optimisation of sample preparation..........................71

Table 2-2. Optimal sample dilution for blue mussels, sea urchin and spider crabs respectively..77

Table 2-3. Buffer conditions tested: NaAc (sodium acetate); PBS (phosphate-buffered saline); TFA (trifluoracetic acid); ACN (acetonitrile); NaCl (sodium chloride), Cu (Copper), HCl (hydro chloride) 78

Table 2-4. Selected binding buffers for the various ProteinChip arrays...................78

Table 2-5. Test of optimal sample incubation volume, incubation time and temperature..80

Table 2-6. List of factors investigated with regard to matrix application.................82

Table 2-7. Final sample preparation protocol...83

Table 2-8. Protein standards and volumes used for calibration of mass spectra.........84

Table 2-9. Body burden of PAHs (as SUM PAH and EPA 18) in mussels from one of the reference sites (Førlandsfjorden, column 1-2) and various sites known to be contaminated. Column 3 (Visnes) represents a metal-contaminated site, while columns 4-8 are from PAH-contaminated sites...92

Table 3-1. The PAH composition recorded in Statfjord B crude oil and some characteristics of the PAH molecules....104

Table 3-2. Composition of APs and PAHs used in the spike....105

Table 3-3. Overview of number of significantly ($p<0.05$, Mann-Whitney-Wilcoxon test) upregulated (Up) and downregulated (Down) peaks in the two exposure regimes....109

Table 3-4. Examples of complex response pattern in mussels exposed to oil and spiked oil (OAP)....110

Table 3-5 Shows all mass peaks that were significantly ($p<0.05$) changed in one or several exposure groups. The table indicate the size (Da) of the altered peaks, as well as where (i.e. exposure group) and how (up or downregulated) it was altered. Because of the size of the table, it has been divided into part A, B and C. OAP = spiked oil, F = females, M = males, UP = upregulated, and DOWN = downregulated....111

Table 3-6. Prediction models (generated with Biomarker Pattern^{TM} software) for classification of controls vs. exposed. Input are the 149 mass peaks with $S/N \geq 5$....112

Table 4-1. Exposure compounds, i.e. BPA, BDE-47 and DAP....113

Table 4-2. Exposure monitoring of DAP, BPA and BDE-47 in water....135

Table 4-3. Acquisition parameters (i.e. laser intensities and detector sensitivities) used for mussels and spider crabs in the low-molecular weight (LM) and high-molecular weight (HM) runs....137

Table 4-4. Summary of significantly ($p < 0.05$) upregulated (Up) and downregulated (Down) peaks in various exposure regimes for spider crabs (*Hyas araneaus*) and mussels (*Mytilus edulis*)........140

Table 4-5. Summary of treatment-specific responses in mussels (*Mytilus edulis*) following exposure to DAP, BDE-47 and BPA (ns = not significant response).........144

Table 4-6 Summary of treatment-specific responses in spider crabs (*Hyas araneuss*) following exposure to DAP, BDE-47 and BPA (ns = not significant response, MXR = multixenobiotic resistance, TOSC = total oxyradical scavenging capacity)............145

Table 4-7. Test of prediction models created with Biomarker Pattern^{TM} Software for comparison of controls versus exposed in spider crabs (*Hyas araneus*) and mussels (*Mytilus edulis*). Prediction models were generated from 100 mussel samples and 80 samples from crabs, and subsequently tested with 60/40 new samples from mussels and crabs, respectively........149

Table 5-1. Nominal exposure concentrations of dispersed oil compared to measured water concentrations of THC (n = 5) and PAH (n = 1) in the exposure tanks..........166

Table 5-2. Measured concentrations of PAHs in biota (i.e. sea urchin gonads and whole-mussel tissue) at the end of the exposures (n = 3 replicates per sample type)........166

Table 5-3. Overview of number of significantly ($p < 0.05$, Mann-Whitney-Wilcoxon test) upregulated (Up) and downregulated (Down) peaks in various exposure regimes for sea urchins (*Strongylocentrotus droebachiensis*) and mussels (*Mytilus edulis*)...168

Table 5-4. Test of prediction models, created in Biomarker Pattern^{TM} Software, for comparison of controls versus exposed in sea urchins (*Strongylocentrotus droebachiensis*) and mussels (*Mytilus edulis*). 174

Table 6-1. Bioaccumulation of metals in *Mytilus edulis* deployed for 18 days along a copper gradient........191

Figures

Figure 1-1. Illustration of hormone-signal mediation via cell surface (membrane) receptors (A) and intracellular receptors (B) for water soluble and lipophilic hormones respectively. Adapted from (Helmreich 2001; Lodish et al. 2000). HRE = Hormone response element in DNA 30

Figure 1-2. Shows how ecotocicology, ERA and risk management depend upon each other for a most efficient management of environmental pollutants. Figure courtesy of Steinar Sanni...36

Figure 1-3. Complexity of the different 'omics' levels are influenced by genetic predisposition, environmental factors and regulatory interactions within and between the 'omes'(adapted from Lay et al. 2006). Figure courtesy of Prof. Charles Wilkins...42

Figure 1-4. 'Omics' data categorised into components, interactions and functional-states data, describing most of the components and interactions within a cell (adapted from Joyce and Palsson 2006). Figure courtesy of Dr. Andrew Joyce and Dr. Bernhard Palsson...43

Figure 1-5. Simplified schematic representation of the workflow in a 'top-down' 'expression-proteomics' study. Samples are first separated using chromatography or electrophoreses (1), the resulting data are then processed using uni and multivariate statistics (2A), statistics may reveal putative biomarkers (protein expression signatures) specific to compound, gender, species, dose, time. When interesting proteins have been selected, they are purified using multidimensional chromatography and sequenced using tandem mass spectrometry (3). To obtain protein identifications, sequence data have to be matched to genome information in databases (2B). Validation (4) is important at all steps, and particularly before a commercial assay (5) is made based on the identified biomarkers...52

Figure 1-6. Selected study organisms: (A) the blue mussel (*Mytilus edulis*), (B) a spider crab (*Hyas araneus*), and (C) the green sea urchin (*Strongylocentrotus droebachiensis*). Pictures courtesy of Kåre Telnes (i.e. Picture B and C)....................................63

Figure 2-1. Body fluid sampling: the red rings indicate where haemolymph (mussels and crabs) and coelomic fluid (sea urchins) was collected; (A) haemolymph from *Mytilus edulis* was collected from the posterior adductor muscle, (B) haemolymph from *Hyas araneus* was collected from the arthrodial membrane at the base of the 4th pair of walking legs, (C) samples of coelomic fluid from *Strongylocentrotus droebachiensis* were collected by inserting the syringe through the peristomial membrane............69

Figure 2-2. The three phases of separation used for profiling complex biological mixtures such as cell lysate or biofluids. The first phase is surface-type (e.g. Reverse phase, ion exchange etc.), the second is wash stringency (pH, salt, detergent modulation etc.), and the final phase is the mass spectrometry analysis. For each chip surface and wash condition, a different protein fingerprint is achieved that can be used to look for differential protein analysis of control and test samples. Figure courtesy of Ciphergen Biosystems/Bio-Rad...71

Figure 2-3. The two categories of ProteinChip® arrays: (A) Chromatographic arrays that extract proteins through hydrophobic, anionic, cationic, metal ions and hydrophilic surfaces (analogue to conventional chromatographic sorbents); these array surfaces are best suited for profiling broad subset of proteins from crude biological mixtures to compare differential protein expression. (B) Biological preactivated surfaces that are designed to capture specific biomolecules such as antibodies, receptors, or oligonucleotides (through covalent bindings) to study biomolecular interactions. Figure courtesy of Ciphergen Biosystems/Bio-Rad...73

Figure 2-4. Interaction chemistries for selected ProteinChip® arrays: (A) strong anion exchange array (SAX/Q10), (B) weak cation exchange array (WCX/CM10), (C) Metal affinity array (IMAC-30), and (D) Reverse phase/hydrophobe interactions array (H4). Figure courtesy of Ciphergen Biosystems/Bio-Rad...74

Figure 2-5. Species-specific (partial) protein profiles. Analyses of plasma from 3 individuals of (A) blue mussels, (B) sea urchins, and (C) spider crabs showing unique species-specific MS traces when captured on the same chip surface (with equal buffer conditions); (D) shows an 'overlay' of the three species to elucidate similarities and differences of the species. In addition, individual variation can be seen in (A), (B) and (C)..75

Figure 2-6. Example of sample dilution (i.e. 1:5, 1:10, 1:50, 1:100) of sea urchin plasma on weak cation exchange arrays (and sodium acetate buffer pH 4.5). The figure shows an "overlay" of MS traces for each dilution. Black = 1:5, red = 1:10, blue = 1:50, and green = 1:100..76

Figure 2-7. Average number of peaks (± SD) detected on WCX, SAX, IMAC-Cu and H50-RP arrays (with a signal to noise greater than or equal to 5, present within the mass range of 1500 to 200000 Da) for sea urchin plasma, mussel plasma and spider crab plasma, when analysed with optimal buffer conditions. The arrows indicate the best chip surface for each species (i.e. WCX)..77

Figure 2-8. (A) Sample processing using the "direct on-spot" method of a ProtenChip array; (B) a 96- well Bioprocessor from Bio-Rad loaded with 12 protein arrays. Figure courtesy of Ciphergen /Bio-Rad..78

Figure 2-9. A PBS-IIc time-of-flight mass spectrometer with (left) and without (right) automatic loading of protein arrays. Figure courtesy of Ciphergen Biosystems/ Bio-Rad..85

Figure 2-10. Shows array spots – how they are divided into sections, allowing repeated analysis of each sample, to optimise analysis of both small and large molecules in the same sample. For each analysis at least 60 - 100 laser shots are collected, and the average presented in the corresponding protein profile. Figure courtecy of Ciphergen Biosystems/Bio-Rad..87

Figure 2-11: Classification tree example. Figure courtesy of Dr. Eric T. Fung (Ciphergen Biosystems)..90

Figure 2-12. Sampling sites for mussels (*Mytilus edulis*) in Førlandsfjorden (Norway), spider crabs (*Hyas araneus*) at Krokaneset (Norway), and sea urchins (*Strongylocentrotus droebachiensis*) in Lysefjorden (Norway) Map photos are from http://norgeibilder.no/ 93

Figure 2-13. (A) Collection of crabs by traps baited with fish, and (B) collection of mussels from stones below the low water mark........ 94

Figure 2-14. Diagram of how the CFS was used in the first laboratory study (Chapter 3). Figure courtesy of Rolf Sundt........ 95

Figure 3-1. Treatment-specific (A), and gender-specific (B) responses. Each ring contains the number of proteins significantly ($p < 0.05$) altered only by the indicated groups. The overlapping areas indicate the numbers of proteins that were affected by both groups. OAP = oil spiked with alkylphenols and extra poly aromatic hydrocarbons. Data illustrates male and female responses combined........ 113

Figure 3-2. Gender-specific protein forms. Partial protein expression profile showing examples of mass peaks primarily expressed in either females or males; visualised as "gel-view" (top) and "spectra-overlay" (bottom). A: M/Z 5866 Da, 25 fold up-regulated in males when males and females are compared independent of exposure. B: M/Z 5970 Da, expressed in females (3.5 fold up-regulated in average) and males exposed to spiked oil........ 120

Figure 3-3. Classification models created with Biomarker Pattern™ software when data from 150 plasma samples (grouped by gender independent of exposure) were fed to the software. Each of the blue squares represent a classification model with a certain number of predictors/classifiers (indicated by the number of nodes on the x-axis) needed for accurate classification of test samples. The relative cost (y-axis) indicates how accurate the model is........ 121

Figure 3-4. The best classification model (learning model) created with Biomarker Pattern™ software when data (i.e. 149 peaks) from 150 plasma samples (grouped by gender independent of exposure) were used as input. The model was tested with 60 new samples (i.e. 30 males and 30 females). ↑= upregulated, ↓= downregulated 122

Figure 3-5. Example of array-to-array reproducibility. Two plasma samples from control mussels (sample 1 and 2) were applied on three different ProteinChip arrays to check the reproducibility between arrays. The figure shows partial mass spectra and 'gel-views' obtained from SELDI TOF MS analysis of the samples. The coefficient of variance (CV) for M/Z values between 2.5 and 180 kDa (n = 149) was 0.6 – 7.9% for sample 1 and 0.7 – 13.3% for Sample 2. CV was calculated before normalisation of spectra..123

Figure 4-1. Diagram of the continuous flow system as used in this study...............133

Figure 4-2. Venn diagrams showing treatment-specific responses in mussels (A and B) and spider crabs (C and D) as the number of altered peaks by one or several exposures. Each ring contains the number of protein peaks altered only by the indicated compound (i.e. BPA, DAP or BDE-47). The overlapping areas indicate the numbers of peaks that were affected by two compounds, i.e. either BPA + DAP, BPA + BDE-47, DAP + BDE-47, or all compounds (indicated in the middle). Results were based on n = 80 mussel and 60 spider crab samples per group..143

Figure 4-3. Multidimensional scaling representation (MDS) of similarities and differences between exposure groups (females and males combined) and species. The analysis is based on a similarity matrix constructed using normalised Euclidian Distances. Data inputs were all plasma-protein peaks with S/N ≥5 for (a) spider crabs and (b) blue mussels, where each symbol represents mean peak intensity for all the individuals in one exposure group...149

Figure 4-4. PRIMER MDS plots revealing gender-specific response patterns after exposure to three EDCs. The response patterns are based on plasma- protein expression analysis with ProteinChip technology and SELDI TOF MS. Inputs were 328 mass peak for spider crabs and 227 peaks for mussels (A) = female mussels, (B) = male mussels, and (C) = spider crabs, females and male. ♀ = female, ♂= male........................152

Figure 4-5. Pie charts showing the percentage distribution of gender-specific responses of all peaks with significant different expression (in males and females combined) after exposure in mussels (*Mytilus edulis*) and spider crabs (*Hyas araneus*). Significant = $p < 0.05$, tested with Mann-Whitney-Wilcoxon test incorporated in the ProteinChip software)..**153**

Figure 4-6. Examples of types of responses observed in male (BPA-M) and female (BPA-F) mussels exposed to bisphenol A: Significant (and equal) responses in both genders (a), opposite responses (b), significant response in only males (c), and significant response in only females. CF = control females; CM = control males......**154**

Figure 5-1. Principle of the system used to supply oil to the main flow of seawater in the continuous flow system, and exposure tanks for sea urchins (125 animals per tank). Figure courtesy of Dr. Thierry Baussant...**161**

Figure 5-2. (A) Exposure tanks (i.e. 60 L) with mussels divided into 2 groups for each treatment: top ≈ 150 mussels and bottom ≈ 500 mussels. The flow rate was about 500 and 800 $ml.min^{-1}$, respectively in the bottom and top aquaria. (B) Mixing bottles for dilution of stock (5 ppm) dispersion of oil Figure courtesy of Dr. Thierry Baussant...**162**

Figure 5-3. Nominal exposure concentrations plotted against measured concentrations of sum-THC in water, sum-PAH in water, and sum-PAH in biota (sea urchins to the left and mussels to the right)..**163**

Figure 5-4. Dose-response for (A) number of significantly affected mass peaks and (B) average fold changes in protein expression for each exposure group. SD = *Strongylocentrotus droebachiensis*, ME = *Mytilus edulis*................................**167**

Figure 5-5. Examples of the different types of dose-response curves observed for mussels and sea urchins following oil exposure. X- axis = doses (i.e. 15, 60 and 250 ppb oil), Y-axis = relative peak intensity for selected M/Z values...............................**170**

Figure 5-6. Pie charts showing percentage distribution of the various types of dose-response relationships observed for all significantly altered protein species. Note. 'Normal' includes both upregulated and downregulated protein forms, and 'Z-shaped' include also 'inverted Z-shape'......171

Figure 5-7. Two-dimensional scaling representation of similarities and differences in response to different exposure concentrations. The analysis is based on a similarity matrix constructed using normalised Euclidian Distances. Data inputs were all plasma-protein peaks with S/N ≥ 5 (n = 212 for mussels and n = 153 for sea urchins), where each symbol represents mean peak intensity for all the individuals in one exposure group (n = 30). Red = Control, green = 15 ppb, dark blue = 60 ppb, and light blue = 250 ppb, ME = *Mytilus edulis*, SD = *Strongylocentrotus droebachiensis*......172

Figure 5-8. Three-dimensional scaling representation of individual similarities and differences in response to different exposure concentrations for sea urchins. The analysis is based on a similarity matrix constructed using normalised Euclidian Distances. Data inputs were all plasma-protein peaks with S/N ≥ 5 (n = 212 for mussels and 153 for sea urchins), where each symbol represents mean peak intensity for one individual animal. A: included individuals from controls, 15, 60 and 250 ppb exposure groups. B: included only controls and the two lowest concentrations......173

Figure 6-1. Location of the three sites along the coppergradient at Visnes (Site 2-4), the gradient reference (Site 1), and the location of the natural population of mussels at Visnes (VIS)......187

Figure 6-2. The type of cages used for the caging study. Dimensions are 64 cm x 32 cm x 28 cm......187

Figure 6-3. T Accumulation of copper in mussel tissue along a pollutant gradient in the vicinity of an old copper mine. Values are based on measurements of one pooled (n = 20 mussels) sample per site......192

Figure 6-4. Pie charts showing percentage distribution of the various types of dose-response relationships observed for all significantly altered protein species in *Mytilus edulis* transplanted from a reference site in Førlandsfjorden (Karmøy, Norway) to a pollutant field gradient (including a gradient reference – site 1); male mussels to the left, and female mussels to the right. Note. 'Normal' includes both upregulated and downregulated protein forms..**195**

Figure 6-5. MDS plots, derived from Euclidean distance similarity, comparing response on protein expression patterns in *Mytilus edulis* along the copper gradient. Each symbol in the plot represents the average response of 30 individuals per group (input = 240/220 mass peaks for males and females, respectively). F = females (A), M = males (B), 1 - 4 = site 1- 4 (site 1 = gradient reference)..**196**

Figure 6-6. MDS plots comparing protein expression patterns in mussels transplanted from Førlandsfjorden to Visnes with protein profiles in indigenous mussels at Visnes. Each symbol in the plot represents the average response of 30 individuals per group (input = 240/220 mass peaks for males and females, respectively). F = females (A), M = males (B), S1- S4 = site 1- 4..**197**

Figure 6-7. Plots of prediction success in classification models created with Pattern^{TM} Software, for male (A) and female (B) mussels, in classifying individuals at site 1 (gradient reference) from those at site 2-4 and Visnes. Graphs illustrate the percentage (values indicated) correct classification of exposed (sensitivity) and reference mussels (specificity). Learning models were created with 40 samples and tested with 20 new samples..**199**

Figure 6-8. MDS plot comparing seasonal variation at the reference site with effect of caging at a copper contaminated site for *Mytilus edulis*: females (A) and males (B). Each symbol in the plot represents the average response on protein expression of 30 individuals per group. Mussels collected at the reference site (Førlandsfjorden) in November (NOV), January (JAN), March (MAR) and May (MAY) were compared with mussels from Førlandsfjorden deployed at Visnes (copper-site) for 18 days in November (NOV-Site 4). M = males, F = females......................................**201**

Figure 6-9. MDS plot of gender similarities and differences in mussels at site 2 - 4 (A-C) and Visnes (D) compared to gender differences in mussels at site 1 (the gradient reference).The analysis is based on a similarity matrix constructed using normalised Euclidian Distances. Data inputs were all plasma protein peaks with S/N ≥5 (n = 240 for males and 220 for females), where each symbol represents mean peak intensity for all the individuals in one exposure group (n = 30). The arrows indicate the relative distance between male and female organisms for each treatment group. S1 - S4 = site 1 - 4, VIS = Visnes, M = males, F = females..**202**

Acknowledgements

This study was undertaken in order to further understand (and predict) the threat posed by natural and anthropogenic compounds (on invertebrates) in the environment. The work was conducted as part of a Strategic Institute Programme at the International Research Institute of Stavanger (IRIS – Biomiljø) funded by the Research Council of Norway (project 133724/420). Additional funding was provided by the European commission (via the BEEP program – EVK3-2000-00543). I gratefully acknowledge the opportunity and funding. In addition, I would like to thank ENI Norge AS, Total Norge E&P AS and the Research Council of Norway for funding the BIOSEA JIP programme.

I am forever indebted to many for their help, encouragement, and advice throughout the course of this study. First of all I would like to thank my two supervisors, Prof. Malcolm Jones (University of Plymouth) and Dr. Odd Ketil Andersen (IRIS) for excellent guidance, motivation, and effort throughout this study. You have both contributed greatly to the realisation of this thesis. I would also like to acknowledge Prof. Michael Depledge, who contributed with many good ideas in the early phase of this project.

I am very grateful to Päivi T. Lædre and Anna Aabø (Vice-president and President of IRIS) for being so supportive, and for granting me leave of absence from my work the past months, in order for me to finalise this thesis. I would also like to thank Arild Johannessen for stepping in as my 'replacement' at work on very short notice. I appreciate all the help and support I have received from Ciphergen Biosystem/Bio-Rad (particularly from Peter Iversen).

Thanks also to all colleagues and great friends at IRIS-Biomiljø for creating such a nice place to work, and for all the good laughs and fun. I will not mention everyone in particular, however, many thanks and warm thoughts goes to Bodil who recently returned to Denmark after many years in Norway (and who spent many late evenings and weekends with me in the lab and field while she was in Norway). I miss you very much! I would also like to thank Anne-Mette for always being so helpful, and the biotechnology group for being such a great team! Furthermore, I would like to thank Renée and Thierry for allowing me to collect samples from their BIOSEA projects, and a big thank you to Mari for the wonderful year on maternity leave, all help with Max, and all fruitful discussions around the lake (Stokkavannet).

Throughout this study I have met many great international friends and colleagues, including my Lithuanian mom – Janina. Thank you so much for being you, for all the laughs, (moral) support, and scientific advisees. Your room in our house is always ready for you! Nadia and Dani (the 'BEEP' girls) will also always have a special place in our house and our hearts.

I would like to tank Jan Baak and his group at the pathology department of Stavanger University Hospital too. I enjoy very much to work with you! Thank you for all encouragement, support and for introducing me to Sinterklaas.

Last, but not least, I want to thank my 'not-work-related' friends and family for always being there for me. Finally, I am so grateful to my closest family, little Max and John. I am sorry for leaving you alone so many evenings and weekends, but it was always great to know that you were waiting for me. I am so privileged to have you in my life. You are more important to me than a 1000 PhDs - THANK YOU!

Presentations and publications

Bjørnstad A, Monsinjon T, Knigge T, and Jones MB, Andersen OK (2002). Proteomics in ecotoxicology. BEEP-project meeting, Athens (Greece) 2002.**Poster and platform presentation.**

Bjornstad A, Larsen BK, Monsinjon T, Knigge T, Lehtio J, Jones MB, Andersen OK (2003). Use of proteomics (SELDI – TOF) in assessment of endocrine disruption in molluscs and crustaceans. PRIMO 12 (Tampa, Florida, US, May 2003). **Poster**

Bjørnstad A, Larsen BK, Jones M, Andersen OK (2003). Altered plasma-protein expression in mussels (*Mytilus edulis*) exposed to known endocrine disrupters; Bisphenol A; DAP and PBDE-47. BEEP project meeting, Barcelona, Spain (4th – 6th December 2003). **Poster and platform presentation.**

Bjørnstad A, Larsen BK, Jones M, Andersen OK (2003). Altered plasma-protein expression in mussels (*Mytilus edulis*) exposed to known endocrine disrupters; Bisphenol A, DAP and PBDE-47. European society for comparative physiology and biochemistry, 22nd Conference, Alessandria (Italy), December 2003. **Platform presentation**

Bjørnstad A., Larsen BK, Skadsheim A, Jones MB, Andersen OK (2004). The potential of ecotoxicoproteomics in environmental monitoring; biomarker profiling in mussel plasma using proteinchip array technology. Symposium for Toxicology and Environmental Health, Oslo (Norway), October 2004. **Platform presentation.**

Bjørnstad A., Larsen BK, Skadsheim A, Jones MB, Andersen OK (2005). The potential of ecotoxicoproteomics in environmental monitoring; biomarker profiling in mussel plasma using proteinchip array technology. SETAC-LILLE (France). May 2005. **Platform presentation**

Bjørnstad A., Larsen BK, Skadsheim A, Jones MB, Andersen OK (2006). The potential of ecotoxicoproteomics in environmental monitoring; biomarker profiling in mussel plasma using proteinchip array technology. J. Toxicol. Environ. Health A 69(1): 77-96

Bjørnstad A., et al. (2008). Invited to be keynote speaker at http://proteomlux2008.lippmann.lu/program.php3 to talk about gender-related responses to environmental pollutants.

Contributions as co-author (related to Chapters 3 and 4):

Gomiero A, Pampanin DM, **Bjørnstad A,** et al. (2006). An ecotoxicoproteomic approach (SELDI-TOF mass spectrometry) to biomarker discovery in crab exposed to pollutants under laboratory conditions. *AQUATIC TOXICOLOGY* 78: 34-41

Sundt RC, Pampanin DM, Larsen BK, Brede C, Herzke D, **Bjørnstad A,** Andersen OK. (2006). The BEEP Stavanger Workshop: Mesocosm exposures. *AQUATIC TOXICOLOGY* 78: 5-12. Suppl. 1.

Larsen BK, **Bjørnstad A,** Sundt RC, et al.(2006). Comparison of protein expression in plasma from nonylphenol and bisphenol A-exposed Atlantic cod (Gadus morhua) and turbot (Scophthalmus maximus) by use of SELDI-TOF. *AQUATIC TOXICOLOGY* 78: 25-33, Suppl. 1.

Barsiene J, Syvokiene J, **Bjornstad A** (2006). Induction of micronuclei and other nuclear abnormalities in mussels exposed to bisphenol A, diallyl phthalate and tetrabromodiphenyl ether-47. *AQUATIC TOXICOLOGY* 78: 105-108. Suppl.

- Minier C, Forget-Leray J, **Bjørnstad A,** Camus L (2008). Multixenobiotic resistance, acetyl-choline esterase activity and total oxyradical scavenging capacity of the Arctic spider crab, Hyas araneus, following exposure to Bisphenol A, Tetra Bromo Diphenyl Ether and Diallyl phtalate. *MARINE POLLUTION BULLETIN* 56 (8): 1410-1415.

Author's declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

This study was financed with the aid of a studentship form the Natural Environment Research Council and carried out in collaboration with Plymouth Marine Laboratory.

A programme of advanced study was undertaken, which included a final year honours course in colloid chemistry, supervised information technology instruction and a postgraduate course on analysis and hazard assessment of marine pollution.

Relevant scientific seminars and conferences were regularly attended at which work was often presented; external institutions were visited for consultation purposes and several papers prepared for publication.

Publications (or presentation of other forms of creative and performing work):

- see list at page 22.

Presentation and Conferences Attended: - see list at page 22.

External Contact: Dr. Odd Ketil Andersen

Word count of thesis: 74560 (including bibliography)

Signed Anne Bjørnstad

Date 15th June 2009

Abbreviations

2D/3D	- Two-dimensional/three-dimensional
2DE	- Two-dimensional gel electrophoresis
ACN	- Acetonitrile
Ag	- Silver
AP(s)	- Alkylphenol(s)
As	- Arsenic
ATPase	- Adenosine triphosphatase
Ba	- Barium
BDE-47	- 2,2`,4,4`Tetra Bromo Diphenyl Ether
BDE-99	- 2,2`,4,4`, 5-Penta Bromo Diphenyl Ether
BEEP	- Biological Effects of Environmental Pollution in Marine Ecosystems
BPA	- Bisphenol A (4, 4-isopropylidene diphenol)
BSA	- Bovine serum albumin
C	- Carbon
c	- Concentration
°C	- Degree Celsius (temperature scale)
Cd	- Cadmium
CF	- Control females
CFS	- Continuous flow system
CHCA	- Alpha-cyano-4-hydroxy cinnamic acid
cm	- Centimeters
CM	- Control males
CM10	- Weak cation exchange arrays
Co	- Cobalt
Cr	- Chromium
Cu	- Copper
$CuSO_4$	- Copper sulfate
CV	- Coefficient of variance
D	
Da	- Dalton (size unit for proteins)

DAP - Diallyl phthalate
DDT - Diklor-difenyl-trikloretan, *(1,1,1-trichloro-2,2-bis-(p-chlorophenyl) ethane)*
DIGE - Difference gel electrophoresis
Down - Down regulated (protein forms)
DNA - Deoxyribonucleic acid
°E - East
EAM - Energy absorbing molecule
EC50 - Median effect concentration
EDC(s) - Endocrine disrupting chemical(s)
EPA - United States Environmental Protection Agency
ERA - Environmental risk assessment
EU - European Union
F - Females
FID - Flame ionization detection
FØR - Førlandsfjorden
g - Gram
GC - Gas chromatography
GC/MS - Gas Chromatography connected to a Mass Spectrometer
GLP - Good laboratory practice
GSH - Intracellular glutathione
GST - Glutathione-S-Transferase
H4/H50 - Reverse phase or hydrophobic interaction(s) arrays
HCl - Hydro chloride
HM - High-molecular weight
HPLC - High performance liquid chromatography
HRE - Hormone response element in DNA
ICAT - Isotope-coded affinity tag
ICPL - Isotope-coded protein label
ICP/MS - Inductively coupled plasma mass spectrometry
IMAC30 - Immobilized metal affinity chromatography arrays
IPCS - International Programme on Chemical Safety
IPG - Immobilized pH gradient

iTRAQ	- Isobaric tag for relative and absolute quantitation
kDa	- Kilodalton (size unit for proteins)
kg	- Kilogram
L	- Liter
LC	- Liquid chromatography
LC50	- Median lethal concentration
LM	- Low molecular weight mass
log K_{ow}	- Partitioning coefficient
M	- Males
m^3	-Cubic meter(s)
MDS	- Multi-dimentional scaling
μg	- Microgram
μl	- Microliters
mm	- Millimeter(s)
mM	- Millimolar
min	- Minute(s)
mRNA(s)	- Messenger ribonucleic acid(s)
MS	- Mass spectrometry
MS/MS	- Tandem mass spectrometry
MT(s)	- Metallothionein(s)
Mw	- Protein size (molecular weight)
MXR	- Multixenobiotic resistence
M/Z	- Mass-to-charge ratio
'N	- North
n	- number
NaAc	- Sodium acetate
NaCl	- Sodium chloride
ng	- Nano
Ni	- Nickel
NMR	- Nuclear magnetic resonance
NOEC	- No Observed Effect Concentration
ns	- Not significant
NTA	- Nitrilotriacetic acid

$^*O_2$-	- Superoxide
OAP	- Oil spiked with alkylphenols and PAHs
OSPAR	- Convention (OSlo and PARis) for protection of the marine environment of the North-East Atlantic Ocean
p	- Probability
PAH(s)	- Polycyclic aromatic hydrocarbon(s)
Pb	- Lead
PBS	- Phosphate buffered saline
PBDEs	- Polybrominated diphenyl ethers
PCB(s)	- Polychlorinated biphenyl(s)
PCR	- Polymerase Chain Reaction
pH-value	- A number denoting the degree of acidity or of basicity
pI	- Protein charge
ppb	- Parts per billion
ppm	- Parts per million
PRIMER	- Plymouth Routines in Multivariate Ecological Research
PTMs	- Posttranslational modifications
Q10	- Strong cation exchange arrays
R & D	- Research and development
REACH	- Registration, Evaluation, Authorisation and Restriction of Chemical substances
REF	- Reference group/site
RNA	- Ribonucleic acid
ROS	- Reactive oxygen species
RP	- Reverse phase
RT–PCR	- Real time Polymerase Chain Reaction
S1 - S4	- Site 1 – site 4
SAGE	- Serial analysis of gene expression
SAX	- Strong cation exchange arrays
SD	- Standard deviation
SDS-PAGE	- Sodium dodecyl sulphate-polyacrylamide gel electrophorese
SELDI	- Surface enhanced laser desorption ionisation
SETAC	- Society of Environmental Toxicology and Chemistry

SFT	- Norwegian pollution control authority (Statens Forurensningstilsyn)
SIM	- Selected ion mode
S/N	- Signal to noise ratio
SPA	- Sinapinic acid
TBT	- Tributyltin
TFA	- Trifluoracetic acid
THC	- Total hydrocarbon concentration
TOF	- Time of flight
TOSC	- Total oxyradical scavenging capacity
Up	- Up regulated (protein forms)
V	- Volt or vanadium
VIS	- Visnes
vs.	- Versus
WCX	- Weak cation exchange arrays
w.w.	- Wet weight
Zn	- Zinc

Chapter 1

General introduction

" I have no data yet
It is a capital mistake to theorise before one has data.
Insensibly one begins to twist facts to suits theories,
instead of theories to suit facts."

- Sherlock Holmes to Dr. Watson in a *Scandal in Bohemia* by Sir Arthur Conan Doyle

The key problem faced by environmental scientists is to recognize the damaging effects of chemical pollutants on natural biota (Depledge and Fossi 1994). The biological consequences of introducing contaminants to the environment are becoming more apparent (Carson 1962), and there is an increasing awareness that man's activities are having detrimental effects on man and wildlife (Colborn et al. 1996). While Rachel Carson (1962), in "Silent Spring", gave an urgent warning about the dangers posed by overuse of pesticides, Theo Colborn and co-workers (1996) reviewed a large and growing body of scientific evidence linking synthetic chemicals to aberrant sexual development and behavioural and reproductive problems in "Our Stolen Future". These two books contributed significantly to the worldwide concern regarding certain groups of chemicals (natural and synthetic) that have the potential ability to interfere with endogenous hormone systems, so-called endocrine disrupting chemicals (EDCs). As a result of this awareness, much international effort have been put into establishing research programs, organising conferences and workshops, and forming expert groups and committees to address and evaluate EDC-related issues. These include: (1) sources, fates and distribution; (2) chemical classes and their potencies; (3) exposure pathways and chemical modes of actions; (4) dose-response relationships, particularly in low-dose regions; (5) consequences of exposures to chemical mixtures; (6) evidence for ecological effects; (7) the importance and relevance of observations in humans and wildlife; (8) chemical testing procedures and guidelines, and (9) risk assessment /management (e.g. Colborn and Clement 1992; Kavlock et al. 1996; DeFur et al. 1999; Vos et al. 2000; IPCS 2002; Matthiessen and Johnson 2007). However, in spite of these efforts, the causal link between exposure to EDCs and endocrine disruption is still unclear, and new methods and test guidelines that could enhance the understanding of EDCs and their potential effects are a constant demand (e.g. Matthiessen and Johnson 2007).

1.1 The challenge – potential EDCs

Humans and wildlife are constantly exposed to a multitude of natural and anthropogenic chemicals. The worldwide use of chemicals has increased rapidly every year (e.g. Colborn 1996). Approximately 100 000 chemicals are currently being produced on an industrial scale (Depledge and Galloway 2005), whereas 1000-2000 new compounds are annually being introduced to the market (Choi et al. 2004). Over the past decades, there has been growing scientific concern and public debate over the potential of some of these compounds to interfere with the normal functioning of the endocrine system in wildlife and humans (e.g. Colborn and Clement 1992). Endocrine

disruptors (EDCs) have emerged as a main focus in medicine (e.g. Sharpe and Skakkebaek 1993; Caserta et al. 2008), (eco)toxicology (e.g. Depledge and Billinghurst 1999; Oberdorster and Cheek 2001; Benninghoff 2007), and regulatory policy (OECD 1997; Durodie 2003; Segner 2003; http://ecb.jrc.it/REACH/). Concerns about EDCs are related to: (1) some compounds, such as those associated with tributyltin (TBT) and oestrogen mimics, are known to cause significant environmental effects (i.e. sex changes in marine snails and riverine fish respectively) which were not predictable by traditional risk assessments (Jobling and Sumpter 1995; Matthiessen and Gibbs 1998); and (2) the modes of action of EDCs were not foreseen when existing chemicals regulations were enacted (Matthiessen and Johnson 2007). EDCs affect organisms by interfering with their endocrine systems instead of by more 'traditional damage' of cellular and physiological processes. Nevertheless, it is becoming increasingly clear that many (potential) EDCs can induce several types of toxicity/effects (e.g. Wetherill et al. 2007). Thus, EDCs constitute a class of chemicals not defined by chemical nature only, but rather by biological effects. As a result, various pollutants can be collectively referred to as EDCs (Roderiguez-Mozaz et al. 2004), including synthetic hormones, phytoestrogens, industrial by-products, pesticides, plasticizers, detergents, pharmaceuticals, disinfectants, and additives used in consumer products and food (IPCS 2002). This diversity of compounds makes it impossible to define a 'typical' EDC. In addition to their structural diversity, EDCs possess a range of different physicochemical characteristics. However, the definition agreed upon by the International Programme on Chemical Safety (IPCS 2002) stated that "An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny or (sub) populations", while "a potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations".

The list of known and potential EDCs is constantly increasing, and has grown beyond what anyone expected when attention was first drawn to this class of chemicals (e.g. http://ec.europa.eu/environment/endocrine/strategy/substances_en.htm#top). A list of chemicals known or suspected to be EDCs, established by the European Community, included more than 500 compounds (EEC 2001). A comprehensive list of compounds, as well as their proposed mechanisms of action, is also provided at http://www.ourstolenfuture.org/Basics/chemlist.htm.

There are many sources and exposure pathways for EDCs in the environment (e.g. Johnson and Jurgens 2003). Humans and wildlife may be exposed through ingestion of contaminated water, air, food or consumer products (IPCS 2002). Exposure can also happen via skin contact (or via the gills) across cell membranes into the bloodstream (IPCS 2002). The fate of EDCs is related to their biophysical properties (Facemire 1999), ranging from persistent to rapidly degraded, lipophilic to hydrophilic, and nontoxic to very toxic (Sculte-Oehlmann et al. 2006). Persistency to degradation, lipophilicity and bioavailability are important factors affecting how and if compounds bioaccumulate/bioconcentrate and potentially biomagnifies via the food web (Facemire 1999). Although a handful of the most notorious chemicals has been taken off the market (e.g. PCB and DDT), and others have been restricted to a narrower range of uses, new research is revealing that many other chemicals (e.g. brominated flame retardants, alkylphenols, phthalates, perfluorooctanes) could be equally or more harmful than the banned ones (e.g. http://www.wwf.org.uk/filelibrary/pdf/causeforconcern01.pdf). Furthermore, relatively little attention have been paid to e.g. heavy metals as potential EDCs, even though recent reports indicate that they might disturb normal neuro-endocrine functions of vertebrates (e. g. Handy 2003), and interfere with hormones that stimulate reproduction and ovarian growth in invertebrates (e.g. Medesani et al. 2004; Rodriguez et al. 2007).

Moreover, due to poor degradation of many chemicals in the environment (IPCS 2002), wildlife and humans are simultaneously suffering contamination by chemicals no longer in production as well as chemicals currently in use, making it almost impossible to predict and understand the potential long-term insidious effects.

1.1.1 Chemical modes of action on (endocrine) systems

Endocrine systems

The basic endocrine strategy to regulate biological processes has been widely conserved (McLachlan 2001), however, due to evolutionary divergence of specific components of endocrine systems, there are distinct differences between the various biological taxa (Oehlmann and Schulte-Oehlmann 2003). It is beyond the scope of this thesis to provide a comprehensive description of endocrinology; however a brief description of endocrine systems will help understand the mode of action of EDCs. Endocrine systems are integrated networks of cells, tissues and organs involved in the release of extracellular signalling molecules or 'chemical messengers' (i.e. hormones) to regulate metabolic, nutritional, behavioural and reproductive processes (IPCS 2002). Equally, the endocrine system plays an important role in regulation of growth, development, gut, kidney and cardiovascular functions, and responses to all forms of stress (e.g. DeFur et al. 1999; Greenspan and Gardner 2006). The primary function of an endocrine system is to transform various exogenous stimuli into chemical messengers and hormones, resulting in appropriate gene expressions and synthesis of proteins and/or activation of already existing tissue-specific enzyme systems

(Greenspan and Gardner 2006). Hormones are synthesised by a group of specialised cells termed glands. Endocrine glands arise during development for all three embryologic tissue layers (i.e. endoderm, mesoderm, ectoderm). The type of hormone(s) produced by the different glands is dependent on the tissue layer a gland originates from. For example, glands of ectodermal and endodermal origin produce peptide and amine hormones, while mesodermal-origin glands secrete hormones based on lipids (e.g. steroids) (http://www.emc.maricopa.edu/faculty/farabee/BIOBK/BioBookENDOCR.html). This arrangement is not true for all organisms. For example, for invertebrates, the arthropods are the only group with true endocrine glands derived from epithelial tissue and functioning the way vertebrate glands do (DeFur 2004). Hormones are, however, involved in endocrine regulation in many invertebrate taxa (e.g Mollusca, Crustacea, and Echinodermata), (Oetken et al. 2004). In general, hormones may be grouped into five main classes based on their chemical properties. These are peptide hormones, glycopeptides, amino acid derivates, fatty acid derivates, and steroid hormones (Greenspan and Gardner 2006). Most hormones circulate in the blood or extracellular fluid, where they exist as soluble molecules or are bound to plasma proteins (Hammond 1995).

How do hormones act?

An important area of study is to determine precisely how the hormone (as well as natural and anthropogenic chemicals) acts to change the physiologic state of its target cells (its mechanism of action). The best known example of hormonal signal transduction is where hormones bind to functional and specific hormone receptors on target cells (Greenspan and Gardner 2006). Hormone receptors can either be located on the surface of the cell or within the cell depending on the hormone (Table 1-1), (http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/index.html).

Table 1-1 The two categories of hormone receptors, classes of hormones that bind to them, and principal mechanisms of action.

Adapted from http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/index.html.

Location of Receptor	**Classes of Hormones**	**Principle Mechanism of Action**
1. Cell surface receptors (plasma membrane)	Proteins and peptides, catecholamines and eicosanoids	Generation of second messengers which alter the activity of other molecules - usually enzymes - within the cell
2. Intracellular receptors (cytoplasm and/or nucleus)	Steroids and thyroid hormones	Alter transcriptional activity of responsive genes.

For example, water-soluble hormones, like growth factors and cytokines, first bind to cell surface receptors that mediate their signals across cellular membranes via the generation of so-called second messenger systems (e.g. protein kinase activity), that subsequently activate intracellular signalling pathways for transduction of the hormonal signal (Lodish et al. 2000; Helmreich 2001). On the other hand, lipophilic, fat-soluble hormones, like steroid hormones, pass through the cell membrane by diffusion, bind to intracellular receptors directly and act upon target cells by affecting gene expression and transcription, and hence potentially protein expression (Figure 1-1). Nevertheless, growing evidence suggests that some hormones (e.g. steroids) have the capability to act through non-genomic pathways (e.g. Janer and Porte 2007).

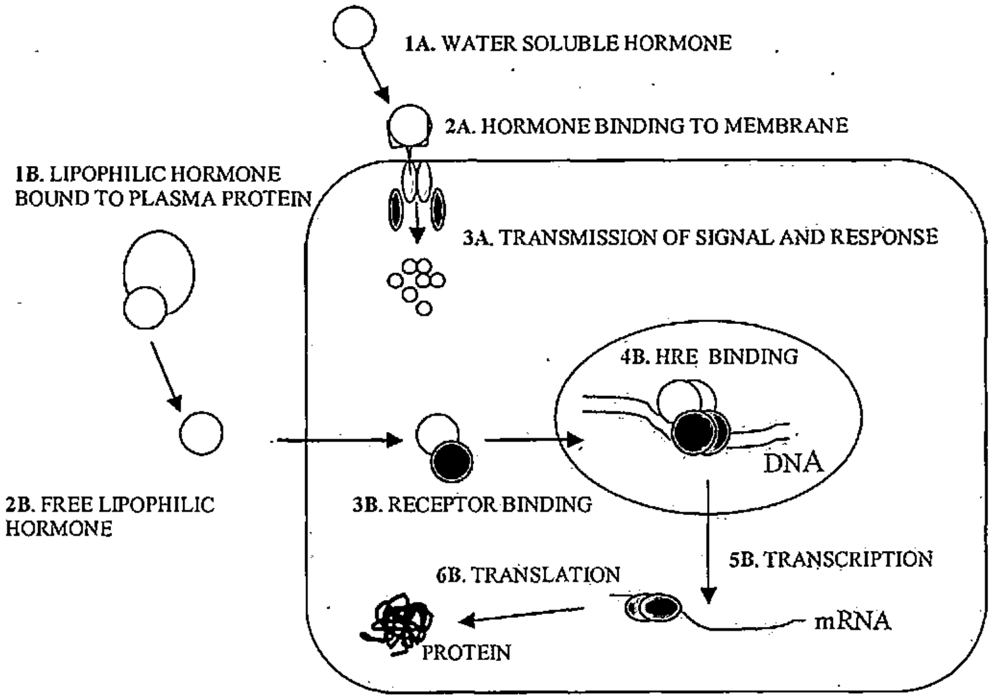

1A. WATER SOLUBLE HORMONE
2A. HORMONE BINDING TO MEMBRANE
1B. LIPOPHILIC HORMONE BOUND TO PLASMA PROTEIN
3A. TRANSMISSION OF SIGNAL AND RESPONSE
4B. HRE BINDING
DNA
2B. FREE LIPOPHILIC HORMONE
3B. RECEPTOR BINDING
5B. TRANSCRIPTION
6B. TRANSLATION
mRNA
PROTEIN

Figure 1-1. Illustration of hormone-signal mediation via cell surface (membrane) receptors (A) and intracellular receptors (B) for water soluble and lipophilic hormones respectively. Adapted from (Helmreich 2001; Lodish et al. 2000). HRE = Hormone response element in DNA.

The physiological effects of hormones are dependent on their concentration in the blood and extracellular fluid (Helmreich 2001). Almost inevitably, disease results when hormone concentrations are either too low or too high (Helmreich 2001). The concentration of a hormone (as seen by the target cell) is determined by: (1) rate of production, (2) rate of delivery, and (3) rate of degradation and elimination (Bentley 1998).The endocrine system regulates its hormone level primarily through negative feedback mechanisms (e.g. Liu et al. 2007). Increase in hormone activity for a specific hormone will automatically lead to a decrease in the production of that hormone to maintain a constant hormone level (and homeostasis). For example, disruption of physiological negative feedback loops in relation to cancer might result in the loss of cell cycle control and lead eventually to increased tumour growth and local breakdown of adjacent stroma (Singer et al. 2003). In addition to signal transduction via hormones, there are other chemical regulators such as neurohormones (secreted by neurons), and cytocrines, that can be divided into intracrines (molecules that act on the same cell that produced them) and paracrines (molecules that diffuse from their source to target neighbouring cells), (e.g. IPCS 2002).

How do EDCs act?

Despite an overall lack of knowledge of mechanisms of action of EDCs, there are several examples of mechanisms of actions related to direct perturbations of endocrine function and, ultimately, to adverse *in vivo* effects (Sonnenschein and Soto 1998; IPCS 2002; Choi et al. 2004). These can be divided into:
(1) agonistic/antagonistic effects ("hormone mimics"); (2) disruption of production, transport, metabolism, or secretion of natural hormones; and (3) disruption of production and/or function of hormone receptors (Goksoyr 2006). Receptor-mediated mechanisms of action, where chemicals act as 'hormone-mimics' by binding to hormone receptors either as agonists (i.e. molecules that bind the receptor and induce all post-receptor events that leads to a biological effect) or as antagonists (i.e. molecules that bind the receptor and block binding of the agonist, but fail to trigger intracellular signalling events), have, by far, attracted the most attention (e.g. Goksoyr 2006). However, research has clearly shown that EDCs can act at multiple sites via multiple mechanisms of action (e.g. Satoh et al. 2001), and hormone synthesis, transport, and metabolism are equally important as receptor-mediated mechanisms of action (e.g. IPCS 2002). Furthermore, endocrine systems are able to communicate with each other as well as with other systems via so-called cross talk (e.g. IPCS 2002; Goksoyr 2006), which does not make it easier to assess/predict all the potential mechanisms of action for EDCs.

1.1.2 Evidence of (ecological) effects of EDCs

Endocrine disruption is a global phenomenon and has been the subject of many reviews and workshops in recent years. Across the world, endocrine-related effects have been reported to cause reproductive failure and population declines in wildlife (e.g. IPCS 2002). In humans, decline in sperm quality, increase in the frequency of developmental abnormalities of the male reproductive tract, precocious puberty, altered neuronal development, and increase in the frequency of mammary, prostate and testicular tumours prompted numerous studies of endocrine disruption in humans and wildlife species (IPCS 2002). A review of the wide and complex field of endocrine disruption in wildlife was outside the scope of this work, however, excellent (and comprehensive) reviews are provided by several authors (e.g. Tyler et al. 1998; Depledge and Billinghurst 1999; DeFur 1999; IPCS 2002; Oetken et al. 2004; Goksoyr 2006; Porte et al. 2006; Ketata et al. 2008). In summary, the greatest attention to endocrine disruption has been paid to estrogenic effects (e.g. IPCS 2002), although one of the best documented cause-effect relationships has been established for the androgenic activities of organotin compounds in molluscs (e.g. Bettin et al. 1996; Oehlmann and Schulte-Oehlmann 2003). In spite of this (or except for this), there has been a general lack of focus on effects of EDCs in invertebrates. As a consequence, a workshop exploring the current status of invertebrate endocrinology and endocrine-mediated effects of (potential) EDCs was held in the Netherlands in 1998 (Endocrine Disruption In Invertebrates: Endocrinology, Testing And Assessment – EDIETA). The workshop culminated in the SETAC publication carrying the same title as the workshop (DeFur et al. 1999), and included overviews of various invertebrate endocrinology and observed effects of EDCs. The volume provided many suggestions for improvement and indicated several research gaps, and thus served as a starting point for further research. More recently, a special issue of *Ecotoxicology* was dedicated to endocrine disruption in invertebrates (Ecotoxicology, volume 16, Number 1/ February 2007). The aim of this special issue (containing 17 papers) was to review some of the efforts undertaken to fill the research gaps outlined in DeFur et al. (1999) (Weltje and Schulte-Oehlmann 2007). For example, the challenge to differentiate between general/reproductive toxicity and the modulation of complex endocrine mechanisms of biological regulation was addressed by Lagadic et al. (2007); LeBlanc (2007) evaluated similarities and differences between the hormone systems of insects and crustaceans; an updated overview of the 'case study' of tributyltin effects in prosobranch snails was given by Oehlmann et al. (2007); and Sugni et al. (2007) provided good arguments for echinoderms as new sentinels for endocrine disruption research. Furthermore, knowledge regarding mechanisms of (endocrine) mediated signalling processes and the potential ways of impairing the functionality of the latter is essential to predict effects of EDCs. Lafont and Mathieu (2007), Janer and Porte (2007), and Kohler et al. (2007) focused on mechanistic aspects of endocrine disruption in invertebrates as a whole, with an emphasis on (sex) steroids. The papers reviewed both receptor-mediated (Kohler et al. 2007) and non-receptor-mediated (non-genomic) effects (Janer and Porte 2007), as well as the roles of steroids in aquatic invertebrates (Lafont and Mathieu 2007). Finally, current (and suggested) guidelines for testing of EDCs were described and discussed by Gourmelon and Ahtiainen (2007); Duft et al. (2007); Kusk and Wollenberger (2007); Tatarazako and Oda (2007); Verslycke et al. (2007); and Taenzler et al. (2007). The combined efforts evaluated in this special issue revealed that much have been achieved regarding improved knowledge about EDCs and their effects on invertebrates, however, many research gaps still remain (e.g. Hutchinson 2007).

1.1.3 Research needs and current concerns regarding EDCs

Based on recommendations and research needs summarised in selected EDC reviews (i.e. IPCS 2002; Sumpter 2005; Hutchinson 2007; Matthiessen and Johnson 2007), the following issues are of high priority to understand and predict potential effects of natural and anthropogenic chemicals on man and wildlife (many of which is not limited to EDCs only):

(1) Elucidate the range of mechanisms by which EDCs may act, at all levels of biological organisation, and at key stages of life cycles, and assess windows of susceptibility to EDCs from mechanistic data.

(2) Develop more specific and sensitive biomarkers for detecting endocrine-mediated effects on individuals and populations.

(3) Clarify the possible roles of non-specific versus endocrine-mediated effects.

(4) Develop improved methodologies for assessing dose-response relationships at environmentally-relevant concentrations.

(5) Improve knowledge regarding biphasic dose-response relationships (at low concentrations).

(6) Improve knowledge regarding mixture toxicity (at low concentrations).

(7) Increase long-term monitoring of 'sentinel' wildlife species to provide baseline data on population status.

(8) Assess species differences and similarities. Elucidate how and why chemicals act differently in different organisms.

(9) Continue to identify chemicals that are likely candidates for high-impact effects in individuals (humans) and populations, and improve post-registration monitoring of chemicals to assess potential longer-term implications.

In addition, some of the aforementioned tasks depend upon a better understanding of endocrinology (e.g. DeFur et al. 2004), as well as a wider assessment of reproductive status (Hutchinson 2007), since reproductive success is a vital parameter for maintaining populations. Furthermore, international collaboration is recommended to assess the exposure and effects of EDCs on man and wildlife populations on a more global basis (IPCS 2002). Moreover, endocrine disturbances must be put into the context of other environmental pressures such as global warming, spread of disease and habitat loss (Jenssen 2006; Jobling and Tyler 2006).

1.2 Current environmental monitoring

The study of potential toxic substances in the environment includes several disciplines such: (1) *environmental chemistry* that focuses on the presence and fate of chemicals in the environment and on their transport between air, soil, and water; (2) *environmental toxicology* focusing on the effect of chemicals in the environment on organisms; and (3) *ecology* focusing on interactions between organisms, and between organisms and their environments, whether or not human influences are present (Francis 1994). All these disciplines are now combined in the term 'ecotoxicology' that was first defined by René Truhaut (1977) as "the branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context". Forbes and Forbes (1994) defined three main objectives in ecotoxicology: (1) Obtaining data for environmental risk assessment (ERA) and environmental management, (2) meeting the legal requirements for the development and release of new chemicals into the environment, and (3) developing empirical or theoretical principles to improve knowledge of the behaviour and effects of chemicals in living systems. The ultimate aim of ERA is to provide sufficient information for decision making with the purpose of protecting the environment (and the organism within it) from unwanted effects of natural and anthropogenic chemicals (Breitholtz et al. 2006). Thus ecotoxicology, ERA and risk management (decision making) should be linked in a proactive approach as shown in Figure 1-2.

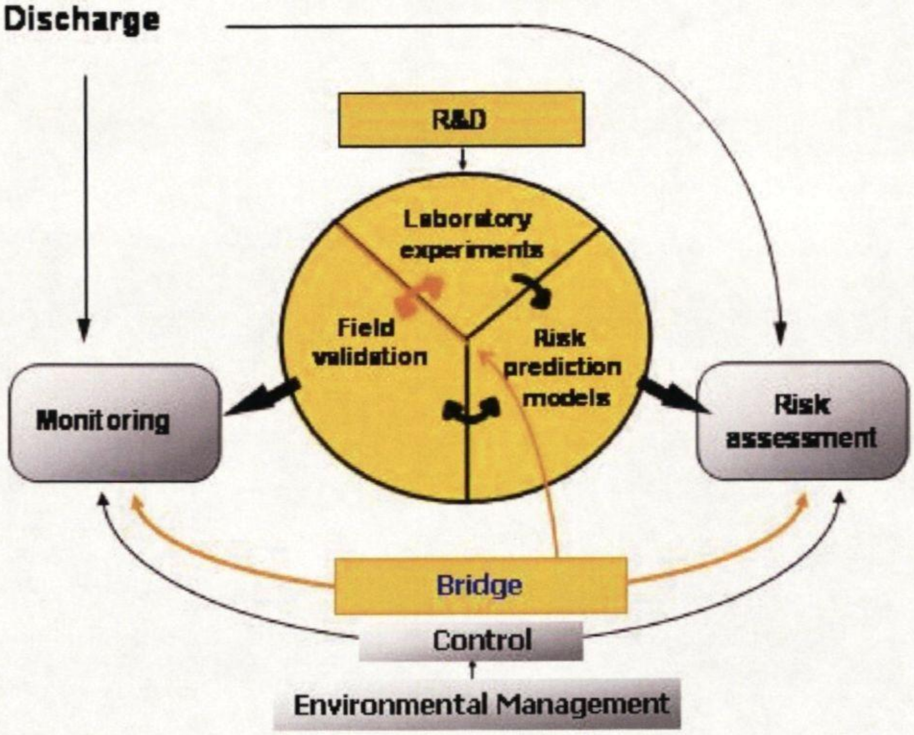

Discharge
R&D
Laboratory experiments
Field validation
Risk prediction models
Monitoring
Risk assessment
Bridge
Control
Environmental Management

Figure 1-2. Shows how ecotocicology, ERA and risk management depend upon each other for a most efficient management of environmental pollutants. R & D = Research and Development. Figure courtesy of Steinar Sanni at IRIS Biomiljø.

Ecological risk assessment, in contrast to human risk assessment, is usually concerned with effects at four different levels of organisation, including individual organisms, populations of organisms, communities and ecosystem, where an ecosystem is the pool

of communities in a given area and depends on the continued functioning of diverse elements to maintain the balance of the system (Francis 1994).

In the early phase of environmental monitoring, the most common approach was to measure physical and chemical variables with the occasional implementation of biological variables (Lam and Gray 2003). This approach was based on ranking the relative toxicities of chemical with laboratory acute tests performed on a few species, generally not ecologically relevant. By measuring the level of chemicals in water, air, soils, sediments and biota, predictions were then made, based on extrapolations from toxicity tests (i.e. LC50), as to whether adverse effects were likely to occur in the considered ecosystem. The drawback of this approach was that it did not focus on the well being of individual organisms, populations and communities *in situ*. Another approach to measure the impact of pollution on the aquatic ecosystem has been the 'bioindicator' method which measured contaminant impact *in situ* at the community or ecosystem level (e.g. Gray et al. 1990). Nevertheless, 'bioindicators', relying on the presence or absence of a species, provide information on the impact only after the ecosystem equilibrium has been disrupted (e.g. Lam and Gray 2003). However, with technical developments that enhanced the ability to detect and quantify biological changes at the molecular, cellular and physiological levels following exposure to environmental chemicals, the 'biomarker-concept' emerged (e.g. Peakall 1992; Depledge 1993). 'Biomarker' is a commonly-used term within a variety of scientific fields (e.g. http://en.wikipedia.org/wiki/Biomarker), thus the term has many definitions depending on the application. However, in ecotoxicology, it has been defined as "biochemical, cellular, physiological, or behavioural changes that provide evidence of exposure to, and/or toxic effects of, one or more contaminants" (Depledge and Fossi 1994). Biomarkers are classified commonly as biomarkers of exposure or biomarkers of effects according to the meaning of the toxicological indication they provide (Fossi et al. 1999), and can help to bridge the gap between the laboratory and the field by giving direct evidence of whether or not a particular animal, plant or ecosystem is being affected by pollution. Biomarkers often provide more reliable evidence of exposure than measurements of the pollutants themselves in the environment, the latter are often short lived and difficult to detect, whereas their effects (detectable via biomarkers) may be much longer term (Fossi et al. 1999). A high-quality biomarker of a specific chemical class or specific mechanism of action (as defined by Benninghoff 2007) should have the following attributes: (1) the biomarker should be inducible or repressible, (2) the measured response should be specific to chemicals within that class, (3) the response should have sufficient sensitivity for routine detection, (4) the biomarker should be highly accurate and reproducible among experiments within a laboratory and among different laboratories and animal models, and (5) the biomarker should be quantifiable so that degree of risk can be estimated.

Implementation of biomarker techniques has, however, not yet been fully incorporated into routine environmental monitoring (e.g. EU monitoring guidelines). The reason for this could be that there is controversy over how biomarker responses at different levels of biological organisation should be interpreted (e.g. Dagnino et al. 2007). As a result, much effort has been spent defining so-called 'expert-systems' or 'biomarker-index systems' that aim to integrate multiple biomarker responses into health indexes that are more easily interpretable, by regulatory authorities, as decision-making tools to direct further actions to ensure environmental quality (e.g. Narbonne et al. 1999; Broeg et al. 2005; Dagnino et al. 2007). Furthermore, a closer collaboration between human risk assessment and ERA has been suggested, to achieve a more balanced view of environmental health issues, as well as to facilitate the acceptance of biomarkers within regulatory risk assessment (e.g. Galloway 2006; Owen et al. 2008). Genomic and proteomic-based approaches are increasingly applied as input to human risk assessment (e.g. Ge et al. 2007), and are thus potential candidates for a closer link between human risk assessment and ERA. Moreover, the research needs outlined in Section 1.1.3 (e.g. understanding of chemical modes of actions) have not been addressed adequately by current methods. Therefore, several authors emphasise the need for supplementing methods that can identify new compound- and mechanism-specific biomarkers, exploiting genomic and proteomic techniques (e.g. Depledge 1993; Rotchelle and Ostrader 2003; Moore et al. 2004; Matthiessen and Johnson 2007). Thus, 'toxicoproteomics' might be the next step in the evolution of environmental biomarkers (Benninghoff 2007).

1.3 The new methods – 'omics' technologies

It has been 64 years since the historic demonstration by Avery et al. (1944) that DNA was the genetic material. Two years later, Auerbach and Robson (1946) demonstrated that chemicals exert powerful mutagenic effects and, hence, seriously alter the genetic codes that control all functions of life. Since then, a comprehensive understanding of the DNA structure, mechanism of DNA replication, the genetic code for proteins and enzymes that control cell structure and function, have been achieved (Aardema and MacGregor 2002). However, the advent of whole-genome sequencing (Fleischmann et al. 1995; Lander et al. 2001) marked a significant phase transition in the history of biological research (e.g. Quackenbush 2007). With tens of thousands of genes, and potentially hundreds of thousands of proteins to identify, correlate and understand, new technology that could study more than one gene, gene product or process at the same time became a necessity (MacGregor 2003). Thus, biological science moved from hypothesis–driven reductionist to holistic science with no *a priori* decisions regarding importance of potential changes (Coulton 2004), and the era of 'omes' and 'omics' came upon us. 'Omics' is derived from the Latin suffix 'ome' which denotes a body or group (Lederberg and McCray 2001), here converted into, genome, transcriptome, proteome, metabolome etc. – the complete set of genes, transcripts, proteins, metabolites, etc. contained in the cellular complement of chromosomes. The respective studies of the 'omes' are termed 'omics' (Lederberg and McCray 2001).

In the past decade, a plethora of new 'omics' technologies has been coined (e.g. http://en.wikipedia.org/wiki/List_of_omics_topics_in_biology) and resulting 'omics' data are providing comprehensive description of nearly all components and interactions within a cell (Joyce and Palsson 2006). For example, DNA (genomics, e.g. Kellis et al. 2003) is first transcribed into mRNA (transcriptomics, e.g. Carter 2006) and translated into proteins (proteomics, e.g. Patterson and Aebersold 2003), which can catalyse reactions that act on and give rise to metabolites (metabolomics, e.g. Dunn and Ellis 2005), glycoproteins and oligosaccharides (glycomics, e.g. Shriver et al. 2004), as well as various lipids (lipidomics, e.g. Wenk et al. 2005). When these components are tagged, they can be localised within the cell (localizomics e.g. Huh et al. 2003) or in tissue cultures (histocytomics, Coulton 2004). Furthermore, the processes that are responsible for generating and modifying these cellular components are generally dictated by molecular interactions (interactomics, e.g. Cusick et al. 2005), such as protein-DNA or protein-protein interactions. Ultimately, the metabolic pathways comprise integrated networks, or flux maps (fluxomics, e.g. Wiechert et al. 2007), which control/dictate the cellular behaviour, or phenotype (phenomics, e.g. Bochner et al. 2003).

The goal of 'omic' approaches is to acquire comprehensive, integrated understanding of biological complexity by a simultaneous study of all biological processes (Lay et al. 2006). Due to the complexity of biological systems and thus at the different 'omics' levels (Figure 1-3), there is still a long way to go before this goal is achieved (e.g. Coulton 2004; Baak et al. 2005; Lay et al. 2006).

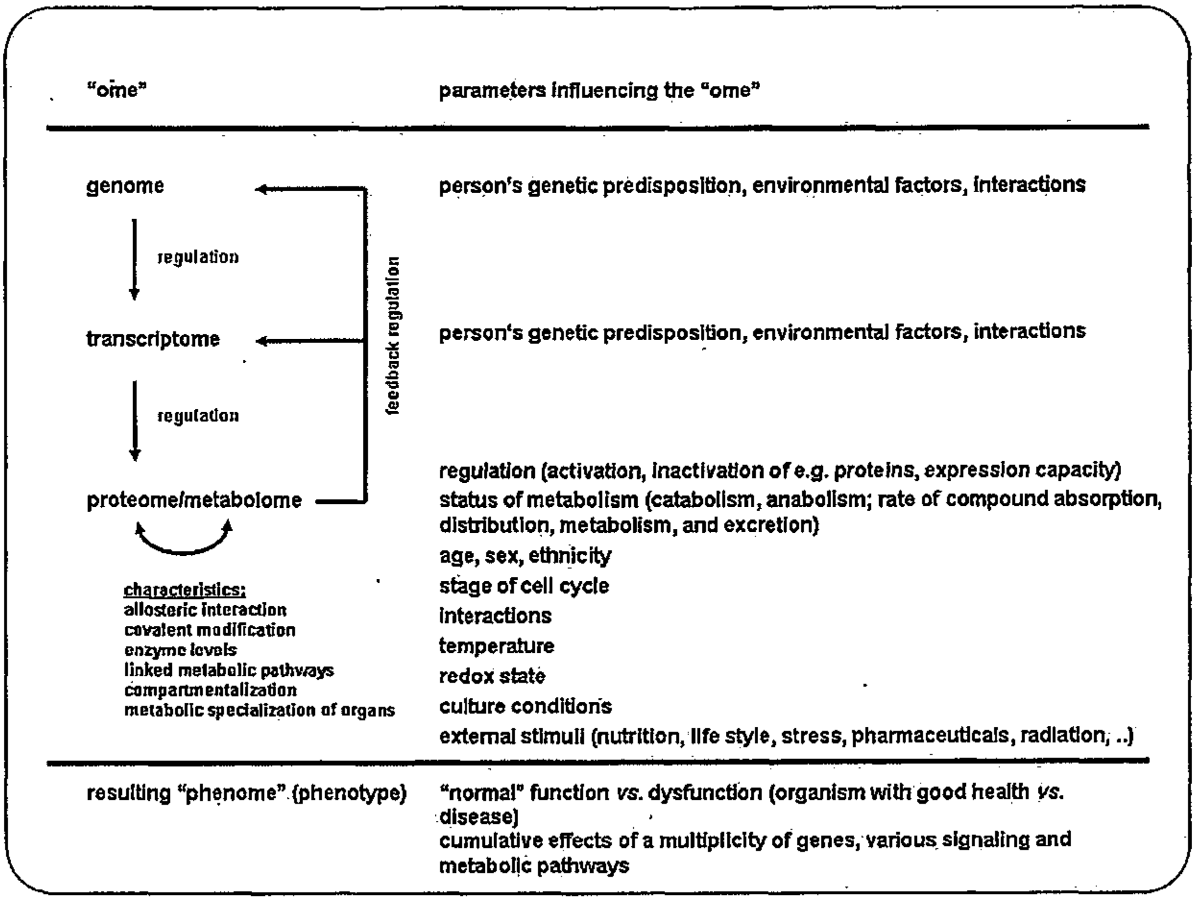

"ome"
parameters influencing the "ome"
genome
regulation
transcriptome
regulation
proteome/metabolome
feedback regulation
characteristics:
allosteric interaction
covalent modification
enzyme levels
linked metabolic pathways
compartmentalization
metabolic specialization of organs
person's genetic predisposition, environmental factors, interactions
person's genetic predisposition, environmental factors, interactions
regulation (activation, inactivation of e.g. proteins, expression capacity)
status of metabolism (catabolism, anabolism; rate of compound absorption, distribution, metabolism, and excretion)
age, sex, ethnicity
stage of cell cycle
interactions
temperature
redox state
culture conditions
external stimuli (nutrition, life style, stress, pharmaceuticals, radiation, ..)
resulting "phenome" (phenotype)
"normal" function vs. dysfunction (organism with good health vs. disease)
cumulative effects of a multiplicity of genes, various signaling and metabolic pathways

Figure 1-3. Complexity of the different 'omics' levels are influenced by genetic predisposition, environmental factors and regulatory interactions within and between the 'omes' (adapted from Lay et al. 2006). Figure courtesy of Prof. Charles Wilkins.

Figure 1-3 simplifies the interaction of the main 'omics' technologies. However, Joyce and Palsson (2006) classified 'omics' data into three categories: components, interactions and functional-states data, where component data describe the molecular content of the cell or system, interactions data specify the links between molecular components, and functional states data provide an intergraded display of all omics data types by revealing the cellular phenotype (Figure 1-4).

1.3.1 Genomics

The genome is defined as "all the genetic information, the entire genetic complement, all of the hereditary material possessed by an organism" (http://www.medterms.com). Humans and (some) higher animals have 'two' genomes, a chromosomal genome (inside the nucleus of the cell in the form of chromosomes) and a mitochondrial genome (the mitrochondrial chromosome, in the cytoplasm of the cell) which together make up the total genome (e.g. Saavedra and Bachere 2006). The mitochondrial compartment of the genome is especially interesting in bivalves because some species show a particular type of inheritance called ' doubly uniparantal', which is two types of mitochondrial genomes, transmitted through females and males, respectively (reviewed in Zouros 2000).

The term 'genomics' has been known since 1920 when it was used to describe the complete set of chromosomes and their associated genes (Snape et al. 2004). Today, the term is used to describe scientific disciplines like genome sequencing, identification of gene function and determining genome architecture (Snape et al. 2004). Since 1995, 639 genome-sequencing projects have been completed (as of September 2007, Liolios et al. 2008), which is more than double the number reported 2 years earlier (Liolios et al. 2006). In addition to the complete projects, there are currently 2158 ongoing sequencing projects; 1328 of those are bacterial, 59 archaeal and 771 eukaryotic projects (Liolios et al. 2008). Although marine species have not been highly prioritised in genome-sequencing projects, interest in marine wildlife genomics has emerged in recent years (e.g. http://www.marinegenomics.org; Venter et al. 2004; McKillen et al. 2005; Peck et al. 2005). For example, bivalve genomics (e.g. Meyer et al. 2003; Saavedra and Bachere 2006; Jenny et al. 2007) and echinoderm genomics (e.g. Cameron 2002; Bottjer et al. 20006; Sodergren et al. 2006) have received much attention lately, mainly due to their utility as a model organism for the study of the genomic control circuitry underlying embryonic development (e.g. Samanta et al. 2006) or importance in aquaculture industry (e.g. Saavedra and Bachere 2006). Nevertheless, these sequence data could become very useful in ecotoxicological studies to understand the mechanisms involved in toxic responses following anthropogenic insults.

Much of the current sequencing effort is directed towards the construction of microarrays (e.g. Schena et al. 1995; 1998), which have proven to be useful tools to study different physiological functions (e.g. Stoughton 2005). For example, for bivalves (farming species), an international collaboration has been initiated to produce multispecies microarrays constructed from relevant genes (i.e. expressed sequence tags) of haemocytes, gills, gonads and digestive in oysters (*C. gigas* and *C. virginica*), mussels (*Mytilus* sp.) and clams (*Ruditapes* sp.) (see Hedgecock et al. 2005; http://www.marine-genomics-europe.org/). The use of microarrays leads to the next category of 'omics', transcriptomics.

1.3.2 Transcriptomics

The genome is only a source of information. In order to function, the genes must be expressed. The transcription of genes to produce RNA is the first stage of gene expression. When a cell is affected by changes in the environment it responds by accessing the genes encoding instructions for the production of new cellular 'building blocks'. These instructions, to make messenger RNA (mRNA) molecules (known as transcripts), are read from the genes in a process called transcription (Campell 1991). The field of transcriptomics, thus, provides information about both the presence and the relative abundance of RNA transcripts, thereby indicating the active components within the cell (e.g. Joyce and Palsson 2006). Since 1990, many studies have examined the dynamics of gene expression in various model systems (e.g. reviewed by Stoughton 2005; Jaluria et al. 2007). The most applied approaches in transcriptomics are microarrays (e.g. Brown and Botstein 1999; Hardiman 2004) and serial analysis of gene expression (SAGE), (e.g. Velculescu et al. 2000; Harbers and Carninci 2005). Furthermore, transcriptomics have been suggested to be the most true 'omics' (i.e. global method - because of its ability to investigate the entire genome simultaneously; Coulton 2004), due to the existence of microarrays that cover almost all the genome (e.g. Samanta et al. 2006). Transcriptomics (and whole-genome covering microarrays) provides an opportunity to identify statistically valid changes in pattern of gene expression (Coulton 2004), as well as an organism's non-protein-coding transcriptome (e.g. He et al. 2006).

Gene-expression analyses have gained popularity in ecotoxicology as well. For example, a literature search at 'ISI Web of knowledge' (http://apps.isiknowledge.com), using the search strings 'gene-expression' and 'ecotoxicology', generated 561 hits in 2008, which is a 10-fold increase from 2002 (i.e. 56 hits). Applications cover various biological questions, however, they are concerned predominantly with so called '(eco)toxicogenomic' (Nuwaysir et al. 1999; Snape et al. 2004) issues, i.e. identification of gene sets predictive of toxic outcome (e.g. reviewed by Neumann and Galvez 2002; Letteri et al. 2006).

Nevertheless, even though transcriptomics data provide useful information regarding gene expression, it has been shown that the level mRNA coding for particular proteins does not necessarily correlate with level of expressed proteins (e.g. Anderson and Anderson 1998; Gygi et al. 1999). Moreover, various levels of posttranslational modifications (PTMs) of proteins are not captured by transcriptomics analyses (e.g. Joyce and Palsson 2006).

1.3.3 Proteomics

It is the proteins, and not mRNAs, that are the functional units of the cell (Gygi et al. 1999). The word protein is defined as "A large molecule composed of one or more chains of amino acids in a specific order determined by the base sequence of nucleotides in the DNA coding for the protein" (http://www.medterms.com). The process of making proteins starts with the cells transcribing DNA into mRNAs. Ribosomes then translate mRNAs into proteins by matching each codon (a set of any three adjacent bases in the DNA or RNA) with the correct amino acid to form the full protein chain (Campbell 1991). After translation, proteins can undergo modifications in several ways; for example, through specific cleavage (proteolyse, e.g. Arguello-Morales et al. 2005) or by the addition of biological molecules, such as sugar (glycosylation, e.g. reviewed by Morelle et al. 2006) and phosphates (phosphorylation, e.g. reviewed in Paradela and Albar 2008). There are more than 100 different types of known PTMs and each plays an important role in the functioning of proteins (Morelle et al. 2006).

The term proteome was coined from the PROTEin complement of the genOME by Marc Wilkins (Wilkins et al. 1996), and is defined as "the complete set of proteins expressed and modified following their expression by the genome". Proteomics is defined as "the study of proteins, how they're modified, when and where they're expressed, how they're involved in metabolic pathways and how they interact with one another." Although initially defined as the effort to catalogue the protein complement of cells and tissues, proteomics now covers nearly any type of technology focusing on protein analysis, including the systematic analysis of protein expression, structure, molecular interactions, function, folding, purification, and structural genomics on a parallel scale (Carbonaro 2004). Consequently, proteomics are often categorised into: (1) 'expression proteomics', i.e. the comparison of protein abundance between different conditions; (2) 'structural proteomics', e.g. analysing protein complexes and protein-protein interactions; and (3) 'functional proteomics' e.g. analyses of PTMs (Graves and Haystead 2002). Compared to genomics/transcriptomics, applications of proteomics in ecotoxicology has been limited. An 'ISI web of knowledge search' using the search strings 'protein-expression' and 'ecotoxicology', generated 50 hits, in contrast to the 561 hits that were generated if 'protein expression' was replaced by 'gene expression'. Nevertheless, the number of publication released within ecotoxicoproteomics is increasing within 'expression proteomics' (i.e. the comparison of protein abundance between different conditions) being the predominant application within the field, comprising 85% of the available publications (reviewed by Monsinjon and Knigge 2007).

1.3.4 Metabolomics/Metabonomics

The discipline of metabolomics (also termed metabonomics in pharmacological studies) was defined as "the quantitative measurement of the multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification" by Nicholson et al. (1999), and is thus the study of the small molecules, the metabolites. According to a widely-used definition, a metabolite is a product of metabolism or is necessary for metabolism (Dettmer and Hammock, 2004). Metabolites range from sugars, lipids, amino acids and nucleotides, to more novel structures, and represent the substrates and products of the chemical reactions occurring in a cell. Most of the present metabolomics studies rely on nuclear magnetic resonance, NMR (e.g. Nicholson et al. 2002; Robosky et al. 2002; Viant et al. 2003), or mass spectrometry coupled to (liquid or gas) chromatography (e.g. Plumb et al. 2002; 2003; Dunn and Ellis 2005). One of the greatest challenges in biology is to understand how phenotypes map on to the genotype (e.g. Depledge 1994; Snape et al. 2004). As the changes in the metabolome is the ultimate measure of an organism to genetic alterations, disease, or environmental influences, metabolomics will be the best predictor of the phenotype (Fiehn 2002). However, the study of genotype-phenotype relationship has its own category of 'omics', phenomics (e.g. Bochner et al. 2003).

At present, metabolomics is the least applied (i.e. published) 'omics' technology in ecotoxicology. Nevertheless, is has been proposed as an alternative approach to histology for gender determination in (spawned) bivalves (Hines et al. 2007). Viant et al. (2003) investigated withering syndrome in the shellfish red abalone (*Haliotis rufescens*) using a NMR-based metabolomic approach. Metabolomics has also been applied to study the impact of chemical exposures (e.g. Stentiford et al. 2005; Samuelsson et al. 2006; Ekman et al. 2007) and anoxic tolerance (e.g. Podrabsky et al. 2007) in fish.

1.3.5 Which is the preferred 'omic' technology?

Genomics, proteomics and metabolomics are complementary approaches that contribute unique information (e.g. Joyce and Palsson 2006). It has been acknowledged that the best approach to fully understand cell physiology and an organism's strategy of life would be to simultaneously carry out a comprehensive analysis of transcripts, proteins and metabolites, combined with bioinformatics/chemometrics and other relevant (clinical) data in a so-called 'systems biology'/'biomics' approach (e.g. Morel et al. 2004; Coulton 2004; Joyce and Palsson 2006; Lin and Qian 2007). Each of the currently available technologies, standing alone, has particular advantages and disadvantages for specific applications, and there is also a strong synergy between them. For example, the genome is, in contrast to the other 'omes', of static nature and is 'only' a source of information that cannot give complete insight into cellular processes

(Campbell 1991). Transcriptomics provide additional information as to which genes are being expressed. However, not all mRNA is transcribed into proteins (e.g. Gygi et al. 1999), and analysis of gene expressions will only be able to predict the proteins that can potentially be generated, but not where, when or at what level (Anderson and Anderson 1998). Furthermore, it is not possible to tell which cells the proteins will be expressed in or at what stages of development, differentiation or environmental stimulus this will happen. Moreover, the enormous diversification of structure that results from alternative splicing of mRNA, as well as various posttranslational modifications (that can significantly alter the function of a protein), must also be taken into account (Pandey and Mann 2000; Tyers and Mann 2003). Nevertheless, a proteomic approach is dependent on genomic data (sequence database information) to fulfil its potential. Metabolomics is, as proteomics, suitable for identification of gene products and cellular constitutes in both various body fluids (allowing non- invasive testing) and tissue compartments. Additionally, metabolome analysis allows a direct link to phenotype (Fiehn 2002). An advantage of metabolomics over transcriptomics and proteomics is that the complexity is less, because there are fewer metabolites than genes and proteins (Raamsdonk et al. 2001). MacGregor (2003) predicts that genomics/transcriptomics will play a 'discovery' role with respect to biomarkers for *in vivo* monitoring, by identifying those genes induced in response to given stimulus, while peptides, proteins, and metabolites, will emerge as those biomarkers of functional status or damage response used in routine toxicological practise.

As a systems biology approach was not possible in the present study, a proteomics approach was chosen with the aim of detecting biomarker patterns indicative of conditions/effects in selected species following exposure to environmental pollutants.

Further 'omics' descriptions and discussions in this report will be restricted to 'expression' proteomics.

1.4 The current state of 'expression' proteomics

Proteomics is probably the most complex and daunting of the 'omics' technologies, given the sample's complexity and dynamic range of protein abundance (e.g. Meyer and Stuhler 2007). Proteomics encompasses knowledge of the structure, function and expression of all proteins as a function of time, state, age and external factors (e.g. Wilkins et al. 1996; Reynolds 2002). Multiple choices of technologies and approaches are available for the different categories of proteomic research. Currently, two basic approaches are used for the characterisation of proteins in proteomic protocols. The so-called 'top-down' approach starts with analyses of native proteins (complex mixtures), while in the 'bottom –up' approach (i.e. MudPit - multidimensional protein identification technique) or "shotgun approach", the complex protein mixtures are digested to the peptide level and analysed directly (e.g. MacCoss et al. 2002). In the present study a 'top-down' approach, as outlined in Figure 1-5, has been utilised. In addition to the selected variables outlined (e.g. gender, species, chemical, dose, season), 'age' and 'reproductive stage' of the organisms could also be considered as important variables especially whilst considering the susceptibility to environmental endocrine disrupting agents (IPCS 2002). However, as information regarding age (and reproductive status) is not easily assessable in the selected test species (i.e. *Mytilus edulis*, *Hyas araneus* and *Strongylocentrotus droebachiensis*), these variables were not prioritised and taken into consideration in the present study.

Furthermore, identification of putative biomarkers has not been attempted within the frames of this project, due to lack of adequate instrumentations (e.g. tandem mass spectrometer) and resources.

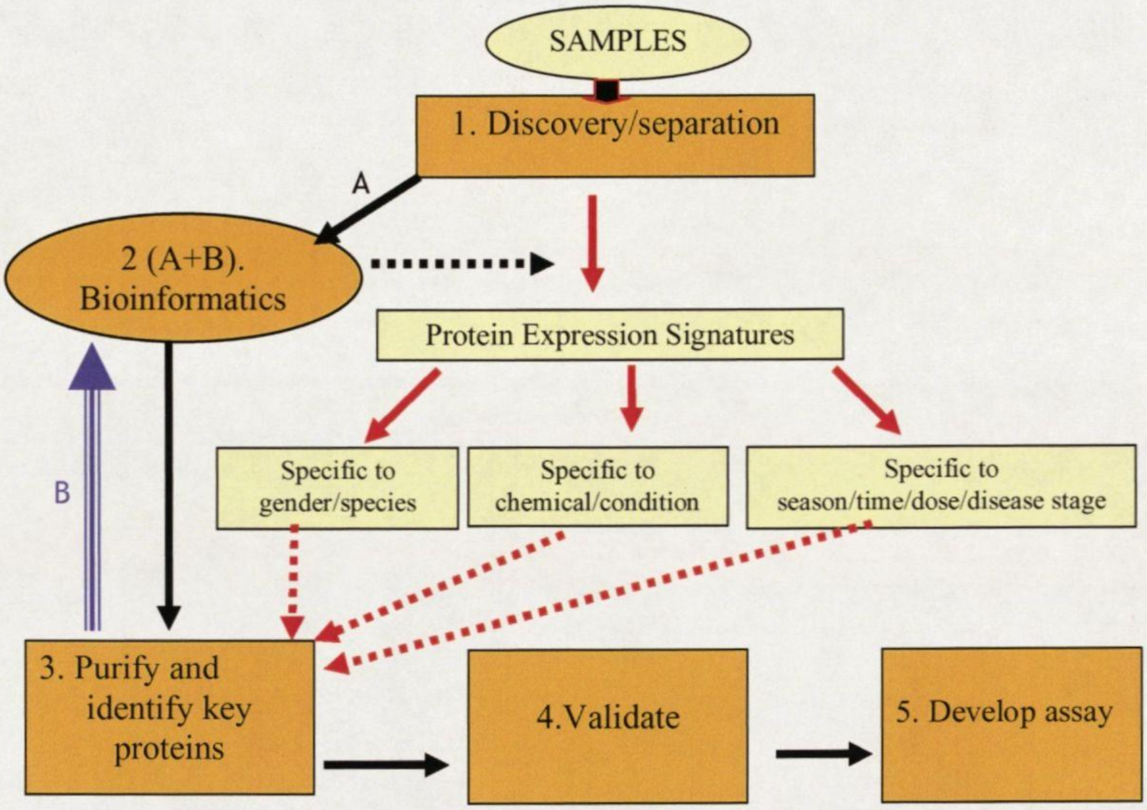

SAMPLES
1. Discovery/separation
A
2 (A+B). Bioinformatics
Protein Expression Signatures
Specific to gender/species
Specific to chemical/condition
Specific to season/time/dose/disease stage
B
3. Purify and identify key proteins
4.Validate
5. Develop assay

Figure 1-5. Simplified schematic representation of the workflow in a 'top-down' 'expression-proteomics' study. Samples are first separated using chromatography or electrophoreses (1), the resulting data are then processed using uni and multivariate statistics (2A), statistics may reveal putative biomarkers (protein expression signatures) specific to compound, gender, species, dose, time/season. When interesting proteins have been selected, they are purified using multidimensional chromatography and sequenced using tandem mass spectrometry (3). To obtain protein identifications, sequence data have to be matched to genome information in databases (2B). Validation (4) is important at all steps, and particularly before a commercial assay (5) is made based on the identified biomarkers.

1.4.1 Protein separation/ quantitation – the discovery phase

There is no doubt that two-dimensional gel electrophoresis (2DE) is the technique that is most widely used in global proteome analysis (Rabilloud 2002), thus

combined with MS (for protein identification) the current 'workhourse' of proteomics (Görg et al. 2004). Some of the many applications are reviewed by Barany et al. (1998), Guo et al. (2002), Bouchal and Kucera (2002), and Lau et al. (2003). Recently, 2DE-proteomics have also been applied, in ecotoxicology, to investigate effects of environmental pollutants on protein expression in fish and bivalve molluscs (e.g. Shepard and Bradley 2000; Bradley et al. 2002; Lopez et al. 2002; Shrader et al. 2003; Rodriguez-Ortega et al. 2003; Olsson 2004; Apraiz et al. 2006; Jonsson et al. 2006b; Amelina et al. 2007) and, more specifically, oxidative stress (Redox-proteomics) in bivalves (e.g. McDonagh et al. 2005; McDonagh and Sheehan 2007). 2DE technology (combined with MS) has also been applied to investigate (microbial) pathogen-host relationships in crustaceans (e.g. Herbiniere et al. 2008). Furthermore, Kimmel and Bradley (2001) assessed specific protein responses in the calanoid copepod *Eurytemora affinis* to salinity and temperature variation using 2DE. Roux et al. (2006; 2008) analysed unfertilized and fertilized sea urchin (*Strongylocentrotus purpuratus*) eggs to gain more insight into signalling pathways implicated in egg activation.

The history of the 2DE technique goes back to Stegemann (1970). A modern version was described by O'Farell (1975) where denaturing conditions during sample preparation was introduced, to gain increased resolution in the analysis. The principles of the method are that proteins are separated by charge (pI) using isoelectric focusing (IEF) in the first dimension, followed by size (Mw) in the second dimension, using Sodium dodecyl sulphate-polyacrylamide gel electrophorese (SDS-PAGE) (Görg et al. 2004; O'Farrell 1975). Depending on the gel size and pH gradient used, 2DE can resolve more than 5000 proteins simultaneously (ca. 2000 proteins routinely) and detect and quantify < 1 ng of protein per spot (Görg et al. 2004). Nevertheless, some of the limitations associated with the technology include poor representation of very acidic/basic (i.e. below pH 3 and above pH 10), lipophilic, and low abundance proteins (e.g. Görg et al. 2002). Adoption of thiourea to improve solubilisation of membrane proteins (Rabilloud 1998), implementation of prefractionation steps (e.g. Görg et al. 2002; Cho et al. 2005), use of overlapping narrow range strips (e.g. Hoving et al. 2000), and development of broader range immobilized pH gradient (IPG) strips (Görg et al. 2000) have helped to improve some of the limitations of the 2DE technology. As evaluation and comparison of the complex 2D patterns from gels 'with the eye' is impossible, 2DE technology is used in combination with image analysis systems (e.g. Miura 2001). However, this can be a laborious procedure (Görg et al. 2004), that are greatly shortened by the use of difference gel electrophoresis (DIGE), developed and described by Unlu et al. 1997).

Although 2DE is as yet unchallenged in its ability to resolve and display thousands of protein (e.g. Görg et al. 2004) challenges like, for example, difficulties with automation (and thus high throughput) have brought other 'gel-free' - high throughput alternatives for protein profiling analyses to the surface. One such alternative is the ProteinChip® array technology combined with surfaced-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI TOF MS). The SELDI concept was first introduced by Hutchens and Yip (1993), and was later commercialised by Ciphergen Biosystems (Palo Alto, USA). The technology is distributed by Bio-Rad (www.Bio-Rad/proteinchip/). ProteinChip® array technology includes a suite of analytical tools such as retentate chromatography, on-chip protein characterisation and multivariate analysis, which allows the user to examine patterns of protein expression and modification (Fung and Enderwick 2002; Tang et al. 2004). The principle of the technology is that sample fractionation is accomplished by retentate chromatography, while protein detection is accomplished using SELDI TOF MS (e.g.

Issaq et al. 2003). The chromatography is performed on protein arrays with varying chromatographic properties; for example, anion and cation exchange, metal affinity and reverse phase (Merchant and Weinberger 2000). These properties enable capture of different subsets of proteins according to their physiochemical properties (e.g. Fung et al. 2000). By a combinational use of arrays with different surface chemistry, in parallel and in series, a complex mixture of protein, from cells or bodily fluid, can be resolved into subset of proteins with common properties (Fung and Enderwick 2002). SELDI TOF MS analysis of protein forms retained on the chip arrays generate mass spectra containing peaks with unique mass-to-charge ratio (M/Z) values, and since most of the peaks are single charged during the analysis, each peak usually correspond to a single peptide/protein with a molecular weight equivalent to the M/Z value (Poon 2007).

The flexibility and versatility of the technology enables its use in a wide range of research areas, including oncology, toxicology, neurology, drug discovery and environmental monitoring (e.g. see list of SELDI applications at (www.Bio-Rad/proteinchip/ - SELDI applications). Most of these applications are profiling for protein patterns (biomarkers) of disease, disease progression or remission, as well as biomarker(s) that predict the toxicity of certain compounds on different organs/species. However, other possible applications of the ProteinChip® array technology include investigations of transcription factors (e.g. Forde et al. 2002), receptor ligand interactions (Tassi et al. 2001), and protein-protein interactions/protein complexes (e.g. e.g. Shen et al. 2002; Favre-Kontula et al. 2008). These applications utilize protein chip arrays pre activated with bait molecules such as for example, enzymes, antibodies or biotinylated DNA. Furthermore, SELDI technology has recently demonstrated its use in detecting phosphorylated proteins (e.g. Akashi et al. 2007). Compared to 2DE, applications of ProteinChip® array technology and SELDI TOF MS in ecotoxicology are limited to a few studies investigating effect of anthropogenic compounds on protein expressions in fish (Hogstrand et al. 2002; Larsen et al. 2007; Walker et al. 2007), bivalves (Knigge et al. 2004; Bjørnstad et al. 2006; Monsinjon et al. 2006) and arthropods (Gomiero et al. 2006), to find new biomarkers indicative of endocrine disruption (e.g. Walker et al. 2007; Gomiero et al. 2006; Larsen et al. 2006). Furthermore, Provan et al. (2006) applied SELDI TOF MS to investigate effects of density stress in farmed Atlantic salmon (*Salmo salar*), while Ward et al. (2006) utilised the technology to assess geographical origin and liver tumor status of flatfish (*Limanda limanda*). As with 2DE technology, there are certain advantages and diatvantages with this technology. The greatest advantage with ProteinChip® array technology is its ability to rapid screen and generate proteomic patterns for hundreds of crude samples (thus allowing large sample sets that can provide results robust against e.g. individual variation), from as little as one micro litre body fluid or as few as 25 – 50 cells (Paweletz et al. 2000, 2001). Furthermore, the technology can be used for the development of rapid, sensitive and high throughput assays. The latter can be done by immobilising antibodies to the specific biomarker (or set of biomarkers) on the protein array surface (e.g. Xiao et al. 2000; 2001). One of the major disadvantages with the technology is poor resolution for larger molecules, i.e. intact or modified proteins greater than 20 kDa (e.g. Kiehntopf et al. 2007). Furthermore, even a slight shift in the methodology/reagents has been reported to affect the results across experiments leading to poor intra-experiment reproducibility (e.g. Poon 2007). The latter can be addressed by improved study design. Moreover, the SELDI analysis itself does not provide a direct sequence based identification (there being many proteins with close to a given mass), and hence additional effort is needed to identify a potential biomarker and subsequent translate the measurements into a classical immunoassay format (e.g.

Kiehntopf et al. 2007). However, direct identification of peptides in the low-molecular range (< 3 kDa) is possible through the use of the ProteinChip tandem-MS interface (e.g. Caputo et al. 2003).

Although not frequently applied (i.e. reported) in ecotoxicological studies, other examples of gel-free technologies in quantitative protein profiling are those based on metabolic or chemical labelling strategies, whereas isotope mass differences are used mainly for relative quantitation of proteins in different samples. For example, in the isotope-coded affinity tag (ICAT) peptide labelling method, originally described by Gygi et al. (1999), the desired mass difference between two samples was achieved by modification of cystein side chains with either a light or a heavy-isotope labelled biotin tag. The isolated tagged peptides are further separated and analysed by capillary high performance liquid chromatography (HPLC)-tandem MS (Gygi et al. 2000; Islam et al. 2003). One of the greatest advantages of the ICAT strategy is its high throughput potential, with the ability to scan more than 1200 peptide pairs in an hour (Gygi et al. 1999). Furthermore, the method is compatible with any amount of proteins from bodily fluids, cells or tissues under any growth conditions, and the success of the analysis is not compromised by the presence of salts, detergents and stabilisers in the samples (Gygi et al. 1999). Moreover, any type of sample fractionation is compatible with ICAT analysis, increasing the chance of detecting alterations in low abundance proteins. Nevertheless, the technology fails to detect protein forms that contain no cysteines (unless ICAT reagents with specificities to groups other than thiols are synthesized), (Gygi et al. 2000). Additionally, the relatively large modification of the ICAT label (~ 500 Da) remains on each peptide throughout MS analysis and can potentially complicate the database searching algorithms, especially for peptides containing less than seven amino acids (Gygi et al. 2000). The drawback of ICAT not being applicable to cysteine-free proteins have been addressed in recent years by the development of alternative chemical labelling methods such as the isotope-coded protein label method (ICPL) described by Schmidt et al. (2005), and the isobaric tag for relative and absolute quantitation (iTRAQ) technology (Hunt et al. 2004; Ross et al. 2004). The latter technique has also been proven useful in (pre)validation of putative biomarkers discovered by 2DE (Gluckmann et al. 2007). The authors proposed iTRAQ as an alternative, more reliable, method to real time PCR for verification of protein markers, because of the poor correlation between mRNA and protein expression. Incorporation of stable isotopes (e.g. Kolkman et al. 2005) or stable isotope-encoded amino acids (e.g Ong et al. 2003) into proteins during growth of organisms are other available approaches; however, metabolic labelling approaches are best applicable to cell cultures under defined conditions (Beynon et al. 2005).

As with the combined use of 'omics' technologies, it would also be beneficial to use several proteomics technologies simultaneously as there currently is no single technology available capable of resolving and displaying the complex mixture of proteins in a cell or tissue (e.g. Issaq 2001; Patterson 2004; Boschetti 2007).

1.4.2 Datamining/bioinformatics

One of the greatest challenges in expression proteomics is datamining (e.g. Biron et al. 2006). Statistically evaluation of data and interpretation of results require distinct bioinformatics expertise (e.g. Lay et al. 2006). The term bioinformatics incorporates the application of computer science and informatics to molecular biology, and will, in addition to statistical evaluation of expression-data, help to map amino acid sequences to databases for protein identification, create models for molecular interactions, evaluate structural compatibility (Debes and Urrutia 2004). The (mis)use

of statistics for interpretation of proteomic data has caused much debate due to the high frequency of overfitting of data and thus false discoveries (e.g. Baggerly et al. 2004; Lay et al. 2006). Nevertheless, bioinformatics coupled with biological knowledge is the only way to draw meaningful conclusions from the huge amount of data generated from (protein) expression analysis, emphasising the importance of statistical and mathematical skills, as well as appropriate study design (e.g. with an adequate number of replicates).

1.4.3 Protein purification and identification

There are several 'routes' to protein identity depending on the technology platform used in the 'discovery-phase', as well as characteristics of protein to be purified and identified. For example, if the putative protein biomarkers were discovered by 2DE, common procedure would be to excise the interesting protein spot from the gel either manually or automatically by a robot, and thereafter prototypically cleave the protein using enzymes of known specificity (e.g. trypsin). During the process, peptides are formed, and thereafter extracted, for subsequent analysis with (tandem) mass spectrometry (e.g. Westermeister and Naven 2002). There are multiple choices of tandem MS technologies (e.g. reviewed by Gygi and Aebersold 2000) and the choice of technology should ideally reflect the properties of the protein to be identified, however in most cases it is restricted to what is available in the lab. If the interesting proteins were discovered by ProteinChip® array technology, however, a more comprehensive protein purification procedure would be required prior to tandem MS analysis. There are several options for protein purification (including commercially available purification kits), however, one alternative is the use of multidimensional (e.g. ion exchange followed by reverse phase) chromatography until the sample has been purified to a satisfactory level of homogeneity. Purified samples are then digested with a site-specific protease such as trypsin, and the products of the digest analysed by tandem MS (e.g. Caputo et al. 2003; Diamond et al. 2003; Walker et al. 2006). For organisms where there is limited or no genetic information, it is essential to identify exact sequence of amino acids that make up a protein in order to match to previously characterised proteins or genes. Some of the relevant database searching programs is Sequest, MASCOT, PeptideSearch, and PROWL (Gygi and Aebersold 2000). Once a reliable match is found in one of the databases, the sequence can be used to identify the protein and predict its full sequence.

1.4.4 Promises of a proteomic approach in ecotoxicology

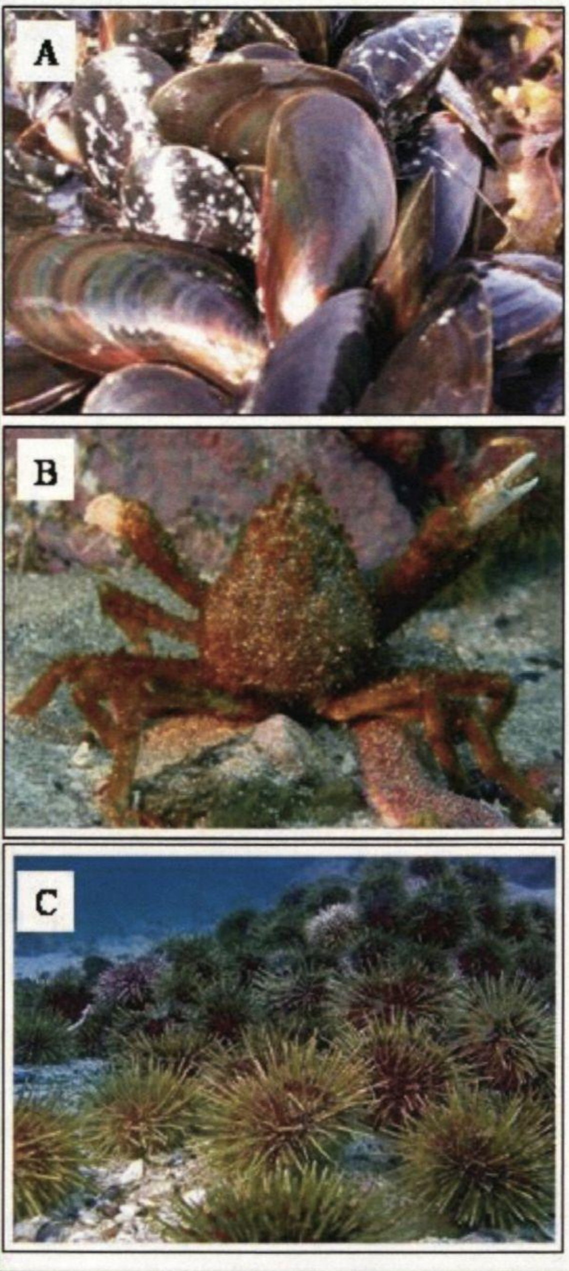
The greatest utility for proteomics in the field of ecotoxicology is to bridge the limitations imposed by either exposure or effects assessment (by traditional methods), by potentially identifying the mechanisms of action of a contaminant on an organism, and thus help in predicting the overall toxicological effect that contaminant exposure may have on host physiology (e.g. Aardema and MacGregor 2002). Improved knowledge regarding cellular control and defence mechanisms will allow a more robust extrapolation between model species and target species (MacGregor 2003), and reduce uncertainties involved in predicting threshold levels of various types of toxicity (and thus a better understanding of dose-response relationships). Furthermore, it has long been acknowledged that single-endpoint analyses or chemical monitoring alone is not adequate to address biological questions (e.g. Galloway et al. 2006; Dagnino et al. 2007). For example, Futreal et al. (2004) reported that more than 1 % of all genes are causally involved in oncogenesis. It is likely that any toxic response involves changes not only in a single gene but rather a cascade of gene interactions (e.g. Nuwaysir et al.

1999). Thus, proteomics provide a relatively high throughput (depending on choice of technology) approach for screening of multiple endpoints.

1.5 Study organisms

Representatives from three invertebrate phyla were selected for this study: the blue mussel *Mytilus edulis*, a spider crab, *Hyas araneus*, and the green sea urchin *Strongylocentrotus droebachiensis* (Figure 1-6).

Marine invertebrates comprise more than 95% of the faunal species in the oceans and, therefore, play a vital role in the functioning of marine ecosystems (DeFur 1999). Many invertebrates are also economically important as a food resource, directly or as part of the food chain (e.g. Saavedra and Bachere 2006). Thus, the efforts to understand the diverse biology of invertebrates come from many directions, including Nobel Prize winning developmental biology, research to control insects that threaten human health and food supplies, aquaculture opportunities and ecotoxicology (Hutchinson 2007). The blue mussel was chosen as the 'main' focus of this study, because it has been an established bioindicator species for global environmental monitoring programmes (e.g. Mussel Watch; Goldberg 1975) for more than 30 years. Its biology and physiology is well documented (Gosling 1992). It is a sessile organism that filters food and other particles from surrounding water, thereby, making it susceptible to accumulation of environmental pollutants. Crabs and sea urchins were included to provide information on the comparability of vulnerability of species and phyla with different feeding modes (i.e. grazing and omnivore).



Phylum: Mollusca
Class: Pelecypod/Bivalvia
Order: Mytiloida
Family: Mytilidae
Species: *Mytilus edulis*

Habitat: Below mid-tide level on rocky shores

Feeding strategy: Sessile filter feeder

Phylum: Crustacea
Order: Decapoda
Class: Malacostraca
Family: Majidae
Species: *Hyas araneus*

Habitat: Hard and sandy substrates, among rocks and seaweed at depths of 10-1200m

Feeding strategy: Omnivore

Phylum: Echinodermata
Class: Echinoidea
Family: Echinidae
Species: *Strongylocentrotus droebachiensis*

Habitat: Kelp beds and rocky areas in the low intertidal zone and subtidal zone to depths of 1200 m

Feeding strategy: Grazing

Figure 1-6. Selected study organisms: (A) the blue mussel (*Mytilus edulis*), (B) a spider crab (*Hyas araneus*), and (C) the green sea urchin (*Strongylocentrotus droebachiensis*). Pictures courtesy of Kåre Telnes (i.e. Picture B and C).

Furthermore, crustaceans are a particular focus of endocrine disruption research, reflecting their abundance in nature, commercial importance and their inclusions in the regulatory assessment schemes for active pharmaceutical ingredients (DeFur et al. 1999). An additional advantage with crustaceans is that their endocrinology is more

extensively described than for many other invertebrate phyla (e.g. LeBlanc 2007), making interpretation of proteomic data and potential endocrine effects easier.

There are many relevant aspects which prove echinoderms to be good candidates as useful test species in ecotoxicology. For example, from a proteomic point of view, the genome of the sea urchin *Strongylocentrotus purpuratos* has been sequenced (e.g. Sodergren 2006) increasing the chance of linking potential protein biomarkers found in this study (for *Strongylocentrotus droebachiensis*) to genome information, improving understanding of the function and relevance of the biomarker(s). Furthermore, echinoderms are deuterostomes, the same evolutionary lineage as vertebrates, making a possible link between invertebrates and vertebrates with regard to response patterns (DeFur et al. 1999). In addition, many echinoderms are second or third level predators, and therefore susceptible to biomagnification processes (Sugni et al. 2007).

1.5.1 Mytilus edulis

Mytilus edulis is widespread in European waters from beyond the polar circle to the MediterraneanSea and is found, typically, on rocky coasts from the upper shore down into the shallow sublittoral. The species normally occurs in dense beds of one or more layers, with individuals bound together by their byssus threads. Shell colour varies from deep blue to purple and is sometimes greenish/brown (Figure 1-5-1A). Size (and shell shape) varies with environmental conditions (e.g. temperature, salinity, food availability, tidal exposure, parasitism) as well as genotype. Usually, individuals range from 5 – 10 cm shell length, although some may reach 15 – 20 cm. They filter feed on algae, detritus and organic material. Because they filter out other contaminants in the process (with 80 - 100% efficiency), they are likely to serve as a vector for any water-born disease or pollutant. Breeding occurs in spring and early summer, although certain populations of *M. edulis* exhibit some spawning all year round (information obtained from Gosling 1992).

1.5.2 *Hyas araneus*

Hyas araneus is a temperate species of spider crab with a geographical distribution from Northern Spain to Svalbard; crabs occur at depths between 10 to 1200 meters on various type of bottom.

They are yellow-brown with a pear-shaped carapace, up to 10 cm long and 8 cm wide (see Figure 1-5-1B). At the anterior end of the carapace, the rostrum projects between the eyes and is split into 2 tapering parts that touch at their tips. The first pair of abdominal limbs, or pleopods, bear pincers and are shorter and stouter than pairs 2-5, which are long, slender and spider like. The carapace has a few large tubercles and may be covered with encrusting invertebrates. Eggs are incubated in winter, larvae occur in the plankton from May (*zoea*) to September (*megalopa*), although breeding may change due to latitude. (Sources of information:

http://www.marlin.ac.uk/species/Hyasaraneus.htm;

http://www.seawater.no/fauna/Leddyr/sandpyntekrabbe.htm.)

1.5.3 *Strongylocentrotus droebachiensis*

Strongylocentrotus droebachiensis is distributed widely in northern waters in both sheltered and exposed kelp beds and rocky areas; in the Atlantic Ocean, individuals occur in the low intertidal zone down to 1200 meters. Usually, this urchin is green with reddish/brown tones (see Figure 1.5.1 C). The tube feet (podia) are generally darker than the spines and are usually purple. They are radially symmetrical with a test (i.e. the skeleton) diameter of around 8.5 cm; spines reaching around 2.5 cm in length. They feed on large algae such as bull kelp, green algae and laminarians, but will also consume a wide variety of organisms including mussels, sand dollars, barnacles, whelks, periwinkles, sponges, bryozoans, and dead fish. Reproduction occurs on an annual cycle with spawning occurring in the spring, generally between February and May, but sometimes as late as June (sources of information: http://www.marlin.ac.uk/species/strongylocentrotusdroebachiensis.htm; http://www.racerocks.com/racerock/eco/taxalab/ensy02/aldoc.htm).

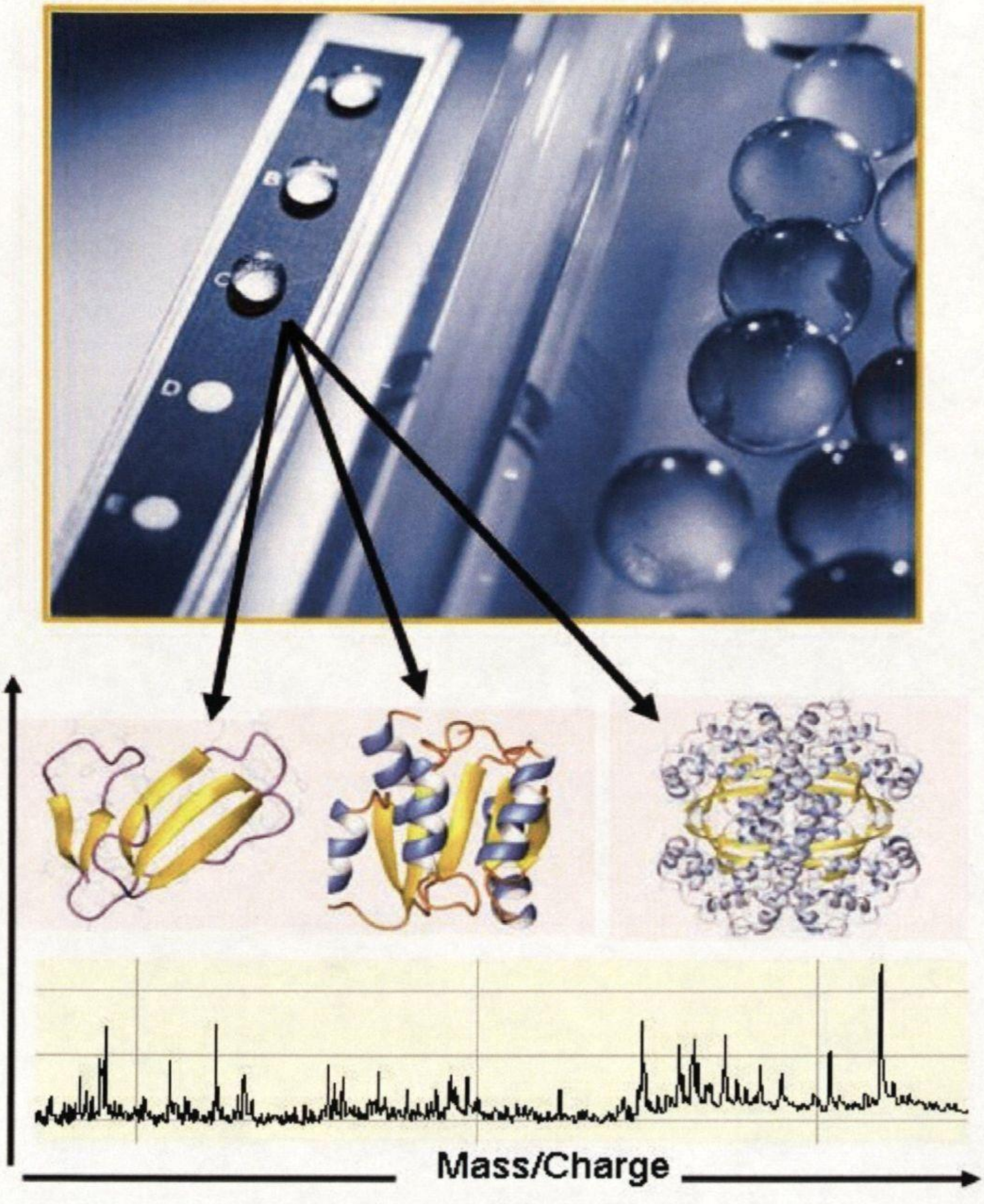
1.6 Aim of the study

The aim of this thesis was to evaluate the potential for proteomics in ecotoxicology and environmental risk assessment. The hypothesis was that proteomics technologies (i.e. ProteinChip technology in combination with mass spectrometry) could be a useful supplement to existing methods, by providing a sensitive, non-invasive, rapid multi-endpoint assessment of effects of anthropogenic chemicals on organisms *in vivo*. To test this hypothesis, three controlled laboratory experiments (Chapters 3, 4 and 5) and one field validation study (Chapter 6) were undertaken, using invertebrates as 'model-organisms' and potential EDCs as 'case-study'. Each of the experiments addressed one or several of the concerns and research gaps described in recent EDC literature:

1. The first laboratory study investigated mixture effects (Chapter 3).
2. The second study assessed species and gender differences/similarities in response to EDCs (Chapter 4).
3. In the third laboratory study, dose-response relationships at low doses were (in two species) addressed (Chapter 5).
4. The field validation study assessed dose/site-response relationship in a contaminated fieldgradient, as well as gender-specific responses. Furthermore, the results were compared to seasonal variation in reference organisms (Chapter 6).

Chapter 2

Material and methods



> *"Currently, with the combination of systems biology and comparative genomics, the possibilities are beyond imagination. As more new omics levels and dimensions of data are examined, biological scientists will better understand and model the miraculous intricacies of life."*
>
> - *Jimmy Lin & Jiang Qian 2007*

2.1 Method development - proteomics

The completion of the Human Genome Project (Venter et al. 2001), and the subsequent "omics revolution" in biological research, has stimulated dramatic technological advances to meet present and upcoming challenges within life sciences. For proteomic research, a number of complementary technologies have been developed attempting to separate, identify and characterise a global set of proteins in an effort to provide information about protein abundance, location, modification and protein-protein interactions in a proteome of a given biological system. The present study utilised SELDI TOF MS and ProteinChip® array technology (Ciphergen Biosystems/Bio-Rad) to evaluate the potential for proteomics in ecotoxicology. The choice of this system was based on its simplicity of experimental procedures and its high-throughput nature, allowing use of large sample sets with the added advantages of robust statistical interpretation of results and evaluation of individual variability. Furthermore, the technology operates with very small sample volumes, being sensitive at the femtomole level or less (Merchant and Weinberger 2000). In addition, the procedure has features that are complementary to 2DE analysis but, unlike 2DE, the technology enables profiling of proteins regardless of their intrinsic hydrophobicity, and is very sensitive in detecting proteins in the lower molecular weight range (< 20 kDa). The flexibility and versatility of the SELDI TOF MS and ProteinChip® array technology enables its use in a wide range of research areas, however, the majority of applications, to date, have been within medicine, drug discovery and toxicology. The challenge of this study was to develop suitable and reproducible protocols to measure protein expression in marine invertebrate plasma samples. Plasma was chosen because of the ability for non-invasive sampling. Using the manufacturer's recommendations for human samples as a starting point, numerous variables (including study design, sample preparation, MS analysis, data pre and post processing) were tested to optimise proteomic analysis of plasma samples from mussels, crabs and sea urchins.

2.1.1 Study design and sampling

Study design has great influence on proteomic analyses and can, potentially, give rise to significant pre-analytical variability. For example in sampling, care must be taken to avoid pre-analytical variability (that refers to any experimental variability introduced into the analysis prior to the actual assay). Even slight variations in sample collection procedure, such as blood sampling, time before freezing and centrifugation speed, can potentially generate inconsistent results. In the present study, sample procedures were consistent through all laboratory and field experiments, including first measurements of size and blood (haemolymph) sampling. Haemolymph was withdrawn by needle aspiration (Figure 2-1) from individual organisms (needles and syringes were changed for each individual) and immediately centrifuged for 10 min at 3000 g and 4°C.

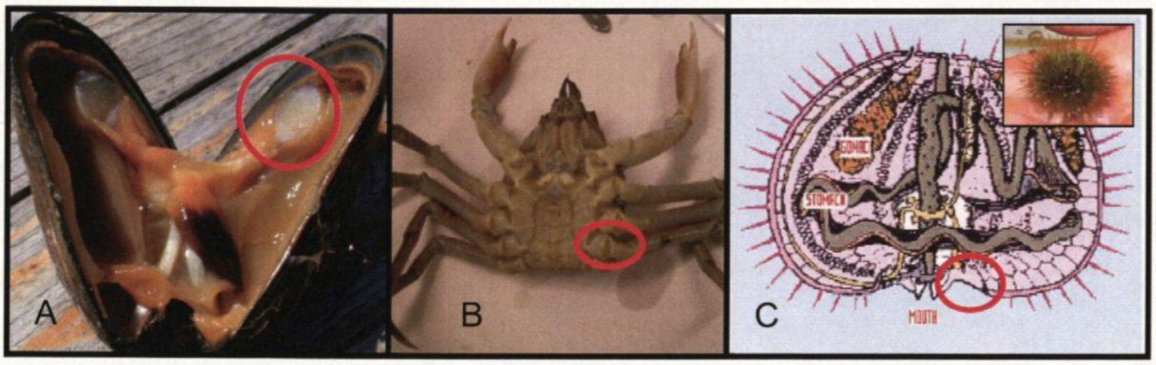


Figure 2-1. Body fluid sampling: the red rings indicate where haemolymph (mussels and crabs) and coelomic fluid (sea urchins) was collected; (A) haemolymph from *Mytilus edulis* was collected from the posterior adductor muscle, (B) haemolymph from *Hyas araneus* was collected from the arthrodial membrane at the base of the 4th pair of walking legs, (C) samples of coelomic fluid from *Strongylocentrotus droebachiensis* were collected by inserting the syringe through the peristomial membrane.

The supernatant (plasma) was harvested, a protease inhibitor cocktail (P2714 - Sigma-Aldrich) was added, and the sample was snap frozen in liquid nitrogen and stored at -

80°C until analysed. Robust stratification of samples into the appropriate classes is important, since data analyses were based on the class to which a sample was assigned. As treatment groups are given by the exposure or sampling locations, the most essential action for the present study was to correctly sub-group control and treatment groups into males and females, for evaluation of gender-specific responses. While crabs have external secondary sexual characteristics, mussels and sea urchins have no external sexual dimorphism. To determine gender, a gonad smear from each mussel and sea urchin was taken and examined under a light microscope. Furthermore, all descriptive information (including abnormalities or any prior sample treatment) associated with a given sample was noted. A minimum of 30 individual samples were collected for each exposure group. When possible, samples from 30 males and 30 females were collected from each group. The sample group size was decided following discussions with statisticians (from Ciphergen Biosystems). To avoid 'overfitting' of data when using multivariate statistical programmes such as e.g. Biomarker Pattern™ Software (Ciphergen Biosystems) it was suggested to use sample sets of minimum 30 samples per group. Thus, in order to have at least 30 males and 30 females for each treatment group 60-70 organisms per treatment groups were collected when possible.

2.1.2 Sample preparations with ProteinChip array technology

As with any other proteomic analysis (e.g. Issaq 2001) only a subset of the protein forms present in the original sample was detected and examined. The protein subset in the final analysis is dependent on a multitude of factors including chip type(s), sample quality, sample dilutions, incubation, wash conditions, type of matrix used and method of application (Figure 2-2).

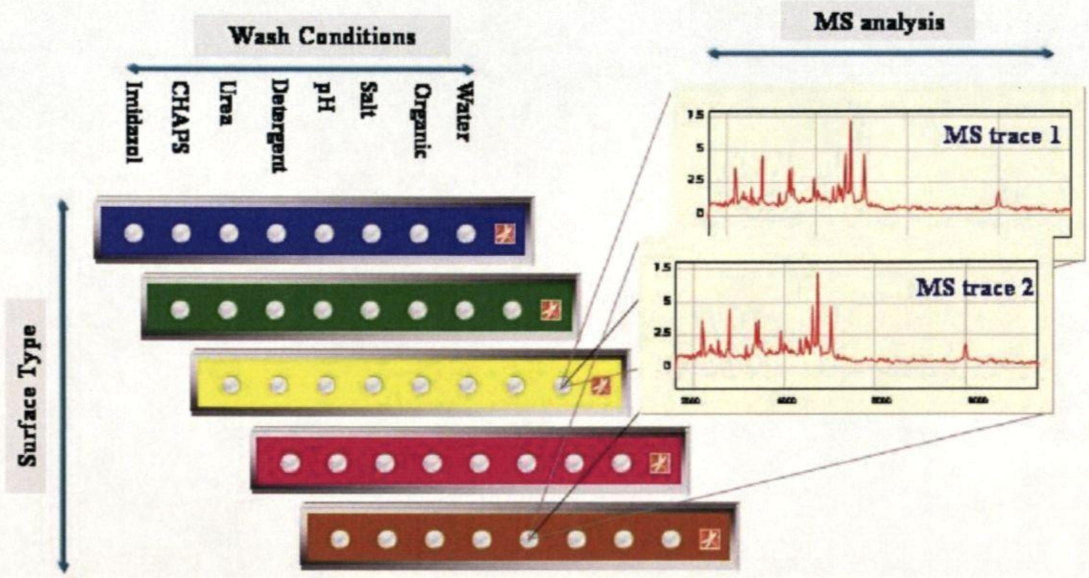


Figure 2-2. The three phases of separation used for profiling complex biological mixtures such as cell lysate or biofluids. The first phase is surface-type (e.g. Reverse phase, ion exchange etc.), the second is wash stringency (pH, salt, detergent modulation etc.), and the final phase is the mass spectrometry analysis. For each chip surface and wash condition, a different protein fingerprint is achieved that can be used to look for differential protein analysis of control and test samples. Figure courtesy of Ciphergen Biosystems/Bio-Rad.

Additionally, settings on the SELDI mass spectrometer (e.g. laser intensity and detector sensitivity) can affect the final subset of proteins examined in the analysis. The aim, therefore, was to identify the best experimental method for proteomic profiling of invertebrate-plasma samples, based on the assessment of the quality and reproducibility of different combinations of factors mentioned above. Since the aim was not to capture any specific peptide/protein, the experimental protocols were optimised to capture as many protein species as possible to maximise the probability of finding statistically significant biomarker candidates for effects of EDCs on mussels, crabs and sea urchins. Factors addressed in sample preparation are listed in Table 2-1 and are discussed in subsequent sections.

Table 2-1. Factors addressed in optimisation of sample preparation.

ID	Factor	Description
A	ProteinChip type	Ion exchange, metal affinity, reverse phase.
B	Sample dilution	A dilution series including 4 dilutions depending on the total protein concentration of samples from each species.
C	Binding conditions	Type of buffer (pH & ionic strength, detergent).
D	Sample incubation	Incubation volume, time and temperature. Direct "on chip" versus bioprocessor.
E	Wash conditions	Type of buffer (pH & ionic strength, detergent), number and length of washes.
F	Air drying of chip arrays	Post washing/pre-matrix: time between last wash and addition of matrix.
G	Matrix type	Sinapinic acid (SPA), alpha-cyano-4-hydroxy cinnamic acid (CHCA), energy absorbing molecule (EAM-1).
H	Matrix application	Number and volume of matrix depositions.

A. ProteinChip arrays

Prior to ProteinChip analysis, no pre-preparation of samples (e.g. fractionation, denaturation, removal of prominent proteins) was done. Various ProteinChip arrays (from Ciphergen Biosystems/Bio-Rad Laboratories) were tested to find the optimal arrays for the analysis of invertebrate body fluids. The principle of protein array surfaces is to selectively retain a specific subset of proteins, allowing others to be washed away to reduce sample complexity. ProteinChip arrays are aluminium strips (10-mm x 80-mm long) with 8 2-mm spots (allowing 8 samples or replicates to be analysed per array) that incorporate the full range of surface properties (Figure 2-3), extending from classical chromatographic separation media (e.g. ion exchange, reverse phase and immobilised metal affinity capture) to more specific bio-molecular affinity probes (e.g. antibodies, receptors, enzymes, and ligands). While the latter is used in protein interaction studies, the present study focused on the first category of chip arrays (i.e. with chemically-treated surfaces) for protein expression profiling and biomarkers

3. IMAC30: immobilized metal affinity to capture molecules that bind divalent cationic metals such as Ni, Cu and Zn (e.g. histidin tagged proteins, phosphorylated proteins and metal binding proteins). Active spots contain nitrilotriacetic acid (NTA) groups on the surface that chelate the metal ions. Proteins applied to the surface may bind to the chelated metal ions through histidine, tryptophan, cysteine, and phosporylated amino acids.
4. H4/H50: to capture molecules through reverse phase or hydrophobic interactions. The active spots contain metylene chains that closely mimic the characteristics of C6 to C12 alkyl chromatographic sorbent.

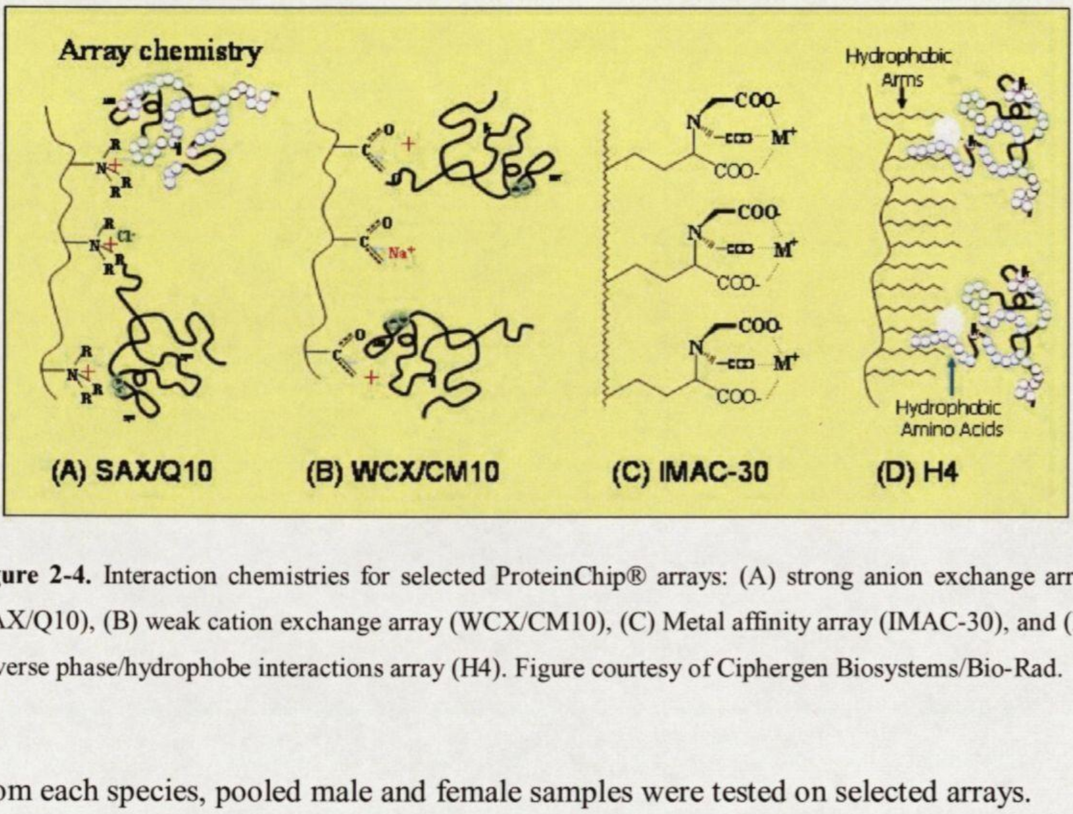


Figure 2-4. Interaction chemistries for selected ProteinChip® arrays: (A) strong anion exchange array (SAX/Q10), (B) weak cation exchange array (WCX/CM10), (C) Metal affinity array (IMAC-30), and (D) Reverse phase/hydrophobe interactions array (H4). Figure courtesy of Ciphergen Biosystems/Bio-Rad.

From each species, pooled male and female samples were tested on selected arrays. Results revealed that each surface retained a unique set of proteins, although some protein features were retained by several surfaces. When samples from sea urchins, mussels and crabs were profiled on the same array type and with identical buffer conditions, each revealed unique (species-specific) protein profiles. Profiles from mussels and sea urchins, however, had more peaks in common than spider crabs (Figure

2-5). Although each species showed significant individual variation, it was possible to determine the species origin of the sample from the partial protein profile.

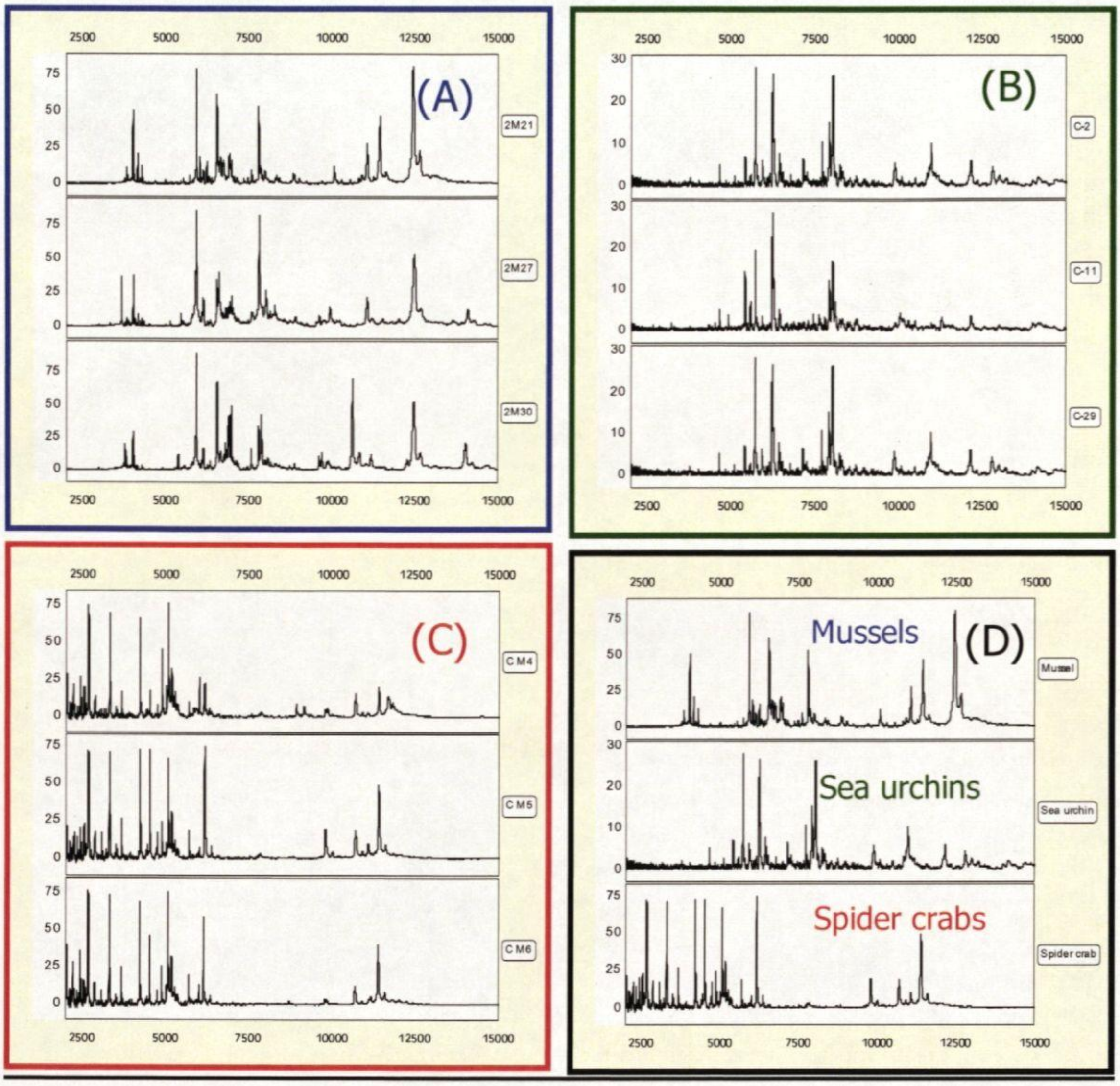


Figure 2-5. Species-specific (partial) protein profiles. Analyses of plasma from 3 individuals of (A) blue mussels, (B) sea urchins, and (C) spider crabs showing unique species-specific MS traces when captured on the same chip surface (with equal buffer conditions); (D) shows an 'overlay' of the three species to elucidate similarities and differences of the species. In addition, individual variation can be seen in (A), (B) and (C).

B. Sample dilution

Correct sample dilution was achieved by balancing several factors. For example, the sample must not be diluted such that the proteins will not bind to the surface.

However, if the sample is too concentrated, the more abundant proteins will saturate the surface, preventing less abundant proteins (that are potential biomarkers) from being detected. Manufacturer's recommendation is 0.05- 2 mg/ml total protein per spot. Dilution series experiments were conducted with samples from each species (e.g. Figure 2-6).

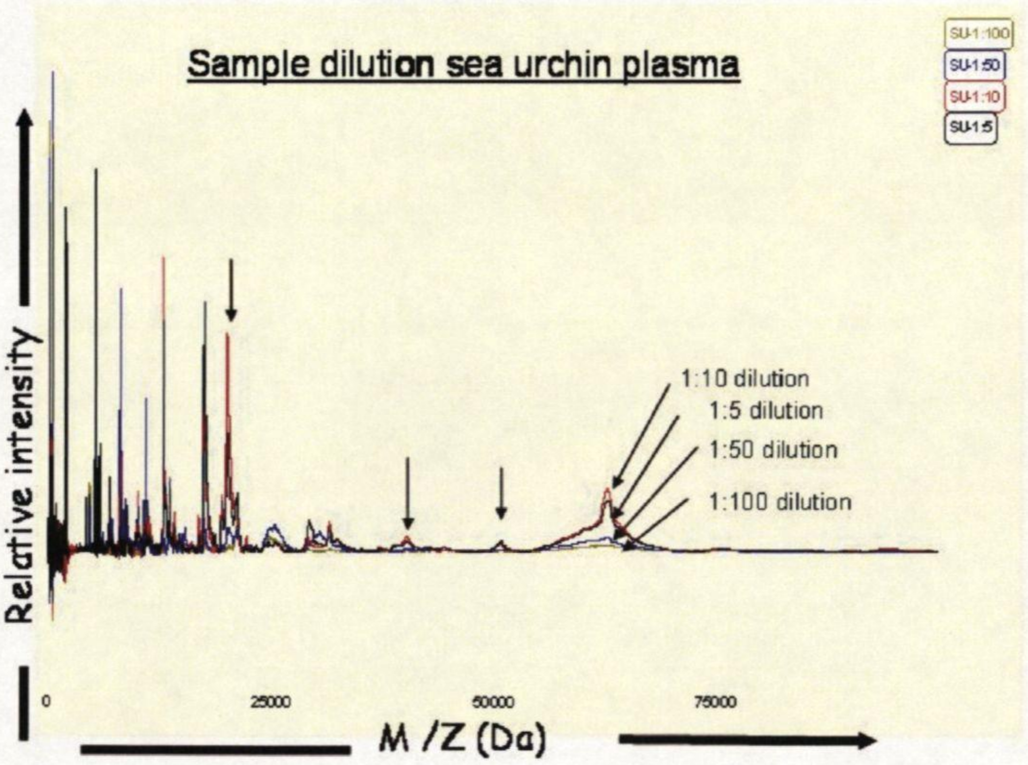


Figure 2-6. Example of sample dilution (i.e. 1:5, 1:10, 1:50, 1:100) of sea urchin plasma on weak cation exchange arrays (and sodium acetate buffer pH 4.5). The figure shows an "overlay" of MS traces for each dilution. Black = 1:5, red = 1:10, blue = 1:50, and green = 1:100.

Results shown in Figure 2-6 indicate that many molecules bind in a dose-dependent manner up to a dilution of 1:10 (see arrows). At 1:5 dilution, the peak intensity is decreasing again, and some protein features are lost compared to 1:10 dilution.

Four dilutions, within the recommended protein range, were tested to find the dilution that resulted in the best MS trace, with respect to number and quality of detected peaks (Table 2-2). The total protein concentrations of the samples were determined by a

procedure based on the Bradford method (Bradford, 1976). Samples were diluted and pipetted onto microplates in 4 replicates. A dilution series of 8 standards was made from a 5 % Bovine Serum Albumin (BSA) solution, and all standards were run in 3 replicates, on each microplate analysed. A 1:5 solution of Bradford reagents (Bio-Rad Laboratories) in distilled water was prepared shortly before use and added to each well. After 5 min reaction time, the absorbance at 595 nm was measured (Labsystems, Multiscan RC) (Table 2-2).

Table 2-2. Optimal sample dilution for blue mussels, sea urchin and spider crabs respectively.

Species	**Plasma protein c (mg/ml)**	**Dilutions tested**	**Best dilution**	**Corresponding protein c added per spot**
Mussels	2.5 ±0.6	1:5, 1:10, 1:50, 1:100	1:10	≈0.25 mg/ml
Crabs	51 ±9.0	1:20, 1:50, 1:200, 1:500	1:50	≈1,02 mg/ml
Sea urchins	2.1 ±0.7	1:5, 1:10, 1:50, 1:100	1:10	≈0.21 mg/ml

Note. c = concentration.

Results from Table 2-2 show that the best protein profiles (i.e. in terms of number of peaks) were obtained with a 1:10 dilution of sea urchin and mussel plasma, while a 1:50 dilution was best for crabs. The explanation for the 4 times higher protein concentration required to obtain good-quality MS traces for crabs compared to mussels and sea urchins have not been investigated, however, it could be due to different levels of abundant plasma proteins, such as e.g. hemocyanin (i.e. a copper-containing protein with an oxygen-carrying function similar to that of hemoglobin, present in the blood of certain molluscs and arthropods), in the selected species.

C. Buffer conditions for sample binding

Several factors were addressed to find buffer conditions for optimal binding of samples to the chip surfaces. For example, the operating mechanism of ionic exchange arrays is the reversible binding of charged molecules to the surface, and the property of a peptide/protein that govern its binding is its net surface charge. Since surface charge is the result of weak acidic and basic amino acids within the protein, binding of the protein to the array is highly pH dependent. In addition, the ionic strength of the buffer, or the total salt concentration, is important in defining surface- binding selectivity. By increasing the ionic strength, competition is generated between the charged protein on the surface and the buffer ions, causing weakly- bound proteins to elute from the array surface (i.e. more specificity). The optimal salt concentration will, therefore, be a matter of the degree of non-specific binding that can be tolerated. The various combinations of binding buffers tested are listed in Table.2-3.

Table 2-3. Buffer conditions tested: NaAc (sodium acetate); PBS (phosphate-buffered saline); TFA (trifluoracetic acid); ACN (acetonitrile); NaCl (sodium chloride), Cu (Copper), HCl (hydro chloride).

Array	**Surface properties**	**Buffers tested**	**pH**	**Ionic strength (mM)**	**Detergent i.e. 0.05% Triton X-100**
CM10/WCX	Cation exchange	NaAc	3.5, 4.5, 5.5, 6.5	10, 50, and 100	yes/no
Q10/SAX	Anion exchange	Tris-HCl	6.5, 7.5, 8.5	10, 50, and 100	yes/no
		Hepes	6.0, 7.0, 8.0		
IMAC-30(Cu)	Metal affinity	*PBS*	7.2	*50 and 100*	yes/no
		+ NaCl		100 and 500	
H4-H50	Hydrophobic interaction	(10%, 20%, or 30 %) ACN + 0.1 % TFA	-	-	-

The buffer conditions found to bind invertebrate plasma most efficiently (i.e. that gave protein profiles with the highest number of resolved peaks) for the various array types are shown in Table 2-4.

Table 2-4. Selected binding buffers for the various ProteinChip arrays.

Array	Binding buffer
CM10/WCX	- 50 mM NaAc, pH 4.5 + 0.05 % Triton X-100
Q10/SAX	- 50 mM Tris-HCl, pH 8.5 + 0.05 % Triton X-100
IMAC-30(Cu)	- 50 mM PBS, pH 7.2 + 0.5M NaCl + 0.05% Triton X-100
H4-H50	- 10% Acetoneitrile + 0.1% TFA

Due to the large number of samples analysed in this study, it was decided to select only one array type. To choose the best array for all species, 2 pooled plasma samples (i.e. from males and females, respectively) from each species were analysed in 3 replicates on the arrays/buffer conditions listed in Table 2-4. Detected peaks with a signal to noise greater than or equal to 5 (i.e. this choice was based on recommendations provided by Ciphergen Biosystems), present within the mass range of 1500 to 200000 Da, were counted and compared for each array type (Figure 2-7). For each species, ProteinChip arrays coated with carboxylate groups, providing weak cation exchange (WCX) properties in combination with a binding buffer containing 50 mM Sodium Acetate (NaAc) and 0.05% Triton X-100 at pH 4.5, were found to bind plasma proteins most efficiently, therefore, this combination was chosen for all experiments in this study.

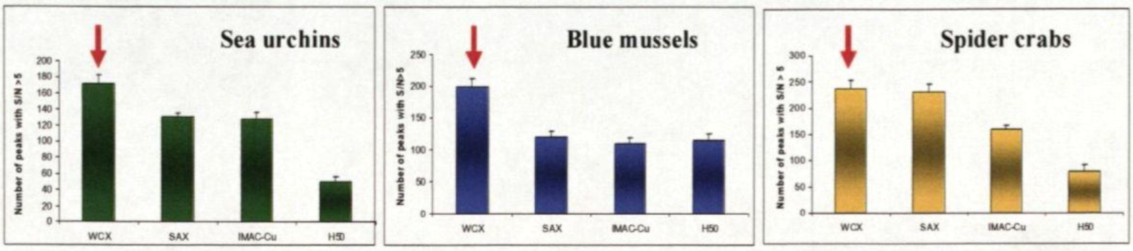


Figure 2-7. Average number of peaks (± SD) detected on WCX, SAX, IMAC-Cu and H50-RP arrays (with a signal to noise greater than or equal to 5, present within the mass range of 1500 to 200000 Da) for sea urchin plasma, mussel plasma and spider crab plasma, when analysed with optimal buffer conditions. The arrows indicate the best chip surface for each species (i.e. WCX).

D. Sample incubation

Sample processing and incubation can be conducted either by direct 'on-spot', or using Bioprocessors (Figure 2-8), each holding 12 arrays, and hence allowing 96 samples to be processed in parallel. The bioprocessors form separate wells above each spot on the arrays, enabling loading of volumes of up to 500 μl per well. Although some tests were run 'direct on-spot', it was decided to use the bioprocessor method throughout the study due to the large number of samples in the different experiments, as well as the increased reproducibility and sensitivity due to application of greater sample volumes.

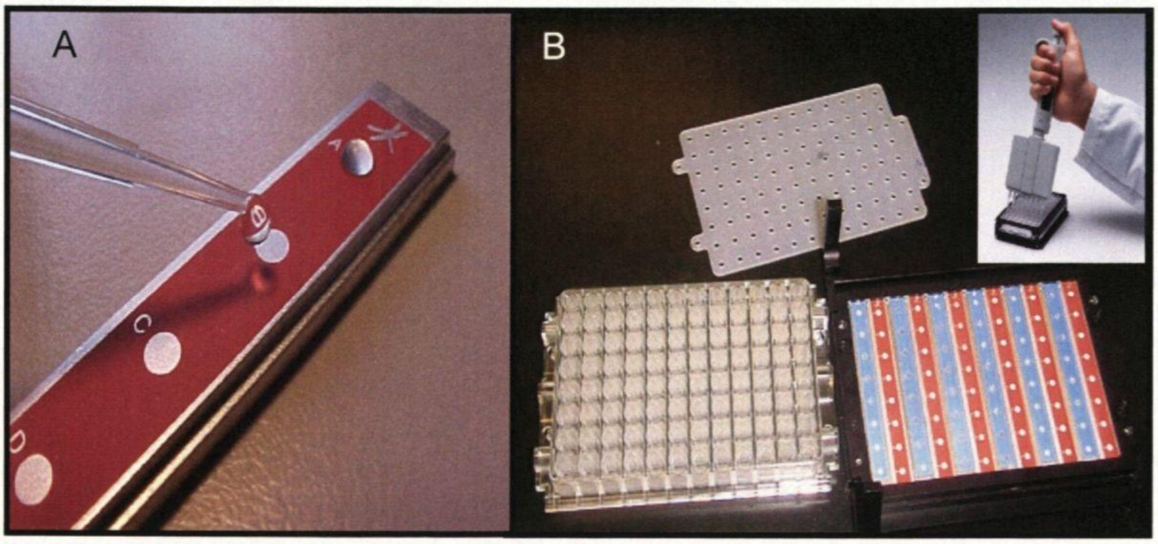


Figure 2-8. (A) Sample processing using the "direct on-spot" method of a ProtenChip array; **(B)** a 96-well Bioprocessor from Bio-Rad loaded with 12 protein arrays. Figure courtesy of Ciphergen/Bio-Rad.

Other factors associated with sample incubation that were tested included the volume of diluted sample per spot/well, the incubation time and the influence of temperature (Table 2-5). The number of peaks and peak intensity is influenced by the amount of time that the sample is left to interact with and bind to the array surface. Allowing the binding interaction to reach equilibrium, improved reproducibility as it minimised differences in peak number and intensities amongst replicates. Best reproducibility was observed with 60 min incubation at room temperature and with incubation overnight at 4°C (Table 2-5). Furthermore, sample incubation volumes of 100 and 150 μl/spot showed (equally) better spectral reproducibility and quality than the 50μl/spot. The combination used for this study was therefore: overnight incubation at 4°C and 100 μl/spot for all sample types.

Table 2-5. Test of optimal sample incubation volume, incubation time and temperature.

Factors	Test	Choice for this study
Sample incubation volume	- 50, 100, or 150 (μl per spot)	- 100 μl per spot
Incubation time and temperature	- 30, 60, or 120 min at room temperature - 16 hours (over night) at 4°C	- over night at 4 °C

E. Wash conditions

After incubation, unbound proteins and other contaminants were washed off the surface of the ProteinChip array using buffers and other wash solutions as required by the array chemistry. Only proteins interacting with the chemistry of the array surface were retained for analysis. Following manufactures recommendations, the same buffers used for binding (see Table 2-4) were used for stringency washing, but wash buffers were without detergent_(i.e. Triton X-100). The following wash procedure was used throughout this study: arrays were washed 3 times for 10 min with 50 mM NaAc pH 4.5 and rinsed quickly with ultra pure water twice to remove weakly-bound proteins.

F, G & H. Energy absorbing molecule (matrix)

After stringency washing, protein arrays were removed from the bioprocessor and air dried before application of matrix/energy absorbing molecules (EAMs). EAMS are essential to facilitate desorption and ionization of the sample in the ProteinChip reader (SELDI TOF MS). Application of the matrix causes the proteins on the chip surface to solubilise and co-crystallise with the matrix as the solution dries. These crystals absorb the energy from the laser and generate the ionised protein forms that are detected by the mass spectrometer. Several factors are likely to affect the final proteomic output trace, including type of matrix, age of matrix, age of organic solvents (i.e. acetonitrile-ACN and trifluoracetic acid-TFA), application volume, number of depositions, and the time over which the chip was allowed to dry before addition of matrix. A list of factors investigated (and results) in this study is shown in Table 2-6.

Table 2-6. List of factors investigated with regard to matrix application.

Factors	**Test**	**Choice for this study**
Matrix type	Sinapinic acid (SPA), alpha-cyano-4-hydroxy cinnamic acid (CHCA), energy absorbing molecule (EAM-1).	- SPA
Volume	0.5, 0.6, 1.0 µl/spot	0.6/1.0 µl/spot*
Number of depositions	1,2	2
Time for airdrying of arrays	10, 15 ,30 ,60 ,120 min	15 min

* In the middle of the PhD study, manufacturer produced a new improved generation of protein arrays, with the same surface properties as the old ones e.g. WCX → CM10, however, while 0.6 µl/spot was sufficient for the WCX chips, the new CM10 chips allowed 1.0 µl/spot.

SPA and CHCA are recommended (by Ciphergen Biosystems/ Bio-Rad) for analysis of peptides and small molecules, while EAM-1 is best for glycoproteins. Results from the optimisation experiments revealed that SPA and CHCA gave equally good traces in the low mass region (i.e. 1500 – 10 000 Da); however, SPA also gave good traces in the mass region 10 000 – 200 000 Da contrary to CHCA. EAM-1 did not work at all for invertebrate plasma samples. The reproducibility and peak intensity was improved by 2 depositions of matrix compared to only one. Regarding time for air drying of protein arrays prior to matrix application, only 60 and 120 min gave significantly reduced spectral quality. Based on the results from this method development study, a sample preparation protocol for use throughout this study was established (see Table 2-7):

Table 2-7. Final sample preparation protocol.

Steps/materials	Description
Protein array	- Weak cation exchange - WCX (old version) /CM10 (new version)
Binding buffer	- 50 mM Sodium Acetate (NaAc) and 0.05% Triton X-100 at pH 4.5
Wash buffer	- 50 mM NaAc pH 4.5
Matrix	- SPA (Ciphergen/BioRad), resolved in 50% (v/v) ACN/ 0.1% (v/v) TFA
Protein standards for mass calibration	- Standard Proteins from Calibrant Kit, Protein MW Standards; Ciphergen/Bio-Rad Part C100-0001) – see Table 2-8 for content and preparation of calibration solutions
1. Array pre-treatment	- Spots were pre-activated with 100 µl of 10 mM HCl for 5 minutes, rinsed quickly with ultra pure water (3 x 100 µl), and incubated with 100 µl of binding buffer for 5 minutes.
2. Sample dilution	- 1 : 10 (mussels and sea urchins) or 1 : 50 (crabs) in binding buffer
3. Sample incubation	- 100 µl per spot overnight at 4°C with vigorous agitation.
4. Post-binding stringency washing	- 3 x 10 min (200 µl/spot) with wash buffer, followed by quick rinsing with ultra pure water twice (200 µl/spot).
5. Air drying of arrays *	- 15 min
6. Matrix application	- 2 x 0.6/1.0 µl per spot for WCX/CM10 arrays respectively, allowing the applied solution to dry (15 min) between applications.
7. Preparation of mass standards	- 15 µL of the saturated SPA solution is added to a 5.0 µl aliquot of the protein standards and mixed.
8. Application of mass standards	- 2 x 0.6/1.0 µl per spot for WCX/CM10 arrays respectively, allowing the applied solution to dry (15 min) between applications. Mass standards were applied in 3 replicates, to dry spots, that have not been preactivated or washed with buffer.

***Note: It is important to ensure that the spots are dry before adding matrix.**

All buffer solutions were freshly made for each new experiment, while new matrix solutions were prepared every second day. The 1% TFA solution, used for preparation of the matrix, was changed every week. Additionally, protein standards for mass calibration of SELDI spectra were included in all analyses. The protein standards were prepared according to the package instructions and then mixed together in the volumes listed in Table 2-8.

Table 2-8. Protein standards and volumes used for calibration of mass spectra.

Standard	**Mass** Da	**Concentration** pmol/µl	**Volume** µl	**Final Concentration** pmol/µl
Low molecular weight mass standards				
Dynorphin A (209-225), porcine	2147.5	10	5	0.29
ACTH (1-24), human	2933.5	10	10	0.57
Beta-endorphin (61-91), human	3465.0	10	5	0.29
Insulin bovine	5733.6	10	5	0.29
Ubiquitin	8564.8	10	30	1.71
Cytochrome C, bovine	12230.9	10	40	2.29
Bovine β-Lactoglobulin A	18363.3	10	80	4.57
High molecular weight mass standards				
Insulin bovine	5733.6	10	15	0.54
Ubiquitin	8564.8	10	22.5	0.81
Cytochrome C bovine	12230.9	10	30	1.08
Bovine β-Lactoglobulin A	18363.3	10	60	2.16
Horseradish Peroxidase	43240.0	20	30	2.16
Serum Albumin bovine	66433.0	20	90	6.49
IgG, bovine	147 300	50	30	5.41

The final solution was aliquoted out into 5.0 µl portions and frozen at -80 °C until use.

2.1.3 Data collection with SELDI TOF MS

The protein arrays were analysed on a PBS-IIc time-of-flight mass spectrometer (Figure 2-9) using ProteinChip Software version 3.1 (Ciphergen Biosystems/Bio-Rad). The instrument uses acquisition protocols, which need to be defined, for acquiring data from a spot or portion of a spot on the protein arrays. Important data acquisition parameters that require optimisation include: laser intensity, detector sensitivity, focus mass range, sampling rate, and number of shots to acquire into the spectrum average. The ProteinChip reader uses a nitrogen laser to desorb and ionise the sample. Ionisation of the analyte results from an interplay between the laser energy, the matrix, and the analyte.

With autolauder (auto BMS)
CIPHERGEN

Figure 2-9. A PBS-IIc time-of-flight mass spectrometer with (left) and without (right) automatic loading of protein arrays. Figure courtesy of Ciphergen Biosystems/ Bio-Rad.

The laser energy induces both protein ionization and a change of state from the solid, crystalline phase, into the gas phase, and is hence one of the most critical parameter to optimise. For example, the laser intensity settings that are too low cause insufficient extraction of ions from the array surface, resulting in low peak counts and low peak intensities; excessive laser intensity yields off-scale readings, an unstable baseline, and broad, flat-topped peaks that make accurate quantitation of expression differences impossible. Acquisition parameters, optimised for each new experiment in this study, yielded protein profiles with optimal numbers of sharp and well-resolved peaks. Optimisations were done by manually collecting data from a sample type by shooting at

the sample with various laser intensities, starting low (e.g. at 150) and then increasing the laser until MS traces were of best possible quality. Simultaneously, detector sensitivity and mass focus areas were varied until satisfactory traces were obtained. Following optimisation, 'spot' and 'chip' analysis protocols were made to ensure similar settings for analyses of all samples to be compared. These protocols were not the same in all experiments, and details are provided as appropriate in the following chapters. However, as instruments/lasers can drift or change over time, a reference sample was included in each analysis to ensure that peak intensities did not vary with changes in the laser over time and differences reflected biological differences. Additionally, samples to be compared were analysed on the same day (as far as possible), and all exposure groups were mixed so that samples were distributed randomly on the protein arrays. To ensure sufficient coverage of the entire mass range (i.e. 0 - 200 kDa), data acquisition settings were optimised for both low (i.e. 2.0 – 15 kDa) and high molecular weight ranges (> 15 kDa), as larger molecules require more energy to 'fly' than small ones. The array spots are divided into sections (Figure 2-10), allowing analysis of each sample several times, but with different settings (i.e. collecting data from unique positions at the spot), to maximise the number of detected protein species over the whole mass range.

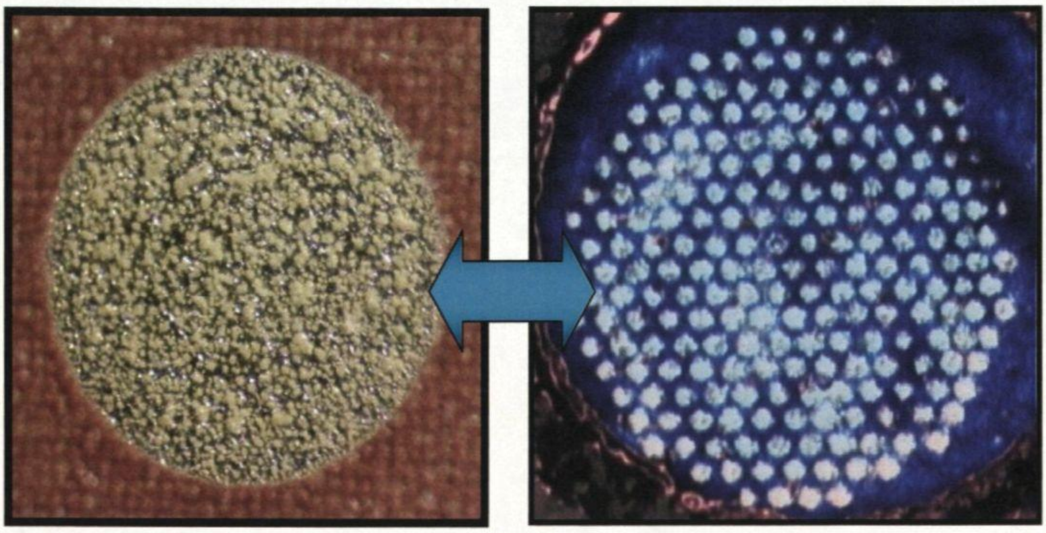


Figure 2-10. Shows array spots – how they are divided into sections, allowing repeated analysis of each sample, to optimise analysis of both small and large molecules in the same sample. For each analysis at least 60-100 laser shots are collected, and the average presented in the corresponding protein profile. Figure courtesy of Ciphergen/Bio-Rad.

In this study, each sample was analysed twice to give two mass spectra, one where detection of small molecules was optimised, and one favouring larger molecules. Results from the two analyses were combined for further data analysis (peaks in the mass range 2.0 – 15 kDa were kept from the 'low-mass run', while peaks from 15 – 200 kDa were kept from the 'high-mass run'. However, if no extra peaks were gained in the high-mass run, only the full-range spectra obtained in the low-mass run were used for analysis.

2.1.4 Data handling and statistics

Data handling (pre and post-processing of data) is one of the most challenging parts of proteome analysis, and numerous bioinformatics tools are being developed around current proteomic platforms to help handle, process, and meaningfully interpret the large body of data that is emerging. A similar approach to that developed successfully in medical SELDI-TOF MS studies was chosen for data collected in the

present study. Preliminary data were processed and interpreted using the Biomarker Wizard feature of the ProteinChip Software (version 3.1, Ciphergen Biosystems) followed by multivariate analysis. The multivariate analyses undertaken in this study were regression tree-based methods (Breiman et al. 1984) embodied in the Biomarker Pattern^{TM} Software (version 4.0.1, Ciphergen Biosystems). In addition, multi-dimentional scaling (MDS) from the Plymouth Routines in Multivariate Ecological Research (PRIMER) software v6 was used for illustration of 'trends' in the datasets.

Preprocessing of data ensures that all the data can be compared together, and includes e.g. baseline subtraction, mass calibration and total ion current normalisation. In Biomarker Wizard, mass spectra from all treatment groups were imported into one experimental file. Baseline subtraction was performed and the spectra were mass-aligned using three mass peaks (intact or modified proteins/peptides) prominent in all spectra, and further normalised to the total ion content for the amplitudes of all peaks detected in the mass range of interest (e.g. 2.0 - 15 kDa for a low-mass analysis, and 15 - 200 kDa for a high-mass analysis) in the spectra compared. Background noise was subtracted from the same M/Z region prior to data collection. Additionally, all spectra were externally mass calibrated using standards listed in Table 2.9. Separate calibration equations were calculated for low-mass and high-mass ranges to obtain best possible mass accuracy. Protein/peptide peaks, with similar M/Z values (peak closeness 0.5 % of mass), were automatically grouped across all the spectra into peak clusters, if present, in a minimum of 50% of the samples from one treatment group. The peaks used to generate these clusters had to meet a minimum signal to noise ratio ($S/N \geq 5$). Not being aware of the intensity of a potential biomarker, and hence risking to loose diagnostic information, only peaks with $S/N \geq 5$ were collected and evaluated in this study as a general rule (based on Ciphergen Biosystems recommendations).

Once the data were collected, they were tested for differences between treatment groups using Mann-Whitney-Wilcoxon test (incorporated in ProteinChip software). The statistical test provided a *p* value for each peak. Differences at $p < 0.05$ were considered significant; however, *p* values alone are not always indicative of the utility of a peak as a biomarker. A highly predicative diagnostic test relies on minimising the overlap (in response) between exposed organisms and controls in terms of value distribution for a given parameter. This underlines the need for multivariate analyses that are based on multiple variables to find the best possible biomarkers.

After peak detection and preliminary statistical analysis, raw data were exported from Biomarker Wizard to Microsoft Excel to determine the presence/absence/fold changes of peaks between treatment groups, and to examine the data for potential exposure or gender-specific alterations of proteins. The data were exported simultaneously to Biomarker Pattern^{TM} software and/or PRIMER for identification of potential multivariate patterns classifying exposed and control organisms. The algorithm in the Biomarker Pattern^{TM} software examines each peak cluster present in the spectra and assesses its quality as a classifier (described in Fung and Enderwick 2002 and Figure 2-11). Each node (i.e. black square in the figure) is a decision point. Each sample is sifted down the tree based on how it answers the question in each node. Terminal nodes are stopping points and the majority of the samples determine the classification of each terminal node. Sensitivity is calculated as the ratio of correctly-classified treated samples (true positives) to the total number of treated samples. Similarly, specificity is calculated as the ratio of the number of controls correctly classified (true negatives) to the total number of controls. Figure 2-11(B) shows how the rules in the classification tree manifest themselves in the raw data, and how the classification model uses the peak intensity to classify each sample.

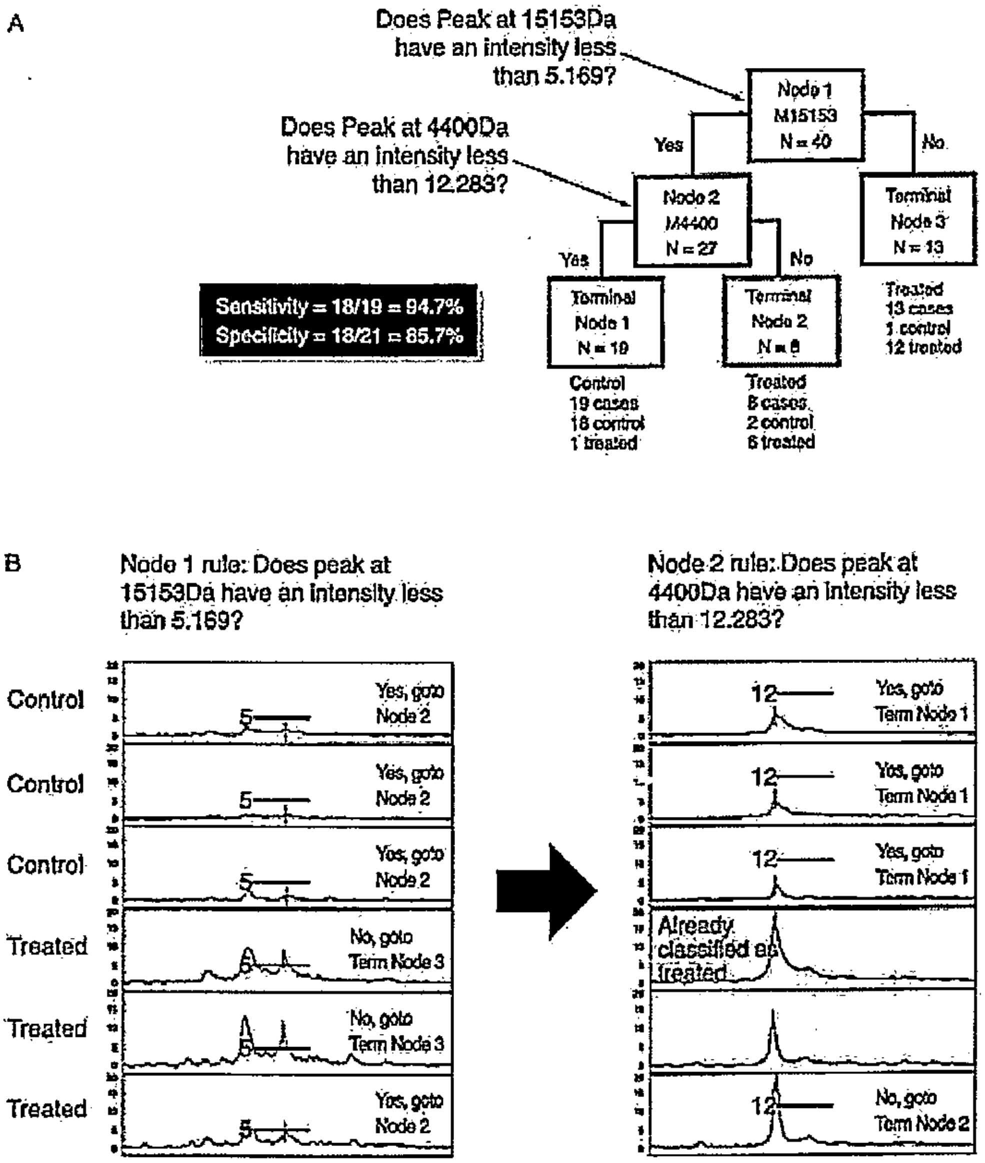

A
Does Peak at 15153Da have an intensity less than 5.169?
Does Peak at 4400Da have an intensity less than 12.283?
Node 1
M15153
N = 40
Yes
No
Node 2
M4400
N = 27
Terminal
Node 3
N = 13
Treated
13 cases
1 control
12 treated
Yes
No
Sensitivity = 18/19 = 94.7%
Specificity = 18/21 = 85.7%
Terminal
Node 1
N = 19
Control
19 cases
18 control
1 treated
Terminal
Node 2
N = 8
Treated
8 cases
2 control
6 treated
B
Node 1 rule: Does peak at 15153Da have an intensity less than 5.169?
Node 2 rule: Does peak at 4400Da have an intensity less than 12.283?
Control
Control
Control
Treated
Treated
Treated
Yes, goto Node 2
Yes, goto Node 2
Yes, goto Node 2
No, goto Term Node 3
No, goto Term Node 3
Yes, goto Node 2
Yes, goto Term Node 1
Yes, goto Term Node 1
Yes, goto Term Node 1
Already classified as treated
No, goto Term Node 2
5
12

Figure 2-11: Classification tree example. Figure courtesy of Dr. Eric T. Fung (Ciphergen Biosystems).

Based on the selected classifiers, the software generates and tests different classification models, using a cross-validation method that randomly picks 10% of the samples. The models (classification trees) with the best prediction success and lowest error costs were chosen for further testing. Mass spectra from unknown samples were classified subsequently by likeness to the pattern found in the plasma mass spectra used to create the classification model. A percentage of samples from each treatment group (depending on the total number of samples available, i.e. specified in respective sections) were used to build the classification models, while the remaining samples from each of the three treatment groups were kept for blind-testing of the models. Multidimensional scaling (using PRIMER software), i.e. non-metric MDS plots, derived from Euclidean distance similarity matrices were used to visualise similarities/dissimilarities between treatment groups in laboratory and field studies, where close points in the MDS plot are reflecting similar response patterns (Galloway et al. 2004). Nevertheless, it must be emphasised that these plots (based on data averages from all individuals in one exposure group) cannot be used for statistical interpretation of differences, but merely for visualisation of response-trends in the datasets.

2.2 Collection and maintenance of animals

All study organisms were collected at (relatively) clean sites outside Stavanger, western Norway (Rogaland), (Figure 2-12). These sites have been used in several other projects (e.g. Knigge et al. 2004), and contamination levels are monitored regularly (e.g. Table 2-9).

Table 2-9. Body burden of PAHs (as SUM PAH and EPA18) in mussels from one of the reference sites (Førlandsfjorden, column 1-2) and various sites known to be contaminated. Column 3 (Visnes) represents a metal-contaminated site, while columns 4-8 are from PAH-contaminated sites (adapted from Andersen et al. 2003a). PAH concentrations were analysed using gas chromatography connected to a mass spectrometer in selected ion mode (GC/MS-SIM).

	Reference		Contaminated sites →					
Mussels µg/kg w.w.	1998 Førland. µg/kg	2003 Førland. µg/kg	1998 Vistnes µg/kg	1998 Bukkøy µg/kg	2003 Bukkøy µg/kg	1998 Høgevarde µg/kg	1999 Høgevarde µg/kg	2003 Høgevarde µg/kg
Naphthalene	0.0	0.8	0.1	4.9	0.7	10.0	0.0	3.8
C1-naphthalene	1.5	3.0	1.4	8.3	2.4	0.0	0.0	9.4
C2-naphthalene	4.9	29.0	6.3	18.3	14.4	0.0	94.0	106.0
C3-naphthalene	7.5	10.0	9.9	25.4	13.0	0.0	119.0	41.5
Acenaphthylene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7
Acenaphthene	0.0	0.6	9.9	0.0	6.3	53.7	195.0	100.1
Fluorene	0.0	0.2	0.0	0.0	2.8	64.3	193.0	75.6
Phenanthrene	1.0	1.6	1.2	72.0	22.9	511.2	1439.0	671.4
Anthracene	0.0	0.2	0.0	6.7	3.5	97.6	73.0	58.9
C1-phen/anthr	1.2	1.0	0.8	75.1	35.6	880.6	1989.0	1333.1
C2-phen/anthr	3.9	1.0	3.6	74.6	55.8	1182.0	1734.0	1486.3
Dibenzothiophene	0.0	0.0	0.0	4.2	1.0	45.5	67.0	33.7
C1-dibenzothiophene	0.1	0.0	0.0	6.8	3.8	109.0	121.0	86.4
C2-dibenzothiophene	0.8	0.0	1.1	22.6	13.5	299.2	229.0	196.7
Fluoranthene	5.0	7.2	2.3	357.0	182.8	6917.0	10823.0	14150.0
Pyrene	0.8	4.4	0.6	175.9	68.1	3174.0	5229.0	7140.0
Benzo(a)anthracene	0.6	1.8	0.7	67.3	31.9	4395.0	3268.0	4130.0
Chrysene	3.1	3.4	1.6	91.5	58.8	5460.0	4220.0	6050.0
C1-chrysene	0.4	1.0	0.0	32.4	10.7	1523.6	1599.0	1857.0
C2-chrysene	0.5	0.4	0.0	0.0	3.7	605.5	411.0	574.7
Benzo(b)fluoranthene	1.1	2.1	1.0	84.3	28.1	3529.0	3708.0	5300.0
Benzo(k)fluoranthene	0.8	0.6	0.4	22.3	7.5	950.0	924.0	1460.0
Benzo(b+k)fluoranthene	1.9	2.6	1.2	114.9	35.2	3925.0		6290.0
Benzo(a)pyrene	0.0	0.2	0.0	0.0	4.3	890.0	971.0	1112.8
Indeno(1,2,3,cd)pyrene	0.0	0.3	0.7	0.0	6.0	545.0	388.0	507.8
Benzo(g,h,i)perylene	0.0	0.4	0.1	0.0	7.2	400.1	370.0	505.3
Dibenzo(a,h)anthracene	0.0	0.0	0.0	0.0	1.1	242.2	135.0	137.1
Sum PAH	33	69	42	1150	586	31884	38299	47129
EPA16	12	24	18	894	433	26731	32003	40968

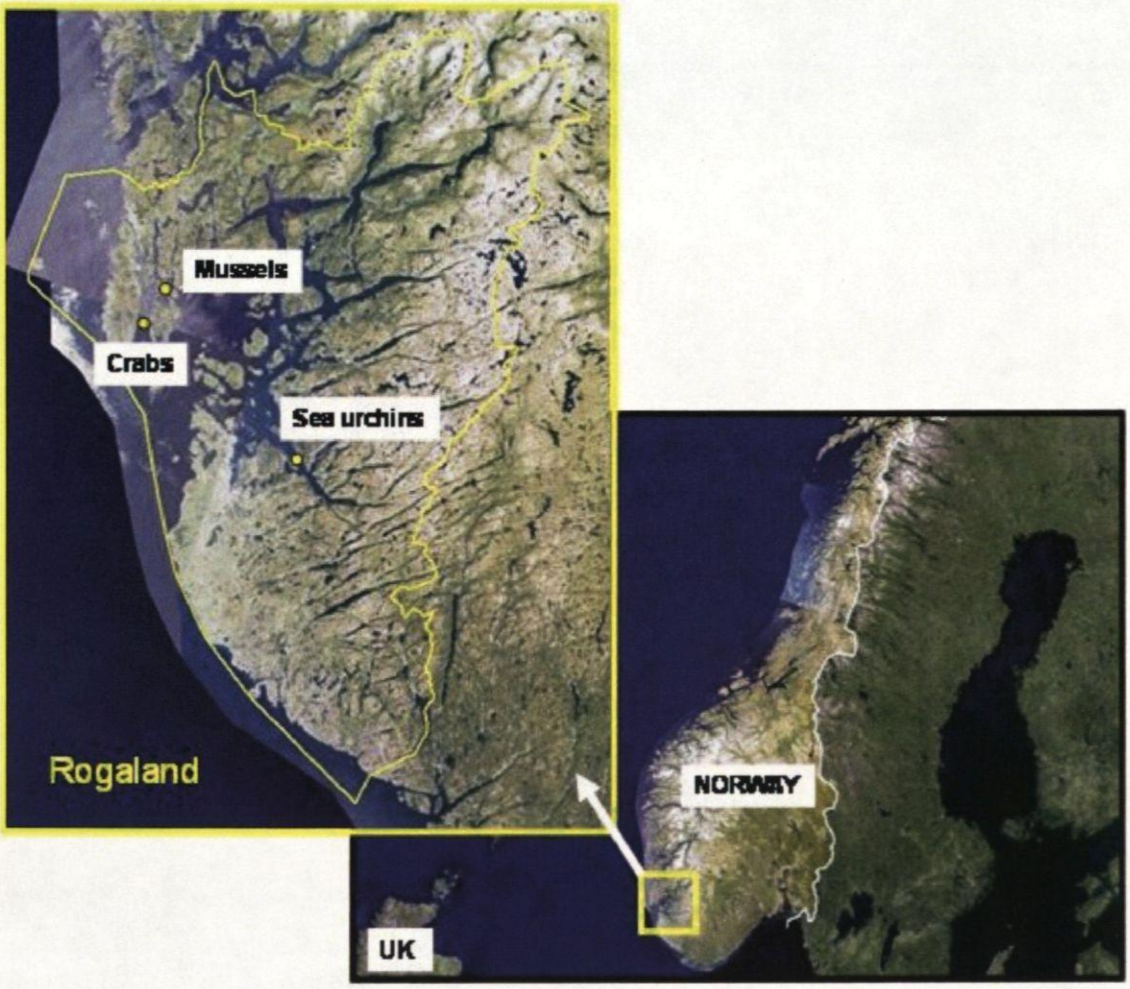


Figure 2-12. Sampling sites for mussels (*Mytilus edulis*) in Førlandsfjorden (Norway), spider crabs (*Hyas araneus*) at Krokaneset (Norway), and sea urchins (*Strongylocentrotus droebachiensis*) in Lysefjorden (Norway) Map photos are from http://norgeibilder.no/.

Spider crabs (*Hyas araneus*) were collected from Karmøy (i.e. Krokaneset), Norway, with traps (Figure 2-13) that were baited with fish (e.g. Atlantic cod), blue mussels (*Mytilus edulis*) were picked from stones below the low water mark (Figure 2-13) in Førlandsfjorden (Norway), while sea urchins *(Strongylocentrotus droebachiensis)* were collected by divers.

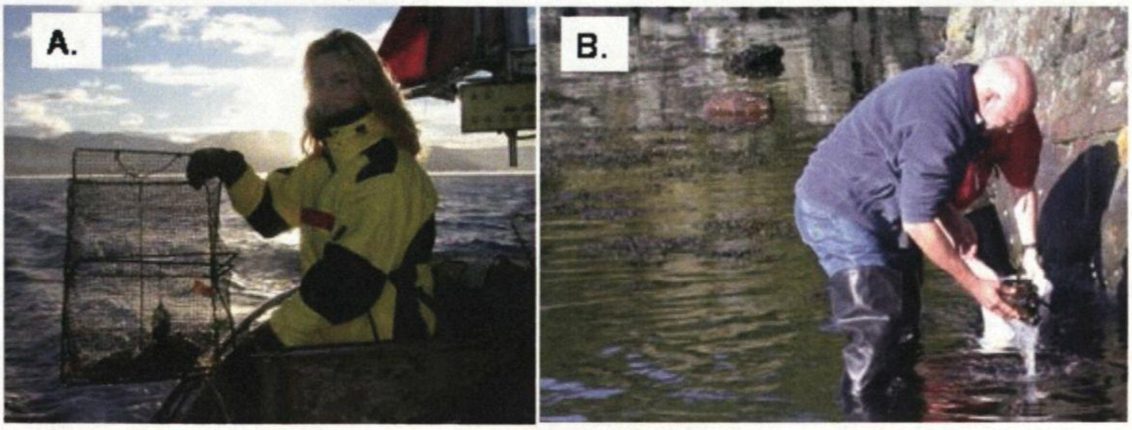


Figure 2-13. (A) Collection of crabs by traps baited with fish, and (B) collection of mussels from stones below the low water mark.

Animals were immediately transported to the laboratory, transferred to clean running sea water (salinity of 34, temperature 10 - 12 °C), and kept isolated for two weeks prior to the exposure experiments. All specimens were fed throughout the acclimation and exposure periods. Crabs were fed raw shrimps (*Pandalus borealus*), mussels were fed a 1:1 mixture of algae (*Isochrysis* and *Rhodomonas* sp.), and sea urchins were fed a combination of raw shrimps (*Pandalus borealus*) and freshly-collected brown algae (*Laminaria sp.*) *ad libitum.* Mussels and crabs were fed daily, while sea urchins were fed 3 times per week. However, the feeding was stopped one day prior to sampling.

2.3 Exposure system (used in laboratory studies)

All laboratory exposures (Chapters 3 - 5) were performed using a continuous flow system (CFS), designed for performing studies of chronic (steady-state) exposures of marine organisms to mixtures of poorly-water soluble chemicals (detailed description provided by Sanni et al. 1998; Baussant et al. 2001). Originally, the system was built to study the impact of oil, however, any chemical, whether water miscible or not, can be tested under long-term exposure in this system. It is a state-of-the-art exposure system for chronic toxicity testing of aquatic organisms. The design is flexible

and can be connected to a wide range of test chambers (e.g. Figure 2-14). The laboratory facility has continuous collection (by pumps) of sea water from 80 meters depth (below the thermocline). The water passes through a sand filter before it reaches the water taps. The CFS system was adapted to fit the requirements for each experiment included in this study, and specific details regarding the exposures are given in Chapters 3 - 5.

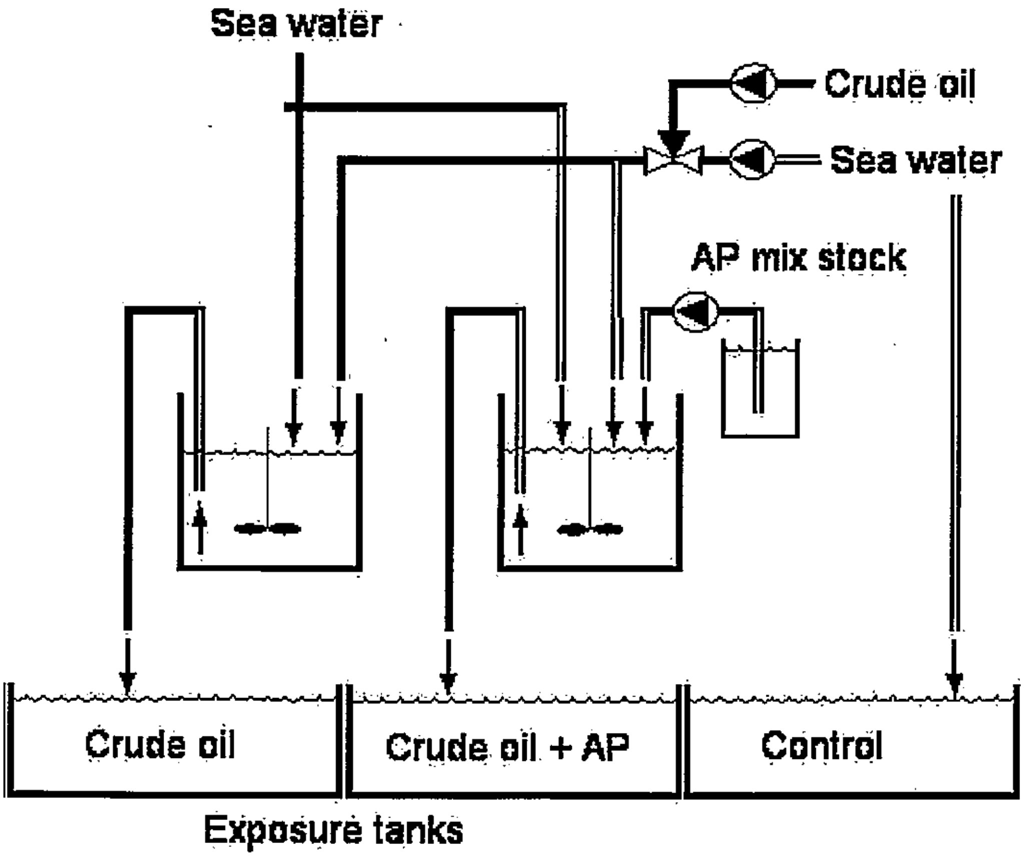

Sea water
Crude oil
Sea water
AP mix stock
Crude oil
Crude oil + AP
Control
Exposure tanks

Figure 2-14. Diagram of how the CFS was used in the first laboratory study (Chapter 3). Figure courtesy of Rolf Sundt, IRIS Biomiljø.

2.4 Laboratory and field experiments

To evaluate the potential of proteomics in ecotoxicology and environmental risk assessment, three laboratory experiments and one field study were undertaken. Two of the laboratory studies (i.e. Chapters 3 and 4), as well as the field validation study (Chapter 6) were conducted as part of the 'BEEP' project (i.e. Biological Effects of Environmental Pollution in Marine Ecosystems) project (http://www.iris.no/Internet/akva.nsf/wvDocID/D9265337DF6A70E4C1256EB90069ABC1) partly funded by European Commission and partly by the Research Council of Norway. The third laboratory study (Chapter 5) was part of a research programme BIOSEA JIP, funded by ENI Norge AS, Total Norge E&P AS and the Research Council of Norway (http://www.iris.no/Internet/student.nsf/5CD78704522281FBC12567F60051ECEE/431C334F2973BFEAC12572570057F7DD?opendocument)

2.4.1 Laboratory study 1 (mixture effects)

In November/December 2002, mussels (*Mytilus edulis*) were exposed for three weeks to either (a) a control of filtered seawater (salinity of 34 and temperature 11 ± 1 °C), (b) 0.5 ppm of dispersed North Sea crude oil (Statfjord B oil), or (c) 0.5 ppm crude oil spiked with 0.1 ppm Alkylphenol (AP) mix.

2.4.2 Laboratory study 2 (species and gender-related effects)

In March/April 2003, mussels (*Mytilus edulis*) and spider crabs (*Hyas araneus*) were exposed for 3 weeks to nominal concentrations of either (a) 50 ppb Diallyl phthalate (DAP, from Fluka EC No 2050163, purity > 98%), (b) 5 ppb 2,2`,4,4`Tetra Bromo Diphenyl Ether (BDE-47, Chiron Product No 1688.12, purity > 95.6%), or (c)

50 ppb Bisphenol A (BPA, Merc, EC No 201-245-8, purity > 97%). Acetone was used as carrier for all components. Concentration of carrier in the exposure units was kept lower than 2 ppb. Control animals received only filtered seawater (salinity of 34 and temperature 11 ± 1 °C).

2.4.3 Laboratory study 3 (Dose-response relationships)

In November/December 2002 Green sea urchins *(Strongylocentrotus droebachiensis)* and blue mussels (*Mytilus edulis*) were exposed for 4 and 5 weeks, respectively, to nominal concentrations of 0, 15, 60, and 250 ppb dispersed North Sea crude oil at water temperatures of 7 ± 0.5°C.

2.4.4 Field validation study (Copper gradient)

In November/December 2003 (*Mytilus edulis*) were collected in Førlandsfjorden (59°20´N, 5°13´E) and then directly transferred to four sites along a fieldgradient in the vicinity of an old copper mine at Visnes (59°22´N, 5°13´E). The mussels were deployed in the coppergradient for 18 days, and then brought to the laboratory the evening before sampling (and kept in filtered, running seawater prior to sampling). Simultaneously, indigenous mussels from Visnes were collected for comparison of results of the caging study. In addition, mussels from Førlandsfjorden were collected at four timepoints (i.e. November 2003, January 2004, March 2004, and May 2004) to indicate the level of seasonal variation in plasma-protein profiles of the mussels.

2.5 Exposure monitoring

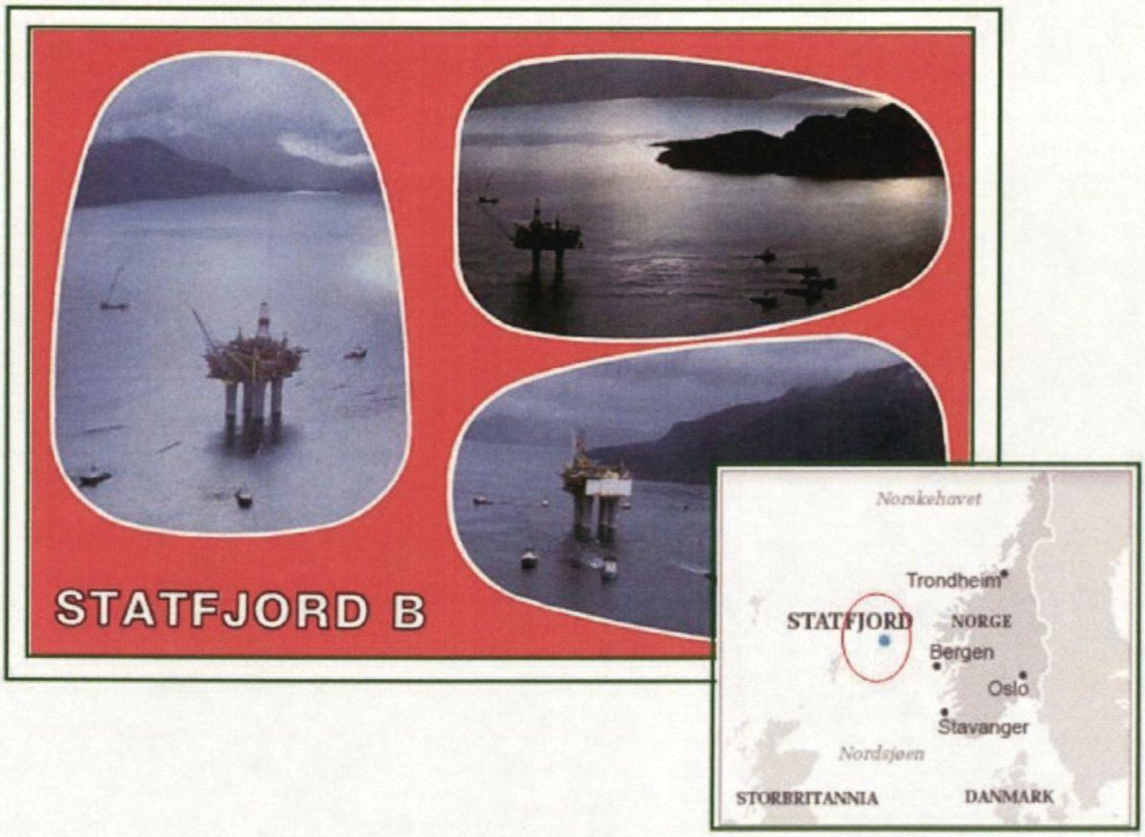
Various methods have been employed for exposure monitoring in the experiments undertaken, including water and tissue concentrations measured by Gas Chromatography connected to a Mass Spectrometer (GC/MS), as well as measurements of particle number and size (i.e. oil droplet size) in water by a Coulter® II particle size analyser. The exposure monitoring is unique for each experiment, and will be described separately in Chapters 3-6. Detailed description regarding exposure monitoring in laboratory study 1 and 2 (Chapter 3 and 4) have also been published in Aquatic Toxicology (Sundt et al. 2006).

2.6 Chemicals

If not stated otherwise, all chemicals were analytical grade and purchased from Sigma-Aldrich.

Chapter 3

Effects of oil and produced-water compounds on protein expression in the mussel *Mytilus edulis*


STATFJORD B
Norskehavet
Trondheim
STATFJORD
NORGE
Bergen
Oslo
Stavanger
Nordsjøen
STORBRITANNIA
DANMARK

Statfjord is one of the oldest producing fields on the Norwegian continental shelf Statfjord B (the source of oil in this study) started production on 5th November 1982, and are still producing roughly 150-160 000 barrels of oil per day.

3.1 Introduction

The increasing number of chemicals released into the environment provides greater potential for impact on terrestrial and aquatic wildlife (IPCS 2002). Since the late 1960s, significant effort has been directed to develop the best possible tools, at different levels of biological organisation, for assessing how these pollutants affect ecosystems and the organisms constituting them. In the early stages of environmental monitoring, the most common approach was to measure physical and chemical variables with the occasional implementation of biological variables (Lam and Gray 2003). With the realisation that some environmental pollutants, so-called endocrine disrupting compounds (EDCs), also caused deleterious effects that were not foreseen when existing chemicals regulations were enacted (e.g. Matthiessen and Johnson 2007) attention moved away from contaminant monitoring to measuring biological effects. In the mid 1980s, biomarkers representing molecular, cellular and physiological changes in an organism following exposure to various types of pollutants (Peakall 1992) emerged as promising and useful monitoring tools. New biomarkers are being developed constantly; however, it has become clear that no single biomarker will serve to indicate the full effect of environmental pollutants (e.g. Galloway et al. 2004). Analogous to diagnosis in human medicine, it is recognised that most pollutant effects will depend upon the determination of suites of responses, rather than any pollutant-specific or disease-specific response.

A new trend in ecotoxicology and biomedical research is the application of so-called 'omics' technologies. These are methods that have the potential to monitor complete classes of cellular molecules such as messenger RNAs, proteins and intermediary metabolites in a single analysis (Morgan et al. 2002; Nicholson et al. 2002; Lau et al.

2003; Botstein and Risch 2003; Clish et al. 2004; Petricoin and Liotta 2004a), compared to traditional analyses that rely on only one endpoint. By allowing simultaneous analysis of thousands of genes, proteins and metabolites, these new global technologies have enabled a wider approach to biological questions, since toxicity generally involves changes not only in a single gene but rather a cascade of gene interactions (Nuwaysir et al. 1999; Aardema and MacGregor 2002). To date, few ecotoxicological studies have utilised ’omics’ technologies. Snape et al. (2004) proposed the term ’ecotoxicogenomics’ to describe the integration of genomics (transcriptomics, proteomics and metabolomics) into ecotoxicology, and defined it as “the study of gene and protein expression in non-target organisms that is important in responses to environmental toxicant exposures”. The authors emphasised the need for ecotoxicology to move towards a more holistic approach which integrated high throughput ’omics’ technologies. Identification of endpoints and responses from such an approach could potentially improve risk assessment through a clearer insight into mechanisms of actions gained by an increased level of revealed information at the molecular level. Improved knowledge regarding cellular control and defence mechanisms will allow a more robust extrapolation between model-species and target species (MacGregor 2003), and reduce uncertainties involved in predicting threshold levels of various types of toxicity. It has also been suggested that genetic variation is the major cause for variation in susceptibility to disease and toxicant exposure variants (Aardema and MacGregor 2002; Ashton et al. 2002; Botstein and Risch 2003), indicating that a certain set of genes or proteins could be used to discover sensitive species and (sub) populations. Furthermore, current environmental risk assessment and regulation of chemicals does not take formal account of any mixture effects which might occur when chemicals are released into the environment (Matthiessen and Johnson 2007), although there is an increasing interest in the assessment of mixture ecotoxicology (e.g. DeLorenzo and Fleming 2008; Jukoskv et al. 2008; Soto et al. 2008). Mixtures are often described to follow one of two models, concentration addition or response addition (e.g. Dardenne et al. 2008). However, mixtures that deviate from these models exist; they typically exhibit phenomena like synergism, ratio or concentration dependency, or inhibition (Dardenne et al 2008). At present, mixture-effects have mainly been evaluated for acute toxicity responses (e.g. Manzo et al. 2008; Escher and Hermens 2002) and there is a general lack of information on modes/mechanisms of toxic action or of mechanisms of toxicity interactions (McCarty and Borgert 2006). Genetic responses are considered to be the primary reaction in case of toxicant exposure and carry valuable mechanistic information; hence analysis of gene, protein and metabolite expression could provide valuable insight into mixture-toxicity-modes of action.

The aim of the present study was to evaluate the effects of crude oil alone, and oil in a mixture with other toxic compounds, on protein expression in mussels (*Mytilus edulis*). Mussels were exposed to environmentally-relevant concentrations of oil alone, or oil spiked with short-chained alkylphenols (APs) and extra polycyclic aromatic hydrocarbons (PAHs). The spike was made to reflect the composition of APs and PAHs found in produced water from offshore platforms and installations in the North Sea oil fields. Produced water, a by-product from oil and gas production, is a highly complex mixture of water, dispersed oil (microdroplets of oil in water) and chemicals. Trace amounts of oil, PAHs, and APs are only some of the compounds present, however, the aromatic compounds are assumed to be the most important contributors to toxicity (Utvik 1999). Various effects of oil and produced-water compounds have been reported for marine organisms, including effects on reproduction (Krause 1994; Giesy et al. 2000), development (Baldwin et al. 1992), genotoxicity (Harvey et al. 1999; Aas et al.

2000; Taban et al. 2004; Bolognesi et al. 2006, Barsiene et al. 2006b), metabolism (Narvia and Rantamaki 1997; Martin-Skilton et al. 2006; Jonsson et al. 2004; 2008), steroid levels (Lavado et al. 2006) and alterations in protein expression (Gimeno et al. 1998; Hasselberg et al. 2004; Gomiero et al. 2006; Monsinjon et al. 2006; Sturve et al. 2006). In spite of much effort, knowledge of the fate and effects of effluents related to oil and gas production is limited, indicating a need for continued research, as well as an evaluation of appropriate assessment tools. In the present study, therefore, the influence of environmental pollutants on plasma protein expression in mussels has focussed on the particular protein forms either induced or suppressed following exposure. The hypothesis was that exposing mussels to oil alone, or oil spiked with APs and extra PAHs would result in unique combinations of expressed protein species that could be indicative of either exposure or effect.

3.2 Material and methods

Blue mussels (*Mytilus edulis*) were colleted in late October 2002 in Førlandsfjorden (Figure 2-12). Following two weeks acclimation, they were exposed in the continuous flow system shown in Figure 2-14 for 3 weeks, to nominal concentrations of either 0.5 ppm dispersed North Sea crude oil (i.e. Statfjord B oil) or 0.5 ppm oil spiked with a mixture of 0.2 ppm APs and 0.1 ppm extra PAHs (nominal concentrations); control mussels received only filtered seawater (salinity of 34, 10-12°C). Oil dispersions were made mechanically by passing oil and seawater through a high pressure mixing valve. The composition of the AP and PAH mixtures used in the spike, as well as the composition of PAHs in Statfjord B oil, are listed in Tables 3-1 and 3-2. The spike was made by mixing equal amounts of the two solutions. Acetone (grade > 99.5) was used as a carrier, and high precision peristaltic pumps (model "33", Harvard

Apparatus, Massachussets, USA) were used to ensure correct doses in the exposure tanks. The spike was added to the oil emulsion via a 5 litre mixing flask with strong spin-bar mixing. This flask fed a diluted spike solution into the oil emulsion. The acetone carrier enhanced the dissolution of APs in water.

Table 3-1. The PAH composition recorded in Statfjord B crude oil and some characteristics of the PAH molecules.

Compound/quantity	**Statfjord µg/g oil**	**Mass (M/Z)**	**Log K_{ow}**
Naphthalene	1147,0	128,2	3,3
C1-Naphthalenes	3787,3	142,2	3,9
C2-Naphthalenes	5288,7	156,2	4,4
C3-Naphthalenes	3830,3	170,2	4,9
Acenaphthylene	10,0	152,2	4,1
Acenapthene	9,7	154,2	4,0
Fluorene	135,9	166,2	4,2
Anthracene	252,9	178,2	4,6
Phenanthrene	0,0	178,2	4,6
C1-Phenanthrenes	460,2	192,2	5,1
C2-Phenanthrenes	439,4	206,0	
Dibenzothiophene	91,9	184,2	4,4
C1-Dibenzothiophene	196,5	198,2	
C2-Dibenzothiophene	232,9	212,2	
Fluoranthene	2,6	202,0	5,1
Pyrene	8,6	202,0	5,1
Chrysene	23,9	228,2	5,7
C1-Chrysene	37,9	242,2	
C2-Chrysene	41,5	256,2	
Benzo(a)antracene	3,3	228,2	5,7
Benzo(b)fluoranthene	7,7	252,3	6,4
Benzo(k)fluoranthene	0,0	252,3	6,5
Benzo(b+k)fluoranthene	6,9		
Benzo(a)pyrene	4,7	252,3	6,3
Indeno(1,2,3,cd)pyrene	0,0	276,3	6,9
Benzo(g,h,i)perylene	1,7	276,3	7,0
Dibenzo(a,h)antracene	0,0	278,3	6,7
Sum PAH	16020,9		
Sum PAH in 1ppm dose (µg)	16,0		

Note. Log Kow is the logarithm of the –octanol to water coefficient Kow. LogKow values on alkylated PAH are hard to find. A thumb rule is to add from 0.3 to 0.5 log units per methyl group added, see also http://logkow.cisti.nrc.ca. The 1 ppm oil dosage would equal a quantity of 1 mg oil/kg seawater.

Table 3-2. Composition of APs and PAHs used in the spike.

PAH mix		AP mix	
Compound	**Gram/L Acetone**	**Compound**	**Gram/L Acetone**
Naphthalene	7,649	p-cresol	33,8
C1-Naphthalenes	13,792	m-ethylphenol	6,54
C2-Naphthalenes	14,42	3,5-dimethyllphenol	6,54
C3-Naphthalenes	7,669	2,4,6-trimethylphenol	3,8
Fluorene	0,867	2-tert-butylphenol	0,236
Phenanthrene	0,779	3-tert-butylphenol	0,236
C1-Phenanthrenes	1,264	4-n-butylphenol	0,236
C2-Phenanthrenes	1,089	4-pentylphenol	1,62
Dibenzothiophene	0,167		
C1-Dibenzothiophene	0,363		
C2-Dibenzothiophene	0,347		
Sum	**48,405**	**Sum**	**53,01**

3.2.1 Exposure monitoring

Exposures were monitored by repeated (n = 15 during the course of the experiment) analyses of the average oil droplet size and number by a Coulter® II particle size (and number) analyser equipped with a 70 mm aperture tube. Oil concentrations were calculated from the estimated particle size and number of particles in the water. Semi-quantitative fluorescence analysis of total hydrocarbon concentration (THC) in the water was measured (as described in Aas et al. 2000) five times during the exposure period. Additionally, THC in water was measured twice by Gas Chromatography (HP5890, Hewlett Packard, USA) connected to a Mass Spectrometer (Finnigan SSQ7000, USA) and analyzed in selected ion mode (GC/MS-SIM) as described in Baussant et al. (2001).

3.2.2 Sample collection

Haemolymph samples from mussels were collected following the method described in Chapter 2 (i.e. 2.1.1) and Figure 2-1. Seventy mussels (shell size 7.5 ± 0.7 cm) were sampled and analysed per treatment.

3.2.3 Sample preparation on ProteinChip arrays

ProteinChip arrays with weak cation exchange surfaces properties (WCX-arrays) were used for protein expression analyses and the protocol outlined in Table 2-7 was used for sample preparation.

3.2.4 SELDI TOF MS analysis

The protein arrays were analysed immediately (after sample preparation and incubation) on a PBS-IIc time of flight mass spectrometer using ProteinChip Software version 3.1. Mass spectra were recorded on the following settings: 91 laser shots/ spot surface in a positive ionisation mode (65 of the shots were collected, starting at position 20 and ending at position 80 of the spot), laser intensity 214, detector sensitivity 8, detector voltage 2900 V, data acquisition from 0 - 180000 Da, and optimum mass range focus from 2500 – 15000 Da. Given the time of flight, the known length of the tube and voltage applied, the mass-to-charge ratio (M/Z value) for each mass peak was estimated automatically. The mass spectra consist of the sequentially recorded number of ions arriving at the detector (the mass peak height or relative intensity) coupled with the corresponding M/Z values. The PBS-IIc instrument was externally calibrated with bovine insulin (5733.58 Da) and bovine IgG (147300.0 Da) mass standards (Ciphergen Biosystems).

3.2.5 Data handling and statistics

Preliminary data processing was done in the Biomarker Wizard feature of ProteinChip Software (version 3.1). Mass spectra from all treatment groups were imported into one experimental file. Baseline subtraction was performed and the spectra were mass aligned using three M/Z values prominent in all spectra (4036 Da, 12470 Da and 27402 Da). Further data processing and biomarker discovery, with uni- and multivariate statistics, were done according to the description given in Chapter 2 (i.e. 2.1.4). Furthermore, data sets for multivariate decision tree classification (with Biomarker Pattern^{TM} Software) were separated randomly into a training set and a test set before analysis. Fifty samples from each treatment group were used to build the classification models, while 20 samples were kept for blind testing of the test models.

3.3 Results and discussions

3.3.1 Chemistry

The average oil concentration in water based on 15 multisizer measurements was 0.56 ± 0.19 ppm in the tanks receiving only oil, and 0.61 ± 0.26 ppm in the tanks with spiked oil. Semi-quantitative fluorescence analysis of THC concentrations in the water showed that water from the oil treatment had an average (n = 5) THC of 0.25 ± 0.04 ppm, while the average concentration in water from the spiked oil treatment was 0.36 ± 0.07 ppm. Total PAH by GC/MS-SIM was only measured for the oil treatment, and average (n = 2) PAH concentration, converted to THC, was 0.38 ± 0.07ppm.

There is no available method for measuring concentrations of APs in water or biota. Sundt and Baussant (2003) cited the following log K_{ow} (partitioning coefficient) for some of the substances: 4-tert-butylphenol (3.04 – 3.31), 4n-pentylphenol (unknown), 4n-hexylphenol (3.60), 4n-heptylphenol (4.00), while Shiu et al. (1994) reported log K_{ow} for p-cresol to be (1.62 – 2.06). The APs will thus have a water solubility ranging from somewhat more than naphthalene to somewhat less than C1-naphthalene, and a liability for uptake in the mussels ranging from somewhat less than naphthalene to somewhat more than C1-naphthalene (see Table 3-1 for comparisons). A conservative estimate of the water-borne exposure would therefore be that ca 80% to possibly ca 95% of the APs were available for uptake from the spike based on how the concentrations of naphthalene and C1-naphthalene were measured in the water (Skadsheim personal observations), giving a total of between 0.16 – 0.19 mg alkylphenols/litre seawater in the spike exposure.

3.3.2 Proteomic response

Profiling of mussel plasma on WCX ProteinChip arrays revealed 2287 distinct peaks and corresponding M/Z values when data were collected at the highest sensitivity. In addition to real peptide and protein peaks, the unfiltered mass spectra contained electronic noise as well as chemical noise due to the ionisation matrix used. Further analysis of data was restricted to those M/Z values with a signal to noise greater than or equal to 5, present within the mass range of 2500 to 180000 Da. Analysis of 70 samples (35 males and 35 females) from each treatment yielded 149 peaks with M/Z values between 2716 and 159764 Da that met the abovementioned criteria. Among the 149 qualifying peaks, 90 had M/Z values between 2500 and 10 000 Da, 30 were between 10 000 and 20 000 Da, and 31 peaks had M/Z values greater than 20 000 Da. The dominance of low-molecular weight protein forms is in accordance with what has been reported for medical studies utilising the same proteomic technology (Petricoin et al. 2002; Li et al. 2002; Rogers et al. 2003; Conrads 2004). Information regarding the

difference between exposed mussels and controls, however, was present throughout the entire M/Z region studied.

In general, results indicated that exposure to spiked oil had a more significant effect on protein expression in mussels than oil alone, indicating an added effect of APs and PAHs in the oil-mixture (Table 3-3). While 83 of the detected proteins were significantly ($p < 0.05$) altered by spiked oil, 49 were altered by oil. Both exposure regimes had a predominantly upregulating effect on protein expression in mussel plasma. When only significantly altered peaks were considered, 68 % were upregulated in both exposure groups. Average changes in protein expression (as the ratio exposed/control) were less than two-fold for mussels exposed to both oil (1.6 fold) and spiked oil (1.9 fold) when all detected peaks were compared (Table 3-3).

Table 3-3. Overview of number of significantly ($p < 0.05$, Mann-Whitney-Wilcoxon test) upregulated (Up) and downregulated (Down) peaks in the two exposure regimes.

Exposure	Up	Down	Σ	%
Oil	33	16	49	33 (1.6)
OAP	56	27	83	56 (1.9)
n = 149 resolved peaks with S/N ≥5				

Note. Σ = total number of significantly altered peaks, S/N = signal-to-noise ratio, % = percentage of peaks that were significantly altered by exposure. OAP = spiked oil. Average fold change in expression is given brackets.

Examination of differences between exposed organisms and controls revealed a complex response patter. Examples of different types of responses are given in Table 3-4, while the complete list of significantly altered mass peaks is given in Table 3-5.

Table 3-4. Examples of complex response pattern in mussels exposed to oil and spiked oil (OAP).

M/Z (Da)	oil - ♀	oil - ♂	OAP - ♀	OAP - ♂	Significant response in
46134	↑	↑	↑	↑	all groups
44988	↑	ns	↑	↑	3 groups
78669	ns	↓	↓	↓	
10834	↓	↓	↓	ns	
3842	↑	ns	ns	ns	only oil exposed
4359	ns	↓	ns	ns	(females, males or both)
6302	↓	↑	ns	ns	
23390	ns	ns	ns	↑	only OAP exposed
27119	ns	ns	↓	ns	(females, males or both)
135202	ns	ns	↓	↓	
4047	ns	↑	ns	↑	males only
5653	ns	↓	ns	↓	
10132	ns	↓	ns	↑	
5481	↑	ns	↑	ns	females only
7048	↓	ns	↓	ns	
3952	↓	↑	ns	ns	females and males;
23690	ns	ns	↓	↑	opposite response

Note. ↑ = up-regulation, ↓ = down-regulation, ♀ = females, ♂ = males, ns = not significant response.

For example, when each treatment group was divided into females and males for comparisons of responses, only one peak (i.e. M/Z 46134 Da) was significantly altered in all 4 groups (oil females, oil males, spiked oil females and spiked oil males) (highlighted in Table 3-5 part C). Furthermore, eight peaks were affected (6 up-regulated and 2 down-regulated) in 3 of the exposure groups, however, not all of them in the same 3 groups (Table 3-5).

Table 3-5 Shows all mass peaks that were significantly ($p < 0.05$) changed in one or several exposure groups. The table indicate the size (Da) of the altered peaks, as well as where (i.e. exposure group) and how (up or downregulated) it was altered. Because of the size of the table, it has been divided into part A, B and C. OAP = spiked oil, F = females, M = males, Up = upregulated, and Down = downregulated.

Part A		Significant ($p < 0.05$) response							
Peaks (Da)		Oil-F		Oil-M		OAP-F		OAP-M	
		Up	Down	Up	Down	Up	Down	Up	Down
3058									3058
3154								3154	
3692						3692			
3770		3770				3770		3770	
3842		3842							
3887				3887					
3952			3952	3952					
3973				3973		3973			
4021								4021	
4047				4047				4047	
4061								4061	
4099									4099
4113		4113				4113			
4155					4155	4155			
4185								4185	
4213						4213			
4359					4359				
4402								4402	
5333						5333			
5423				5423					
5481		5481				5481			
5613		5613							
5653					5653				5653
5923		5923							
6089						6089			
6198						6198			
6248						6248			
6302			6302	6302					
6516									6516
6751					6751				
7017				7017			7017		
7048			7048				7048		
7336								7336	
7406		7406				7406		7406	
7479				7479					
7558							7558		
7640						7640			
8025						8025			
8378								8378	
8492				8492					
8604					8604				

Table 3-5 Continues (PART B).

Part B		Significant ($p < 0.05$) response							
Peaks (Da)		**Oil-F**		**Oil-M**		**OAP-F**		**OAP-M**	
		Up	Down	Up	Down	Up	Down	Up	Down
8797						**8797**		**8797**	
8881		**8881**							
8958		**8958**				**8958**		**8958**	
9091		**9091**		**9091**		**9091**			
9169					**9169**			**9169**	
9660						**9660**			
9793								**9793**	
9868		**9868**				**9868**		**9868**	
9941								**9941**	
10015					**10015**				
10132					**10132**			**10132**	
10467				**10467**				**10467**	
10544								**10544**	
10744					**10744**				
10834			**10834**		**10834**		**10834**		
10934								**10934**	
11026				**11026**				**11026**	
11114				**11114**				**11114**	
11809				**11809**				**11809**	
12109						**12109**		**12109**	
13417									**13417**
13726									**13726**
14176							**14176**		
14715								**14715**	
16845			**16845**						
17672		**17672**				**17672**			
17818		**17818**				**17818**			
17990		**17990**				**17990**			
18282						**18282**			
18546						**18546**			
19494		**19494**							
19993						**19993**			
20950								**20950**	
21169				**21169**				**21169**	
21731							**21731**		
22344								**22344**	
23390								**23390**	
23690							**23690**	**23690**	
27119							**27119**		
27479							**27479**		
27931							**27931**		
28402							**28402**		
41398		**41398**							
44019		**44019**				**44019**			

Table 3-5 Continues (PART C).

Significant ($p < 0.05$) response									
Peaks (Da)		**Oil-F**		**Oil-M**		**OAP-F**		**OAP-M**	
PART C		Up	Down	Up	Down	Up	Down	Up	Down
44988		**44988**				**44988**		**44988**	
46134		**46134**		**46134**		**46134**		**46134**	
55403							**55403**		**55403**
64580		**64580**							
69213							**69213**	**69213**	
78669					**78669**		**78669**		**78669**
82461									**82461**
97676									**97676**
107566							**107566**		**107566**
135202							**135202**		**135202**
159764									**159764**

While certain protein features were affected by only one exposure condition, others responded to both exposures, (Table 3-5 and Figure 3-1A) where the response pattern could be either similar or opposite with regard to induction or suppression (e.g. Table 3-4). Some protein forms were affected in only one gender (e.g. Figure 3-1 B), and some revealed opposite responses in males and females (e.g. Tables 3-4 and 3-5).

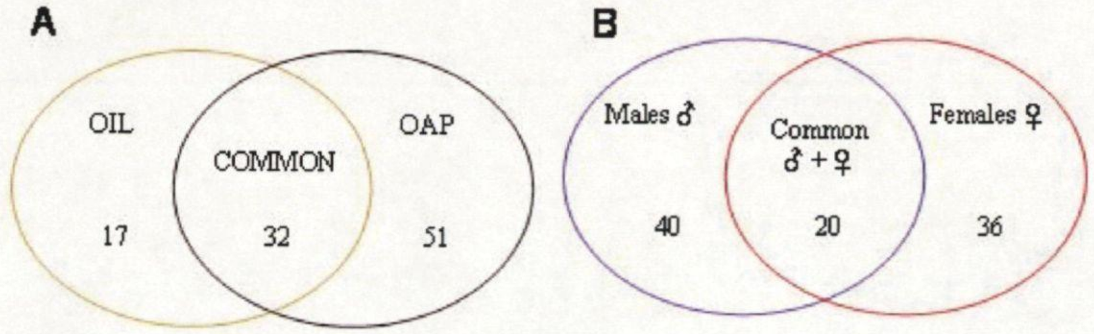


Figure 3-1. Treatment-specific (A), and gender-specific (B) responses. Each ring contains the number of proteins significantly ($p < 0.05$) altered only by the indicated groups. The overlapping areas indicate the numbers of proteins that were affected by both groups. OAP = spiked oil. Data in (A) illustrate male and female responses combined.

3.3.3 Treatment-specific response

Pollutants with different chemical features are likely to affect distinct physiological or biochemical processes through binding to endogenous structures. Depending on which molecules or mechanisms are affected the impact will, theoretically, give rise to patterns of changes that are specific to the various pollutants. Zachariassen et al. (1991) reported that a range of physiological parameters responded differently in mussels according to which pollutant the mussels were exposed to and suggested that the combinational use of these physiological parameters might serve as a pollutant-specific fingerprint in environmental monitoring. Results from the present study support this hypothesis, as mussels exposed to oil alone revealed a totally different plasma protein expression pattern from mussels exposed to oil spiked with APs and extra PAHs. For example, 17 mass peaks were significantly altered only in samples from oil exposed mussels, while 51 peaks showed specific response to spiked oil (Figure 3-1 A). Although common responses were also observed in the two treatment groups, the degree of response was markedly higher in the spiked oil group for 25 out of 32 peaks that were significantly altered by both treatments. Pollutant-specific changes in protein expression have also been observed in, for example, mussel gills (Shepard and Bradley 2000), in mussel digestive gland (Rodriguez-Ortega et al. 2003; Knigge et al. 2004; Monsinjon et al. 2006), in mussel peroxisomes (Apraiz et al. 2006; Mi et al. 2007), in crab hepatopancreas (Gomiero et al. 2006), in fish liver (Shrader et al. 2003), in fish plasma (Larsen et al. 2006), and in fish gills (Hogstrand et al. 2002).

Since the pollutants used in this study were complex mixtures, it is not easy to determine which components contributed most to the observed effects. However, if robust protein patterns are identified for specific chemicals and mixtures, these patterns may be used to identify a pollutant in cases where its identity is not known. To test this hypothesis, the present data (the 149 mass peaks and corresponding M/Z values) were exported to Biomarker Pattern^{TM} Software for identification of potential multivariate patterns classifying exposed from control mussels. Two comparisons were made, control vs. oil and control vs. spiked oil. All mass spectra were divided into training sets (to create prediction models) and test sets (for subsequent blind-testing of the models). For comparison of control vs. oil, 9 distinct models were generated. The best performing model, utilising a combination of 11 mass peaks, classified randomised blind-samples with 90% sensitivity (correct classification of exposed) and 90% specificity (correct classification of controls). The best prediction model for comparison of control vs. spiked oil needed 10 peaks to predict controls with 95% accuracy, and individuals from the spiked oil treatment with 90% accuracy (Table 3-6).

Table 3-6. Prediction models (generated with Biomarker Pattern^{TM} software) for classification of controls vs. exposed. Inputs are the 149 mass peaks with S/N ≥5.

Groups compared	**Prediction success (%), model: n = 100**		**Prediction success (%), test of model: n = 40**		**Classifiers - M/Z (Da)**
	Control	Exposed	Control	Exposed	
Control vs. oil	100	98	90	90	6302 (↑) 3973 (↑) 5423 (↑) 6815 (↑) 11301 (↓) 4061 (↑) 5333 (↑) 3887 (↑) 159763 (↓) 8958 (↑) 9091 (↑)
Control vs. OAP	100	100	95	90	69213 (↓) 3692 (↑) 3810 (↑) 3770 (↑) 4213 (↑) 4185 (↑) 13057 (↓) 4021 (↑) 4300 (↑) 10544 (↑)

Note. Importance of classifiers is decreasing from top to bottom (i.e. M/Z 6302 Da and M/Z 69213 Da being the most important peaks for the classification of mussels exposed to oil or the OAP mixture, respectively. OAP = Oil spiked with alkylphenols and PAHs, ↑ = up-regulated, ↓ = down-regulated.

Data were also tested to see if it was possible to generate good prediction models that could distinguish controls from exposed individuals as well as discriminate individuals from the two exposure groups. The best classification model was able to classify all 3 groups with >80% accuracy (i.e. control = 90%, oil = 80% and spiked oil = 85%). Although the prediction success for the masked test samples in the present study was good, it is not certain that another set of samples from mussels exposed to oil and spiked oil collected at, for example, a different time of the year, would perform equally well if tested on the same prediction model. It is important to bear in mind the dynamic nature of the proteome. For example, different biomarker profiles are found at the onset of a disease versus the late stage where symptoms and indirect effects are prominent (Van der Greef et al. 2004). Similarly, factors like seasonal changes, reproductive cycles, nutrient availability, age etc. are likely to affect protein expression in an organism. Thus, an ideal protein biomarker profile for environmental pollutant monitoring should consist of a subset of proteins that are robust against external factors other than the one under investigation.

3.3.4 Gender-specific responses

Protein expression was altered differently in males and females following exposure. For example, M/Z 3952 Da, 6302 Da, 7017 Da, 23690 Da, and 69213 Da were down-regulated in females and up-regulated in males, while M/Z 4155Da was up-regulated in females and down-regulated in males (Table 3-5) Another 40 peaks were significantly altered only in males, 9 of which showed similar response to each treatment, 9 that responded only to oil exposure and 22 that responded only to the spiked oil treatment (Table 3-5) Similarly, 36 peaks were affected only in females, 8 by both exposures, 8 by oil and 20 by spiked oil. Common response in males and females were observed for 20 peaks (Table 3-5).

An attempt was made to create a prediction model for classification of mussels from 6 different treatment groups (i.e. mussels from the 3 treatment groups divided into males and females). The best model included 31 M/Z values as classifiers and managed to predict blind samples of control males and females, oil-exposed males, and females exposed to spiked oil with 80% accuracy. While 70% of the oil-exposed females were classified correctly, 30% were misclassified as control males. Only 60% of males exposed to spiked oil were correctly classified. The remaining 40% were misclassified as control females. However, when the number of classifiers are so high, there is a great risk of overfitting the data (e.g. Lay et al. 2006) leading to false results. The most trustable models are those with only 2-3 classifiers, unless the sample group is sufficiently large (Lay et al. 2006). Furthermore, without knowledge of the protein identity and function, it is difficult to interpret the functional relevance of these findings. For example, are gender-specific responses an indication of endocrine disruption? Oil-production related effluents, PAHs and particularly APs have been associated with endocrine disruption and the estrogenic activity of APs in fish has been well established both in vitro and in vivo (White et al. 1994; Nimrod and Benson 1996; Arukwe et al. 2000, 2001). Modulated steroid metabolism in echinoderms (Den Besten et al.1993), as well as effects on moulting and reproduction in male grass shrimps, and offspring of exposed mothers (Oberdorster et al. 2000), has been observed following exposure to PAHs. Krause et al. (1994) found that the reproduction of caged sea urchins was impaired up to 100 metres from a produced water outfall. Similarly, the reproductive performances and growth rate of caged mussels were disrupted between 100 and 1000 metres from an active produced water discharge (Osenberg et al. 1992). Negative effects of gonadal development in mussels from the present exposure study, as well as an induction of vitellogenin-like proteins in both males and females have been reported by Aarab et al. (2004). However, even though both oil and compounds in the spiked oil mixtures clearly have the potential to interfere with endocrine functions, the explanation for gender-specific responses may be another than endocrine disruption. Alternatively, males and females may have different susceptibility to one or several of the different toxicities potentially induced by oil, or oil in combination with APs and PAHs; this remains to be investigated.

Finding the function and full identity of the discriminating protein and peptide features was beyond the scope of this study. Nevertheless, it is important to link key molecules and their functions to provide essential mechanistic information of the underlying toxicity, as well as to provide a basis for antibody production of a set of biomarkers as robust alternatives or complements to single endpoint biomarkers. Despite the precision of the mass information yielded by the SELDI technique, the analysis does not directly provide a sequence-based identification. Furthermore, information regarding how post-translation modifications might have altered the protein size is not provided. Additional effort is therefore necessary to reveal the true identity of the protein(s) of interest. Several biomedical studies have identified key proteins discovered utilising ProteinChip® array technology in combination with SELDI TOF MS (e.g. Thulasiraman et al. 2001; Zhang et al. 2002; Diamond et al. 2003; Sanchez et al. 2004). A detailed description of methodology for protein purification and characterisation that is applicable to ProteinChip arrays is described in Caputo et al. (2003).

3.3.5 Gender-specific protein patterns

Grouping all 210 samples by gender, independent of exposure, identified 64 distinct mass peaks differing between the two genders with a p-value less than 0.05. The number of female-specific and male-specific peaks was identical (i.e. 32). These peaks were predominant in one of the genders in all treatment groups. Examples of gender-specific peaks are shown in Figure 3-2. For example, M/Z 5866 Da revealed a 25 fold higher expression in males when samples were compared as either males or females independent of exposure. Another peak, M/Z 5970 Da, primarily expressed in females, was also induced in males exposed to spiked oil.

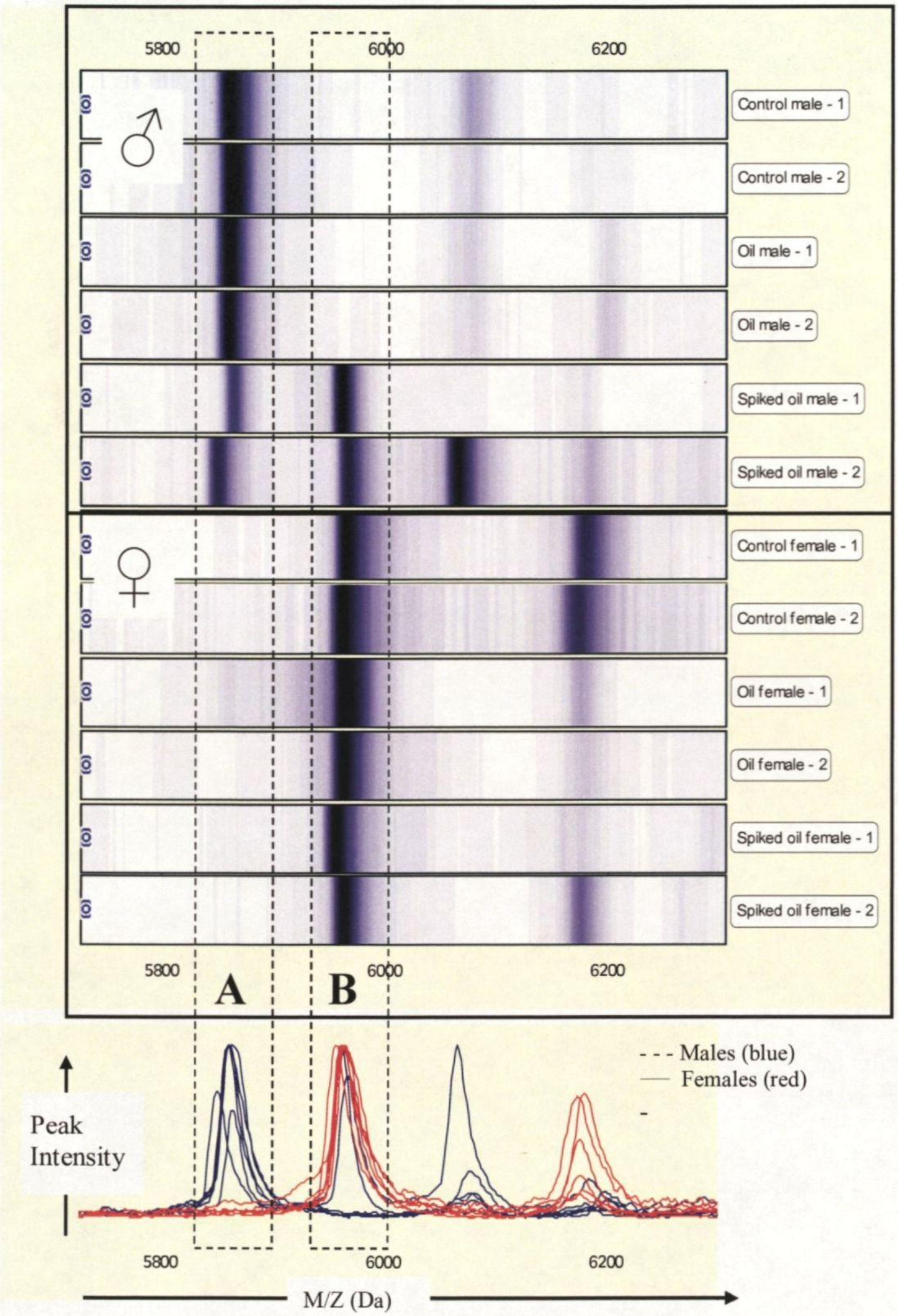


Figure 3-2. Gender-specific protein forms. Partial protein expression profile showing examples of mass peaks primarily expressed in either females or males; visualised as "gel-view" (top) and "spectra-overlay" (bottom). A: M/Z 5866 Da, 25 fold up-regulated in males when males and females are compared independent of exposure. B: M/Z 5970 Da, expressed in females (3.5 fold up-regulated in average) and males exposed to spiked oil.

Classification tree analysis showed that no single protein or peptide peak was able to completely separate the two genders (Figures 3-3 and 3-4). When data from 150 plasma samples were used as inputs for analysis with Biomarker Pattern^{TM} software, the software created 4 different classification models (i.e. the blue squares in Figure 3-2) based on 1- 6 peaks, respectively (for classification of males versus females). The software automatically highlights the 'best' model, which is usually the one with the least number of nodes (classifiers) in combination with the lowest 'relative cost'. The 'relative cost' is a measure of the ration between the misclassification rate and the number of nodes (classifiers/peaks) in the classification tree. Thus, the best model (algorithm) will be the one that achieves the relative best classification of the dataset using the lowest number of nodes. The output of this evaluation is given as the cost value, and hence a low cost value indicates a good classification model (Ciphergen Biosystems).

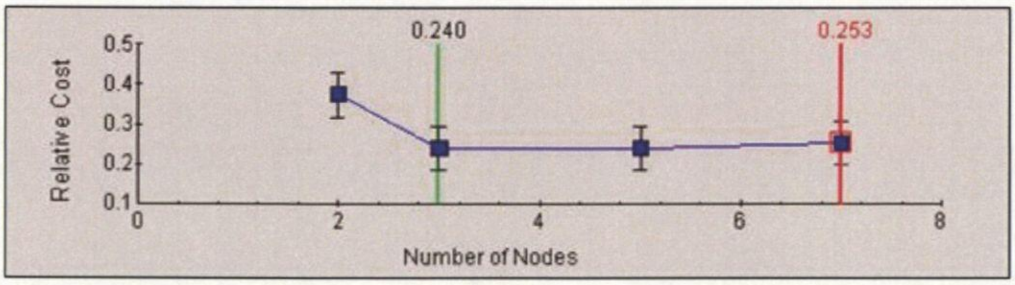


Figure 3-3. Classification models created with Biomarker Pattern^{TM} software when data from 150 plasma samples (grouped by gender independent of exposure) were fed to the software. Each of the blue squares represent a classification model with a certain number of predictors/classifiers (indicated by the number of nodes on the x-axis) needed for accurate classification of test samples. The relative cost (y-axis) indicates how accurate the model is.

The best model for gender determination (i.e. green line in Figure 3-3) used a combination of only 2 peaks at M/Z 5866 Da and 7853 Da respectively to classify males with 92 % and females with 85% accuracy. The model is illustrated in Figure 3-4.

3.3.6 Reproducibility

A great challenge with any new method is to ensure that it is reproducible and, hence, reliable. To monitor the reproducibility between ProteinChip arrays in the present study, two control samples were divided into three replicates that were incubated and analysed on three different arrays (Figure 3-5). The coefficient of variance (CV) calculated for 149 M/Z values (between 2.5 and 180 kDa) in the two samples varied between 0.6 – 7.9% in sample 1 and between 0.7 – 13.3% in sample 2. CV calculations were performed before mass and intensity normalisation of spectra. Results revealed that spot-to-spot variability for a sample is much less than the individual variability (see Figure 3-5, comparison of 'gel-views' from control sample 1 and 2) emphasising the need for large sample groups in order to obtain robust (statistical) results.

3.3.7 Use of (prote)omic data in risk assessment

Any effective environmental management strategy is dependent on accurate risk assessment to minimise potential harmful consequences of man-made and natural environmental impacts on ecosystem and human health. There is general agreement that current procedures, including determination of physical and chemical variables or measurements of whole-organism responses (e.g. mortality, growth, reproduction) of generally sensitive indicator species, have limited ability to predict the likely adverse effects of anthropogenic pollutants or activities on complex ecosystems and their components (Peakall 1992; Galloway et al. 2004a; Galloway et al. 2004b; Moore et al. 2004; Snape et al. 2004). Although such approaches are useful for identifying chemicals of potential concern, selected endpoints should also include those capable of evaluating the sublethal toxicity, chemical mode(s) of action, critical "exposure windows", threshold levels of various types of toxicity (e.g. immunotoxicity, genotoxicity, endocrine disruption), variation in response and susceptibility, effect of various factors like genetics, gender, age, diet, reproductive cycles etc. (Schlenk 1999; Gibb et al. 2002; MacGregor 2003; Moore et al. 2004).

Currently, there is no single method available that covers all the factors identified in the previous section. A multidisciplinary approach is, therefore, necessary to provide a more holistic understanding of the fate and effects of chemicals, and hence a more accurate prediction of potential risks. Including proteomics and other 'omics' technologies in existing risk assessment approaches has many advantages. For example, it would allow high throughput screening of potential changes in thousands of cellular molecules simultaneously and, hence, provide a more rapid evaluation of a chemical's toxic potential. As changes in cellular molecules are thought to precede toxic outcomes, the response patterns could be used to predict toxic responses at an early stage for a wide variety of species. Cunningham et al. (2003) suggested two possible approaches to incorporate 'omic' data in (eco)toxicology: (1) a target approach in which one assesses the expression levels of key biochemical pathways identified a priori, and (2) a "shotgun" approach in which gene (protein and metabolite) expression profiling, coupled with bioinformatics techniques, is used to identify those key pathways.

Proteomic approaches are now actively used, for instance, in cancer risk and response assessment in human medicine, as the technology allows detection of cancers at their earliest stages, even in the pre-malignant state, and ultimately translates into a higher cure rate (Petricoin and Liotta 2004b). Additionally, the information obtained by these global analyses is used to identify high-risk patients, cause or consequence of disease processes, and how each individual patient responds to therapy.

In spite of the potential of 'omic' data, regulatory bodies will need to gain confidence in the accuracy, reproducibility, sensitivity, and robustness of these new methods in order to fully integrate 'omic' information into risk and safety assessment.

3.4 Conclusions

The hypothesis tested in this chapter was that exposing mussels to oil alone, or oil spiked with APs and extra PAHs would result in unique combinations of expressed protein species that could be indicative of either exposure or effect. The results revealed specific protein expression profiles that could be used to distinguish: (1) controls from exposed organisms, (2) type of exposure, (3) degree of response, and (4) males from females independent of exposure. Additionally, males and females responded differently to exposure, in the sense that exposure affected different protein forms in the two genders. The latter indicates the necessity of knowing the gender of the test organisms, as well as having a balanced distribution of males and females in the different exposure groups. The potential mechanisms of actions behind the complex response-patterns have not been investigated in this study, however, certain trends were observed (e.g. females exposed to oil showed similar protein expression patterns as control males, while males exposed to spiked oil revealed patterns similar to control females). Additionally, results indicated that mixing crude oil with APs and PAHs enhanced the effect of crude oil alone, as some of the protein features altered by both exposure regimes, showed a higher degree of response (as fold change in expression) in mussels treated with the mixture. Results from the present study clearly indicate that the proteome contains information about both specific stressors and their effects, highlighting that an ecotoxicoproteomic approach has the potential to make a valuable contribution to environmental research, as well as environmental risk assessment that complements existing methodologies. However, for ecotoxicoproteomics to fulfil its potential, national and international collaboration will be essential in order to obtain necessary baseline information, genomic sequence information (particularly for non-model species), as well as a standardisation of methods to ensure both inter-experiment and inter-laboratory reproducibility. Furthermore, more knowledge is required on species and gender-specific susceptibility to environmental pollutants before cause-effect relationship for any chemical can be ascertained and true 'safe'-levels assigned for such chemicals.

4.1 Introduction

Endocrine disrupting chemicals (EDCs) are a major problem in the aquatic environment with clear negative impacts for wildlife (e.g. IPCS 2002). Some EDCs known to exert profound and deleterious effects on wildlife (and humans) are prohibited and others have very restricted use (e.g. DDT, PCBs and TBT). Nevertheless, as many of these EDCs are persistent and bioaccumulative, they are still found in relatively high concentrations in both sediment and food webs globally, and continue to have an impact on life and ecological processes (e.g. Breivik et al. 2002; Liu et al. 2008). Additionally, numerous ECDs are still in use including, for example, phthalates, brominated flame retardants (polybrominated diphenyl ethers - PBDEs) and bisphenol A (BPA).

The phthalates represent a class of chemicals used widely and diversely in industry in the production of polyvinyl chloride and, to a lesser degree, in paints, lacquers and cosmetics (Harris et al.1997). They are released into the environment both during the manufacturing processes and during the life time of the manufactured products, and have been detected in sediment, water and air (Fatoki and Vernon 1990). Epidemiological studies with humans have shown that phthalates induce adverse health effects such as disorders in the male reproductive tract and breast, are responsible for testicular cancers and disrupt the neuroendocrine system (e.g. Spelsberg and Riggs 1987; Sharpe and Shakkebaek 1993). Most studies assessing the toxic effects of phthalates on aquatic species have been restricted to acute and chronic toxicity tests including the LC50 (median lethal concentration), EC50 (median effect concentration, that is the concentration that produces a defined effect on 50% of the population) and the NOEC (No Observed Effect Concentration) (e.g. reviewed by Staples et al. 1997). Recent studies have reported both genotoxic effects (Barsiene et al. 2006b) of diallyl phtalate (DAP), and effects on cell signalling systems (i.e. tyrosine kinase activity) in bivalve molluscs (Burlando et al. 2006). Furthermore, mussels (*Mytilus edulis*) exposed to DAP showed altered ovocyte development and inhibited expression of vitellogenin-like proteins (Aarab et al. 2006).

Polybrominated diphenyl ethers (PBDEs) are a group of flame retardants that involve 209 different congeners, varying in both number and position of bromination (Birnbaum and Staskal 2004). They are used as a flame retardant in plastics and in textile coating (WHO 1994). PBDEs are structurally comparable to PCBs and DDT, and have similar chemical properties, persistence and distribution in the environment (Gustafsson et al. 1999; Helleday et al. 1999). PBDEs have been detected in wildlife samples around the world, including the Arctic (e.g. DeWit 2002; Law et al. 2003; 2006), and their fate and potential effects on man and wildlife have raised major concerns (McDonald 2002; Darnerud 2003; Birnbaum and Staskal 2004; McDonald 2005). Currently, one of the greatest concerns for potential adverse effects of PBDEs relates to their developmental neurotoxicity (e.g. Birnbaum and Staskal 2004; Costa and Giordana 2007), although there are conflicting reports regarding the genotoxicity of PBDEs. Evandri et al. (2003) did not find BDE-99 to be genotoxic *in vitro*, however, a recent study reported genotoxic effects in bivalves following exposure to BDE-47 (Barsiene et al. 2006b). Some PBDEs may have endocrine disrupting effects (Legler and Brouwer 2003; Vos et al. 2003), as they have been shown to interact as antagonists or agonists at androgen, progesterone, and estrogen receptors (Meerts et al. 2001; Hamers et al. 2006). Additionally, hydroxylated PBDEs are structurally very similar to thyroid hormones, and have been reported to disrupt thyroid homeostasis (e.g. Meerts et al. 2000; Boas et al. 2006).

Bisphenol A (BPA; 4, 4-isopropylidene diphenol) is a chemical intermediate used primarily in the production of epoxy resins and polycarbonate products. Because of its extensive use in the manufacture of consumer goods and products, including polycarbonate food containers and utensils, dental sealants, protective coatings, some flame retardants, and water supply pipes, there is a widespread and well-documented human exposure to BPA (reviewed in Kang et al. 2006). Although very few studies have reported concentration levels of BPA in wildlife, Basheer et al. (2004) recorded between 13.3 and 213.1 ng g^{-1} w.w. of bisphenol-A in supermarket seafood from Singapore (i.e. prawn, crab, blood cockle, white clam, squid, and fish). Pojana et al. (2007) reported the occurrence and distribution of several EDCs, including BPA, in water, sediment and biota (Mediterranean mussel, *Mytilus galloprovincialis*) in the Venice lagoon. Moreover, BPA has a widespread distribution in surface waters and sediment worldwide (reviewed by Crain et al. 2007), with the highest concentrations of BPA found at landfill leachate and sewage treatment effluents. Numerous studies have investigated the effect of BPA on wildlife, and extensive evidence indicates that BPA induces feminization during gonadal ontogeny of fishes, reptiles and birds, although at concentrations above those found in the environment (Crain et al. 2007). Most of the reported effects of BPA on vertebrate wildlife species can be attributed to BPA acting as an estrogen receptor agonist, but the action of BPA extends beyond its ability to mimic, enhance or inhibit the activity of endogenous estrogens and/or disrupt estrogen nuclear hormone receptor action. Other observed effects and action of BPA are extensive and include disruption of thyroid hormone function, diverse influences on development, differentiation and function of the central nervous system, influence on the immune system, and modification of cytochrome P450 enzyme expression and activity (reviewed in Wetherill et al. 2007). Furthermore, species-specific effects on

protein expression in fish, following exposure to BPA, have been reported (Larsen et al. 2006). Little is known regarding both the effects and chemical modes of action for BPA in invertebrates (Crain et al. 2007). However, an increasing number of invertebrate studies have been undertaken as the importance of this group of animals (in both ecosystem functioning and aquaculture, e.g. Saavedra and Bachere 2006) is becoming more apparent. For example, Canesi et al. (2007) demonstrated that BPA altered the gene expression, the activities of enzymes involved in the redox balance, and the lysosomal function in molluscs (i.e. *Mytilus galloprovincialis*), and concluded that BPA, at environmentally-relevant concentrations, potentially can have similar estrogen-like effects (and other effects) in invertebrates as those reported in vertebrates.

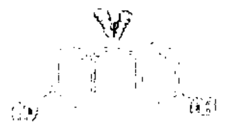
While the exposure of wildlife species and humans to phthalates, PBDEs and BPA has been reported increasingly in the literature, there are significant gaps regarding their general ecological effects. Moreover, very little is known regarding their molecular mechanisms of action, dose-response relationships, critical developmental windows of exposure, or species and gender-specific impacts of exposure (e.g Crain 2007; Wetherill 2007: Vos et al. 2003). This is particularly true for invertebrate species (e.g. extensively addressed in a special issue of Ecotoxicology; Volume: 16 Issue: 1 Published: February 2007). Consequently, there are many concerns and challenges in the risk assessment of EDCs in the aquatic environment (Breitholtz et al. 2006). For example, to determine potential risk of EDCs, current toxicity studies are conducted on a selected few 'suitable' model animals (sentinels), and results are subsequently extrapolated to other wildlife species and humans. In almost all cases, quantitative differences in dose-response relationship exist between model species and other target species (e.g. Aardema and MacGregor 2003; IPCS 2002; DeFur et al. 1999). In some cases, biological responses to a given exposure may also differ qualitatively (e.g. as observed in Chapter 3, certain protein forms were upregulated in male organism while they were downregulated in females, and vice versa, following the same exposure regime). Given the great differences in, the endocrinology of males and females, vertebrates and invertebrates (as well as between various invertebrate taxa and phyla), there are most likely numerous of species and gender-specific modes of action for environmental chemicals. Thus there is a need for so-called 'bridging biomarkers' (Aardema and MacGregor 2003) that can be used to compare toxic responses between species. One promising approach is to compare the gene and protein expression in 'model' and 'target' species, where similar expression patterns could indicate similar molecular damage and response (and hence support the relevance of chosen model species). Likewise, dissimilar pattern could indicate the lack of relevance of extrapolation of effects between model and target species.

The aim of the present study was to use a proteomics approach to assess the potential species and gender differences between invertebrates in response to exposure to EDCs, and thus evaluate the utility of proteomics in providing useful information for environmental assessment regarding, for example, species (and gender) susceptibility to EDCs. To achieve this aim, the effects of BPA, diallyl phthalate (DAP) and 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), on protein expression profiles in two invertebrate species, i.e. blue mussels (*Mytilus edulis*) and spider crabs (*Hyas araneus*) were investigated. The hypothesis was that protein expression signatures would contain information that was sensitive and specific to species and gender for each of the selected test compounds.

4.2 Material and methods

Mussels (*Mytilus edulis*) and spider crabs (*Hyas araneus*) were collected in March 2003 from Førlandsfjorden and Krokaneset, respectively (see Figures 2-12 and 2-13), and then exposed for three weeks in the continuous (steady-state) system described in Chapter 2.3 (Figure 2-14) and shown in Figure 4-1, to nominal concentrations of 50 ppb DAP, 5 ppb BDE-47, 50 ppb BPA, or filtered seawater (i.e. control groups). Chemical structures of the compounds as well as supplier information are shown in Table 4-1. It would have been beneficial to the experiment to test more than one dose of each compound (e.g. in order to discover potential threshold levels for effects of the selected compounds). However, given the space available in the test facility, this was not possible in the present study.

Table 4-1. Exposure compounds, i.e. BPA, BDE-47 and DAP.

Component	**Structure**	**Purity**	**Supplier**	**Duration of exposure**
Bisphenol A (BPA) (Diphenylopropane)		> 97%	Merck EC No. 201-245-8	3 weeks
BDE-47 (2,2',4,4'Tetra Bromo Diphenyl Ether)		> 95,6%	Chiron AS Product no 1688,12	3 weeks
Diallyl phthalate (DAP)		> 98%	Fluka EC No: 2050163	3 weeks

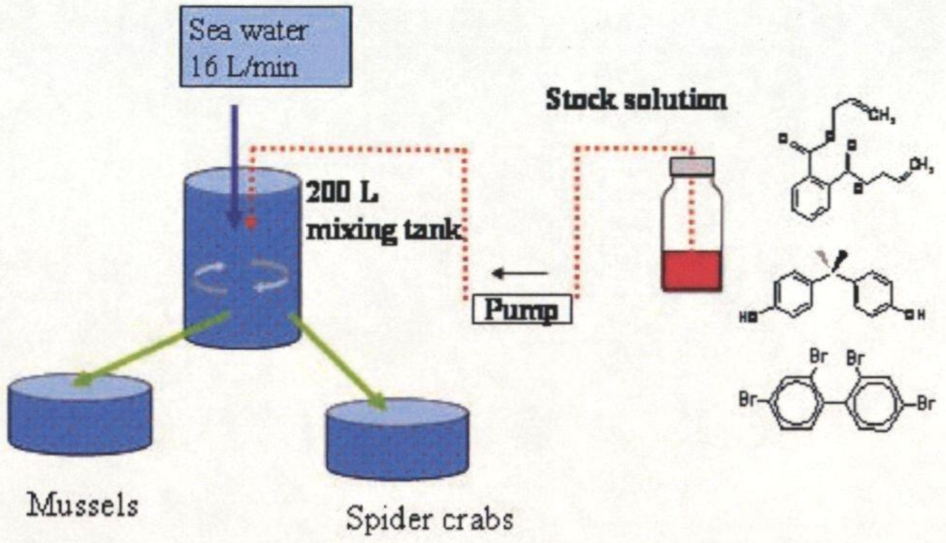


Figure 4-1. Diagram of the continuous flow system as used in this study.

4.2.1 Monitoring of ECDs during exposure

Exposure concentrations of ECDs in water were monitored during the course of the experiment as described in Table 4-2. Additionally, PBDEs were measured once (at the end of the experiment) in whole mussel tissue. All chemical analyses in this study have been done by Dr. Cato Brede (at Stavanger University Hospital) and Dr. Dorte Herzke (at Norwegian Institute for Air Research), and analytical details are given in Sundt et al. 2006.

Table 4-2. Exposure monitoring of DAP, BPA and BDE-47 in water

Component	Nominal concentrations	Mean measured concentrations	Number of measurements	Analysis method
Bisphenol A	50 μg/L	59,4 ± 10,659 μg/L	7	GC-MS
BDE 47	5 μg/L	0,23 ± 0,189 μg/L*	8	GCP, Florisil chromatography GC-MS-EI (DB5MS)
DAP	50 μg/L	38,3 ± 9,693 μg/L	6	GC-MS

Exposure monitoring revealed that the average water concentration of BDE-47 was only 4.6% of nominal concentration (5 ppb). However, uptake of BDE-47 in mussels was confirmed by analysis of whole soft tissues. The results revealed that 225 µg/g soft tissues (lipid normalised) had been uptaken by the mussels. The low concentration measured in water could be explained by the fact that the pure BDE-47 was adsorbed to particles, e.g. food reminders and faeces, or the container walls, and by this very easy accessible for the animals leading to direct intake of the substance (Sundt et al. 2006).

4.2.2 Sample collection

Haemolymph samples from mussels and crabs were collected according to the description given in Chapter 2.1.1 and Figure 2-1. A total of 80 mussels (40 females and 40 males; shell size 7.2 ± 0.8 cm) and 60 crabs (30 females and 30 males; carapace width 4.6 ± 0.7 cm) was sampled and analysed per treatment.

4.2.3 Sample preparation on ProteinChip arrays

ProteinChip arrays with weak cation exchange surfaces properties (WCX-arrays) were used for protein profiling of both mussel and spider crab plasma. Analyses were conducted according to the sample preparation protocol outlined in Table 2-7.

4.2.4 SELDI TOF MS analysis

Following sample preparation and incubation, all arrays were analysed immediately on a PBS-IIc time of flight mass spectrometer using ProteinChip Software version 3.1 Mass spectra were recorded twice from each sample on the following settings: 91/112 laser shots/sample in a positive ionisation mode (65/80 of the shots were collected, starting at position 20 and ending at position 80 of the spot), detector voltage 2850 V, data acquisition from 0- 200000 Da, and optimum mass range focus from 2500-15000 Da /10000-200000 Da. Laser intensities and detector sensitivities for mussels and crabs are given in Table 4-3.

Table 4-3. Acquisition parameters (i.e. laser intensities and detector sensitivities) used for mussels and spider crabs in the low-molecular weight (LM) and high-molecular weight (HM) runs.

Species	Laser intensity		Detector sensitivity	
	LM run	HM run	LM run	HM run
Mussels	190	195	7	8
Spider crabs	187	190	7	8

The PBS-IIc instrument was calibrated externally with bovine insulin (5733.5 Da) and bovine IgG (147300 Da) mass standards (Bio-Rad). In addition, each mass spectrum was calibrated using calibration equations prepared by four standards for low mass spectra [i.e. Dynorphin A (2147.5 Da), Insulin bovine (5733.6 Da), Ubiquitin (8564.8 Da), Cytochrome C bovine (12230.9 Da)] and four standards for high mass spectra [i.e. Bovine β-Lactoglobulin A (18363.3 Da), Horseradish Peroxidase (43240.0 Da), Serum Albumin bovine (66433.0 Da) and IgG bovine (147300 Da)].

4.2.5 Data handling and statistics

Preliminary data processing was done in the Biomarker Wizard feature of ProteinChip Software (version 3.1). Mass spectra from all treatment groups were imported into one experimental file. Baseline subtraction was performed and the spectra were mass aligned using three M/Z values prominent in all spectra (4036 Da, 12470 Da and 27402 Da for mussels, and 4225 Da, 11377 Da, 36600 Da for spider crabs). Further data processing and biomarker discovery, with uni- and multivariate statistics, were done according to the description given in Chapter 2.1.4. Furthermore, data sets for decision tree classification (with Biomarker Pattern^{TM} Software) were separated randomly into a training set and a test set before analysis. The training sets consisted of data from 50 mussels and 40 crabs per group, while the test sets included data from 30 mussels and 20 crabs per group. For each dataset, all acquired data (mass peaks with signal-to-noise ratios greater than or equal to 5) from both the low-mass run and the high-mass run were compiled. In addition, multidimensional scaling analyses (MDS) using PRIMER software version 6, were performed with all acquired data from 80 mussels or 60 crabs as input. MDS was used to indicate the similarities (and dissimilarities) between pairs of units. Samples are presented as points in low-dimensional space (normally 2D or 3D) so that the relative distances apart from all points are in the same rank order as the relative dissimilarities of the samples (Galloway et al. 2004; Gomiero et al. 2006).

4.3 Results and discussions

The hypothesis tested in this study was that protein expression signatures would contain information that was sensitive and specific to species and gender for each of the selected test compounds. Results obtained with plasma proteome analysis of mussels and spider crabs confirmed this hypothesis. When data were collected at the highest sensitivity (and not filtered for electronic and chemical noise), profiling of mussel and spider crab plasma on WCX ProteinChip arrays revealed 2349 and 3023 distinct peaks, and corresponding M/Z values for mussels and crabs respectively. Removal of all peaks other than those with a signal to noise greater than or equal to 5 (present within the mass range of 2000 to 200000 Da) resulted in 257 peaks for mussels and 328 peaks for spider crabs. These were further analysed and were predominantly low-molecular weight protein forms (i.e. 82 % of the detected protein forms in mussel plasma had M/Z values less than 20000 Da) which is in accordance with findings in Chapter 3, where 81% of the detected peaks were below 20000 Da (Bjørnstad et al. 2006). Similarly, mass spectra of spider crab plasma revealed that 89% of the protein features had M/Z values less than 20000 Da. The reason for the uneven distribution of masses for detected protein features is most likely technology-related, as the ProteinChip technology is most effective at profiling low mass molecules (i.e. < 20000 Da), in contrast to 2D gel electrophoresis that favours detection of protein forms greater than 20000 Da (e.g. Issaq et al. 2002). However, information regarding the difference between exposed organisms and controls was clearly present in the subset of the proteomes resolved by SELDI TOF MS.

4.3.1 General response

SELDI TOF MS analyses of plasma samples obtained from Mytilus *edulis* and *Hyas araneus* following exposure to three different EDCs, indicated that all chemicals tested had an effect on protein expression in mussels and spider crabs. For both species, a significant alteration ($p < 0.05$) in expression was observed in a high percentage of the peaks that were successfully resolved and displayed by the ProteinChip technology (Table 4-4). While 32 - 70% of the total numbers of detected peaks revealed significantly altered expression in mussels, 40 - 69% of the peaks were affected in crabs (see Table 4-4 for summery of affected peaks in the different exposure groups). All exposure regimes had a predominantly upregulating effect on protein expression in spider crab plasma. In mussels, however, both BDE-47 and BPA exposure caused reduced expression of the majority of detected protein forms (Table 4-4) with, for example, 85% (females) and 83% (males) down-regulated peaks in BPA-exposed mussels.

Table 4-4. Summary of significantly ($p < 0.05$) upregulated (Up) and downregulated (Down) peaks in various exposure regimes for spider crabs (*Hyas araneaus*) and mussels (*Mytilus edulis*).

Spider crabs (*H. araneaus*)					Mussels (*M. edulis*)				
Exposure	Gender	Up	Down	Σ	Exposure	Gender	Up	Down	Σ
DAP	♀	129	98	227	DAP	♀	59	33	92
	♂	79	74	153		♂	47	36	83
BDE-47	♀	82	45	127	BDE-47	♀	49	93	142
	♂	86	72	158		♂	48	73	121
BPA	♀	136	84	220	BPA	♀	20	114	134
	♂	67	86	153		♂	30	149	179
n = 328 resolved peaks with S/N ≥5					n = 227 resolved peaks with S/N ≥5				

Note. ♀ = female, ♂ = male, Σ = total number of significantly altered peaks, S/N = signal-to-noise ratio

Average changes in protein expression (as the ratio of protein expression in exposed organisms/ protein expression levels in controls) were similar for all exposure groups (i.e. approximately two-fold, when all peaks detected for males and females were combined). The greatest average change in expression was observed in mussels exposed to BPA (2.3-fold), and the lowest in spider crabs exposed to BDE-47 (1.8-fold). Moreover, examination of differences in protein expression levels between exposed organisms and controls, males versus females, and mussels versus crabs revealed very complex responses to EDCs that are discussed below.

4.3.2 Treatment-specific response

Results from the present study, revealed that all exposure regimes affected protein expression in mussels and spider crabs in a treatment-specific manner (in the sense that both the quantity and quality of mass peaks that were changed after exposure were unique for each compound), although similar responses (e.g. protein features that were affected by 2 or 3 compounds) were also observed (Figure 4-2). In Figure 4-2, each ring contains the number of proteins significantly ($p < 0.05$) altered only by the indicated groups. These protein forms could be specific biomarker candidates used to predict and recognize damaging effects of that particular compound. The overlapping areas, however, reveals the numbers of peaks that were changed by two compounds. These 'shared' responses could be indicative of similar modes of chemical actions for the two compounds, and thus the peaks could be potential biomarkers of chemical class-specific cellular perturbations (e.g. Hughes et al. 2000). The area in the middle of the diagram shows those protein species that were altered by all compounds, and could reflect more general toxic responses. In female crabs, for example, 100 peaks were significantly induced or repressed by all exposures (Figure 4-2 C), while 80 peaks were significantly altered by all compounds in male crabs (Figure 4-2 D). There is evidence that expression of multiple proteins is closely related to stressors and can be used to diagnose not only exposure to a particular chemical or toxic effect, but also its level of severity (Bradley et al. 2002). Different levels of severity (i.e. as number of significantly affected protein forms) were also observed after exposure to DAP, BPA and BDE-47 for both mussels and crabs, although not the same in the two species (Table 4-4 and Figure 4-2).

crabs (Figure 4-2 C). While BPA exhibits multiple mechanisms of toxicity (reviewed in e.g. Wetherill et al. 2007; Crain et al. 2007), very little is known regarding mechanisms of action for phthalates, in general. However, both Bisphenol A and phthalates have been associated with estrogenic effects (e.g. Jobling et al. 1995) although the mechanisms of action for phthalates are debated (e.g. Gray et al. 2001). Furthermore, steroid molecules are present in all invertebrates (Lafont and Mathieu 2007) and some have hormonal roles (e.g. ecdysteroids in arthropods). This implies that they are susceptible to disruption by compounds with similar chemical structure as the steroid hormones. Moreover, many of the observed common effects could be non-specific stress responses, which may be found in many types of exposures at certain exposure concentrations, or compound related responses that are not associated with endocrine disruption. Recent studies have shown that DAP, BDE-47 and BPA affected several types of toxicity in a compound-specific manner in mussels (summarised in Table 4-5) and spider crabs (summarised in Table 4-6). For example, in mussels, while BDE-47 showed the greatest effect on genotoxic endpoints like micronuclei formation and other nuclear abnormalities (Barsiene et al. 2006b), as well as DNA strand breaks in sperm cells (Taban et al. 2003), BPA had a more significant effect on stress proteins (Jonsson et al. 2006a), general protein expression (Apraiz et al. 2006, and present study), and cell signalling (Burlando et al. 2006), (Table 4-5). DAP; on the other hand, generated the most significant effect on multixenobiotic resistance, acetyl-choline esterase activity and total oxyradical scavenging capacity in spider crabs (Minier et al. 2008). Furthermore, DAP had the greatest impact (i.e. as number of differently expressed peaks compared to controls) on protein expression in spider crabs (e.g. Table 4-6).

Table 4-5. Summary of treatment-specific responses in mussels (*Mytilus edulis*) following exposure to DAP, BDE-47 and BPA (ns = not significant response).

		Treatment				
Biomarker response	Organ	DAP	BDE-47	BPA	Strength of response when significant in more than one exposure group	Reference
Expression of microsomal actin	Digestive gland	ns	$p < 0.02$	ns	-	Jonsson et al. 2006a
Expression of cytosolic Hsp70	Digestive gland	$p < 0.05$*	$p < 0.05$*	$p < 0.05$*	BPA >BDE-47>DAP	Jonsson et al. 2006a
Protein tyrosine phosphorylation	Gills	$p < 0.05$*	$p < 0.05$*	$p < 0.05$*	BPA>DAP,BDE-47	Burlando et al. 2006
Protein tyrosine phosphorylation	Mantle	ns	ns	$p < 0.05$*	-	Burlando et al. 2006
Expression of phosphor-proteins	Mantle	$p < 0.05$*	$p < 0.05$*	ns	DAP>BDE-47	Aarab et al. 2006
Peroxisomal protein expression	Digestive gland	$p < 0.05$	$p < 0.05$	$p < 0.05$	BPA>BDE-47>DAP	Apraiz et al. 2006
Induction of micronuclei	Gills	$p < 0.05$	$p<0.0001$	$p<0.0001$	BDE-47>BPA>DAP	Barsiene et al. 2006b
Induction of bi-nucleated cells	Gills	ns	$p < 0.001$	ns	-	Barsiene et al. 2006b
Induction of bi-nuclear buds	Gills	ns	$p < 0.05$	ns	-	Barsiene et al. 2006b
Induction of fragmented apoptotic cells	Gills	ns	$p<0.0001$	$p < 0.05$	-	Barsiene et al. 2006b
DNA damage (by Comet assay)	Blood (haemocytes)	ns	ns	$p<0.0001$	-	Taban et al. 2003
DNA damage (by Comet assay)	Mantle (sperm cells)	$p<0.0001$	$p<0.0001$	$p<0.0001$	BDE-47>BPA, DAP	Taban et al. 2003
Histopathology – increased peroxisomal volume density	Digestive gland	$p < 0.05$	$p < 0.05$	ns	DAP>BDE-47	Cajaraville et al. 2006
Histopathology – reduced size and number of ovocytes	Mantle	$p < 0.05$*	ns	ns	-	Aarab et al. 2006

Note. When specific significance levels are not provided in the reference, $p < 0.05$* are used to signal significant response in exposed organisms.

Table 4-6 Summary of treatment-specific responses in spider crabs (*Hyas araneus*) following exposure to DAP, BDE-47 and BPA (ns = not significant response, MXR = multixenobiotic resistance, TOSC = total oxyradical scavenging capacity).

Biomarker response	organ	Treatment			Strength of response when significant in more than one exposure group	Reference
		DAP	BDE-47	BPA		
General protein expression	Hepatopancreas	$p<0.05$	$p<0.05$	$p<0.05$	BPA>BDE-47>DAP	Gomiero et al. 2006
MXR protein expression	Hepatopancreas	$p<0.05$	ns	ns	-	Minier et al. 2008
Acetylcholine esterase activity	Muscles (from claws)	$p<0.01$	$p<0.05$	$p<0.01$	DAP>BPA>BDE-47	Minier et al. 2008
Cytosolic TOSC toward peroxyl, hydroxyl and peroxynitrite	Hepatopancreas	$p<0.05$	ns	ns	-	Minier et al. 2008
Cell viability (by Neutral Red assay)	Blood	ns	$p<0.01$	$p<0.01$	BDE-47>BPA	Unpublished data from University of Plymouth
Phagosytosis index	Blood	$p<0.01$	$p<0.01$	ns	BDE-47>DAP in males, while DAP>BDE-47 in females	Unpublished data from University of Plymouth

Note. When specific significance levels are not provided in the reference, $p < 0.05^*$ are used to signal significant response in exposed organisms

The cause-effect relationships for the observed treatment-related effects on both protein expression and other biomarker endpoints (Tables 4-5 and 4-6) cannot be identified from present results or available scientific literature and need further investigation. Furthermore, species and gender-specific vulnerability to adverse effects must be taken into consideration before any cause-effect relationship for any chemical can be ascertained.

4.3.3 Species similarities and differences

While risk assessment for human health is focussed on one species, environmental risk assessment (ERA) should ideally consider all the species present in

the impacted environment, each with specific trophic, physiological and ecological requirements (e.g. Breitholtz et al. 2006). Clearly this is impractical and, as a result, it is unclear whether information on the effects of a given chemical for one species (or taxonomic group) can be extrapolated to protect other species, as one animal's poison may not be another's (Sumpter and Johnson 2005). Therefore, it is of great importance to identify (the most) sensitive species and subpopulations before guidelines and regulations are made regarding 'safe' levels of anthropogenic chemicals.

In the present study, plasma protein expression profiles for mussels and spider crabs were different even in control organisms (e.g. see Figure 2-5). This was not surprising since even related mussel species (i.e. *Mytilus edulis*, *Mytilus galloprovincialis* and *Mytilus trossulus*) have different protein/peptide expression profiles (Lopez et al. 2002). Species comparisons in this study were therefore based on 'degree of response' (as percentage of affected protein features) and 'response pattern' (as up/downregulation and most affected exposure groups).

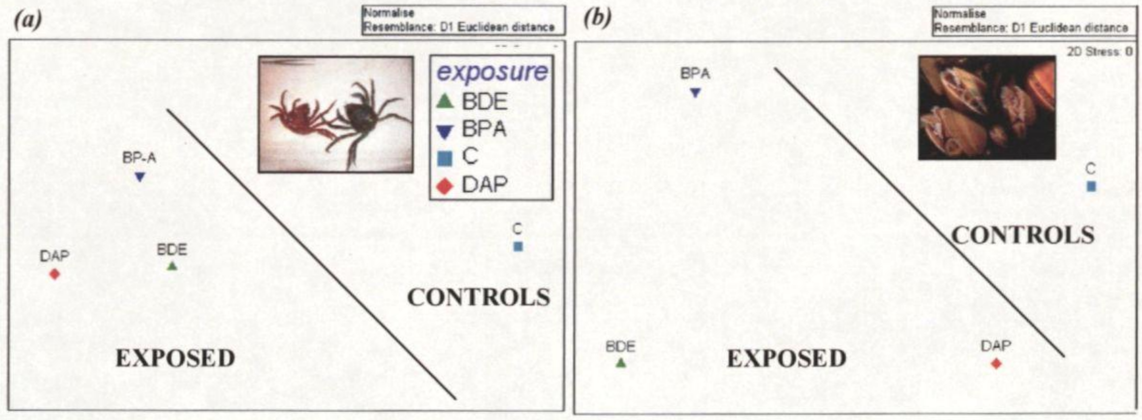


Figure 4-3. Multidimensional scaling representation (MDS) of similarities and differences between exposure groups (females and males combined) and species. The analysis is based on a similarity matrix constructed using normalised Euclidian Distances. Data inputs were all plasma-protein peaks with S/N ≥ 5 for (a) spider crabs and (b) blue mussels, where each symbol represents mean peak intensity for all the individuals in one exposure group.

To visualise which of the species that showed the greatest discrimination between controls and exposed organisms, a multidimensional scaling analysis was performed using PRIMER software (Figure 4-3). Similar to results in Table 4-4 and Figure 4-2, the MDS plots indicate that *Mytilus edulis* and *Hyas araneus* have different susceptibility to DAP. While DAP-exposed crabs have the most dissimilar protein expression pattern compared to controls (Figure 4-3 A), DAP-exposed mussels had the most similar profile to controls (based on relative distances in the plot and thus degree of similarity/differences). Other than that its not possible to interpret much from these plots as they are based on average data, and as such only represents the 'trends' in the response pattern. Nevertheless, the spread of data in the MDS plots in Figure 4-3 indicate that the different compounds have dissimilar protein patterns, and that the two species have different response patterns towards the selected compounds in this study, which has also been indicated by univariate analysis (e.g. Figure 4-2).

Species-related differences in response to environmental pollutants have also been reported by Larsen et al. (2006), who found that while Nonyltnol and Bisphenol A affected protein expression in a similar manner in Atlantic cod (*Gadus morhua*), the same compounds induced very different responses in turbot (*Scophthalmus maximus*), indicating that nonylphenol and BPA only have similar modes of actions in cod, or that the compounds have different metabolism rates via the phase II detoxification system in turbot (Larsen et al. 2006) Recently, species-specific alteration of cellular energy allocation was reported for Arctic crustaceans and molluscs (i.e. *Gammarus setosus, Onisimus litoralis,* and *Liocyma fluctuosa*) after exposure to oil-related compounds (Olsen et al. 2007). Werner and Nagel (1997) found species-specific alteration of stress proteins (i.e. hsp 60 and hsp 70) in crustaceans (i.e. *Ampelisca abdita, Rhepoxynius abronius* and *Hyalella azteca*) following metal exposure. However, there are generally few studies (on any wildlife specie) that have investigated species-specific alterations of protein expression following antropogenic insults, thus a detailed comparison of the results from this study is not possible. However, to test whether multivariate classification algorithms could be indicative of species-specific susceptibility to EDCs, all MS data (i.e. resolved mass peaks and corresponding M/Z values with S/N ≥ 5) were exported to Biomarker Pattern^{TM} Software for testing of prediction models classifying exposed from control organism. The prediction models were created with 100 mussel and 80 spider crab samples (as described in Chapter 3.3.5). The best models were tested with 60/40 (mussels/crabs) new samples. The results are summarised in Table 4-7.

Table 4-7. Test of prediction models created with Biomarker Pattern^{TM} Software for comparison of controls versus exposed in spider crabs (*Hyas araneus*) and mussels (*Mytilus edulis*). Prediction models were generated from 100 mussel samples and 80 samples from crabs, and subsequently tested with 60/40 new samples from mussels and crabs, respectively.

H. araneus	Prediction success(%) in test samples		
Compared groups	Controls	Exposed	Classifiers, M/Z (Da)
C versus DAP	92	100	2432, 4510
C versus BDE-47	72	75	5556, 3150, 2172, 4887, 48996, 36238
C versus BPA	79	85	2432, 4725, 48996, 2071, 6439
M. edulus			
C versus DAP	80	79	6835, 7874, 3896, 4466, 44005, 2650, 2139, 2013
C versus BDE-47	87	90	2169, 6787, 5921, 40520
C versus BPA	89	92	2169, 3675, 3557

Note. Importance of classifiers is decreasing from left to right. C = controls, DAP = diallyl phthalate, BDE-47 = 2,2',4,4'-tetrabromodiphenyl ether, and BPA = bisphenol A

Results showed that species sensitivity was highly compound related. While plasma samples from crabs generated the best prediction model for DAP exposure (i.e. 100% correct classification of exposed and 90% correct classification of controls), the best prediction models for BDE-47 and BPA were generated with mussel samples (Table 4-7). Compound-related species-sensitivity was also reported by Larsen et al. (2006) for cod and turbot exposed to BPA and Nonylphenol. Another interesting observation (related to both species) was that very few classifiers (i.e. intact or modified proteins that are predominantly expressed in one of the test groups, and thus used to classify the test-samples into appropriate groups based on expression level of these protein forms) were common for the prediction models in Table 4-7. However, in spider crabs, the most important classifier for both controls versus DAP-exposed and control versus BPA-exposed organisms was M/Z 2432 Da (3 fold upregulated in BPA and 4 fold upregulated in DAP, males and females combined). Similarly in mussels, M/Z 2169 Da was the most important classifier in both controls versus BDE-47 and controls versus BPA (up-regulated 5 fold by BPA and 3 fold by BDE-47). These observations support the results presented in Figure 4-2, where 73% of the significantly altered protein forms in crabs exposed to DAP and BPA are the same (only in females though), while 48% (females) and 44% (males) of the responses were similar in mussels exposed to BDE-47 and BPA. It must emphasised, however, that 'similar' response here only means that mass peaks with the same M/Z values have been significantly altered by two different EDCs. To confirm that responses are truly the same in both exposure groups, mechanistic studies of modes of actions are required. Such studies could start with successful identification (with tandem MS) of some of the protein forms affected by both compounds. Successful identification of key proteins will depend on several factors such as availability of genome information, and resolution and mass accuracy in mass-spectrometric analyses (e.g. Hu et al. 2005; 2006). The latter is particularly important, when identification is based on *de novo* sequencing and genomic homology to other species, because necessary genomic sequence information is not available for the actual species.

4.3.4 Gender-specific responses

Recently, gender-related responses and susceptibility to environmental pollutants (particularly EDCs) have been addressed in several reviews and reports (e.g. Burger et al. 2007; Fossi et al. 2007; Gochfeld 2007; Orlando and Guillette 2007; McClellan-Green et al. 2007; Vahter et al. 2007). An overall conclusion is that chemical effects often differ by gender, but knowledge about why the effects differ is limited, although there are many hypothesis and possible explanations (e.g. Burger et al. 2007). The (prote)omics approach is recommended as one way forward, because of its ability to elucidate chemical modes of action, and thus improve the understanding of gender-related responses to chemicals (Burger et al. 2007). The results of this study revealed that a proteomic approach indeed managed to detect gender-specific plasma protein profiles in both mussels and spider crabs following exposure to DAP, BDE-47 and BPA. Clear effect of gender in proteomic response patterns was observed for both species (Figure 4-4 A, B and C), but it was particularly apparent for spider crabs, where the compound-specific response patterns appeared to be more similar in males than in females (Figure 4-4 C). The latter could be a reflection of different types of toxicity induced in the two genders following exposure, indicating that threshold levels for effect are different in male and female crabs, or that the modes of action for these compounds are gender-specific. It could, however, also reflect gender-specific uptake and/or metabolism of the chemicals (McClellan-Green et al. 2007). Furthermore, the MDS plots indicated that gender-differences were increased with exposure to EDCs, where exposed crabs were separated into distinct male and female clusters independent of exposure regime (Figure 4-4 C). The latter could be an indication of endocrine disruption, however, with different modes of action involved in males and females.

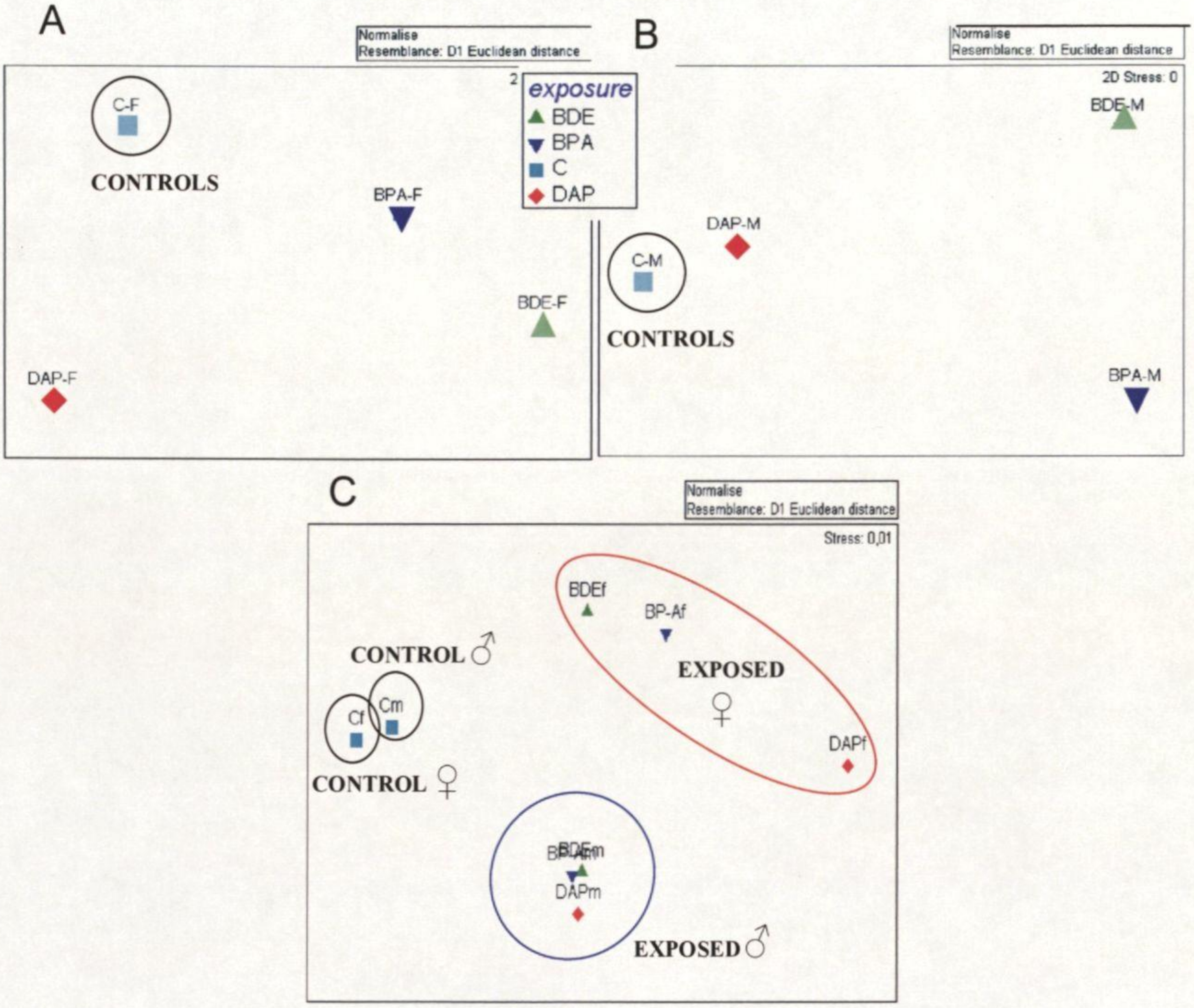


Figure 4-4. PRIMER MDS plots revealing gender-specific response patterns after exposure to three EDCs. The response patterns are based on plasma- protein expression analysis with ProteinChip technology and SELDI TOF MS. Inputs were 328 mass peak for spider crabs and 227 peaks for mussels (A) = female mussels, (B) = male mussels, and (C) = spider crabs, females and male. ♀ = female, ♂ = male.

In general, the degree of response (as the total number of altered protein features) in males and females were different in all exposure groups (Table 4-4), although the degree of difference between genders was highly compound and species related.

The same gender-related response trend was present in *Hyas araneus* hepatopancreas protein profiles after exposure to DAP, BPA and BDE-47 (Gomiero et al. 2006). More specifically, all exposures affected a unique set of mass peaks in males and females (Figure 4-5), in the sense that some protein forms were only significantly affected ($p < 0.05$, using Mann-Whitney-Wilcoxon test incorporated in the ProteinChip software) in females and others only in males. Surprisingly, in all exposure groups, except BPA treated mussels, less than 50% of the significantly affected protein forms were altered in both genders (Figure 4-5). Furthermore, mussels and crabs showed dissimilar gender-related response patterns (i.e. gender effects) for all compounds tested (Figure 4-5). In male organisms, for example, DAP affected 34% of the resolved peaks in mussels and only 13% in crabs. Similarly, BPA had a significant effect on 35% of the peaks in mussels, while only 18% of the peaks in crabs were affected by exposure. Exposure to BDE-47, on the other hand, had a greater effect (i.e. as number of altered protein forms) on male crabs (i.e. 41%) than on male mussels (i.e. 24%). In female organism, however, it was mainly BPA that revealed species-specific gender effects with 43% affected peaks in crab and only 11% in mussels (Figure 4-5). The explanation for the observed species-specific gender effects is not clear, however, a better insight into the endocrinology of the two species could perhaps unravel some more information (e.g. DeFur et al. 1999). Nevertheless, these findings indicate the importance of testing more than one species when evaluating the potential harmful effects of a compound. The biocomplexity, e.g. as shown in this study, is one of the greatest post-genome challenge in ecotoxicology (Moore 2002), as it is almost impossible to assess the true risk of any chemical, provided both species and gender-specific responses.

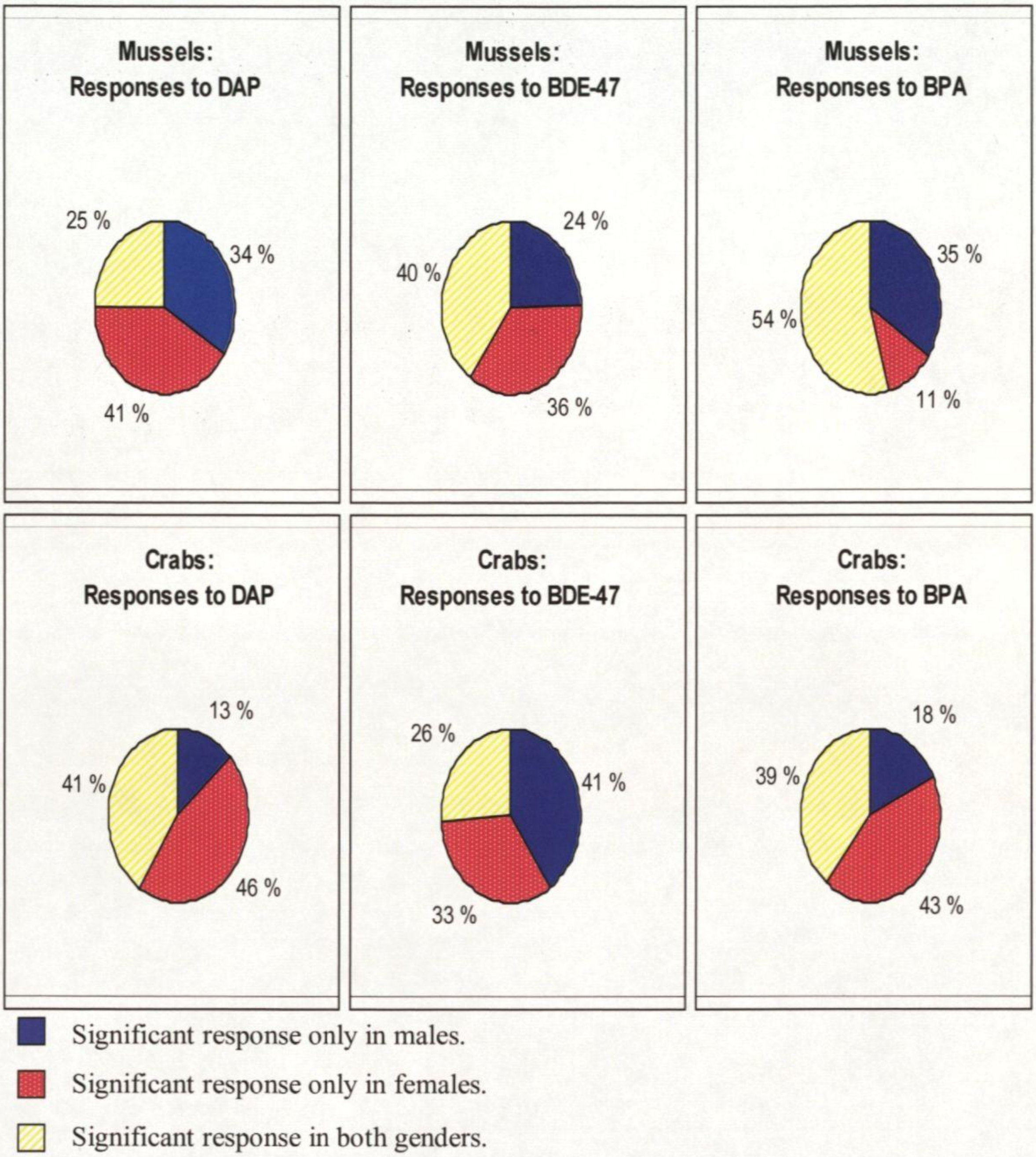


Figure 4-5. Pie charts showing the percentage distribution of gender-specific responses of all peaks with significant different expression (in males and females combined) after exposure in mussels (*Mytilus edulis*) and spider crabs (*Hyas araneus*). Significant = $p < 0.05$, tested with Mann-Whitney-Wilcoxon test incorporated in the ProteinChip software.

For example, even among the 'common responses' (e.g. the same protein feature significantly altered in both genders, in one species) the response could be different in the two genders. M/Z 6011 Da, for instance, was upregulated in female mussels and downregulated in males (Figure 4-6 b).

gender-specific fashion by contaminant exposure (e.g. Janer et al. 2006). This complexity makes the task of understanding how gender relates to chemical susceptibility (and endocrine disruption) very difficult, particularly as the results from the present study show that both species and gender-specific sensitivity is compound related. As a consequence, it is not easy to confirm that an observed (gender-specific) effect is in fact endocrine disruption, and not elicited by another mode of toxicity. Furthermore, because some EDCs have been shown to possess mutagenic and carcinogenic activity (Choi et al. 2004), it is possible that there could be a link between endocrine disruption and other types of toxicity. For example, Hagger et al. (2006) found a strong correlation between the degree of imposex (masculinisation of females) and the extent of DNA damage (micronucleus formation) in haemocytes of the dog whelk (Mollusca: Gastropoda) *Nucella lapillus.*

4.4 Conclusions

To understand the mechanisms of the effects of ECDs to wildlife populations and subpopulations, both gender and species-specific sensitivities must be taken into consideration. The present study assessed species and gender similarities and differences in proteomic responses to bisphenol A (BPA), diallyl phthalate (DAP) and 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) in blue mussels (*Mytilus edulis*) and spider crabs (*Hyas araneus*). The results revealed that the overall response pattern (e.g. number of affected protein forms, induced or suppressed expression after treatment etc.) was clearly different in mussels and crabs. Furthermore, species sensitivity was found to be highly compound related. For example, while protein expression patterns from crabs generated the best multivariate classification models for DAP exposure (i.e. 100%

correct classification of exposed and 90% correct classification of controls), prediction models based on protein profiles in mussels enabled superior classification of BDE-47 and BPA exposure (in masked samples). The ratio of gender-specific versus 'gender-common' responses varied for each treatment and species. However, as few as 25 - 54% of all the protein forms, with significantly changed expression after exposure, were significantly altered in both genders, indicating that combined analysis of males and females might result in inaccurate inputs to environmental risk assessment, and hence limited capability to predict true insults of anthropogenic compounds on wildlife. At present, there is very limited evidence for vertebrate and invertebrate species that EDCs cause gender and species-related effects. Results from the present study indicate that protein expression analysis, and subsequent identification of key-molecules, could be a way forward in determining the presence and consequences of species and gender-specific responses to contaminants. Nevertheless, much research is still required in order to improve our current understanding of the latter. For example, species and gender-specific toxicity threshold levels should be investigated at environmentally relevant exposure concentrations in order to guide decision makers regarding true 'safe-levels' of environmental pollutants.

Chapter 5

Dose-response relationships of oil on protein expression in molluscs and echinoderms

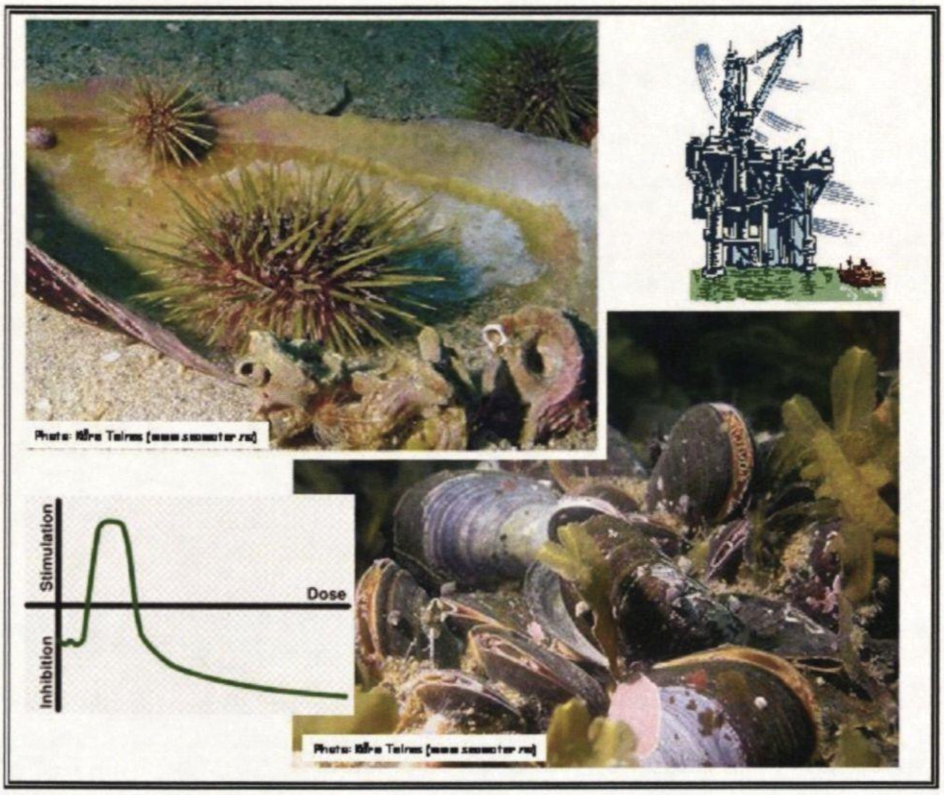


Photo: Kåre Telnes (www.seawater.no)
Stimulation
Inhibition
Dose
Photo: Kåre Telnes (www.seawater.no)

Note. This study was part of the BIOSEA JIP programme, and the project leader for the echinoderm study was Dr. Renée K. Bechmann (International research institute of Stavanger, Norway), while Dr. Thierry Baussant (International research institute of Stavanger, Norway) was the project leader for the mollusc study. For more information about the BIOSEA JIP programme, experimental designs, and additional results from other biomarker and fitness studies, refer to Baussant (2004), Beckmann (2004), Bechmann et al. (2004).

5.1 Introduction

The classical dose-response relationship is the fundamental basis of understanding the toxic effects of chemicals in toxicology, and is generally measured as the chemical concentration that elicits a specified biological effect over a fixed time interval (Forbes and Forbes 1994). Often, biological effects are a log-normal function of chemical dose, and traditional environmental risk assessments (ERA) are founded on the assumption that, below a certain dose, no effects can occur due to the action of repair and excretory mechanism (Matthiessen 2003). Furthermore, for a biomarker (molecular, cellular and physiological changes in an organism following exposure to various types of pollutants) to be useful in ERA, it should respond to pollutants in a dose-dependent manner over a concentration range of the pollutant that is environmentally meaningful (Peakall 1992). However, there are growing concerns that many chemicals do not have definable toxicological thresholds levels (below which effects are negligible or non existent), and cause effects even at very low concentration levels (e.g. IPCS 2002); endocrine disrupting compounds (EDCs) are a good example of these chemicals. Moreover, dose-response curves do not always fit the classical log-normal (or Sigmoid), monotonic response (e.g. http://www.ourstolenfuture.org/). Some dose-response curves show a stimulatory response at low doses and an inhibitory response at higher doses; this phenomenon is termed hormesis' (Stebbing 1982; Calabrese and Baldwin 1997). Typically, hormesis may be graphed as U-shaped, an inverted U-shaped or a J-shaped dose–response curve depending on the endpoint evaluated (Calabrese and Baldwin 2002). Although there is clear evidence that the concept of hormesis is real and reproducible, and has been reported for hundreds of different endpoints and for many chemically-diverse agents (reviewed in Calabrese

2005), there is still considerable debate and reluctance in the scientific community to accept the phenomenon (e.g. Calabrese and Baldwin 2000; Kaiser 2003; Van der Woude 2005; Murado and Vázquez 2006; Mushak 2007; Kefford et al. 2008). Much of the latter is related to the lack of knowledge and understanding of mechanisms to account for hormesis (e.g. Tayer et al. 2005; Mushak 2007). Furthermore, hormesis is difficult to distinguish from background biological variation, as it normally displays only a modest (typically 30-60% above/under the control level) response (Calabrese 2005). In addition, traditional (eco)toxicology evolved as a 'high-dose/few doses testing discipline', and this philosophy may have resulted in many instances of hormesis not being recorded because tests were not conducted with a sufficiently low range of chemical exposures (Chapman 2002).

Understanding dose-effect relationships and low-dose effects remains a most important task for science, industry and regulatory authorities. For example, discharge from the oil and gas industry has received increased attention in recent years due to the potential ecological risks posed by some of the chemicals in the produced water (e.g. Durell et al. 2006; Neff et al. 2006). Usually, produced water is the largest volume of waste from offshore oil and gas production facilities; for example, in the North Sea, discharge reached an annual volume of close to 400 million m^3/year in 2003 (Durell et al. 2006). The chemical composition of produced water is complex and varies from one well to another (Røe Utvik 1999); however, the main components include dispersed oil, dissolved hydrocarbons, organic acids, alkylphenols, metals, and traces of production chemicals added to the oil or produced water during production and treatment (Neff 2002). Many of these components have been associated with effects on wildlife (e.g Krause 1994; Harvey et al. 1999; Giesy et al. 2000; Aas et al. 2000; Taban et al. 2004, Barsiene et al. 2006a; Meier et al. 2007; Hansen et al. 2008). As a consequence, the oil industry in Norway has established a strategy (that has been adapted by the OSPAR convention) that there should be no harmful discharges into the environment (SFT 1999; Johnson et al. 2000). The strategy requires identification of any environmentally harmful discharge, or chemical component of discharges, in order to develop effective approaches to treat or dispose of the discharge to remove its adverse impacts on the environment (Neff et al. 2006). As a result, current regulations state that the concentration of dispersed oil permitted in produced water discharges must be limited to 30 ppm (OSPAR 2006). Although discharge chemicals are diluted in the vicinity of offshore installations just after their discharge (Utvik et al. 1999), the effluents may still exert low dose effects in a gradient from the platforms. Therefore, to be certain that present discharges from the oil and gas industry actually result in 'zero harmful effects' it is essential to investigate effects at doses below 30 ppm.

The present study investigated the effects of low doses of dispersed oil on protein expression in the blue mussel *Mytilus edulis* and the green sea urchin *Strongylocentrotus droebachiensis*. The chosen test concentrations of dispersed oil (i.e. 15, 60, and 250 ppb) were 120-2000 times lower than the current discharge limit for dispersed oil. Since so-called 'omics' technologies allow simultaneous analysis of thousands of genes, proteins and metabolites (e.g. Clish et al. 2004; Joyce and Palsson 2006), these technologies have enabled a wider approach to biological questions compared to single endpoints analyses (e.g. Aardema and MacGregor 2002), and can potentially reflect both the 'quantity' and the 'quality' of effects (Nesatyy and Suter 2007) following environmental pollution. The aim of this study was to investigate dose-response relationships at low doses of oil on protein expression in marine mussels and sea urchins. The rationale for investigating a range of oil concentrations instead of just one is the potential for improved insight into effect-thresholds and dose-effects in the

The continuous flow system, described by Sanni et al. (1998) and Baussant et al. (2001), was used to create the dispersion of crude oil in seawater (Figures 5-1 and 5-2).

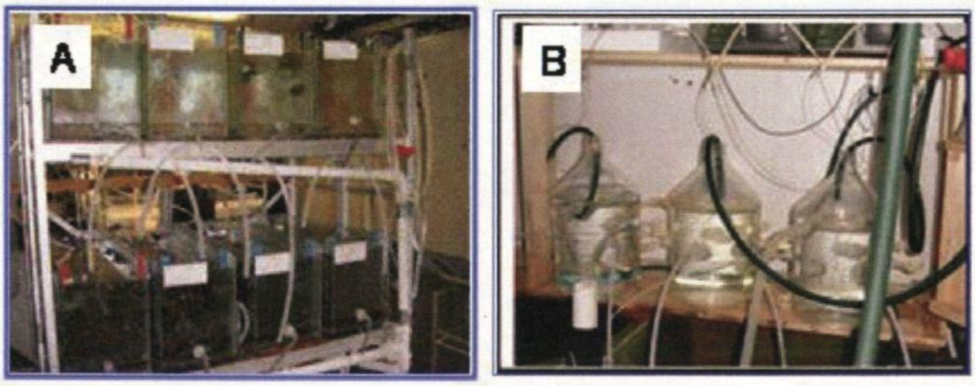


Figure 5-2. (A) Exposure tanks (i.e. 60 L) with mussels divided into 2 groups for each treatment: top ≈ 150 mussels and bottom ≈ 500 mussels. The flow rate was about 500 and 800 ml.min^{-1}, respectively in the bottom and top aquaria. (B) Mixing bottles for dilution of stock (5 ppm) dispersion of oil. Figure courtesy of Dr. Thierry Baussant.

The oil dispersion was created using a mixing valve located close the injection point of the oil (Figure 5-1). The valve position was controlled by pressure readings on the line with the stream of seawater and adjusted so that the mean size of the oil droplets was close to 10 μm. A dispersion equivalent to a nominal concentration of 5 ppm was first made by injection of 42 μl oil/min in the flow of seawater (7 L/min) using a precision syringe pump (ISCO model 260D, USA). A capilar teflon © tubing with an inner size of 0.5 mm was used to conduct oil from the cylinder to the seawater. The dispersion was first conducted to a header tank. Thereafter, it was diluted by means of peristaltic pumps (Watson-Marlow Model 205 and 505, England) with seawater into 3 different 10 L mixing DURAN glass bottles to obtain the desired nominal concentration (250, 60 and 15 ppb) in the exposure tanks (Figure 5-2 A). Mixing bottles were equiped with outlets (hole drilled, then a gummy stopper with glass tubing through it) to allow the water to flow to the exposure tanks (Figure 5-2 B).

5.2.1 Exposure monitoring

During the course of the experiment, the seawater was monitored for total hydrocarbon concentration (THC), poly aromatic hydrocarbons (PAH) concentration and particle size/number; seawater samples were siphoned out using silicon tubing, placed carefully, to avoid inclusion of the top layer of seawater where the presence of oil film could affect the final analysis. A Coulter counter ©, equipped with a 70 mm aperture tube, was used to control oil particle size/number and estimate water concentration in the inlet of the tanks. The counting was made in a 80 ml beaker and the volume analysed was 2x500 µl. The presence of particles other than oil in the seawater (faeces, micro-organisms) influenced the background signal and, hence, compromised the ability to estimate the relative oil concentration in the exposure tanks based on measurement of oil particles. However, the Coulter counter was used regularly (every third day) as a check of the exposure system at the inlet to the tanks to control both particle size and the relative levels in the highest exposure concentration. The PAH concentration in water was measured once during the experiment (i.e. in the middle of the exposure period). Seawater samples were collected in 1-L glass bottles containing hydrochloric acid to ensure low pH (< 2) and prepared for analysis within 48 h after sampling. Analysis of the PAH content in water was done using Gas Chromatography (HP5890, Hewlett Packard, USA) connected to a Mass Spectrometer (Finnigan SSQ7000, USA) and analyzed in selected ion mode (GC/MS-SIM) as described in Baussant et al. (2001). Originally, it was planned to measure THC in water by GC-FID (flame ionization detection); however, this was not successful as the method was developed for oil concentrations above 100 ppb. In three different analyses, all quantifiable concentrations (even in the 250 ppb tanks) were below 100 ppb; therefore, further analyses of THC in water were done, once a week, by semi-quantitative fluorescence analyses (as described by Aas et al. 2000). In addition, the PAH content of whole mussels (soft tissue) and sea urchin gonads was measured at the end of the exposure (3 replicates) by GC/MS-SIM (as described for PAH content in water samples). All tissue samples were transferred into glass vials (pre-heated at 500°C) with teflon© lock and stored at -80°C prior to analysis.

5.2.2 Sample collection

Haemolymph samples from mussels and samples of coelomocytes from sea urchins were collected following the methods described in Chapter 2 (2.1.1) and Figure 2-1. Thirty mussels (shell size 6.0 ± 0.5 cm) and 30 sea urchins (test diameter 5.1 ± 1.2 cm) were sampled and analysed per treatment.

5.2.3 Sample preparation on ProteinChip arrays

ProteinChip arrays with weak cation exchange surfaces properties (WCX-arrays) were used for protein expression analyses and the protocol outlined in Table 2-7 was used for sample preparation.

5.2.4 SELDI TOF MS analysis

The protein arrays were analysed on the PBS-IIc time of flight mass spectrometer using ProteinChip Software version 3.1. Mass spectra were recorded using the following settings: 91 laser shots/spot surface in a positive ionisation mode (65 of the shots were collected, starting at position 20 and ending at position 80 of the spot), laser intensity 185, detector sensitivity 8, detector voltage 2850 V, data acquisition from 0 -

200000 Da, and optimum mass range focus from 2000 – 15000 Da. The same acquisition protocol was used for mussel and sea urchin plasma samples.

The spectra were calibrated with the following molecular weight standards: Dynorphin A (2147.5 Da), Insulin bovine (5733.6 Da), Ubiquitin (8564.8 Da), Cytochrome C bovine (12230.9 Da), Bovine β-Lactoglobulin A (18363.3 Da), Horseradish Peroxidase (43240.0 Da), and IgG bovine (147300 Da).

5.2.5 Data handling and statistics

Data processing and statistical analyses followed the description given in Chapter 2 (i.e. 2.1.4)

5.3 Results

Because of the limited availability of samples (i.e. only 30 samples per group), and an uneven gender-ratio for each species, all results are based on male and female data combined. Further, all proteomic analyses were restricted to M/Z values with a signal to noise greater than or equal to 5 (to ensure that biomarker discovery was not conducted on background noise), present within the mass range of 2000 to 200000 Da (i.e. n = 153 for sea urchins, and n = 212 for mussels).

5.3.1 Chemistry

Measured concentrations of total hydrocarbon concentrations (THC) and poly aromatic hydrocarbons (PAHs) in the exposure tanks, as well as PAHs in biota (i.e. sea urchin gonads and whole mussel tissue), are given in Tables 5-1 and 5-2. Measured water and biota concentrations of THC and PAH increased with increasing nominal concentrations (Figure 5-3 and Tables 5-1, 5-2).

Table 5-1. Nominal exposure concentrations of dispersed oil compared to measured water concentrations of THC (n = 5) and PAH (n = 1) in the exposure tanks.

Species	Nominal concentrations (in ppb)	Sum THC in water (in ppb)	Sum PAHs in water (in ppb)
Sea urchin	15	4	0,170
Mussel	15	3	0,154
Sea urchin	60	29	0,980
Mussel	60	15	0,812
Sea urchin	250	85	4,690
Mussel	250	63	3,758

Note. The THC and PAH concentrations in control tanks were below the detection limits.

Table 5-2. Measured concentrations of PAHs in biota (i.e. sea urchin gonads and whole-mussel tissue). at the end of the exposures (n = 3 replicates per sample type).

Species	Nominal concentrations (in ppb)	Sum PAHs in biota (in µg/kg wet-weight)
Sea urchin	15	63
Mussel	15	272
Sea urchin	60	723
Mussel	60	1750
Sea urchin	250	4766
Mussel	250	13236

While measured water concentrations of sum-THC ranged from 4 - 85 ppb for sea urchin tanks and 3 – 63 ppb for mussel tanks, sum-PAHs in water were 0,17 – 4,69 ppb and 0,15 – 3,76 ppb in sea urchin and mussel tanks, respectively. The lower concentrations measured in the mussel tanks compared to concentrations in the sea urchin tanks, is most likely explained by the fact that mussels are filter feeders and would rapidly remove oil (droplets) from the water; however, this hypothesis needs to be tested and validated.

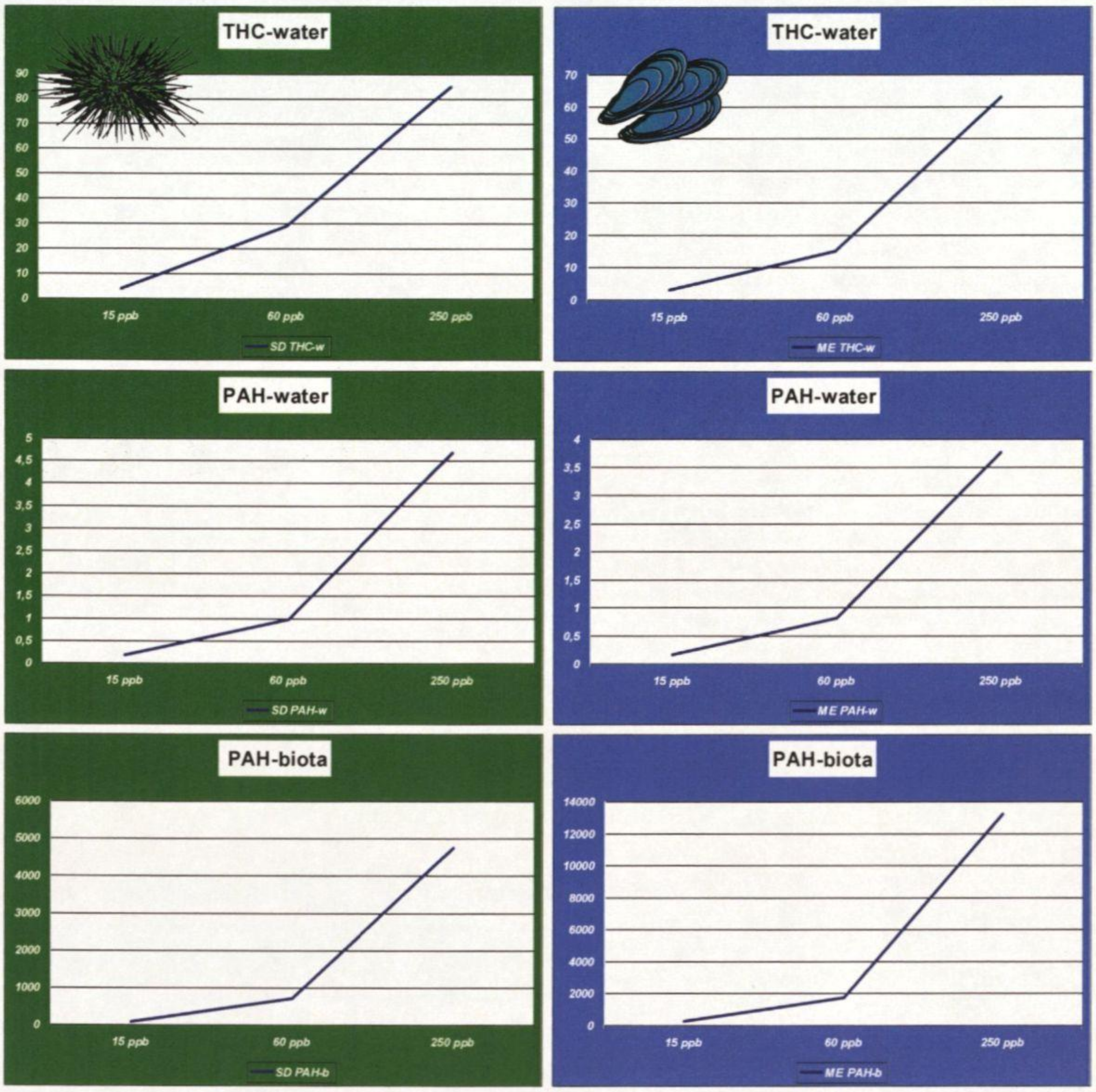


Figure 5-3. Nominal exposure concentrations plotted against measured concentrations of sum-THC in water, sum-PAH in water, and sum-PAH in biota (sea urchins to the left and mussels to the right).

5.3.2 Proteomic response

All tested concentrations of dispersed oil affected the protein expression in *Mytilus edulis* and *Stongylocentrotus droebachiensis*.

Expression was changed for 26 - 50% of all resolved mass peaks in sea urchin plasma, and for 23 - 32% of the resolved peaks in mussel plasma (Table 5-3). For each species, all exposure regimes had a predominantly down-regulating effect on protein expression

with 49 - 80% significantly downregulated mass peaks detected in exposed sea urchins and 54 - 69% in exposed mussels compared to controls (Table 5-3). Average changes in protein expression (as the ratio exposed/control or control/exposed) were relatively low (i.e. in the range 1.8 – 2.5 fold) when only significantly altered peaks were compared (Table 5-3, indicated in the brackets). No 'extreme' responses were observed as none of the detected peaks was changed by more than 7 fold in comparison to the level in controls for either mussels or sea urchins.

Table 5-3. Overview of number of significantly ($p < 0.05$, Mann-Whitney-Wilcoxon test) upregulated (Up) and downregulated (Down) peaks in various exposure regimes for sea urchins (*Strongylocentrotus droebachiensis*) and mussels (*Mytilus edulis*).

Strongylentrotus droebachiensis					*Mytilu edulis*				
Exposure	Up	Down	Σ	%	Exposure	Up	Down	Σ	%
15 ppb	23	22	45	29 (2.0x)	15 ppb	22	26	48	23 (2.0x)
60 ppb	36	46	76	50 (2.5x)	60 ppb	21	47	68	32 (2.3x)
250 ppb	8	32	40	26 (1.8x)	250 ppb	20	37	57	27 (2.2x)
n= 153 resolved peaks with S/N ≥5					n= 212 resolved peaks with S/N ≥5				

Note. Σ = total number of significantly altered peaks, S/N = signal-to-noise ratio, % = percentage of peaks that were significantly altered by exposure. Average fold changes in expression is indicated in brackets.

5.3.3 Dose-response relationships

In general, results revealed that protein expression was altered in a non-monotonic manner, where the middle concentration (60 ppb oil) had the greatest effect on both the number of affected protein features and the average fold changes in expression for all significantly changed peaks following exposure (Figure 5-4).

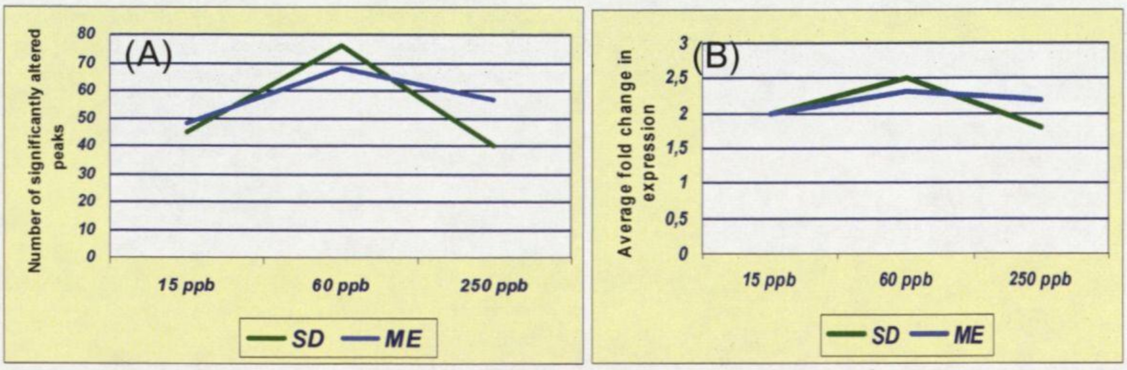


Figure 5-4. Dose-response for (A) number of significantly affected mass peaks and (B) average fold changes in protein expression for each exposure group. SD = *Strongylocentrotus droebachiensis*, ME = *Mytilus edulis.*

Furthermore, the majority of individually affected protein species were altered in a hormetic-like (non-monotonic) dose-response manner (Figure 5-5 and 5-6). For example, in sea urchins, protein expression changed in a non-linear manner for 95% of the significantly ($p < 0.05$) affected mass peaks, while 87% of peaks that were changed in mussels following oil-exposure had a non-linear dose-response curve (Figure 5-6). The form of the does-response curves were diverse (Figure 5-5), and included 'normal' linear dose-response with upregulation, 'normal' linear dose-response with downregulation, V-shaped (similar to the defined U-shaped response curve), inverted V(U)-shape, Z-shaped, and inverted Z shaped response-curves.

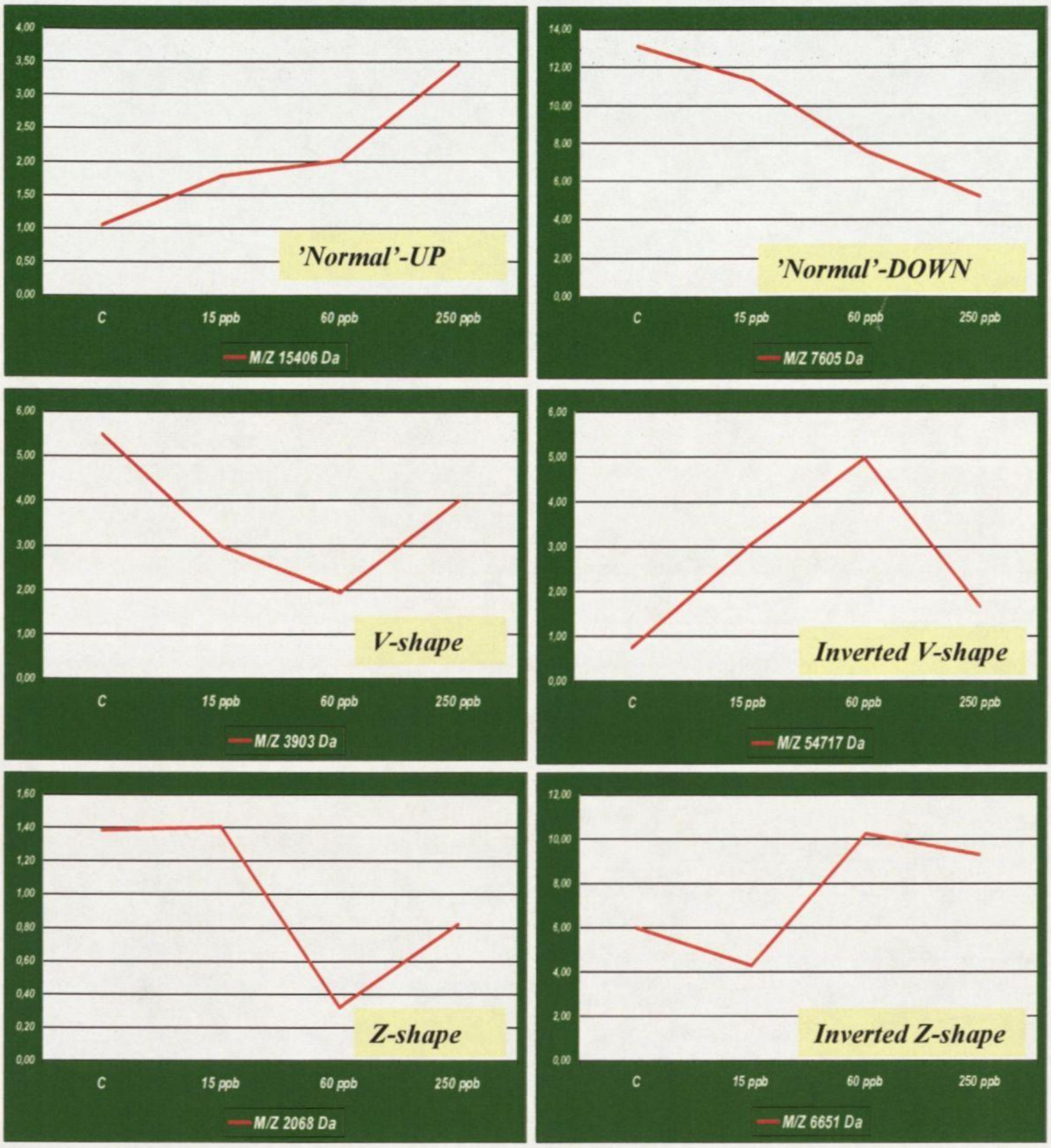


Figure 5-5. Examples of the different types of dose-response curves observed for mussels and sea urchins following oil exposure. X- axis = doses (i.e. 15, 60 and 250 ppb oil), Y-axis = relative peak intensity for selected M/Z values.

individuals in each exposure group (n = 30 for all groups). For mussels and sea urchins, the response pattern indicated that the middle concentration (i.e. 60 ppb) had the most severe effect on protein expression (i.e. the greatest distance from the control group in the plots, indicated by the arrows), (Figure 5-7).

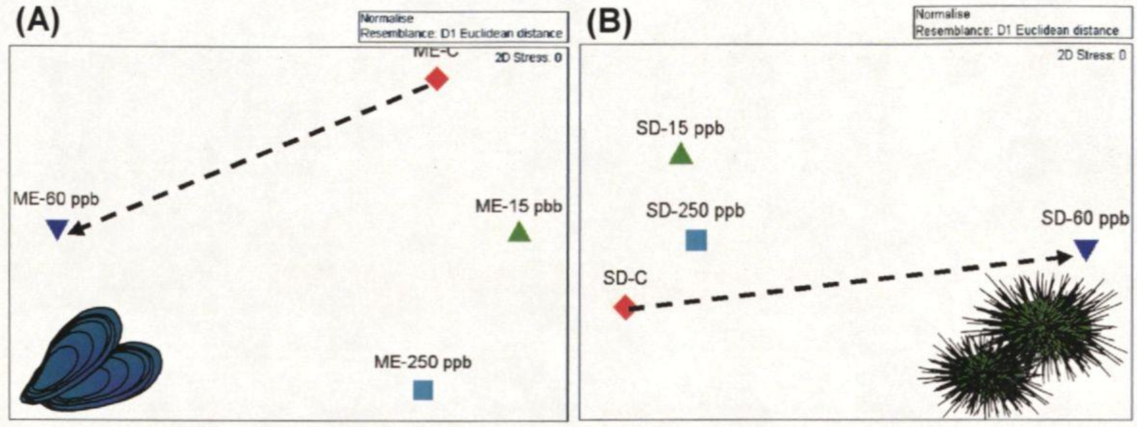


Figure 5-7. Two-dimensional scaling representation of similarities and differences in response to different exposure concentrations. The analysis is based on a similarity matrix constructed using normalised Euclidian Distances. Data inputs were all plasma-protein peaks with S/N ≥5 (n = 212 for mussels and 153 for sea urchins), where each symbol represents mean peak intensity for all the individuals in one exposure group (n = 30). Red = Control, green = 15 ppb, dark blue = 60 ppb, and light blue = 250 ppb, ME = *Mytilus edulis*, SD = *Strongylocentrotus droebachiensis.*

However, the tendency of an inhibitory effect at 250 ppb oil was strengthened by a 3-dimmensional MDS plot created with individual responses in each exposure group (Figure 5-8).

software, was investigated further with multivariate prediction models using the Biomarker Pattern^{TM} Software. The software was used to identify potential multivariate patterns that would separate exposed from control organisms, and also separate the different exposure groups. The hypothesis tested was that the protein expression profiles in the 250 ppb groups would be more similar to those of the control groups (due to the inhibition in response), and hence classification models made with samples from the 250 ppb groups would have less discriminatory power than models created with, for example, 60 ppb samples.

Table 5-4. Test of prediction models, created in Biomarker Pattern^{TM} Software, for comparison of controls versus exposed in sea urchins (*Strongylocentrotus droebachiensis*) and mussels (*Mytilus edulis*).

S. droebachiensis	Prediction success (%) in test samples		
Compared groups	Controls	Exposed	Classifiers, M/Z (Da)
	Specificity	Sensitivity	
C vs. 15 ppb oil	67	90	4274 , 50874, 5608, 7934
C vs. 60 ppb oil	93	93	6225, 7934, 5103
C vs. 250 ppb oil	90	93	6225, 7934, 38464
M. edulus			
C vs. 15 ppb oil	93	77	5899, 25701, 6091, 7852, 3648
C vs. 60 ppb oil	93	90	151702, 6091, 4493
C vs. 250 ppb oil	90	80	9343, 4235, 6091, 3952

Note. Prediction models were generated with 40 samples and tested with 20 new samples. Importance of classifiers decreases from left to right.

Multivariate analysis (regression tree-based methods) detected protein patterns associated with exposure that correctly classified masked samples with 67 - 90 % (sea urchins) or 90 - 93 % (mussels) specificity and 90 - 93 % (sea urchins) or 77 - 90 %

(mussels) sensitivity (Table 5-4). However, the difference in discriminatory power for the various exposure groups were more similar than results shown e.g. Table 5-3 and Figure 5-8. The ability to correctly classify controls from exposed samples was relatively similar for mussels and sea urchins (Table 5-4), although the percentage of affected protein features in sea urchins was approximately twice as high for all exposure groups compared to mussels (Table 5-3). Nevertheless, the best discriminatory power was obtained with the classification model created with samples from sea urchins exposed to 60 ppb oil and sea urchin controls, each classified with 93 % accuracy (Table 5-4). For mussels, although the two most important classifiers for controls vs. 60 ppb and 250 ppb oil were the same (i.e. M/Z 6225 Da and M/Z 7934 Da), the specificity was 3% better in the classification model made with 60 ppb samples compared to the model made with samples from the highest oil-concentration, supporting the conclusion that protein expression in both mussels and sea urchins exposed to 60 ppb oil was most dislike the protein profiles of controls.

The set of classifiers selected in the different prediction models (Table 5-4) were unique for each model, even though there were some overlaps. Furthermore, the classifiers used for mussel samples in this study were not the same as those chosen for controls versus mussels exposed for 3 weeks to 500 ppb oil in the first laboratory study (Chapter 3, Table 3-4). This is not necessarily unexpected, given the sensitivity of proteomic analyses and the dynamic nature of the proteome.

5.4 Discussion

Stress response is a universal defence mechanism against a wide variety of stress conditions (e.g. exposures to environmental pollutants) which adversely affect protein expression, denaturation of proteins, protein folding, and hence protein function (e.g. Schaefer et al. 1998). The hypothesis tested in this study was that protein expression signatures would reveal dose-response relationships and the complexity therein. An assumption for biological effects caused by oil, in this study, was that the oil would be dissolved in exposure tanks and bioavailable to the test organisms. Chemical analysis revealed that measured water and biota concentrations of PAH and THC increased with increasing nominal concentrations (Figure 5-3 and Tables 5-1, 5-2). However, the concentration of PAHs measured in mussels was much higher than in sea urchins (Table 5-2). The most likely explanation for that is that whole-mussel-soft-tissues (including digestive gland) were used for chemical analysis in mussel, while only gonads were used in sea urchins. Zorita et al. (2006) reported that the digestive gland was the main organ for accumulation of environmental pollutants in blue mussels. Nevertheless, the motive for using only gonads for chemical analysis in sea urchins was that the project leader (i.e. Dr. Renée K. Bechmann) wanted to correlate PAH concentrations in gonads with effects of exposure on sea urchin larvae (since larvae/fitness studies was part of the BIOSEA project; see Bechmann 2004 and Bechmanne et al. 2004).

Results from protein expression analyses showed that exposure to concentrations of dispersed oil; 120 - 2000 times lower than the current discharge limit for dispersed oil (i.e. 30 ppm, OSPAR 2006) from offshore installations, affected protein expression in mussels and sea urchins. Furthermore, although different dose-response relationships occurred, protein expression was predominantly altered in a non-monotonic dose-response manner (Figure 5-5 and 5-6). Thus, the hypothesis of this experiment was confirmed, in the sense that protein expression signatures revealed dose-response information, and, additionally, showed that all altered 'endpoints' were not affected in the same dose-response-manner (e.g. Figures 5-5 and 5-6). The latter is, perhaps, not surprising, as the proteome reflects the biological functioning of the total organism (e.g. Anderson and Anderson 1998) and each endpoint (i.e. protein form) has potentially its own unique dose–response relationship (e.g. Calabrese 2005). Finding that a concentration of dispersed oil, as low as 250 ppb (or 63 - 85 ppb total THC), inhibited many protein species, however, was not expected. Unfortunately, as there are no similar studies of dose-response relationships of oil on protein expression (e.g. http://apps.isiknowledge.com), a detailed comparative discussion of present results with literature values is impossible. However, Walker et al. (2007) reported various types of dose-response relationships for different SELDI-TOF MS peaks in fish (*Cyprinodon variegates*) following exposure to 5 concentrations of 17□-estradiol. In addition, other biomarker studies conducted within the BIOSEA JIP programme have reported similar non-linear results. For example, oxidative stress parameters such as Glutathione-S-Transferase (GST) activity, total oxyradical scavenging capacity (TOSC) and Catalase activity were all affected in a non-monotonic dose-response manner following oil exposure in *Mytilus edulis* (Baussant 2004); Clamys islandica (Baussant 2004) and *Pandalus borealis* (Larsen 2004), with some inhibitory effect at the highest compared to the two lowest concentration. The non-monotonic responses were even more pronounced after prolonged exposures of 5 and 7 months (Larsen 2004; Baussant 2004). Although no results are available on GST, TOSC and Catalase activity in sea urchins (*Strongylocentrotus droebachiensis*) after 4 weeks exposure (Bechmann et al. 2004), after 7 months exposure to dispersed oil, Catalase activity was altered in a bell-shaped manner (i.e. only the 60 ppb exposure group was statistically different from the control group), and GST activity had a Z-shaped response curve (Bechmann 2004). Furthermore, Taban et al. (2004) found a bell-shaped dose-response relationship for DNA-damage (as measured by the comet assay) in the haemocytes of *Mytilus edulis* after 5 weeks' exposure to 15, 60 and 250 ppb dispersed oil. Mussels exposed to 60 ppb oil had a higher percentage of DNA in the comet tail than the mussels exposed 250 ppb oil. However, DNA damage in *Strongylocentrotus droebachiensis* was induced in a linear dose-response manner following 4 weeks exposure to the same concentrations of oil. The different types of response pattern for the two species may be explained by the 1week difference in exposure time, or, more likely, by species-specific sensitivity to DNA damage. Fourie et al. (2007) reported species-specific sensitivity to DNA damage (as measured by the comet assay) in five earthworm species following cadmium exposure.

The explanation for the observed dose-response relationships reported here is not clear, as none of the affected protein species have been identified. Non-monotoric, 'hormetic-like' effects have been reported previously in a highly diverse array of biological models for numerous organs and endpoints and chemical/physical stressors (e.g. Calabrese and Baldwin 2001; Li et al. 2007; Widarto et al. 2007), and it is evident that no single mechanism can account for these phenomena. In addition, attempts to assess the underlying mechanisms of 'hormetic-like' dose responses in (eco)toxicology have not reached any agreed conclusion (Calabrese 2005; Murado and Vázquez 2007; Mushak 2007). 'Unexpected' results tend to be ignored, not published or regarded as artefacts of experimental design (Chapman 2002b; Johansson 2003), and terms like 'autoprotection' (Mehendale et al. 1994; Mehendale 1995), 'adaptive response' (e.g. Leonard 2007; Calabrese 2008), 'conditioning' (Calabrese et al. 2007) and

'overcompensation' (e.g. Calabrese 1999) have been used to explain non-monotonic dose-response phenomenon. For example, Calabrese (1999) and Calabrese and Baldwin (2001b) suggested that hormesis represented an 'overcompensation' response to a disruption in homeostasis; 'autoprotection' has been associated with the stimulation of tissue repair (e.g. Mehendale 1995). The proposed principal mechanism common to 'conditioning', 'adaptive response' and 'autoprotection' is that low levels of stress activate or upregulate existing cellular and molecular pathways that enhance the ability of the cell and organism to withstand more severe stress (Calebrese et al. 2007). Some authors have been more explicit in trying to explain the mechanisms involved in non-monotonic responses. For example, Li et al. (2007), using a computational modelling approach, reported a novel mechanism for U-shaped (and inverted U-shapes) dose-response behaviours observed with certain steroid-hormone mimicking chemicals (EDCs). Their results revealed that non-monotonic dose-responses in gene expression can arise within the classical genomic framework of steroid signalling; for example, when the exogenous ligand is an agonist, a U-shaped dose-response appears as a result of the inherently nonlinear process of receptor homodimerization. This U-shaped dose-response curve can be modulated further by mixed-ligand heterodimers formed between endogenous ligand-bound and exogenous ligand-bound receptor monomers. When the heterodimer is transcriptionally inactive or repressive, the magnitude of U-shape increases; conversely, when the heterodimer is transcriptionally active, the magnitude of U-shape decreases (Li et al. 2007). They also found that an inverted U-shaped dose-response can arise when the heterodimer is a strong transcription activator regardless of whether the exogenous ligand is an agonist or antagonist (Li et al 2007).

Nonetheless, hormesis/non-monotonic responses are clearly not well understood, and may be ignored/overlooked and misunderstood (i.e. anomalous dose-response

curves ascribed to hormesis, when it is not; Chapman 2002b;.Mushak 2007). Chapman (2002b) exemplified the general misunderstanding of hormesis with the reported case of increasing water hardness and metal-bioavailability/toxicity. Although there may be no toxicity at the highest test exposures in very hard water, toxicity can appear at intermediate exposures due to dilution effects (not only of metals concentrations, but also of water hardness), and hence the corresponding hormetic-like exposure-response curves are a result of extrinsic factors, rather than intrinsic factors like, for example, attempts to maintain homeostasis. Furthermore, it can be difficult to distinguish/interpret whether stimulatory effects of, for example, EDCs on fecundity and growth-rate are results of disruption of specific hormonal processes or due to general overcompensation to low-levels of stress (Widarto et al. 2007).

Overall, there seems to be a need for a better definition of the 'hormesis concept' and a clearer consideration of whether all biphasic dose-response curves should be considered representative for hormesis or not. For example, Thayer et al. (2005) stated that the use of the term 'hormesis', with its associated descriptors, distracted from the broader and more important questions regarding the frequency and interpretation of non-monotonic dose responses in biological systems, and emphasised that the assumption that hormesis is generally an adaptive response is an oversimplification of complex biological processes. The latter statement illustrates the need for a better understanding of whether hormesis (or low-dose, non-monotonic responses) has positive, neutral, or adverse effects on model organisms, populations, and communities (Chapman 2001). To elucidate would require a massive research effort, focusing on individual variation, species sensitivity (Chapman 2001), and judgement of what is the 'normal' response of a species to a toxicant (Kefford et al. 2008). Improvement of risk assessment strategies may include taking into account biphasic dose-response curves

but should also include consideration of proper physiologically-based pharmacokinetic models for better extrapolation of differences in toxicokinetics going from high- to low-dose exposure, as well as taking into account kinetics for gene repair systems (Van der Woude et al. 2005).

For hormesis-like phenomena to be accepted fully in ecotoxicology and environmental risk assessment, it is important to gain better insight into the mechanisms involved in such processes (Van der Woude et al. 2005), and thus a better understanding of the phenomenon itself. Some of the protein forms that were altered in a biphasic manner in this study could hold essential mechanistic information about hormesis if purified and identified. Therefore, a proteomic approach has the potential to elucidate some of the knowledge gaps in relation to non-monotonic response relationships at low doses.

5.5 Conclusions

In conclusion, the application of protein expression analysis with SELDI TOF MS and ProteinChip array technology has identified specific plasma protein profiles in two invertebrate species (*Mytilus edulis* and *Strongylocentrotus droebachiensis*) that could be used to distinguish controls from exposed organisms and different exposure concentrations from each other. Additionally, unique dose-response relationships were observed for the different protein species detected by MS. Most of the affected protein forms were changed in a non-monotonic manner, where the 60 ppb group (in each species) appeared to be more affected than the 15 and 250 ppb groups.

There is no obvious explanation for the observed effects; however, future identification of key molecules (e.g. some of those protein forms that were altered in a non-monotonic manner) by tandem mass spectrometry could, potentially, provide the essential mechanistic insight that is needed to enlighten why most of the significantly altered protein species were affected in a non-monotonic manner. Furthermore, such mechanistic insight might also assess if the observed responses are ‘positive’, ‘neutral’, or ‘negative’ for the organisms.

6.1 Introduction

Environmental risk assessment (ERA) is the link between ecotoxicology and risk management (Breitholtz et al. 2006). ERA aims at assessing or predicting the potential effects of any chemical on the structure and function of ecosystems (e.g. Galloway et al. 2004; De Laender et al. 2008), and to provide sufficient information for decision-making with the purpose of protecting the environment from unwanted effects of anthropogenic chemicals (Breitholtz et al. 2006). A major challenge for ERAs is to link harmful effects of environmental pollutants in individual sentinel animals to their ecological consequences (Moore et al. 2004). However, risk assessment includes dealing with many sources of uncertainties (Brouwer and Blois 2008). For example, even for well-investigated chemicals there are significant uncertainties about their actual impact on ecosystems, since 'knowledge' regarding effects is often based on extrapolations from the effects seen in a few test species (e.g. Forbes et al. 2001) under controlled laboratory conditions to the entire ecosystem(s) where numerous biotic and abiotic factors interact in a complex fashion (Breitholz et al. 2006). To have a more accurate prediction of possible effects of chemicals on ecosystem structure and function, methods and laboratory results should (as a start) be validated in field experiments (e.g. Martin-Diaz et al. 2008). Furthermore, responses due to chemical exposure must be distinguished from natural sources of variability (e.g. ecological and physiological variables, gender and species-specific differences and individual variability) (Peakall 1992).

In the present study, the SELDI TOF MS-based approach, developed for protein expression analysis in marine invertebrates, was validated in a field study, where mussels (*Mytilus edulis*) collected at the reference site used in Chapters 3 and 4 (i.e.

Førlandsfjorden) were deployed along a copper gradient in the vicinity of an old copper mine (http://www.showcaves.com/english/no/mines/Visnes.html). Although the mining activity stopped in 1972, leakage of metals (e.g. copper) from landfills around the mining area continues to be a source of pollution and cause for concern (Andersen et al. 2003b). Bivalve mussels have been used widely as a monitoring organism (e.g. 'The Mussel Watch' programme, Goldberg 1975) for many reasons, one of them being their ability to readily bio accumulate pollutants such as metals (e.g. Viarengo, 1985), and hence reflect the amount of metals present in the environment. Copper is an essential element for all organisms, it is a constituent part of enzymes and proteins and is essential for their activity and function (reviewed by Linder 1991). Furthermore, copper plays a central physiological role in living organisms because of its ability to exist in multiple oxidation states, and yet, its redox property can also be the cause of toxic effects (Stohs and Bagchi 1995). For example, free excess copper ions are believed to participate in the formation of reactive oxygen species (ROS) (e.g. Manzl et al. 2004; Nawaz et al. 2006; Knauert and Knauer 2008). In the presence of superoxide ($*O_2$-) or reducing agents such as ascorbic acid or intracellular glutathione (GSH), Cu^{2+} can be reduced to Cu^{+}, which is capable of catalyzing the formation of hydroxyl radicals from hydrogen peroxide (Bremer 1998; Kadiiska et al. 1993). The hydroxyl radical is a powerful oxidizing radical capable of reacting with practically every biological molecule (Buettner 1993), and thus potentially initiating oxidative damage and Cu-induced cellular toxicity (Gaetke and Chow 2003). Moreover, copper has been implicated as an endocrine disrupting chemical (Schantz and Widholm 2001; Handy 2003) due to the intimate physiology of copper in the normal neuro-endocrine functions of vertebrate animals and in the endocrinology of fish (Handy 2003). Metals, including copper, have also been shown to interfere with hormones that stimulate reproduction, such as methyl farnesoate, as well as with secretion of the gonad inhibiting hormone, therefore affecting, for example, ovarian growth in crustaceans (e.g. Rodriguez et al. 2007). Although many approaches have been used to assess metal-induced stress (in mussels) including, for example, cellular, biochemical, histochemical and histological approaches (e.g. Almeida et al. 2003; Burlando 2006; Zorita et al. 2006; Labieniec and Gabryelak 2007), the most frequently reported biomarker of metal exposure is the metallothioneins (MTs). MTs are non-enzymatic, low-molecular weight (approximately 60 amino acids), metal-binding proteins that are thought to be involved in homeostatic control of essential metals, as well as detoxification of both non-essential and essential trace metals (e.g. reviewed in Amiard et al. 2006). However, there is still controversy regarding the biological functions of MTs and their utility as biomarkers (Amiard et al. 2006). For example, variations in MT levels may result from factors other than exposure to metals (i.e. organism handling, starvation, anoxia, freezing, presence of antibiotics, vitamins or herbicides, food abundance, reproductive cycle, and seasonal/spatial factors) (e.g. Viarengo et al. 2000; Cotou et al. 2001; Ivankovic et al. 2005; Erk et al. 2008). However, the utility of protein biomarkers could be enhanced if a robust suite of proteins indicative of, for example, copper-induced stress was used in combination as protein expression profiles (Bradley et al. 2002). After all, gene transcription and protein expression lies at the beginning of a response of a cell to, for example, a pollutant (e.g. Pennie et al. 2004). Thus, a response on gene transcription/protein expression could give a preliminary indication of the biochemical or biological mechanism being affected by the pollutant and data from such analyses provide starting points in a toxicological examination. Furthermore, protein synthesis depression is a life-saving mechanism for many organisms faced with environmental stress (Storey and Storey 2004). During environmental stress, organisms can limit protein synthesis by storing inactive ribosomes that are rapidly reactivated when conditions improve (Pytharopoulou et al. 2006). Consequently, downregulation as well as upregulation of protein expression may be early indicators of stress (e.g. Werner and Hinton 1999).

The aim of this study was to determine the utility of SELDI TOF MS proteomics in detecting a fieldgradient of contamination. The hypothesis was that protein expression signatures, obtained with SELDI technology, would contain information that reflected the level of contamination at each site (e.g. increasing number of affected protein species with elevated level of pollution). To test the hypothesis, the protein expression in *Mytilus edulis* deployed along a known gradient of copper was measured and response patterns observed in the field were compared with results (dose-response relationships and gender-sensitivity) observed in the laboratory (described in Chapters 3, 4 and 5). The rationale for choosing a metal field gradient over an organic gradient (given that all laboratory exposures were conducted with organic compounds) was that these sites were primarily affected by metals (see Table 2-9; Column 3-Visnes), while the potential field sites polluted with organic compounds, were also affected by metals and/or TBT, which would complicate the interpretation of results.

6.2 Material and methods

In November 2003, *Mytilus edulis* were collected in Førlandsfjorden (59°20'N, 5°13'E) and transferred directly to four sites along a coppergradient at Visnes (59°22'N, 5°13'E) (Figure 6-1). Mussels were deployed in the coppergradient for 18 days using cages (Figure 6-2), and then brought to the laboratory the evening before sampling (and kept in filtered, running seawater prior to sampling). Simultaneously (i.e. November 2003), indigenous mussels from Visnes were collected for comparison of

6.2.1 Monitoring of the metal gradient

The degree of the copper gradient will depend on local weather and hydrographic condition during the deployment, however, pooled samples of whole mussel tissues were taken for body burden analysis of metals, including vanadium (V), chromium (Cr), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), silver (Ag), cadmium (Cd), barium (Ba) and lead (Pb), in mussels from the various sites (n = 20 mussels per site). Analyses were conducted at an accredited laboratory (i.e. M-lab, www.eurofins.no) using inductively coupled plasma mass spectrometry (ICP/MS). Historical data on metal concentrations in sediment, water and mussel-tissue at Visnes were reported by Andersen et al. (2003b).

6.2.2 Sample collection

Haemolymph samples from the mussels were collected following the method described in Chapter 2 (i.e. 2.1.1 and Figure 2-1). Sixty mussels, with even gender distribution (shell size 7.4 ± 0.9 cm for mussels originating from Førlandsfjorden, and shell size 6.2 ± 0.7 cm for indigenous mussels at Visnes) were sampled and analysed per treatment. All samples were analysed after the last sampling in Førlandsfjorden (i.e. in May). Samples were kept at -80 °C prior to analysis.

6.2.3 Sample preparation on ProteinChip arrays

The new generation of ProteinChip (arrays from Ciphergen Biosystems/Bio-Rad) (http://www.bio-rad.com/proteinchip/) with weak cation exchange surfaces properties (CM10 arrays) were used for protein expression analyses and the protocol outlined in Table 2-7 was used for sample preparation.

6.2.4 SELDI TOF MS analysis

The protein arrays were analysed on the PBS-IIc time of flight mass spectrometer using ProteinChip Software version 3.1 (Ciphergen Biosystems). Mass spectra were recorded twice from each sample (i.e. corresponding to the low molecular weight region/ and the high molecular weight region) on the following settings: 147 laser shots/sample in a positive ionisation mode (105 of the shots were collected, starting at position 20 and ending at position 80 of the spot), laser intensity 170/185 (low mass run/high mass run, respectively), detector sensitivity 7/8 detector voltage 2850 V, data acquisition from 0- 200000 Da, and optimum mass range focus from 1500-20000 Da /15000-200000 Da. The spectra were calibrated with the following molecular weight standards: Dynorphin A (2147.5 Da), Insulin bovine (5733.6 Da), Ubiquitin (8564.8 Da), Cytochrome C bovine (12230.9 Da) for low mass spectra, and Bovine β-Lactoglobulin A (18363.3 Da), Horseradish Peroxidase (43240.0 Da), Serum Albumin bovine (66433.0 Da), and IgG bovine (147300 Da) for high mass spectra.

6.2.5 Data handling and statistics

Data processing and statistics was done according to the description given in Chapter 2.1.4.

6.3 Results

Primarily, results are compared and discussed for mussels deployed at the four sites constituting the pollutant gradient. However, to put the observed effects into perspective, the results have been compared also with data from indigenous mussels collected close to the old copper mine at Visnes (i.e. close to Site 4 in the gradient), as well as data from reference mussels collected in Førlandsfjorden at three additional time points (i.e. January, March and May). Further, to ensure that observed mass peaks were real protein forms (and not chemical or electronic background noise), all analyses of results have been restricted to those M/Z values with a signal to noise greater than or equal to 5, present within the mass range of 2000 to 200000 Da (i.e. n = 240 for males, and n = 220 for mussels). With the exception of body burden analysis of metals (which have been analysed in pooled tissue extracts from mussels that have not been sexed), all comparisons of effects on protein expression have been done separately for male and female individuals (due to observations regarding gender-specific sensitivities found in laboratory studies).

6.3.1 Chemistry

Chemical analysis of whole mussel tissues showed that mussels collected in Førlandsfjorden, and further deployed for 18 days along a copper gradient in the vicinity of an old copper mine (i.e. at Visnes, Karmøy, Norway), bioaccumulated metals to (and over) the same concentration as that detected in the natural population of mussels at Visnes (Table 6-1). The assumed coppergradient was confirmed by the metal analyses (Figure 6-3). The content of copper in the deployed mussels (from sites 1-4) ranged between 6.2 and 52.3 mg kg^{-1} dry weight, with the highest concentration detected in mussels deployed at site 4. The copper concentration in mussels from Førlandsfjorden was 6.4 mg kg^{-1} dry weight, while the concentration in indigenous mussels from Visnes was 44 mg kg^{-1} dry weight (Table 6-1). In addition, the chromium concentration was elevated at site 2; zinc concentrations were high but relatively similar at all sites, including Førlandsfjorden.

Table 6-1. Bioaccumulation of metals in *Mytilus edulis* deployed for 18 days along a coppergradient.

Metal concentrations in mussels (in mg/ kg dry weight)

	Sites					
Compound	1 (ref)	2	3	4	FØR	VIS
Vanadium	1,42	1,27	0,99	1,00	1,71	0,21
Chromium	1,08	11,25	0,93	1,00	0,74	0,07
Cobalt	0,38	0,30	0,45	0,34	0,246	0,06
Nickel	1,84	1,64	3,04	0,49	0,407	0,04
Copper	6,23	6,88	17,10	52,27	6,40	44,0
Zinc	77,24	78,48	68,05	80,04	66	120
Arsenic	10,73	10,74	8,09	10,80	9,7	1,89
Silver	0,15	0,03	0,05	0,04	0,078	0,05
Cadmium	1,73	1,51	1,26	1,46	0,79	0,42
Barium	0,69	0,78	1,54	0,78	0,116	0,266
Lead	1,51	1,07	0,77	0,90	0,75	0,13

Note. Concentrations of metals were measured by ICP-MS in pooled tissue-samples (n = 20 per analysis). FØR = reference mussels from Førlandsfjorden, VIS = indigenous mussels at Visnes (collected close to site 4).

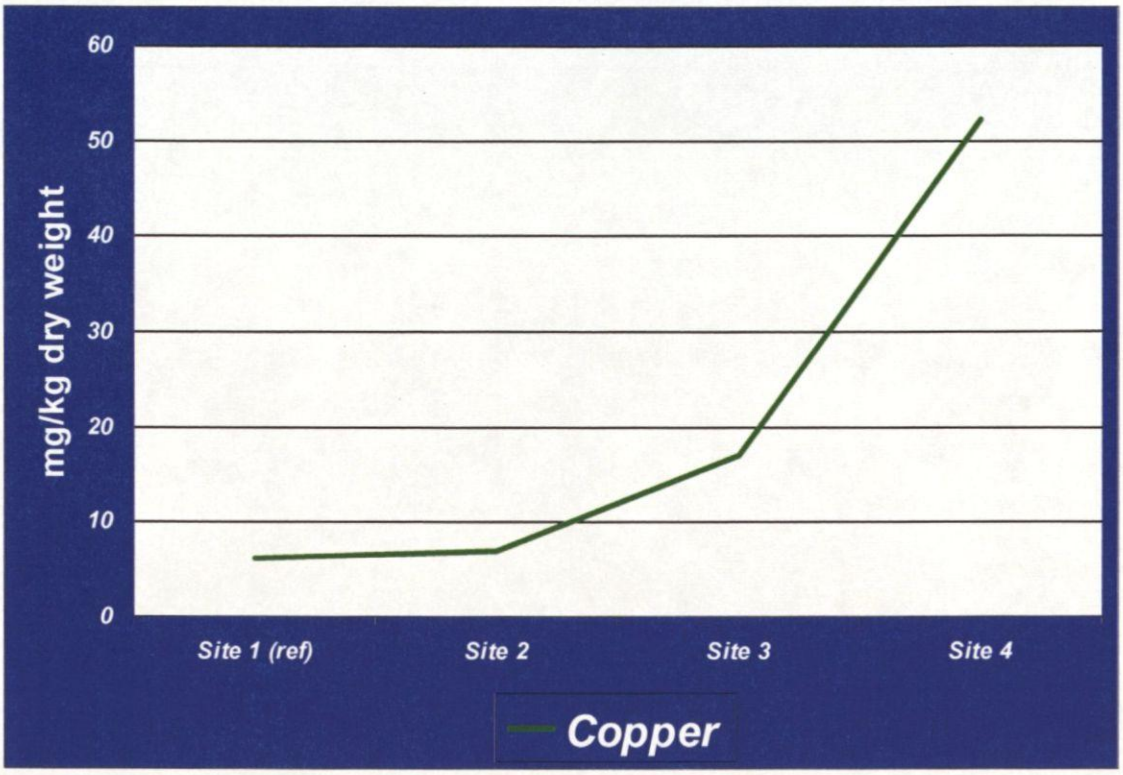


Figure 6-3. Accumulation of copper in mussel tissue along a pollutant gradient in the vicinity of an old copper mine. Values are based on measurements of one pooled (n = 20 mussels) sample per site.

6.3.2 General effect on protein expression

In general, results revealed that protein expression was altered at all sites (i.e. sites 2-4) compared to the gradient reference at site 1 (Table 6-2). Expression was significantly ($p < 0.05$, Mann-Whitney-Wilcoxon test) changed for 11 - 33% of all resolved mass peaks in male plasma and for 18 - 32% of the resolved peaks in female plasma (Table 6-2). For indigenous mussels from Visnes, 36 and 42% (males and females, respectively) of the detected mass peaks were differentially expressed compared with the protein expression patterns found in mussels from site 1 (Table 6-2). The environmental conditions present at Visnes (as compared to site 1) had a predominantly downregulating effect on protein expression in deployed as well as in indigenous Mytilus *edulis* for both genders (Table 6-2). While 81 - 93% of the protein

forms significantly affected in male mussels was downregulated, 68 - 79% was downregulated in female mussels.

Table 6-2. Number of significantly ($p < 0.05$) upregulated (Up) and downregulated (Down) peaks in mussels deployed in the copper gradient at Visnes, as well as indigenous mussels from Visnes, compared to mussels from site 1 (gradient reference).

Males					Females				
Site	Up	Down	Σ	% (fold)	Site	Up	Down	Σ	% (fold)
Site 2	2	25	27	11 (1.9 ± 0.9)	Site 2	9	31	40	18 (2.1 ± 0.8)
Site 3	5	43	48	20 (2.2 ± 1.6)	Site 3	8	17	25	11 (2.2 ± 0.9)
Site 4	12	66	78	33 (2.9 ± 4.0)	Site 4	13	57	70	32 (2.5 ± 2.3)
Visnes	16	70	86	36 (4.3 ± 6.2)	Visnes	20	73	92	42 (5.0 ± 7.6)
n = 240 resolved peaks with S/N ≥5					n = 220 resolved peaks with S/N ≥5				

Note. Σ = total number of significantly altered peaks, S/N = signal-to-noise ratio, % = percentage of peaks that were significantly altered by field exposure. Average fold changes (as induction or repression) in expression ± standard deviation is indicated in brackets.

For the deployed mussels, the average changes in protein expression (as the ratio exposed/control or control/exposed) were similar to those observed in the laboratory studies (see Chapters 3, 4 and 5). The average fold changes in protein expression for deployed mussels were in the range of 1.9 – 2.9 (± standard deviation, see Table 6-2) for male mussels, and 2.0 – 2.5 (± standard deviation) for female mussels (Table 6-2). However, the changes in expression for indigenous mussels at Visnes were markedly higher than in deployed mussels (Table 6-2).

6.3.3 Site/dose-response relationships

The 'dose-response concept' is based on controlled laboratory studies where doses of contaminants to which the organisms have been exposed are known (and exposures include only one compound at the time). However, in this study 'dose' refers to the quantifiable amount of chemicals (e.g. copper) that were detected in the deployed mussels (i.e. Table 6-1). SELDI TOF MS results revealed a complex combination of dose-response relationships, including a non-monotonic hormetic-like response. The forms of the dose-response curves were similar to those observed in Chapter 5 (i.e. dose-response effects of oil in laboratory exposed organisms) including: 'normal' linear dose response with upregulation, 'normal' linear dose response with downregulation, V/U-shaped, inverted V(U)-shaped, Z-shaped, and inverted Z shaped response curves (see Figure 5-5). However, in contrast to results in Chapter 5, the largest individual group of dose-response relationships for deployed mussels was the normally/linear up or downregulated protein forms, with 45 and 44% of all significantly altered mass peaks being up or down regulated in a linear manner in male and female mussels, respectively (Figure 6-4). Nevertheless, protein expression was changed in a non-linear manner for over 50% of the significantly affected mass peaks for both male and female mussels (Figure 6-4).

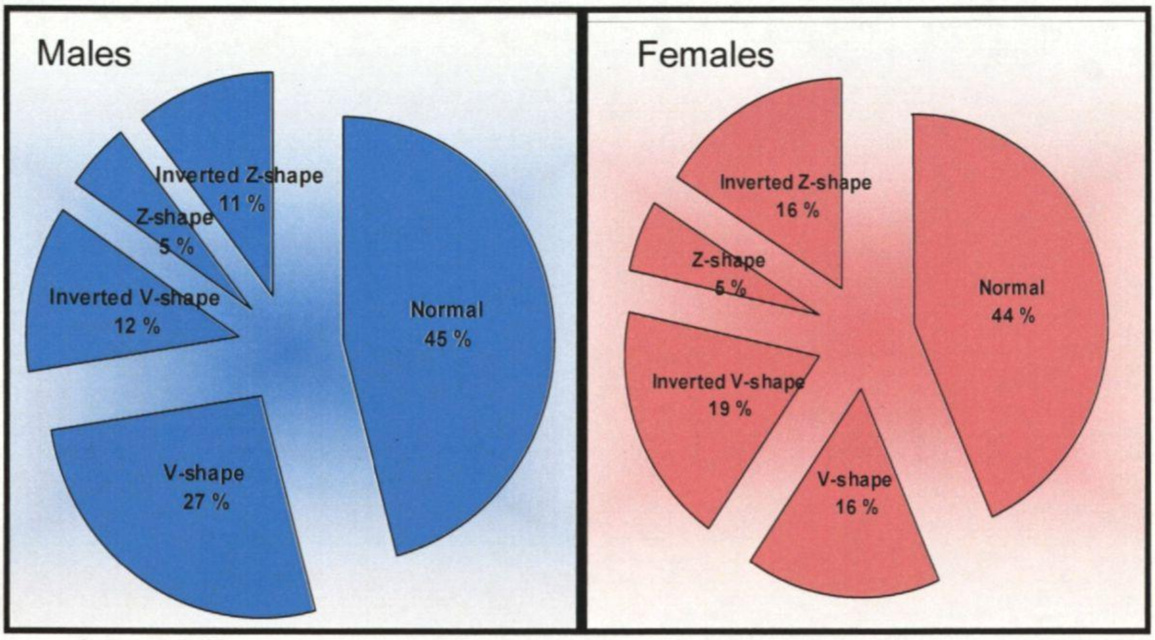


Figure 6-4. Pie charts showing percentage distribution of the various types of dose-response relationships observed for all significantly altered protein species in *Mytilus edulis* transplanted from a reference site in Førlandsfjorden (Karmøy, Norway) to a pollutant field gradient (including a gradient reference – site 1); male mussels to the left, and female mussels to the right. Note. 'Normal' includes both upregulated and downregulated protein forms.

To further investigate site/dose-response 'tendencies' obtained with univariate statistics (Table 6-2 and Figure 6-4), the MS data were tested with multidimensional scaling (using PRIMER software) and multivariate prediction models (using Biomarker Pattern^{TM} Software). Firstly, non-metric MDS plots, derived from Euclidean distance similarity matrices, were used to visualise similarities/dissimilarities between sites in the coppergradient; close points in the plot reflecting similar protein expression patterns. Thus, the relative distance from site 1 to the more contaminated sites (site 2 - 4) can be seen as a measure of dissimilarity and degree of response at the various sites. In spite of the high percentage of non-monotonic response curves observed (Figure 6-4), the results indicate an overall site/dose-related responsepattern for male organisms (when all responses are combined), as the relative distance from site 1 increases along the copper gradient (Figure 6-5 B). The response gradient was not so clear for female

mussels; however, with the exception of site 2, there was a general dose/site-related response pattern (Figure 6-5 A). To compare the observed results from the field gradient with protein patterns of indigenous mussels collected at Visnes, new MDS plots were created with MS data from Visnes mussels (Figure 6-5).

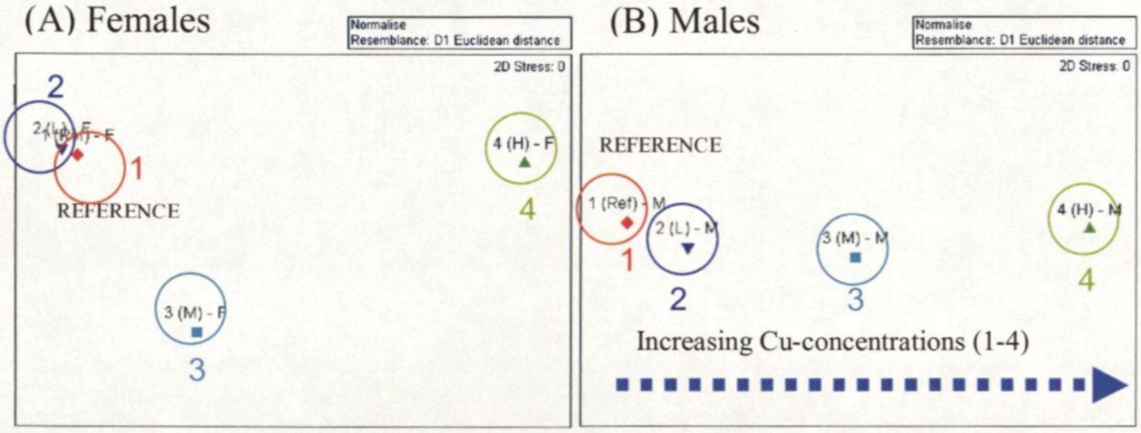


Figure 6-5. MDS plots, derived from Euclidean distance similarity, comparing response on protein expression patterns in *Mytilus edulis* along the copper gradient. Each symbol in the plot represents the average response of 30 individuals per group (input = 240/220 mass peaks for males and females, respectively). F = females (A), M = males (B), 1- 4 = site 1- 4 (site 1 = gradient reference).

The new MDS plots (Figure 6-6) confirmed the gradient response (seen in Figure 6-5), but revealed that protein patterns in indigenous mussels were markedly different from patterns in transplanted mussels (even at site 4, that was very close to the location of the natural population of mussels at Visnes). Furthermore, a site/dose-related response was observed with classification model analyses (analysed with the Pattern^{TM} Software) based on biomarker/protein patterns in each treatment group (i.e. sites 1- 4 and Visnes), (Figure 6-7). The prediction models were created with data from 40 mussels (20 exposed and 20 reference samples for each model), and tested with 20 new samples (10 exposed and 10 reference). The discriminatory power (in classifying site 1 from the other sites) was increasing from sites 2 - 4 for both genders and was greatest for indigenous Visnes-mussels (Figure 6-7).

6.3.4 Comparison to seasonal variation in reference mussels

Seasonal changes in environmental factors may (most likely) influence normal metabolic activities, sensitivity to stress and, thus, the general protein pattern in an organism. To identify seasonal changes, the protein expression patterns detected in mussels deployed and indigenous at the metal-contaminated area of Visnes (in November), were compared with protein profiles in mussels from Førlandsfjorden (i.e. the original site for those mussels deployed at Visnes) collected in January, March and May. MDS plots were used to illustrate the similarities of protein patterns for Førlandsfjorden mussels collected at different timepoints in comparison to peak pattern in Førlandsfjorden mussels transplanted to Visnes (site 4) for 18 days (Figure 6-8). The MDS plots were constructed by including all detected mass peaks with a signal-to-noise ratio greater than or equal to 5 (n = 240/220 for male and female mussels, respectively). Results indicated that protein expression varied with season for male and female mussels from Førlandsfjorden (Figure 6-8). However, the greatest relative distance (and thus dissimilarity) between two data points in the MDS plot (indicated by the arrows in the plots), was observed for mussels deployed at Visnes (i.e. site 4) in November compared with Førlandsfjorden mussels collected in November (Figure 6-8).

6.4 Discussion

While there is an abundance of field data on concentrations of metals in various organisms and tissues, and many controlled laboratory studies have reported the effects of metals on laboratory animals; relatively few studies have examined the relationship between these two attributes of pollutant exposure (e.g. Burger and Gochfeld 1997), even though such knowledge is essential for environmental risk assessment to protect sensitive species and (sub)populations (e.g. Lam and Gray 2001; Burger et al. 2007). Results from the present study confirmed that following 18 days caging in a metal contaminated area (e.g. Tables 6-1 and 6-2), mussels uptake metals and show changes in plasma protein expression. Body burden analysis of mussels confirmed the presence of a coppergradient in the water surrounding the mining area at Visnes, and that tissue concentrations of copper decreased with increasing distance from the mining site (Figure 6-1 and 6-2). These results are consistent with other studies at these sites; Zorita et al. (2006) detected 14, 19, 52 and 184 g Cu/g dry weight in the digestive gland of mussels from sites 1-4, respectively, and reported higher copper concentrations in the digestive gland than in whole soft-tissue extracts, indicating that the digestive gland was the main target organ for Cu accumulation. The hypothesis tested in this study was that protein expression signatures, obtained with SELDI technology, would contain information that reflected the level of contamination at each site. Results of the present study confirmed the hypothesis, as plasma proteome profiles was shown to contain information that reflected the level of contamination present at the various sites along the copper gradient (e.g. Figures 6-5, 6-6 and 6-7). Although the accumulated copper tissue concentration in mussels deployed at site 4 was higher than that in indigenous mussels at Visnes, results indicated that indigenous mussels were more affected, in terms of number of affected protein species (Table 6-2), average fold change in expression (Table 6-2) and discriminatory power in multivariate statistical models classifying exposed from reference organisms (Figure 6-7), than the deployed mussels at all sites. Tree-building algorithms, based on protein expression patterns of mussels at site 1 (gradient reference) against Visnes-mussels, classified randomised blindsamples with 97/96% sensitivity and 97/91% specificity (female/male organisms, respectively). The best predictive model for comparison of site 1 vs. site 4, however, predicted controls (specificity) with 67/81% accuracy and mussels deployed at site 4 (sensitivity) with 76/79% accuracy (Figure 6-7), which was markedly lower. The sensitivity and specificity values found for prediction of Visnes mussels against Førlandsfjorden mussels in this study is in accordance with values reported by Knigge et al. (2004). The latter authors were able to classify masked samples from indigenous mussels at Visnes and Førlandsfjorden with 96% sensitivity and 88% specificity, based on digestive gland protein profiles of *Mytilus edulis* (male and female data combined). It was perhaps not surprising that the natural population of mussels at Visnes was more affected than mussels exposed for only 3 weeks to the same conditions as the indigenous mussels have been faced with chronic exposure of copper (and other metals) for years (Andersen et al. 2003b). Two studies by Barsiene et al. (2004; *in prep* 2008) show how the exposure regime may influence results and, at the same time, elucidate the vulnerability of (single endpoint) analysis measured at only one time-point and one site/dose. In the first study, indigenous mussels at Visnes were found to have micronucleus (MN) formation close to the background level measured in reference mussels from Førlandsfjorden (Barsiene et al. 2004). In the second study, mussels deployed at Visnes for 18 days showed a 4.8 fold increase in MN frequency compared to the natural population of mussels at Visnes (Barsiene et al. *in prep*. 2008), indicating a inhibitory effect on genotoxicity with long-term exposure to metals (e.g. copper). If the second study had not been undertaken, the (misleading) conclusion could have been that the contaminant load at Visnes did not cause genotoxicity in mussels. Exposure regime also affected the toxic responses in gill extracts of the Chinese mitten crab (*Eriochor sinensis*) exposed to either 500 ppb cadmium for 3 days or 50 ppb for 30 days (Silvestre et al. 2006). Results revealed that, while expression of 6 proteins was significantly changed after the acute 3-days exposure, 31 proteins were affected following the 30-days exposure. Clearly, exposure regime (e.g. exposure time) affects the response patterns in organisms and could (partly) explain why protein profiles of indigenous mussels differed so much from profiles of deployed mussels faced with the same contaminant load (Figure 6-6). Moreover, toxic threat (and thus protein expression) can also be affected by the nutritional and reproductive status of the organisms in the receiving environment (Dowling and Sheehan 2006).

In general, a site/dose-related response pattern was found for number of affected protein species, average fold up or downregulation of protein expression, and discriminatory power in multivariate classification models (Table 6-2 and Figure 6-7). Nevertheless, when comparing site/dose-response curves for all significantly altered mass peaks in male and female organisms, more than 50% of the peaks showed non-monotonic response curves (Figure 6-4). This difference illustrates the complexity of biological systems and their interactions (as well as the complexity in mixture effects). Complex site/dose-response patterns were reported previously for mussels along the copper gradient at Visnes (Zorita et al. 2006; Dondero et al. 2006; Burlando et al. 2006). For example, lysosomal content of neutral lipids and lipofuscin (Dondero et al. 2006; Zorita et al. 2006), and calcium ATPase activity (Dondereo et al. 2006) were altered in a bell-shaped sit/dose-response manner. Similarly, tyrosine kinase-dependent signalling

was significantly induced only at the intermediate contaminated site (site 3), indicating an inhibition of tyrosine kinase mechanisms at site 4 (Burlando et al. 2006). Furthermore, induction of vitellogenin-like protein in the gonads of female mussels were significantly elevated at sites 3 and 4 (Zorita et al. 2006); however, levels at site 4 were slightly lower than at site 3 (indicating an inhibition of response at site 4). Although not statistically significant, the same response trend was observed in male samples (Zorita et al. 2006). Peroxisomal catalase activity and gene expression of mt20 was altered in a V-shaped site/dose-response manner, while metallothionein concentration in digestive glands varied in a Z-shaped manner along the copper gradient (Dondero et al. 2006). Nevertheless, lysosomal membrane stability in the digestive gland (Zorito et al. 2006; Dondero et al. 2006), lysosomal/cytoplasm volume ratio (Dondero et al. 2006), gene expression (as average fold change for all genes), (Dondero et al. 2006) and histological markers (Zorita et al. 2006) all revealed a gradual (linear) effect along the copper gradient, with mussels at site 4 being most affected. In spite of the complexity in response patterns for various biomarker endpoints, when results from individual endpoints were combined in a so called Expert-System (Dagnino et al. 2007, made for policy makers to be able to interpret biomarker results into actual health risks), the system ranked the overall stress levels in deployed mussels in a linear manner along the gradient with site 4 mussels being least healthy, i.e. site 4 < 3 < 2 = 1 (Dondero et al. 2006), similar to the pattern(s) observed in the present study.

These complex response patterns and non-monotonic hormetic-like effects (e.g. Calabrese and Baldwin 1997; Chapman 2002b; Calabrese 2005; Mushak 2007; Kefford et al. 2008), could be explained by differing modes of action at high and low doses, (Matthiessen and Johnson 2007, discussed in further details in Chapter 5), or just reflecting the contaminant mixture present at the sites. In summary, there are great knowledge gaps regarding the mechanisms of biphasic dose-response relationships and a general lack of understanding of why they occur (e.g. Johansson 2003; Thayer et al. 2005; Calabrese et al. 2007; Murado and Vázquez 2007; Mushak 2007). Nonetheless, non-monotonic dose-response relationships appear frequently in the (eco) toxicological literature (e.g. reviewed by Calabrese and Baldwin 2001a). For example, Bradley (2000) reported changes in protein expression in *Mytilus edulis* (gills) following laboratory exposure to low levels of $CuSO_4$. The results indicated that the lowest concentration of copper (20 ppb) had the greatest impact on protein expression (i.e. in terms of numbers of affected protein forms), while mussels exposed to the highest concentration (80 ppb) had the most similar protein profiles to controls, indicating a hormetic-like dose-response relationship for protein expression (Bradley 2006); unfortunately, dose-response curves for individual protein forms were not shown or discussed in the paper. In general, metal-related stress has not been well documented at the (prote)ome level and site/dose-response relationships are rarely reported in field studies. Indeed, no similar studies of uptake and effect (on protein expression) along a contaminated field gradient have been found in literature databases (e.g. http://apps.isiknowledge.com), excluding the possibility of a detailed comparative discussion of present results, and indicating a great need for more research on the topic. Nevertheless, results from the present study show that protein expression patterns contain information on animal health that reflects the level of contamination in the organism (e.g. Figures 6-2, 6-5 and 6-7). Furthermore, the protein expression information appears to be robust against seasonal variation in reference mussels (i.e. from Førlandsfjorden), (Figure 6-8). It was anticipated that protein expression signatures in reference mussels would be affected by seasonal variation as, for example, the metabolic activities of invertebrates are influenced by seasonal changes in biotic and

abiotic factors (e.g. Verlecar et al. 2008). Changes in environmental factors, as a consequence of change in seasons, may therefore influence normal metabolic activities of organisms and the induction of oxidative stress as a consequence of increased generation of reactive oxygen species (ROS) (Verlecar et al. 2008). Exposure to ROS can cause a range of reversible and irreversible modifications of protein amino acid side chains (reviewed by Ghezzi and Bonetto 2003). Some of these changes lead to inactivation of proteins, some are protective of the protein's structural integrity and some can be viewed as a means of the cell "sensing" changes in redox status (McDonagh and Sheehan 2006). Thus, seasonality and variations in ROS levels will influence the overall protein expression profiles (e.g. Dowling and Sheehan 2006; Sheehan et al. 2007) in, for example, reference mussels from Førlandsfjorden. However, the question asked in this study was if the seasonal variation in reference mussels would be greater than the effect of deployment at a metal-contaminated site. The results indicate that for Førlandsfjorden mussels, metal contamination has a greater impact on protein expression than seasonality (Figure 6-8), although other site-specific factors (e.g. salinity, water flux, food availability etc.) might contribute to the observed differences. However, to achieve a better understanding of the influence of seasonality on toxic responses, future studies should include more frequent (at least once a month) analysis of seasonal variation in both reference and exposed organisms.

Another factor that plays a role in the response of animals to toxic chemicals is gender (e.g. McClellan-Green et al. 2007; Burger et al. 2007; results in Chapters 3 and 4 from the present study). However, even unexposed mussels have shown gender-specific protein patterns (e.g. Torrado et al. 2002; Bjørnstad et al. 2006). Hines et al. (2007) proposed genomic and metabolomic technologies as alternative methods to the more traditional histological method for gender-determination in bivalve mussels.

While histology identifies gender based on the presence of ovary or sperm follicles within the mantle matrix (Seed et al. 1969), the alternative methods (genomics and metabolomics) are based on the presence or absence of gender-specific transcripts or polar metabolites extracted from mantle tissue of male and female mussels (Hines et al. 2007). The authors concluded that the genomic method (i.e. reverse transcriptase polymerase chain reaction, RT–PCR) was the most accurate and robust method when both ripe and spawned mussels were tested (Hines et al. 2007). Thus, it is evident that genes and gene products (e.g. proteins and metabolites) hold information regarding gender of an organism, and it is therefore not surprising that unexposed and contaminated animals showed gender-specific protein profiles. The present study, however, indicated that contaminants alter the gender-specific protein patterns in unexposed mussels in a unique manner for the two genders (e.g. Figure 6-9). Exposing mussels to the metal mix present at sites 2-4 increased and decreased the dissimilarity between the genders. While male and female mussels deployed at the intermediate and most contaminated sites (i.e. with regard to copper concentrations) revealed a more similar protein pattern compared to site 1, male and female organisms deployed at the least contaminated site (site 2) showed greater gender differences than the controls. The explanation for the observed site-specific gender differences is not clear. Gender-related effects in wildlife have most often been associated with endocrine disruption (e.g. IPCS 2002), however, copper is not normally regarded as an EDC (e.g. http://www.ourstolenfuture.org/). On the other hand, Handy (2003) concluded that physiological changes observed in fish following chronic exposure to copper (i.e. upregulation of enzymes/metabolism, haematopoietic responses and altered tissue-cellularity) could be explained by e.g. copper interfering with specific neuro-endocrine processes. Furthermore, elevated concentrations of chromium (Cr) were detected in mussels at site 2 (Table 6-1). Chromium has been associated with endocrine disruption in crabs (i.e. *Ucides cordatus*) (Correa et al. 2005), and with reduced sperm quality in humans and animals (Li et al. 2001). Endocrine disruption, as an explanation for the gender-specific responses in mussels deployed at these metal-contaminated sites, can therefore not be completely ruled out, especially not without further insight into chemical modes of action. Nevertheless, gender-specific responses could also be explained by differences in physiology (e.g. Levin 2001), gender-specific susceptibility (e.g. Burger et al. 2007), or gender-related uptake and fate of chemicals (Burger et al. 2003).

6.5 Conclusions

The present study, showing that protein expression profiles of mussels deployed along a contaminated field gradient were altered in a site/dose and gender-specific manner, supported results from the controlled laboratory experiments. In addition, results from this field study revealed that protein expression pattern/toxic responses can be affected by exposure regime (e.g. exposure time) and seasonality. Understanding gender-related effects in wildlife is crucial for protecting and managing wildlife populations, as gender clearly plays a role in the response of animals to anthropogenic insults. While laboratory studies are important for elucidating the chemical mechanisms of toxicity for single-compounds or mixtures, field studies are essential to understand the full range of effects (of chronic exposure and seasonality), at the individual and population level. The SELDI TOF MS approach has proven useful in detecting (gender-specific) dose/site related effects of environmental pollutants in the

field. Thus, used in combination with chemical analysis (for investigation of e.g. gender/species-specific uptake and elimination of pollutants), multidimensional LC-MS (for separation and identification of key-molecules) and histochemistry (for localisation of the sites for molecular action/interaction of key molecules), the SELDI TOF MS approach could provide a powerful supplement to existing methods in environmental risk assessment. Use of the SELDI TOF MS approach could provide better insight into mechanisms of actions involved in toxicity, and hence improve understanding of how gender, seasonality and exposure regime influence species susceptibility to contamination.

Chapter 7

General discussion and future perspectives

> *" Always trust the simplest explanation that fits all the facts unless there's a damn good reason not to do so."*
>
> - Dr. Stuart Hay in *Incarnate* by Ramsey Cambell

7.1 General discussion

The primary aim of this thesis was to evaluate the potential for proteomics in ecotoxicology and environmental risk assessment (ERA). The hypothesis was that proteomics technologies (i.e. ProteinChip technology in combination with SELDI TOF MS) could be a useful supplement to existing methods, by providing a sensitive, non-invasive, rapid multi-endpoint assessment of effects of anthropogenic chemicals on organisms *in vivo*. The hypothesis was tested in three controlled laboratory experiments (Chapters 3, 4 and 5) and in one field validation study (Chapter 6), using invertebrates as model organisms and potential EDCs as a test chemicals.

Ecotoxicology, as originally defined by Truhaut (1977), is 'the branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbes, in an integral context'. Chapman (2002a), however, emphasised the importance of an ecological focus in ecotoxicology, on interaction between organisms, and not only 'single-species testing for screening purposes'. Furthermore, as pointed out by Breitholtz et al. (2006), ecotoxicology is an essential tool in risk assessment and risk management. The main aim of environmental risk assessment (ERA) is to predict the likely adverse effects of anthropogenic pollutants or activity on ecosystems and their components using an 'evidence-based' approach (Galloway et al. 2004; 2006). Given the aforementioned definitions of ecotoxicology, it is important to bear in mind that assessment of risks from anthropogenic chemicals may differ in many ways between humans and wildlife. Differences may include: (1) individual versus population focus, (2) longevity versus reproduction, and (3) one receptor versus hundreds or thousands depending upon the ecosystem (Burger et al. 2007). Moreover, due to the biological complexity present in ecosystems, there is controversy regarding the use of 'ecosystems health' as an analogue to 'human health' (e.g. Calow and Forbes 2003). Nevertheless, for an ecosystem to be healthy (i.e. to maintain its integrity), the individual organisms contained within it should also be healthy (Depledge and Galloway 2005). As it is impractical to attempt a comprehensive assessment of each constituent species within a particular habitat, the health of sensitive species becomes a surrogate for the health (status, function or integrity) of the ecosystem (Depledge and Fossi 1994; Galloway et al. 2006).

7.1.1 Requirements/research needs of ecotoxicology/ERA

There are numerous tools available for environmental managers to achieve the primary aim of ERA (e.g. Peakall 1992; Forbes and Forbes 1994). All the same, determination of physical and chemical variables or measurements of whole-organism responses (e.g. mortality, growth, reproduction) of sensitive indicator species have, historically, been favoured methods (e.g. Lam and Gray 2001; 2003). There has also been growing interest in computer models that simulate ecological risks through, for example, discharge distribution patterns (from the oil industry) based on hydrological data (Durell et al. 2006; Neff et al. 2006), or estimation of fate and effects (including, chemodynamics of neutral and ionized organic chemicals, bioaccumulation as a function of sorption and bioenergetics, biotransformation to daughter products, and sublethal and lethal toxicity) based on the concentration of a toxicant within a given organism (e.g. Park et al. 2008).

Although such approaches may provide useful information regarding chemicals of potential concern, they provide little understanding of the mechanism of chemical toxicity (Snape et al. 2004). It is widely accepted that measurements of biological effects of exposure (i.e. biomarkers) may be the only way to provide the crucial evidence that links pollutants to biological changes and (potential) insight into mechanisms of chemical actions (e.g. Ringwood et al. 1999). In addition, analogous to diagnosis in human medicine, it is recognised that most pollutant effects will depend upon the determination of suites of responses, rather than any pollutant-specific or disease-specific response (e.g. Nuwasir 1999; Narbonne et al. 1999; Broeg et al. 2005; Galloway et al. 2006; Dagnino et al. 2007). In the summary and conclusions of a biomarker workshop organised in 1998 (Ringwood et al. 1999), it was pointed out that all biomarker-responses should be evaluated using the following criteria/questions:

1. Is the biomarkers response compound specific, or specific to classes of pollutants?
2. Does the biomarker response distinguish between exposure and adverse effects?

3. Can transient responses be distinguished from sustained chronic responses that reflect irreversible damage?
4. Is there evidence that biomarker response is linked to adverse effects on fitness components such as growth and reproduction?
5. To what extend is the biomarker response affected by natural environmental variables (salinity, dissolved oxygen, temperature etc.), or physiological variables such as reproductive cycle, age, size, gender? Can adjustments be made for the sources of variation, and is it possible to define normal ranges?
6. Are there quantifiable relationships between chemical dose (in tissue and/or environment) and (biomarker) response?

Although these evaluation criteria for biomarker responses were suggested a decade ago, they are equally relevant and important today (e.g. Sumpter et al. 2005; Breitholtz et al. 2006). Other current concerns and shortcomings in ERA include limited knowledge regarding mixture effects, low dose effects, non-linear dose-response relationships, and accumulated or delayed effects (Matthiessen and Johnson 2007). Furthermore, until we have a much greater understanding of how and why chemicals act differently in different organisms, environmental risk assessment systems cannot be perfect (Matthiessen and Johnson 2007). Currently, many uncertainties exist in the extrapolation of biomarker responses from a few model animals (sentinels), tested under laboratory conditions, to complex ecological situations (e.g. DeFur et al 1999; IPCS 2002; Aardema and MacGregor 2002). It is unclear whether information on the effects of a given substance (e.g. EDC) on one species, or even one taxonomic group, can provide sufficient data to protect a diverse range of other species (including humans) with potentially different endocrine systems (Lam and Gray 2001; 2003; Matthiessen and Johnson 2007). It is, therefore, important to assess species similarities and

differences in response to select relevant model organism to be used in standard protocols (Breitholz et al. 2006).

7.1.2 The potential for proteomic in ecotoxicology/ERA

Given the 'needs' described above, how can proteomics be useful in ecotoxicology and ERA?

All living organisms respond to changes in their environment through changes in the expression of multiple genes and gene products (e.g. proteins), where environmental pollutants potentially represent additional stimuli which can induce expression changes (Monsinjon and Knigge 2007). It has become clear that toxicity generally involves changes not only in a single gene but rather a cascade of gene interactions (Nuwaysir et al. 1999; Pennie et al. 2000; Aardema and MacGregor 2002). This conclusion was supported by the results from this thesis. In all experiments (Chapters 3 - 6), several protein forms were affected/changed simultaneously after exposure. For example, 33 and 56 % of the total number of resolved mass peaks were significantly ($p < 0.05$) changed in mussel plasma after exposure to oil, and oil mixed with PAHs and alkylphenols (Chapter 3). Similarly, 37 - 79 % (in *Mytilus edulis*) and 47 - 70 % (in *Hyas araneus*) of all resolved peaks were altered following exposure to the tree EDCs; BPA, DAP and BDE-47 (Chapter 4). Thus, one of the greatest advantages of proteomics, and other 'omics' technologies, compared to traditional methods which rely on a single endpoint, is that they allow simultaneous analysis of thousands of genes, proteins and metabolites etc., providing a wider approach to biological questions (e.g. Snape et al. 2004).

Exposing organisms to graded levels of contamination under controlled laboratory conditions (Chapter 5) and in the field (Chapter 6), revealed that different subsets of proteomes were affected at different levels of exposure (i.e. and not only the same protein species being more affected with increasing exposure concentrations). This is analogous to human medicine, where different biomarker profiles are found at the onset of a disease versus the late stage where symptoms and indirect effects are prominent (Van der Greef et al. 2004). This finding represents an opportunity for applying proteomics for both prognostic (e.g. early warning of potential adverse effects or assessment of recovery) and diagnostic purposes. Moreover, those protein features that were changed by all exposure concentrations showed complex dose-response relationships, including both linear and various types of biphasic response-curves (Chapter 5 and 6). Given that oil is a mixture of many different compounds (with potentially different toxicity threshold) that is not surprising. Furthermore, biphasic dose-response relationships is not an uncommon phenomenon in (eco)toxicological studies (e.g. Depledge et al. 1993; Calabrese and Baldwin 2001; 2002; 2003), although several generations of toxicologists have been taught that the dose response is fundamentally sigmoidal but with a threshold operating at low doses. Indeed, within any exposure scenario, dose-response relationships are expected, but each set of exposure conditions appears to give rise to unique dose-response characteristics, where each dose-response curve corresponds to different combinations and permutations of interacting chemicals and environmental stress. It is often not apparent which dose-response relationship to select for general use in the assessment of potential adverse biological effects of chemicals (Depledge et al. 1993). Additionally, many environmental compounds, including EDCs, have the potential to induce several types of toxicity (e.g. BPA, Wetherill et al. 2007), thus the complexity of dose-response relationships observed in both laboratory experiments (Chapter 5) and in the field (Chapter 6) could potentially represent different threshold levels for the various types of toxicity induced simultaneously by oil and copper, respectively. However, non-monotoninc dose-response curves (particularly at low-dose exposures) are generally not well understood or accepted (e.g. Calabrese 2005; Mushak 2007). Nevertheless, the results of this thesis indicate that a proteomic approach (including both discovery and subsequent identification of key-molecules and their interactions) could potentially unveil some of the mechanisms and biological interactions involved in biphasic dose-response relationships, especially if put together with other 'omics' technologies, bioinformatics/chemometrics, as well as cyto and histochemistry etc. into a 'systems biology' (e.g. Morel et al . 2004) or a 'biomics' (Coulton 2004) strategy. Another advantage of a mechanistic insight is that it might be possible to determine whether an observed endocrine disturbance is a primary response unrelated to toxicity, or a secondary response that arose as a result of pathological processes caused, for example, by metabolic toxicity. Such insight could improve the overall understanding of the 'endocrine-disruption-phenomenon' (e.g. Depledge and Billinghurst 1999).

Improved understanding of endocrine disruption also requires improved knowledge regarding species-specific responses and susceptibility to EDCs (e.g. Matthiessen and Johnson 2007). Furthermore, identification of sensitive species and subpopulation is essential to avoid both 'overprotection' and 'underprotection' of ecosystems (Lam and Gray 2001). An important observation made in this thesis work, was that not only species (Chapters 4 and 5) but also gender (Chapters 3, 4 and 6) have variation in response and susceptibility to natural and anthropogenic compounds. Thus, proteomic analyses could, potentially, reveal if gender-specific responses are related to endocrine disruption, susceptibility or uptake of pollutants.

7.1.3 The challenges and pitfalls for proteomics in ERA

The ultimate goal for proteomics is to be able to monitor all cellular proteins. This requires that: (1) all proteins must be quantitatively extracted from the original biological material; (2) the proteins must be resolved and displayed; (3) each protein must be accurately quantified; and (4) the identity of each protein must be determined (Godovac-Zimmermann and Brown 2001). These requirements represent some of the basic challenges in proteome research and will also influence the quality of proteomics-based ERA.

Even if the first requirement was achieved, it is evident that no single chromatographic or electrophoretic procedure is capable of resolving and displaying the complex mixture of proteins in a cell or tissue (e.g. Issaq 2001; Patterson 2004; Boschetti 2007). For example, as shown for human plasma, the dynamic range of protein abundance comprises up to ten orders of magnitude and cannot be covered by a single analytical technique (Anderson and Anderson 2002). Although the same dynamic range for invertebrate samples has yet to be shown, results from this thesis, as well as those reported in literature; indicate a similar challenge for non-human samples. For example, SELDI TOF MS (the choice of method for this thesis-work) predominantly resolved and detected low-molecular weight protein forms (i.e. M/Z < 20 kDa) in accordance with what has been reported for other studies utilising the same technology (in ecotoxicological studies) (Knigge et al. 2004; Larsen et al. 2006; Monsinjon et al. 2006; Gomiero et al. 2006). Conversely, 2DE technology favours detection of larger protein forms (e.g. Lopez et al. 2001; Jonsson et al. 2006; Apraiz et al. 2006), and has limited access to low-molecular weight proteins as well as low-abundant and membrane-bound proteins (e.g. Ahmed and Rice 2005; Lay et al. 2006). Accordingly, it is uncertain how much of the proteome(s) are being resolved and displayed by current

methodologies (Patterson 2004). However, a combined use of proteomic technologies and separation methods with different mechanisms of separation (e.g. ion exchange, partition, adsorption, affinity, size exclusion etc.) could improve the chances of resolving and detecting larger parts of the complex protein mixtures constituting the proteome(s) (Issaq 2001).

Nevertheless, after the proteins have been resolved and displayed, the next challenge is how to handle the large datasets resulting from 'omics' analyses. Thus, one of the biggest advantages of 'omics' technologies (i.e. simultaneous analysis of thousands of biomolecules), could be its greatest pitfall (e.g. Lay et al. 2006). Extracting the useful information from these overwhelming datasets (e.g. recognising the molecules with greatest prognostic or diagnostic potential) requires sophisticated bioinformatics/chemometrics tools and knowledge (Biron et al. 2006; Urfer et al. 2006). Otherwise, it could be difficult (impossible) to find the right 'needle' in the 'haystack of needles' (Baak et al. 2005), and we might also be in danger of 'throwing the baby away with the bath-water' (Coulton 2004). Furthermore, even with statistical tools there is a risk of misinterpretation, as well as overfitting of data. Overfitting often happens when statistical models are created with too few samples in combination with too many variables (e.g. Wiemer and Prokudin 2004). The potential consequences of overfitting are poor reproducibility of results, and 'false discovery' (Baggerly et al. 2003) including, for example, high numbers of false-positives (i.e. predicted to be affected when not) and false-negatives (i.e. healthy when not) (e.g. Lay et al. 2006). Moreover, both technical and biological variation (e.g. individual variation, gender variation, species variation etc.) will affect the results, and must be assessed properly and taken into consideration (e.g. Karm and Lilley 2007). These are common concerns for all applications of (prote)omics. However, in ecotoxicology/ERA an extra level of complexity is added by the number of species to understand and protect from environmental insults (e.g. Breitholtz et al. 2006).

Another challenge which is greater in studies of wildlife, than for example, in human studies, is the 4th requirement to achieve the aim of proteomics (described by Godovac-Zimmermann and Brown 2001): identification of all proteins, and thus the link between protein expression profiles and distinct cellular processes or conditions (e.g. Kuster et al. 2005; Dowling and Sheehan 2006). Even though interest in wildlife genomics emerged during the last decade (e.g. bivalve-genomic, Saavedra and Bachere 2006), very few sequence data exist for a number of phyla, and, within the majority of each phylum, less than one single sequence exists per species (Snape et al. 2004). This is reflected in the low number of successful identifications of putative protein biomarkers in ecotoxicological studies (reviewed by Monsinjon and Knigge 2007). For organisms where there is limited or no genetic information, it is essential to identify the exact sequence of amino acids (that make up the protein(s)) to match to previously characterised proteins or genes (e.g. Apraiz et al. 2006). However, the utility of a *de novo* sequencing approach is highly dependent on the precision of mass spectrometry (Frank et al. 2007), indicating that not all instrumentations are equally suitable (e.g. Scigelova and Makarov 2006).

7.1.4 Overall summary

Although the results of this thesis must be considered preliminary, several interesting discoveries were made (in the laboratory and field), supporting the hypothesis that proteomics could make a valuable contribution to ecotoxicology and ERA. These include:

(1) SELDI TOF MS and ProteinChip array technology were applicable to plasma samples from three marine invertebrate species.

(2) 'Protein expression' appeared to be a sensitive endpoint, as all exposure regimes significantly affected protein expression in selected test organisms (Chapters 3-6) even at exposure concentrations that were 2000 times lower than the current discharge limits for the actual compounds (Chapter 5). Being able to detect, assess and understand low-dose effects is particularly important for EDCs, since endocrine disruption has been shown to occur at very low levels of exposure, below the threshold levels for other types of toxicity (e.g. IPCS 2002).

(3) Each test compound and mixture induced an unique protein expression signature in the test invertebrates (Chapters 3-6), although some protein features were affected by several compounds (e.g. Chapters 3 and 4), holding promises for a better understanding and prediction of 'mixture effects' by identification of key molecular pathways for various compounds alone and in combination.

(4) Protein expression signatures contained information that reflected chemical dose in controlled laboratory experiments (Chapter 5) and in the field (Chapter 6). Furthermore, results revealed that a complex combination of dose-response relationships occurred (in the same organism) following exposure to environmental pollutants. These included normal linear dose responses and non-monotonic, hormetic-like dose-response relationships (Chapters 5 and 6), emphasising the strength of a multi-endpoint approach, and the vulnerability of single endpoint and single-time-point analyses if the 'wrong' endpoint and/or time-point was chosen.

(5) Protein expression signatures also reflected species sensitivity (Chapters 4 and 5) and gender sensitivity (Chapters 3, 4 and 6), and these aspects need to be included in monitoring programmes to achieve the right level of environmental protection (Lam and Gray 2003).

(6) In addition, results revealed that protein expression pattern/toxic responses were affected by exposure regime (e.g. exposure time) and seasonality (Chapter 6), confirming the need for establishing baseline data and the potential for proteomics in achieving such data.

(7) Finally, since the datasets are relatively large (compared to most of the studies in published literature), the results are robust and could provide many new biomarkers for environmental monitoring once the identity of interesting peaks have been confirmed.

7.2 Future perspectives

During the course of this study, it became increasingly clear that more research is needed to gain improved understanding and prediction of anthropogenic insults on marine ecosystem utilising a proteomic approach. Ultimately, proteomics analyses should be incorporated into an even more holistic 'systems biology' approach. However, some of the more specific requirements to achieve this aim are discussed in the following four sections (not in any order of priority).

7.2.1 Gather baseline information

As proteomics application in ecotoxicology is in its infancy (e.g. reviewed in Knigge and Monsinjon 2007), knowledge of normal variation of response must be obtained to avoid inappropriate conclusions regarding the toxicity of a chemical and mechanisms of actions involved. The results of this thesis indicated that season, gender and species affect protein expression patterns in invertebrates. Therefore, mapping variations due to (at least) seasonal changes and gender should be done for an ecological relevant selection of species. Furthermore, variations should ideally be assessed on both reference (healthy) and exposed organisms (in combined laboratory and field studies), to see how much seasonality (reproductive cycles, temperature, food supply etc.) affect the toxic responses to environmental pollutants.

7.2.2 Validation of the experiments and results from this thesis

Interesting results obtained in this thesis must be validated against traditional biomarker endpoints and through new experiments with improved experimental design that include: (1) investigations of gender and species-related uptake/bioaccumulation of chemicals in combination with further investigations of species and gender-related effects of exposure; (2) dose-response studies (in laboratory and field) that include a greater concentration range (and thus more doses/exposure groups) for several chemicals (in addition to oil and copper) in order to unveil threshold levels for various types of effect, and improved insight into low-dose effects as well as hormetic-like dose-response relationships; (3) investigation of mixture effects by analysis of effects of compounds alone and in combination with various other relevant compounds (related to a realistic field situation) to understand the mechanisms of mixture-toxicity; (4)

methodical improvements such as pre-fractionation of samples prior to proteomic analysis, combined use of several protein arrays (at least for initial screening), as well as inclusion of other (prote)omic methods; and (5) more focus on Good Laboratory Practice (GLP) in order to have reproducible and trustworthy results.

Furthermore, all experiments should include an even higher number of samples to increase the statistical power (and decreased the risk of overfitting the data). Because of the limited available genomic sequence information for many of the ecological relevant test species (including the ones used in the present study), it could be useful to include a well-characterised model species such as zebrafish (*Danio rerio*) for comparative studies of responses to anthropogenic compounds. Moreover, although not tested in this study, investigation of effects at different life stages (in comparison with morphological endpoints) could be very interesting to reveal 'critical exposure windows' during development.

7.2.3 Protein identification and mechanistic studies

Once potential biomarkers (protein forms) have been discovered and validated (Section 7.2.2), these should be identified, to find new biomarkers for monitoring purposes and to gain improved understanding of mechanisms of action for the selected compounds. Identification will involve use of appropriate protein separation and purification tools (e.g. LC chromatography and/or ready-made purification kits from, for example, Bio-Rad), followed by HPLC-MS/MS (ORBITRAP) analysis to facilitate the protein identification and *de novo* sequencing (which is most likely necessary at least for the marine invertebrate samples).

For mechanistic understanding, it is also important to investigate how the identified molecules interact with other molecules at the cellular level (e.g. proteins, DNA) (Joyce and Pálsson 2006). As posttranslational modifications (PTMs) have a major influence on various essential cell functions such as signal transduction, metabolic maintenance and cell division (e.g. Reinders and Sickmann 2005), investigation of occurrence and effect of PTMs in invertebrates following exposure to environmental compounds should be pursued.

7.2.4 Making the information/knowledge accessible and user-friendly for the 'users'

For results obtained in ecotoxicological research to be useful in ERA, it must be possible for policy makers to interpret these results into actual health risks (e.g. Dagnino et al. 2007). Thus, the development of easy-to-use-kits based on putative protein biomarkers could be a potential use of proteomic information for both decision makers and other industrial users. These kits could, for example, be developed using biosensor techology, (analogue to blood glucose monitoring in diabetes patients) (e.g. Mascini et al. 1987; Zhao et al. 2007; Der and Dattelbaum 2008). Interesting biomolecules (i.e. intact or modified peptides/proteins), identified in Section 7.2.3, could be coupled to biosensors for automated monitoring of general or more specific responses to environmnetal pollutants (or other types of environmental stress). There are many types of biosensors and applications (e.g. reviewed in Mulchandani et al. 2001; Roderiguez-Mozaz et al. 2004; Andreescu and Marty 2006; Ahmed et al. 2008; Grieshaper et al. 2008; Sapsford et al. 2008). However, the principle for a protein-based biosensor-kit for automated environmental monitoring would be based on molecular interaction/recognition (e.g. Cooper and Waters 2005). This would involve immobilising a suite of purified diagnostic/prognostic proteins/antibodies onto a sensor surface and then screening the sensor surface against adequate biological extracts (e.g.

cell and tissue culture conditioned media, cell extracts, biological fluids, skin mucus etc.) to detect a source of binding partners for the target protein(s). The reaction and subsequent electronic signal from the sensor (e.g. Roderiguez-Mozaz et al. 2004) could then be converted into an easy interpretable 'yes or now' result, or alternative into a quantitative result (e.g. concentration level).

Bibliography

Aarab N, Minier C, Lemaire S, Unruh E, Hansen PD, Larsen BK, Andersen OK, Narbonne JF (2004). Biochemical and histological responses in mussel (*Mytilus edulis*) exposed to North Sea oil and to a mixture of North Sea oil and alkylphenols. *MARINE ENVIRONMENTAL RESEARCH* 58 (2-5): 437-441.

Aarab N, Lemaire-Gony S, Unruh E, et al. (2006). Preliminary study of responses in mussel (Mytilus edilus) exposed to bisphenol A, diallyl phthalate and tetrabromodiphenyl ether. *AQUATIC TOXICOLOGY* 78: 86-92. Suppl. 1.

Aardema MJ, MacGregor JT (2002). Toxicology and genetic toxicology in the new era of "toxicogenomics": impact of "-omics" technologies. *MUTATION RESEARCH-FUNDAMENTAL AND MOLECULAR MECHANISMS OF MUTAGENESIS* 499 (1): 13-25.

Aas E, Baussant T, Balk L, et al. (2000). 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* 51 (2): 41-258.

Aas E, Baussant T, Balk L, Liewenborg B, Andersen OK (2000). 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.* 51 (2): 241-258.

Ahmed N, Rice GE, (2005). Strategies for revealing lower abundance proteins in two-dimensional protein maps. *JOURNAL OF CHROMATOGRAPHY B-*

ANALYTICAL TECHNOLOGIES IN THE BIOMEDICAL AND LIFE SCIENCES 815 (1-2): 39-50.

Ahmed MU, Hossain MM, Tamiya E (2008). Electrochemical biosensors for medical and food applications. *ELECTROANALYSIS* 20 (6): 616-626.

Ahsan N, Lee DG, Lee SH, et al. (2007). Excess copper induced physiological and proteomic changes in germinating rice seeds. *CHEMOSPHERE* 67 (6): 1182-1193.

Akashi T, Yamori T (2007). Rapid communication - A novel method for analyzing phosphoproteins using SELDI-TOF MS in combination with a series of recombinant proteins. *PROTEOMICS* 7 (14): 2350-2354.

Almeida EA, Bainy ACD, Loureiro APM, et al. (2003). DNA and lipid damage in the brown mussel *Perna perna* from a contaminated site. *BULLETIN OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY* 71 (2): 270-275.

Amelina H, Apraiz I, Sun W, et al. (2007). Proteomics-based method for the assessment of marine pollution using liquid chromatography coupled with two-dimensional electrophoresis. *JOURNAL OF PROTEOME RESEARCH* 6 (6): 2094-2104.

Andersen OK, Bjørnstad A, Larsen BK (2003a). BEEP WP4 Campaigns: PAH body burden in mussels and shore crab. BEEP project meeting, Barcelona, Spain (4th – 6th December 2003).

Andersen OK, Bjørnstad A, Larsen BK (2003b). BEEP WP4 Campaigns: Heavy metals in water, sediment and biota. BEEP project meeting, Barcelona, Spain (4th – 6th December 2003).

Anderson JE, Hansen LL, Mooren FC, et al. (2006). Methods and biomarkers for the diagnosis and prognosis of cancer and other diseases: Towards personalized medicine. *DRUG RESISTANCE UPDATES* 9 (4-5): 198-210.

Anderson NL, Anderson NG (1998). Proteome and proteomics: New technologies, new concepts, and new words. *ELECTROPHORESIS* 19 (11): 1853-1861.

Anderson NL, Anderson NG (2002). The human plasma proteome - History, character, and diagnostic prospects. *MOLECULAR & CELLULAR PROTEOMICS* 1 (11): 845-867.

Anderson NL, Anderson NG (1998). Proteome and proteomics: New technologies, new concepts, and new words. *ELECTROPHORESIS* 19 (11): 1853-1861.

Andreescu S, Marty JL (2006). Twenty years research in cholinesterase biosensors: From basic research to practical applications. *BIOMOLECULAR ENGINEERING* 23 (1): 1-15.

Apraiz I, Mi J, Cristobal S (2006). Identification of proteomic signatures of exposure to marine pollutants in mussels (*Mytilus edulis*). *MOLECULAR & CELLULAR PROTEOMICS* 5 (7): 1274-1285.

Arguello-Morales M, Sanchez-Gonzalez M, Canedo M, et al.(2005). Proteolytic modification of Leuconostoc mesenteroides B-512F dextransucrase. *ANTONIE VAN LEEUWENHOEK INTERNATIONAL JOURNAL OF GENERAL AND MOLECULAR MICROBIOLOGY* 87 (2): 131-141.

Arukwe A, Thibaut R, Ingebrigtsen K, Celius T, Goksoyr A, Cravedi JP (2000). In vivo and in vitro metabolism and organ distribution of nonylphenol in Atlantic salmon (*Salmo salar*). *AQUATIC TOXICOLOGY* 49 (4): 289-304.

Arukwe A, Kullman SW, Hinton DE (2001). Differential biomarker gene and protein expressions in nonylphenol and estradiol-17 beta treated juvenile rainbow trout

(*Oncorhynchus mykiss*). *COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY* 129 (1): 1-10.

Arumugam TV, Gleichmann M., Tang SC, et al. (2006). Hormesis/preconditioning mechanisms, the nervous system and aging. *AGEING RESEARCH REVIEWS* 5 (2): 165-178.

Ashton GHS, McGrath JA, South AP (2002). Strategies to identify disease genes. *DRUGS OF TODAY* 38 (4): 235-244.

Auerbach C, Robson JM (1946). Chemical production of mutations. *NATURE* 157: 302.

Avery OT, McLeod CM, McCarty M (1944). Studies on chemical nature of the substance inducing transformation of Pneumococcal types. *JOURNAL OF EXPERIMENTAL MEDICINE* 79:137-158.

Ayres AC and Parker M, (2001). Produced Water Waste Management, Canadian Association of Petroleum Producers (CAPP), Calgary, Alberta, Canada (2001) 77.

Baak JPA, Janssen EAM, Soreide K, et al. (2005). Genomics and proteomics - the way forward. *ANNALS OF ONCOLOGY* 16: 30-44 Suppl. 2 .

Baggerly KA, Morris JS, Coombes KR (2004). Reproducibility of SELDI-TOF protein patterns in serum: comparing datasets from different experiments. *BIOINFORMATICS* 20 (5): 777-U710.

Baldwin JD, Pillai MC, Cherr GN (1992). Response of embryos of the sea-urchin *strongylocentrotus-purpuratus* to aqueous petroleum waste includes the expression of a high-molecular-weight glycoprotein *MARINE BIOLOGY* 114 (1): 21-30.

Barany M, Barany K, Giometti CS (1998). Gel electrophoresis for studying biological function. *ANALYTICA CHIMICA ACTA*. 372 (1-2): 33-66.

Barsiene J, Lazutka J, Syvokiene J, et al. (2004). Analysis of micronuclei in blue mussels and fish from the Baltic and North Seas. *ENVIRONMENTAL TOXICOLOGY* 19 (4): 365-371.

Barsiene J, Dedonyte V, Rybakovas A, et al. (2006a). Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish treated with crude oil. *AQUATIC TOXICOLOGY* 78: 99-104, Suppl. 1.

Barsiene J, Syvokiene J, Bjornstad A (2006b). Induction of micronuclei and other nuclear abnormalities in mussels exposed to bisphenol A, diallyl phthalate and tetrabromodiphenyl ether-47. *AQUATIC TOXICOLOGY* 78: 105-108, Suppl 1.

Barsiene J, Syvokiene J, Bjornstad A (*in prep 2008*). Induction of micronuclei and other nuclear abnormalities in mussels caged in a copper field gradient in Norway.

Basheer C, Lee HK (2004). Analysis of endocrine disrupting alkylphenols, chlorophenols and bisphenol-A using hollow fiber-protected liquid-phase microextraction coupled with injection port-derivatization gas chromatography-mass spectrometry. *JOURNAL OF CHROMATOGRAPHY A* 1057 (1-2): 163-169.

Baussant T, Sanni S, Jonsson G, et al. (2001). Bioaccumulation of polycyclic aromatic compounds: 1. Bioconcentration in two marine species and in semipermeable membrane devices during chronic exposure to dispersed crude oil. *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY* 20 (6): 1175-1184.

Baussant T (2004). Biological effects of chronic levels of dispersed oil in North Sea and Arctic conditions: Biomarker responses and early life stage tests in two Mollusc species. Report AM-2004/017.

Bechmann RK (2004). Effects of oil on sea urchins – Biomarker responses and early-life stage tests. AM report 2004/019.

Bechmann RK, Baussant T, Larsen BK, Skadsheim A, Bamber S. and S. Sanni (2004). Biosea JIP summary report of results from laboratory experiments. AM report 2004/026.

Benninghoff AD (2007) Toxicoproteomics - The next step in the evolution of environmental biomarkers? *TOXICOLOGICAL SCIENCES* 95 (1): 1-4.

Bentley PJ (1998). Comparative vertebrate endocrinology: Cambridge University press.

Bettin C, Oehlmann J, Stroben E (1996).TBT-induced imposex in marine neogastropods is mediated by an increasing androgen level. *HELGOLANDER MEERESUNTERSUCHUNGEN* 50(3) 299-317.

Beynon RJ, Pratt JM (2005). Metabolic labeling of proteins for proteomics. *MOLECULAR & CELLULAR PROTEOMICS* 4 (7): 857-872.

Bihari N, Fafandel M (2004). Interspecies differences in DNA single strand breaks caused by benzo(a)pyrene and marine environment. *MUTATION RESEARCH-FUNDAMENTAL AND MOLECULAR MECHANISMS OF MUTAGENESIS* 552 (1-2): 209-217

Birnbaum,LS, Staskal DF, (2004). Brominated flame retardants: Cause for concern? *ENVIRONMENTAL HEALTH PERSPECTIVES* 112 (1): 9-17.

Biron DG, Brun C, Lefevre T, et al. (2006). The pitfalls of proteomics experiments without the correct use of bioinformatics tools. *PROTEOMICS* 6 (20) 5577-5596.

Bjornstad A, Larsen BK, Skadsheim A, et al. (2006). The potential of ecotoxicoproteomics in environmental monitoring: Biomarker profiling in mussel plasma using proteinchip array technology. *JOURNAL OF TOXICOLOGY AND ENVIRONMENTAL HEALTH-PART A-CURRENT ISSUES* 69 (1-2): 77-96.

Boas M, Feldt-Rasmussen,U, Skakkebaek NE, et al. (2006). Environmental chemicals and thyroid function. *EUROPEAN JOURNAL OF ENDOCRINOLOGY* 154 (5):599-611.

Bolognesi C, Perrone E, Roggieri P, et al. (2006). Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions. *AQUATIC TOXICOLOGY* 78: 93-98. Suppl. 1.

Bouchal P, Kucera I (2002). Two-dimensional electrophoresis in proteomics: Principles and applications. *CHEMICKE LISTY* 97 (1): 29-36.

Bochner BR (2003). New technologies to assess genotype-phenotype relationships. *NATURE REVIEWS GENETICS* 4 (4): 309-314.

Boschetti E, Lomas L, Citterio A, et al. (2007). Romancing the "hidden proteome", Anno Domini two zero zero seven. *JOURNAL OF CHROMATOGRAPHY A* 1153 (1-2): 277-290.

Bottjer DJ, Davidson EH, Peterson KJ, et al.(2006). Review - Paleogenomics of echinoderms. *SCIENCE* 314 (5801): 956-960.

Botstein D, Risch N (2003). Discovering genotypes underlying human phenotypes: past successes for mendelian disease, future approaches for complex disease. *NATURE GENETICS* 33: 228-237.

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

Bradley BP, Shrader EA, Kimmel DG, Meiller JC (2002). Protein expression signatures: an application of proteomics. *MARINE ENVIRONMENTAL RESEARCH* 54 (3-5): 373-377.

Breiman L, Friedman J, Olshen R, Stone C (1984). Classification and regression trees. Wadsworth, Pacific Grove.

Breitholtz M, Ruden C, Hansson SO, et al. (2006). Ten challenges for improved ecotoxicological testing in environmental risk assessment. *ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY* 63 (2): 324-335.

Breivik K, Sweetman A, Pacyna JM, Jones KC, (2002).Towards a global historical emission inventory for selected PCB congeners—a mass balance approach. 1. Global production and consumption, *SCIENCE OF THE TOTAL ENVIRONONMENT* 290: 181–198.

Bremner I (1998). Manifestations of copper excess. *AM. J. CLIN. NUTR.* 67: 1069–1073.

Broeg K, von Westernhagen H, Zander S, et al. (2005). The "bioeffect assessment index" (BAI) - A concept for the quantification of effects of marine pollution by an integrated biomarker approach. *MARINE POLLUTION BULLETIN* 50 (5): 495-503.

Brouwer R, De Blois C (2008). Integrated modelling of risk and uncertainty underlying the cost and effectiveness of water quality measures. *ENVIRONMENTAL MODELLING & SOFTWARE* 23 (7): 922-937.

Brown PO, Botstein D (1999). Exploring the new world of the genome with DNA microarrays. *NATURE GENETICS* 21: 33-37 Suppl. S.

Buettner GR, Jurkiewicz BA (1996). Catalytic metals, ascorbate and free radicals: combinations to avoid. *RADIAT. RES.* 145: 532–541.

Burger J, Gochfeld M (1997). Risk, mercury levels, and birds: Relating adverse laboratory effects to field biomonitoring. *ENVIRONMENTAL RESEARCH* 75: 160–175.

Burger J, Campbell KR, Campbell TS (2004). Gender and spatial patterns in metal concentrations in brown anoles (*Anolis sagrei*) in southern Florida, USA. *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY* 23 (3): 712-718.

Burger J (2007). A framework and methods for incorporating gender-related issues in wildlife risk assessment: Gender-related differences in metal levels and other contaminants as a case study. *ENVIRONMENTAL RESEARCH* 104 (1) 153-162.

Burlando B, Berti E, Viarengo A (2006). Effects of seawater pollutants on protein tyrosine phosphorylation in mussel tissues. *AQUATIC TOXICOLOGY* 78: 79-85 Suppl. 1.

Cajaraville MP, Ortiz-Zarragoitia M (2006). Specificity of the peroxisome proliferation response in mussels exposed to environmental pollutants. *AQUATIC TOXICOLOGY* 78: 117-123. Suppl. 1.

Calabrese EJ (1999). Evidence that hormesis represents an "overcompensation" response to a disruption in homeostasis. *ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY* 42 (2) 135-137.

Calabrese EJ, Baldwin LA (2000). Tales of two similar hypotheses: the rise and fall of chemical and radiation hormesis. *HUMAN EXPERIMENTAL TOXICOLOGY* 19: 85–97.

Calabrese EJ, Baldwin LA (2001a). The frequency of U-shaped dose responses in the toxicological literature. *TOXICOLOGICAL SCIENCES* 62 (2): 330-338.

Calabrese EJ, Baldwin LA (2001b). Hormesis: A generalizable and unifying hypothesis. *CRITICAL REVIEWS IN TOXICOLOGY* 31 (4-5) 353-424.

Calabrese EJ, Baldwin LA (2002). Defining hormesis. *HUMAN & EXPERIMENTAL TOXICOLOGY* 21 (2) 91-97.

Calabrese EJ, Baldwin LA (2003). The hormetic dose-response model is more common than the threshold model in toxicology. *TOXICOLOGICAL SCIENCES* 71 (2) 246-250.

Calabrese EJ (2005). Paradigm lost, paradigm found: The re-emergence of hormesis as a fundamental dose response model in the toxicological sciences. *ENVIRONMENTAL POLLUTION* 138 (3): 378-411.

Calabrese EJ, Bachmann KA, Bailer AJ, et al. (2007). Biological stress response terminology: Integrating the concepts of adaptive response and preconditioning stress within a hormetic dose-response framework. *TOXICOLOGY AND APPLIED PHARMACOLOGY* 222 (1): 122-128.

Calabrese EJ, (2008). Converging concepts: Adaptive response, preconditioning, and the Yerkes-Dodson Law are manifestations of hormesis. *AGEING RESEARCH REVIEWS* 7: 8-20.

Calow P, Forbes VE (2003). Does ecotoxicology inform ecological risk assessment? *ENVIRONMENTAL SCIENCE & TECHNOLOGY* 37 (7) 156-151.

Cameron RA (2000). The sea urchin, from genome_to development. BIOFUTUR 2000 (206): 36-37.

Campbell MK (1991). Biochemistry. Saunders College Publishing.

Camus, L, Jones, MB; Borseth, JF, et al. (2002). Heart rate, respiration and total oxyradical scavenging capacity of the Arctic spider crab, *Hyas araneus*, following exposure to polycyclic aromatic compounds via sediment and injection. *AQUATIC TOXICOLOGY* 61 (1-2): 1-13.

Canesi L,Borghi C,Ciacci C, et al. (2007). Bisphenol-A alters gene expression and functional parameters in molluscan hepatopancreas. *MOLECULAR AND ENDOCRINOLOGY* 276: 36-44.

Caputo E, Moharram R, Martin BM (2003). Methods for on-chip protein analysis *ANALYTICAL BIOCHEMISTRY* 321 (1): 116-124.

Carbonaro M (2004). Proteomics: present and future in food quality evaluation. *TRENDS IN FOOD SCIENCE & TECHNOLOGY* 15 (3-4): 209-216.

Carter D (2006). Cellular transcriptomics- the next phase of endocrine expression profiling. *TRENDS IN ENDOCRINOLOGY AND METABOLISM* 17 (5): 192-198.

Caserta D, Maranghi L, Mantovani A, et al. (2008). Impact of dendocrine disruptor chemicals in gynaecology. *HUMAN REPRODUCTION UPDATE* 14 (1) 59-72.

Chankova SG, Dimova E, Dimitrova M, et al. (2007). Induction of DNA double-strand breaks by zeocin in Chlamydomonas reinhardtii and the role of increased DNA double-strand breaks rejoining in the formation of an adaptive response *RADIATION AND ENVIRONMENTAL BIOPHYSICS* 46: 409-416.

Chapman PM (2002a). Integrating toxicology and ecology: putting the "eco" into ecotoxicology. *MARINE POLLUTION BULLETIN* 44 (1): 7-15.

Chapman PM (2002b). Ecological risk assessment (ERA) and hormesis. *SCIENCE OF THE TOTAL ENVIRONMENT* 288 (1-2): 131-140.

Cho SY, Lee EY, Lee JS, et al.(2005). Efficient prefractionation of low-abundance proteins in human plasma and construction of a two-dimensional map. *PROTEOMICS* 5 (13): 3386-3396.

Choi SM, Yoo SD, Lee BM (2004). Toxicological characteristics of endocrine disrupting chemicals: Developmental toxicity, carcinogenicity, and mutagenicity.

JOURNAL OF TOXICOLOGY AND ENVIRONMENTAL HEALTH-PART B-CRITICAL REVIEWS 7 (1): 1-32.

Clearwater SJ, Farag AM, Meyer JS (2002). Bioavailability and toxicity of dietborne copper and zinc to fish. *COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY* 132 (3) 269-313.

Clish CB, Davidov E, Oresic M, Plasterer TN, Lavine G, Londo T, Meys M, Snell P, Stochaj W, Adourian A, Zhang X, Morel N, Neumann E, Verheij E, Vogels JTWE, Havekes LM, Afeyan N, Regnier F, Van Der Greef J, Naylor S (2004). Integrative biological analysis of the APOE*3-Leiden transgenic mouse. *OMICS-A JOURNAL OF INTEGRATIVE BIOLOGY* 8 (1): 3-13.

Colborn T, Clement C, eds (1992). Chemically induced alterations in sexual and development: the wildlife/human connection. Advances in Modern Environmental Toxicology, Vol XXI. Princeton, NJ:Princeton Scientific Publishing, 1992:401p.

Colborn T, Dumanoski D. and Myers JP (1996). Our Stolen Future. Penguin, New York.

Conrads TP, Fusaro VA, Ross S, Johann D, Rajapakse V, Hitt BA, Steinberg SM, Kohn EC, Fishman DA, Whiteley G, Barrett JC, Liotta LA, Petricoin EF, Veenstra TD (2004). High-resolution serum proteomic features for ovarian cancer detection. *ENDOCRINE-RELATED CANCER* 11 (2): 163-178.

Cooper WJ, Waters ML (2005). Molecular recognition with designed peptides and proteins. *CURRENT OPINION IN CHEMICAL BIOLOGY* 9 (6): 627-631.

Cordingley HC, Robets SLL, Tooke P, et al. (2003).Multifactorial screening design and analysis of SELDI-TOF ProteinChip((R)) array optimization experiments. *BIOTECHNIQUES* 34 (2): 364.

Correa JD, da Silva MR, da Silva ACB, et al. (2005). Tissue distribution, subcellular localization and endocrine disruption patterns induced by Cr and Mn in the crab *Ucides cordatus*. *AQUATIC TOXICOLOGY* 73 (2) 139-154.

Costa LG, Giordano G, (2007). Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. *NEUROTOXICOLOGY* 28: 1047-1067.

Cotou E, Vagias C, Rapti T, Roussis V (2001). Metallothionein levels in the bivalves *Callista chione* and *Venus verrucosa* from two Mediterranean sites, *Z. NATURFORSCH. C – J. BIOSCIENCE.* 56: 848–852.

Coulton G (2004). Are histochemistry and cytochemistry 'Omics'? *JOURNAL OF MOLECULAR HISTOLOGY* 35 (6) 603-613.

Crain DA, Eriksen M, Iguchi T, et al. (2007). An ecological assessment of bisphenol-A: Evidence from comparative biology. *REPRODUCTIVE TOXICOLOGY* 24 (2): 225-239.

Cunningham ML, Bogdanffy MS, Zacharewski TR, Hines RN (2003). Workshop overview: Use of genomic data in risk assessment. *TOXICOLOGICAL SCIENCES* 73 (2): 209-215.

Cusick ME, Klitgord N, Vidal M, et al (2005). Interactome: gateway into systems biology. *HUMAN MOLECULAR GENETICS* 14 (2): 171-181.

Dagnino A, Allen JI, Moore MN, et al. (2007). Development of an expert system for the integration of biomarker responses in mussels into an animal health index *BIOMARKERS* 12 (2): 155-172.

Dardenne F, Nobels,I, De Coen,W, et al. (2008). Mixture toxicity and gene inductions: Can we predict the outcome? *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY* 27 (3) 509-518.

Dardenne F, Nobels I, De Coen W, et al. (2007). Dose-response relationships and statistical performance of a battery of bacterial gene profiling assays. *APPLIED MICROBIOLOGY AND BIOTECHNOLOGY* 75 (1): 223-234.

Darnerud PO (2003). Toxic effects of brominated flame retardants in man and in wildlife. *ENVIRONMENT INTERNATIONAL* 29 (6): 841-853.

Debes JD, Urrutia R (2004). Bioinformatics tools to understand human diseases. *SURGERY* 135 (6): 579-585.

DeFur PL, Crane M, Ingershold C, Tattersfield L (1999). *Endocrine Disruption in Invertebrates: Endocrinology, Testing, and Assessment* SETAC, Pensacola, FL, USA.

DeFur PL (2004). Use and role of invertebrate models in endocrine disruptor research and testing. *ILAR JOURNAL* 45 (4): 484-493.

DeLaender F, De Schamphelaere KAC, Vanrolleghem PA, et al. (2008). Validation of an ecosystem modelling approach as a tool for ecological effect assessments.*CHEMOSPHERE* 71 (3): 529-545.

DeLuca-Abbott SB, Richardson BJ, McClellan KE, et al. (2005). Field validation of antioxidant enzyme biomarkers in mussels (*Perna viridis*) and clams (*Ruditapes philippinarum*) transplanted in Hong Kong coastal waters. *MARINE POLLUTION BULLETIN* 51 (8-12) 694-707.

DeLorenzo ME, Fleming J (2008). Individual and mixture effects of selected pharmaceuticals and personal care products on the marine phytoplankton species *Dunaliella tertiolecta*. *ARCHIVES OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY* 54 (2) 203-210.

Den Besten PJ, Lemaire P, Livingstone DR, Woodin B, Stegeman JJ, Herwig HJ, Seinen W (1993). Time-course and dose-response of the apparent induction of the cytochrome-P450 monooxygenase system of pyloric ceca microsomes of the female sea star *asterias-rubens L* by benzo[a]pyrene and polychlorinated-biphenyls. *AQUATIC TOXICOLOGY* 26 (1-2): 23-39.

Depledge MH (1993). The rational basis for the use of biomarkers as ecotoxicological tools. In Fossi, M. C. and Leonzio, C. (Editors). Nondestructive biomarkers in vertebrates. 261-285. FL: Lewis.

Depledge MH, Fossi MC (1994). The role of biomarkers in environmental assessment (2). Invertebrates, *ECOTOXICOLOGY* 3: 161–172.

Depledge MH, Billinghurst Z (1999). Ecological significance of endocrine disruption in marine invertebrates. *MARINE POLLUTION BULLETIN* 39 (1-12): 32-38.

Depledge MH, Galloway TS (2005). Healthy animals, healthy ecosystems. *FRONTIERS IN ECOLOGY AND THE ENVIRONMENT* 3 (5) 251-258.

Der BS, Dattelbaum JD (2008). Construction of a reagentless glucose biosensor using molecular exciton luminescence. *ANALYTICAL BIOCHEMISTRY* 375 (1) 132-140.

Dettmer K, Hammock BD (2004). Metabolomics - A new exciting field within the "omics" sciences. *ENVIRONMENTAL HEALTH PERSPECTIVES* 112 (7): 396-397.

DeWit CA (2002). An overview of brominated flame retardants in the environment. *CHEMOSPHERE* 46 (5): 583-624.

Diamond DL, Zhang YN, Gaiger A, Smithgall M, Vedvick TS, Carter D (2003). Use of ProteinChip (TM) array surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) to identify thymosin beta-4, a differentially secreted protein from lymphoblastoid cell lines *JOURNAL OF THE AMERICAN SOCIETY FOR MASS SPECTROMETRY* 14 (7): 760-765.

Dietel M, Sers C (2006).Personalised medicine and development of targeted therapies: the upcoming challenge for diagnostic molecular pathology. A review Source: VIRCHOWS ARCHIV 448 (6): 744-755.

Dondero F, Dagnino A, Jonsson H, et al. (2006). Assessing the occurrence of a stress syndrome in mussels (*Mytilus edulis*) using a combined biomarker/gene expression approach. *AQUATIC TOXICOLOGY* 78:13-24 Suppl. 1.

Duft M, Schmitt C, Bachmann J, et al. (2007). Prosobranch snails as test organisms for the assessment of endocrine active chemicals - an overview and a guideline proposal for a reproduction test with the freshwater mudsnail Potamopyrgus antipodarum *ECOTOXICOLOGY* 16 (1): 169-182.

Dunn WB, Ellis DI (2005). Metabolomics: Current analytical platforms and methodologies. *TRAC-TRENDS IN ANALYTICAL CHEMISTRY* 24 (4): 285-294.

Durell G, Utvik TR, Johnsen S, et al. (2006). Oil well produced water discharges to the North Sea. Part I: Comparison of deployed mussels (*Mytilus edulis*), semi-permeable membrane devices, and the DREAM model predictions to estimate the dispersion of polycyclic aromatic hydrocarbons. *MARINE ENVIRONMENTAL RESEARCH* 62 (3): 194-223.

Durodie B (2003). The true cost of precautionary chemicals regulation RISK ANALYSIS 23 (2) 389-398.

EEC (2001) Community Strategy for Endocrine Disrupters — a Range of Substances Suspected of Interfering With the Hormone Systems of Humans and Wildlife. COM 262, Bruxelles 46pp.

Ekman DR, Teng Q, Jensen KM, et al. (2007). NMR analysis of male fathead minnow urinary metabolites: A potential approach for studying impacts of chemical exposures. *AQUATIC TOXICOLOGY* 85 (2): 104-112.

Erk M, Muyssen BTA, Ghekiere A, et al. (2008).Metallothionein and cellular energy allocation in the estuarine mysid shrimp *Neomysis integer* exposed to cadmium at different salinities. *JOURNAL OF EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY* 357 (2): 172-180.

Escher BI, Hermens JLM (2002). Modes of action in ecotoxicology: Their role in body burdens, species sensitivity, QSARs, and mixture effects. *ENVIRONMENTAL SCIENCE & TECHNOLOGY* 36 (20): 4201-4217.

Evandri MG, Mastrangelo S, Costa LG, Bolle P (2003). In vitro assessment of mutagenicity and clastogenicity of BDE-99, a pentabrominated diphenyl ether flame retardant. *ENVIRONMENTAL MOLECULAR MUTAGEN* 42: 85–90.

Facemire CF (1999). Bioaccumulation, storage and mobilisation of endocrine altering contaminants. In: Environmental endocrine disruptors: An evolutionary perspective (Guilette LJ Crain DA, eds). New York: Taylor & Francis, 135.

Favre-Kontula L, Sattonnet-Roche P, Magnenat E, et al.(2008). Detection and identification of plasma proteins that bind GlialCAM using ProteinChip (TM) arrays, SELDI-TOF MS, and nano-LC MS/MS. *PROTEOMICS* 8 (2): 378-388.

Fiehn O (2002). Metabolomics - the link between genotypes and phenotypes. PLANT *MOLECULAR BIOLOGY* 48 (1-2): 155-171.

Fleischmann RD, Adams MD, White O, et al. (1995). Whole-genome random sequencing and assembly of haemophilus-influenzae rd. *SCIENCE* 269 (5223): 496-512.

Folch J, Lees M, Stanley GHS (1957). A simple method for the isolation and purification of total lipid from animal tissue. *JOURNAL OF BIOLOGICAL CHEMISTRY* 226: 497-509.

Forbes VE, Forbes TL (1994). Ecotoxicology in theory and practice. *Ecotox Ser 1* Chapman & Hall. 292p.

Forbes VE (2000). Is hormesis an evolutionary expectation? *FUNCTIONAL ECOLOGY* 14 (1) 12-24.

Forbes VE, Calow P, Sibly, RM (2001). Are current species extrapolation models a good basis for ecological risk assessment? *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY* 20 (2): 442-447.

Forde CE, Gonzales AD, Smessaert JM, Murphy GA, Shields SJ, Fitch JP, McCutchen-Maloney SL (2002). A rapid method to capture and screen for transcription factors by SELDI mass spectrometry.*BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS* 290 (4): 1328-1335.

Fossi MC, Casini S, Marsili L (1999). Nondestructive biomarkers of exposure to disrupting chemicals in endangered species endocrine of wildlife. *CHEMOSPHERE* 39 (8): 1273-1285.

Fossi MC, Casini S, Marsill L (2007). Potential toxicological hazard due to endocrine-disrupting chemicals on Mediterranean top predators: State of art, gender differences and methodological tools. *ENVIRONMENTAL RESEARCH* 104 (1): 174-182.

Fourie F, Reinecke SA, Reinecke, AJ (2007). The determination of earthworm species sensitivity differences to cadmium genotoxicity using the comet assay. *ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY* 67 (3) 361-368.

Francis BM (1994). Toxic substances in the environment. John Wiley & Sons, Inc.

Frank AM, Savitski MM, Nielsen ML, et al.(2007). De novo peptide sequencing and identification with precision mass spectrometry. *JOURNAL OF PROTEOME RESEARCH* 6 (1): 114-123.

Fung ET, Wright GL, Dalmasso EA (2000). Proteomic strategies for biomarker identification: Progress and challenges. *CURRENT OPINION IN MOLECULAR THERAPEUTICS* 2 (6): 643-650.

Fung ET, Enderwick C (2002). ProteinChip (R) clinical proteomics: Computational challenges and solutions. *BIOTECHNIQUES* : 34-+ Suppl. S.

Futreal PA, Coin L, Marshall M, et al. (2004). A census of human cancer genes. *NATURE REVIEWS CANCER* 4 (3): 177-183.

Gabbianelli R, Lupidi G, Villarini M, et al. (2003). DNA damage induced by copper on erythrocytes of gilthead sea bream *Sparus aurata* and mollusk *Scapharca inaequivalvis*. *ARCHIVES OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY* 45 (3) 350-356.

Gaetke LM, Chow CK, (2003). Copper toxicity, oxidative stress, and antioxidant nutrients. *TOXICOLOGY* 189 (1-2): 147-163.

Galloway TS, Brown RJ, Browne MA, Dissanayake A, Lowe D, Jones MB, Depledge MH (2004a). A multibiomarker approach to environmental assessment. *ENVIRONMENTAL SCIENCE & TECHNOLOGY* 38 (6): 1723-1731.

Galloway TS, Brown RJ, Browne MA, Dissanayake A, Lowe D, Jones MB and Depledge MH (2004b). Ecosystem management bioindicators: the ECOMAN project- a multi-biomarker approach to ecosystem management. *MARINE ENVIRONMENTAL RESEARCH* 58: 233-237.

Galloway TS, Brown RJ, Browne MA, et al. (2006). The ECOMAN project: A novel approach to defining sustainable ecosystem function. *MARINE POLLUTION BULLETIN* 53 (1-4): 186-194.

Galloway TS (2006). Biomarkers in environmental and human health risk assessment. *MARINE POLLUTION BULLETIN* 53 (10) 606-613.

Ge Y, Preston RJ, Owen RD (2007). Toxicoproteomics and its application to human health risk assessment *PROTEOMICS CLINICAL APPLICATIONS* 1 (12): 1613-1624.

Geffard A, Amiard-Triquet C, Amiard JC (2005). Do seasonal changes affect metallothionein induction by metals in mussels, *Mytilus edulis*? *ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY* 61 (2): 209-220.

Ghezzi P, Bonetto B (2003). Redox proteomics: identification of oxidatively modified proteins, *PROTEOMICS* 3:1145–1153.

Gibb HJ, Checkoway H, Stayner L (2002). Improving risk assessment: Priorities for epidemiologic research. *HUMAN AND ECOLOGICAL RISK ASSESSMENT* 8 (6): 1397-1404.

Giesy JP, Pierens SL, Snyder EM, Miles-Richardson S, Kramer VJ, Snyder SA, Nichols KM, Villeneuve DA (2000). Effects of 4-nonylphenol on fecundity and biomarkers of estrogenicity in fathead minnows (*Pimephales promelas*). ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY 19 (5): 1368-1377.

Gimeno S, Komen H, Gerritsen A.G.M., Bowmer T, 1998: Feminisation of young males of the common carp, *Cyprinus carpio*, exposed to 4-tert-pentylphenol during genderual differentiation. *AQUATIC TOXICOLOGY* 43(2-3): 77-92.

Gluckmann M, Fella K, Waidelich D, et al. (2007). Prevalidation of potential protein biomarkers in toxicology using iTRAQ (TM) reagent technology. *PROTEOMICS* 7 (10): 1564-1574.

Gochfeld M (2007). Gender in toxicology and risk assessment. *ENVIRONMENTAL RESEARCH* 104 (1): 1-1.

Godovac-Zimmermann J, Brown LR (2001). Perspectives for mass spectrometry and functional proteomics. *MASS SPECTROMETRY REVIEWS* 20 (1): 1-57.

Goksoyr A (2006). Endocrine disruptors in the marine environment: Mechanisms of toxicity and their influence on reproductive processes in fish. *JOURNAL OF TOXICOLOGY AND ENVIRONMENTAL HEALTH-PART A-CURRENT ISSUES* 69 (1-2): 175-184.

Goldberg ED, Bertine KK, (2000). Beyond the Mussel Watch - new directions for monitoring marine pollution. *SCIENCE OF THE TOTAL ENVIRONMENT* 247 (2-3): 165-174.

Goldberg ED (1975).The mussel watch – a first step in global marine monitoring, *MARINE POLLUTION BULLETIN* 6:111–132.

Gomiero A, Pampanin DM, Bjornstad A, et al. (2006). An ecotoxicoproteomic approach (SELDI-TOF mass spectrometry) to biomarker discovery in crab exposed to pollutants under laboratory conditions. *AQUATIC TOXICOLOGY* 78: 34-41 Suppl. 1.

Gosling CL (1992) The mussel *Mytilus*: Ecology, physiology, genetics and culture. *ELSEVIER SCIENCE PUBLISHERS*, 25.

Gourmelon A, Ahtiainen J (2007). Developing Test Guidelines on invertebrate development and reproduction for the assessment of chemicals, including potential endocrine active substances - The OECD perspective. *ECOTOXICOLOGY* 16 (1): 161-167.

Graves PR, Haystead TAJ (2002). Molecular biologist's guide to proteomics. *MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS* 66 (1): 39+.

Gray JS, Clarke KR, Warwick RM, et al. (1990). Detection of initial effects of pollution on marine benthos - an example from the Ekofisk and Eldfisk oilfields, North-sea. *MARINE ECOLOGY-PROGRESS SERIES* 66 (3): 285-299.

Gray LE, Ostby J, Furr J, et al. (2001). Effects of environmental antiandrogens on reproductive development in experimental animals. *HUMAN REPRODUCTION UPDATE* 7 (3): 248-264.

Greenspan FS, Gardner DG (2006). Basic and clinical endocrinology. New York.

Grieshaber D, MacKenzie R, Voros J, et al.(2008). Electrochemical biosensors - Sensor principles and architectures. *SENSORS* 8 (3): 1400-1458.

Guo ZJ, Wagner CR, Hanna PE (2004). Mass spectrometric investigation of the mechanism of inactivation of hamster arylamine n-acetyltransferase 1 by N-hydroxy-2-acetylaminofluorene. *CHEMICAL RESEARCH IN TOXICOLOGY* 17 (3): 275-286.

Gustafsson K, Bjork M, Burreau S, et al. (2006). Bioaccumulation kinetics of brominated flame retardants (polybrominated diphenyl ethers) in blue mussels (*Mytilus edulis*). *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY* 18 (6): 1218-1224.

Gygi SP, Rist B, Gerber SA, et al. (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *NATURE BIOTECHNOLOGY* 17 (10): 994-999.

Gygi SP, Aebersold R (2000). Mass spectrometry and proteomics. *CURRENT OPINION IN CHEMICAL BIOLOGY* 4 (5): 489-494.

Gygi SP, Rist B, Aebersold R (2000). Measuring gene expression by quantitative proteome analysis. *CURRENT OPINION IN BIOTECHNOLOGY* 11 (4): 396-401.

Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W (2000). The current state of two-dimensional electrophoresis with immobilized pH gradients. *ELECTROPHORESIS* 21 (6): 1037-1053.

Görg A, Boguth G, Kopf A, et al. (2002). Sample prefractionation with Sephadex isoelectric focusing prior to narrow pH range two-dimensional gels. *PROTEOMICS* 2 (12): 1652-1657.

Görg A, Weiss W, Dunn MJ (2004). Current two-dimensional electrophoresis technology for proteomics. *PROTEOMICS* 4 (12): 3665-3685.

Hagger JA, Depledge MH, Oehlmann J, et al. (2006). Is there a causal association between genotoxicity and the imposex effect? *ENVIRONMENTAL HEALTH PERSPECTIVES* 114: 20-26. Suppl. 1.

Hamers T, Kamstra JH, Sonneveld E, et al. (2006). In vitro profiling of the endocrine-disrupting potency of brominated flame retardants. *TOXICOLOGICAL SCIENCES* 92 (1): 157-173.

Hammond GL (1995). Potential function of plasma-steroid proteins. *TRENDS IN ENDOCRINOLOGY AND METABOLISM* 6(9-10): 298-304.

Handy RD (2003). Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process? *COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY A-MOLECULAR & INTEGRATIVE PHYSIOLOGY* 135 (1): 25-38.

Hansen BH, Altin D, Vang SH, et al. (2008). Effects of naphthalene on gene transcription in *Calanus finmarchicus* (Crustacea : Copepoda). *AQUATIC TOXICOLOGY* 86: 157-165.

Harbers M, Carninci P (2005). Tag-based approaches for transcriptome research and genome annotation. *NATURE METHODS* 2 (7): 495-502.

Hardiman G (2004). Microarray platforms - comparisons and contrasts. *PHARMACOGENOMICS* 5 (5): 487-502.

Harris CA, Henttu P, Parker MG, et al. (1997). The estrogenic activity of phthalate esters in vitro. *ENVIRONMENTAL HEALTH PERSPECTIVES* 105 (8): 802-811.

Harvey JS, Lyons BP, Page TS, Stewart CJ, Parry M (1999). An assessment of the genotoxic impact of the Sea Empress oil spill by the measurement of DNA adduct levels in selected invertebrate and vertebrate species. *MUTATION RESEARCH* 441(1): 103-114.

Hasselberg L, Meier S, Svardal A, Hegelund T, Celander MC (2004). Effects of alkylphenols on CYP1A and CYP3A expression in first spawning Atlantic cod (*Gadus morhua*) *AQUATIC TOXICOLOGY* 67 (4): 303-313.

Hedgecock D, Gaffney PM, Goulletquer P, et al. (2005). The case for sequencing the Pacific oyster genome. *JOURNAL OF SHELLFISH RESEARCH* 24 (2): 429-441.

He HS, Cai L, Skogerbo G, et al. (2006). Profiling Caenorhabditis elegans non-coding RNA expression with a combined microarray. *NUCLEIC ACIDS RESEARCH* 34 (10): 2976-2983.

Helleday T, Tuominen KL, Bergman A, et al. (1999). Brominated flame retardants induce intragenic recombination in mammalian cells. *MUTATION RESEARCH-*

GENETIC TOXICOLOGY AND ENVIRONMENTAL MUTAGENESIS 439 (2): 137-147.

Herbiniere J, Greve P, Strub JM, et al. (2008). Protein profiling of hemocytes from the terrestrial crustacean *Armadillidium vulgare*. *DEVELOPMENTAL AND COMPARATIVE IMMUNOLOGY* 32 (8): 875-882.

Hines A, Yeung WH, Craft J, et al. (2007). Comparison of histological, genetic, metabolomics, and lipid-based methods for sex determination in marine mussels. *ANALYTICAL BIOCHEMISTRY* 369 (2): 175-186.

Hogstrand C, Balesaria S, Glover CN (2002). Application of genomics and proteomics for study of the integrated response to zinc exposure in a non-model fish species, the rainbow trout. *COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY B-BIOCHEMISTRY & MOLECULAR BIOLOGY* 133 (4): 523-535.

Honkanen JO, Holopainen IJ, and Kukkonen JVK (2004). Bisphenol A induces yolk-sac oedema and other adverse effects in landlocked salmon (*Salmo salar m. sebago*) yolk-sac fry. *CHEMOSPHERE* 55(2): 187–196.

Hoving S, Voshol H, van Oostrum J (2000). Towards high performance two-dimensional gel electrophoresis using ultrazoom gels. *ELECTROPHORESIS* 21 (13): 2617-2621.

Hu QZ, Noll RJ, Li HY, et al. (2005). The Orbitrap: a new mass spectrometer *JOURNAL OF MASS SPECTROMETRY* 40 (4): 430-443.

Hu QZ, Talaty N, Noll RJ, et al. (2006). Desorption electrospray ionization using an Orbitrap mass spectrometer: exact mass measurements on drugs and peptides. *RAPID COMMUNICATIONS IN MASS SPECTROMETRY* 20 (22): 3403-3408.

Hughes TR, Marton MJ, Jones AR, et al. (2000). Functional discovery via a compendium of expression profiles. *CELL* 102 (1): 109-126.

Huh WK, Falvo JV, Gerke LC, et al. (2003). Global analysis of protein localization in budding yeast. *NATURE* 425 (6959): 686-691.

Hunt T, Huang Y, Ross P, et al. (2004). Protein expression analysis and biomarker identification and quantification using multiplexed isobaric tagging technology – iTRAQ reagents. MOLECULAR & CELLULAR PROTEOMICS 3 (10): 286-286 Suppl. S.

Hutchens TW, Yip TT (1993). New desorption strategies for the Mass-Spectrometric analysis of macromolecules. *RAPID COMMUNICATIONS IN MASS SPECTROMETRY* 7 (7): 576-580.

Hutchinson TH (2007). Small is useful in endocrine disrupter assessment - four key recommendations for aquatic invertebrate research. *ECOTOXICOLOGY* 16 (1): 231-238.

IPCS (2002).Global assessment of the state-of-science of endocrine disruptors. Assessment: International Programme on Chemical Safety. http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/

Islam N, Tsujimoto H, Hirano H (2003). Wheat proteomics: Relationship between fine chromosome deletion and protein expression. *PROTEOMICS* 3 (3): 307-316.

Issaq HJ, Veenstra TD, Conrads TP, Felschow D (2003). The SELDI-TOF MS approach to proteomics: Protein profiling and biomarker identification. *BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS* 292 (3): 587-592.

Issaq HJ (2001). The role of separation science in proteomics research. *ELECTROPHORESIS* 22 (17): 3629-3638.

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

Jaluria P, Konstantopoulos K, Betenbaugh M, et al.(2007). A perspective on microarrays: current applications, pitfalls, and potential uses. *MICROBIAL CELL FACTORIES* 6: Article 4.

Jamers A, Van der Ven K, Moens L, et al. (2006). Effect of copper exposure on gene expression profiles in *Chlamydomonas reinhardtii* based on microarray analysis. *AQUATIC TOXICOLOGY* 80 (3): 249-260.

Janer G, Bachmann J, Oehlmann J, et al. (2006). The effect of organotin compounds on gender specific androstenedione metabolism in the freshwater ramshorn snail *Marisa cornuarietis*. *JOURNAL OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY* 99 (2-3):147-156.

Janer G, Porte C (2007). Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. ECOTOXICOLOGY 16 (1): 145-160.

Jenny MJ, Chapman RW, Mancia A, et al.(2007). A cDNA Microarray for *Crassostrea virginica* and *C-gigas*. *MARINE BIOTECHNOLOGY* 9 (5): 577-591.

Jenssen BM (2006). Endocrine-disrupting chemicals and climate change: A worst-case combination for arctic marine mammals and seabirds? *ENVIRONMENTAL HEALTH PERSPECTIVES* 114: 76-80 Suppl. 1.

Jobling S, Reynolds, T, White R, et al. (1995). A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *ENVIRONMENTAL HEALTH PERSPECTIVES* 103 (6): 582-587.

Jobling S, Tyler CR (2006). Introduction: The ecological relevance of chemically induced endocrine disruption in wildlife. *ENVIRONMENTAL HEALTH PERSPECTIVES* 114: 7-8 Suppl. 1.

Jock CA, Paulauskis JD, Baker D et al. (2004). Influence of matrix application timing on spectral reproducibility and quality in SELDI-TOF-MS . *BIOTECHNIQUES* 37 (1): 30+.

Johansson L (2003). Hormesis, an update of the present position. *EUROPEAN JOURNAL OF NUCLEAR MEDICINE AND MOLECULAR IMAGING* 30 (6): 921-933.

Johnsen S, Frost TK, Hjelsvold M, Utvik TIR (2000). The environmental impact factor – a proposed tool for produced water impact reduction, management and regulation. SPE paper 61178. SPE International Conference, 26–28 June 2000, Stavanger, Norway.

Johnson A, Jurgens M (2003). Endocrine active industrial chemicals: Release and occurrence in the environment. *PURE AND APPLIED CHEMISTRY* 75 (11-12): 1895-1904.

Jonsson G, Bechmann RK, Bamber SD, Baussant T (2004). Bioconcentration, biotransformation, and elimination of polycyclic aromatic hydrocarbons in sheepshead minnows (*Cyprinodon variegatus*) exposed to contaminated seawater. *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY* 23 (6): 1538-1548.

Jonsson G, Cavcic A, Stokke TU, et al. (2008). Solid-phase analytical derivatization of alkylphenols in fish bile for gas chromatography-mass spectrometry analysis. *JOURNAL OF CHROMATOGRAPHY A* 1183 (1-2): 6-14.

Jonsson H, Schiedek D, Goksoyr A, et al. (2006a). Expression of cytoskeletal proteins, cross-reacting with anti-CYP1A, in *Mytilus* sp exposed to organic contaminants. *AQUATIC TOXICOLOGY* 78: 42-48. Suppl. 1.

Jonsson H, Schiedek D, Grosvik BE, et al.(2006b). Protein responses in blue mussels (*Mytilus edulis*) exposed to organic pollutants: A combined CYP-antibody/proteomic approach. *AQUATIC TOXICOLO*GY 78: 49-56, Suppl. 1.

Joyce AR, Palsson BO (2006). The model organism as a system: integrating 'omics' data sets. *NATURE REVIEWS MOLECULAR CELL BIOLOGY* 7 (3): 198-210.

Jukosky JA, Watzin MC, Leiter JC (2008). The effects of environmentally relevant mixtures of estrogens on Japanese medaka (*Oryzias latipes*) reproduction Source: *AQUATIC TOXICOLOGY* 86: 323-331.

Kadiiska MB, Hanna PM, Jordan SJ, Mason RP (1993). Electron spin resonance evidence for free radical generation in copper-treated vitamin E- and selenium-deficient rats: in vivo spin-trapping investigation. *MOLECULAR PHARMACOLOGY* 44: 222–227.

Kaiser J (2003). Hormesis - Sipping from a poisoned chalice. *SCIENCE* 302 (5644): 376+.

Kang IJ, Yokota H, Oshima Y, Tsuruda Y, Imada N, Tadokoro H, and HonjoT (2002). Effect of bisphenol A on the reproduction of Japanese medaka (*Oryzias latipes*), *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY.* 21: 2394–2400.

Kang JH, Kondo F, Katayama Y (2006). Human exposure to bisphenol A, *TOXICOLOGY* 226 (2-3):79–89.

Kavlock RJ, Daston G P, DeRosa C, Fenner-Crisp P, Gray LE et al. (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA-sponsored workshop. *ENVIRONMENTAL HEALTH PERSPECTIVE.* 104: 714-740, Suppl. 4.

Kefford BJ, Zalizniak L, Warne MSJ, et al. (2008). Is the integration of hormesis and essentiality into ecotoxicology now opening Pandora's Box? *ENVIRONMENTAL POLLUTION* 151 (3): 516-523.

Kellis M, Patterson N, Endrizzi M, et al. (2003). Sequencing and comparison of yeast species to identify genes and regulatory elements. *NATURE* 423 (6937): 241-254.

Ketata I, Denier X, Hamza-Chaffai A, et al. (2008). Endocrine-related reproductive effects in molluscs. *COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY* 147(3) 261-270.

Kiehntopf M, Siegmund R, Deufel T (2007). Use of SELDI-TOF mass spectrometry for identification of new biomarkers: potential and limitations. *CLINICAL CHEMISTRY AND LABORATORY MEDICINE* 45 (11): 1435-1449.

Kimmel DG, Bradley BP. (2001). Specific protein responses in the calanoid copepod Eurytemora affinis (Poppe, 1880) to salinity and temperature variation. *JOURNAL OF EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY* 266 (2): 135-149.

Knakievicz T, Ferreira HB, (2008). Evaluation of copper effects upon Girardia tigrina freshwater planarians based on a set of biomarkers. *CHEMOSPHERE* 71(3): 419-428.

Knauert S, Knauer K, (2008). The role of reactive oxygen species in copper toxicity to two freshwater green algae. *JOURNAL OF PHYCOLOGY* 44 (2): 311-319.

Knigge T, Monsinjon T, Andersen OK (2004). Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry approach to biomarker discovery in blue mussels (*Mytilus edulis*) exposed to polyaromatic hydrocarbons and heavy metals under field conditions. *PROTEOMICS* 4 (9): 2722-2727.

Kohler HR, Kloas W, Schirling M, et al. (2007). Sex steroid receptor evolution and signalling in aquatic invertebrates. *ECOTOXICOLOGY* 16 (1): 131-143.

Kolkman A, Dirksen EHC, Slijper M, et al.(2005). Double standards in quantitative proteomics - Direct comparative assessment of difference in gel electrophoresis and metabolic stable isotope labelling. *MOLECULAR & CELLULAR PROTEOMICS* 4 (3): 255-266.

Krause PR (1994). Effects of an oil production effluent on gametogenesis and gamete performance in the purple sea-urchin (*Stronglyocentrotus-purpuratus stimpson*) *ENVIRONMENTAL TOXICOLOGY & CHEMISTRY.* 13 (7): 1153-1161.

Kusk KO, Wollenberger L (2007).Towards an internationally harmonized test method for reproductive and developmental effects of endocrine disrupters in marine copepods. *ECOTOXICOLOGY* 16 (1): 183-195.

Kuster B, Schirle M, Mallick P, et al. (2005). Scoring proteomes with proteotypic peptide probes. *NATURE REVIEWS MOLECULAR CELL BIOLOGY* 6 (7) 577-583.

Labieniec M, Gabryelak T (2007). Antioxidative and oxidative changes in the digestive gland cells of freshwater mussels *Unio tumidus* caused by selected phenolic compounds in the presence of H2O2 or Cu2+ ions. *TOXICOLOGY IN VITRO* 21 (1): 146-156.

Lafont R, Mathieu M. (2007). Steroids in aquatic invertebrates. *ECOTOXICOLOGY* 16 (1): 109-130.

Lagadic L, Coutellec MA, Caquet T (2007).Endocrine disruption in aquatic pulmonate molluscs: few evidences, many challenges. *ECOTOXICOLOGY* 16 (1): 45-59.

Lam PKS, Gray JS (2001). Predicting effects of toxic chemicals in the marine environment. *MARINE POLLUTION BULLETIN* 42 (3): 169-173.

Lam PKS, Gray JS (2003). The use of biomarkers in environmental monitoring programmes. *MARINE POLLUTION BULLETIN* 46 (2): 182-186.

Lander ES, Linton LM, Birren B, Nusbaum et al. (2001). Initial sequencing and analysis of the human genome. *NATURE* 409 (6822): 860-921.

Larsen BK (2004). Biological effects of long term exposure to a North Sea and a Barents Sea oil: early-life stage tests and biomarker responses in crustacea, version 2 . AM report 2004/016, 111pp.

Larsen BK, Bjornstad A, Sundt RC, et al. (2006). Comparison of protein expression in plasma from nonylphenol and bisphenol A-exposed Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) by use of SELDI-TOF. *AQUATIC TOXICOLOGY* 78: 25-33, Suppl. 1.

Lau ATY, He QY, Chiu JF (2003). Proteomic technology and its biomedical applications. *ACTA BIOCHIMICA ET BIOPHYSICA SINICA* 35 (11): 965-975.

Lavado R, Janer G, Porte C (2006). Steroid levels and steroid metabolism in the mussel *Mytilus edulis*: The modulating effect of dispersed crude oil and alkylphenols. *MARINE ENVIRONMENTAL RESEARCH* 62: 235-236, Suppl. S.

Law RJ, Alaee M, Allchin CR, et al. (2003). Levels and trends of polybrominated diphenylethers and other brominated flame retardants in wildlife *ENVIRONMENT INTERNATIONAL* 29 (6): 757-770.

Law RJ, Allchin CR, de Boer,J, et al. (2006). Levels and trends of brominated flame retardants in the European environment. *CHEMOSPHERE* 64 (2): 187-208.

Lay JO, Borgmann S, Liyanage R, et al. (2006). Problems with the "omics" *TRAC-TRENDS IN ANALYTICAL CHEMISTRY* 25 (11): 1046-1056.

LeBlanc GA (2007). Crustacean endocrine toxicology: a review. *ECOTOXICOLOGY* 16 (1): 61-81.

Lederberg J, McCray AT (2001). 'Ome sweet 'omics - A genealogical treasury of words. *SCIENTIST* 15 (7): 8-8.

Legler J, Brouwer A (2003). Are brominated flame retardants endocrine disruptors? *ENVIRONMENT INTERNATIONAL* 29 (6): 879-885.

Leonard BE, (2007). Adaptive response: Part II. Further modelling for dose rate and time influences. *INTERNATIONAL JOURNAL OF RADIATION BIOLOGY* 83 (6): 395-408.

Lettieri T (2006). Recent applications of DNA microarray technology to toxicology and ecotoxicology. *ENVIRONMENTAL HEALTH PERSPECTIVES* 114 (1): 4-9.

Li H, Chen QY, Li S, et al. (2001). Effect of Cr(VI) exposure on sperm quality: Human and animal studies. *ANNALS OF OCCUPATIONAL HYGIENE* 45 (7): 505-511.

Li JN, Zhang Z, Rosenzweig J, Wang YY, Chan DW (2002). Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer.*CLINICAL CHEMISTRY* 48 (8): 1296-1304.

Li L, Andersen ME, Heber S, et al. (2007). Non-monotonic dose-response relationship in steroid hormone receptor-mediated gene expression. *JOURNAL OF MOLECULAR ENDOCRINOLOGY* 38 (5-6): 569+.

Lin J, Qian J (2007). Systems biology approach to integrative comparative genomics. *EXPERT REVIEW OF PROTEOMICS* 4 (1): 107-119.

Linder MC (1991). The biochemistry of copper. pp 1-151.Plenum press, New York.

Liolios K, Tavernarakis N, Hugenholtz P, et al. (2006). The Genomes On Line Database (GOLD) v.2: a monitor of genome projects worldwide. *NUCLEIC ACIDS RESEARCH* 34: 332-334.

Liolios K, Mavromatis K, Tavernarakis N, et al. (2008). The Genomes On Line Database (GOLD) in 2007: status of genomic and metagenomic projects and their associated metadata. *NUCLEIC ACIDS* RESEARCH 36: 475-479.

Liu PY, Iranmanesh A, Keenan DM, et al. (2007). A noninvasive measure of negative-feedback strength, approximate entropy, unmasks strong diurnal variations in the regularity of LH secretion. *AMERICAN JOURNAL OF PHYSIOLOGY-ENDOCRINOLOGY AND METABOLISM* 293 (5): 1409-1415.

Liu WX, Hu J, Chen JL, et al. (2008). Distribution of persistent toxic substances in benthic bivalves from the inshore areas of the Yellow Sea. *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY* 27: 57-66.

Lodish H, Berk A, et al. (2000). Molecular cell biology. New York: WH Freeman and Company.

Lopez JL, Mosquera E, Fuentes J, et al. (2001). Two-dimensional gel electrophoresis of *Mytilus galloprovincialis*: differences in protein expression between intertidal and cultured mussels. *MARINE ECOLOGY-PROGRESS SERIES* 224: 149-156.

Lopez JL, Marina A, Alvarez G, et al. (2002). Application of proteomics for fast identification ofspecies-specific peptides from marine species. *PROTEOMICS* 2 (12): 1658-1665.

Lu BW, Motoyama A, Ruse C, et al.(2008). Improving protein identification sensitivity by combining MS and MS/MS information for shotgun proteomics using LTQ-Orbitrap high mass accuracy data. *ANALYTICAL CHEMISTRY* 80 (6): 2018-2025.

Lyons C, Dowling V, Tedengren M, et al. (2003). Variability of heat shock proteins and glutathione S-transferase in gill and digestive gland of blue mussel, *Mytilus edulis*. *MARINE ENVIRONMENTAL RESEARCH* 56 (5): 585-597.

MacCoss MJ, McDonald WH, Saraf A, et al. (2002). Shotgun identification of protein modifications from protein complexes and lens tissue *PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA* 99 (12): 7900-7905.

MacGregor JT (2003).The future of regulatory toxicology: Impact of the biotechnology revolution. *TOXICOLOGICAL SCIENCES* 75 (2): 236-248.

Manduzio H, Monsinjon T, Galap C, et al. (2004). Seasonal variations in antioxidant defences in blue mussels Mytilus edulis collected from a polluted area: major contributions in gills of an inducible isoform of Cu/Zn-superoxide dismutase and of glutathione S-transferase. *AQUATIC TOXICOLOGY* 70 (1): 83-93.

Manzl C, Enrich J, Ebner H, Dallinger R, Krumschnabel G. (2004). Copper-induced formation of reactive oxygen species causes cell death and disruption of calcium homeostasis in trout hepatocytes. *TOXICOLOGY* 196: 57–64.

Manzo S, Buono S, Cremisini C (2008). Predictability of copper, irgarol, and diuron combined effects on sea urchin Paracentrotus lividus. *ARCHIVES OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY* 54 (1): 57+.

Martin-Diaz ML, Blasco J, Sales D, et al. (2008). Field validation of a battery of biomarkers to assess sediment quality in Spanish ports. *ENVIRONMENTAL POLLUTION* 151 (3): 631-640.

Martin-Skilton R, Thibaut R, Porte C (2006). Endocrine alteration in juvenile cod and turbot exposed to dispersed crude oil and alkylphenols. *AQUATIC TOXICOLOGY* 78: 57-64, Suppl. 1.

Mascini M, Mazzei F, Moscone D, et al. (1987).. Lactate and pyruvate electrochemical biosensors for whole-blood in extracorporeal experiments with an endocrine artificial pancreas. *CLINICAL CHEMISTRY* 33 (4): 591-593.

Matthiessen P, Gibbs PE (1998). Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY* 17 (1): 37-43.

Matthiessen P (2003). Historical perspective on endocrine disruption in wildlife. *PURE AND APPLIED CHEMISTRY* 75 (11-12): 2197-2206.

Matthiessen P, Johnson I (2007). Implications of research on endocrine disruption for the environmental risk assessment, regulation and monitoring of chemicals in the European Union. *ENVIRONMENTAL POLLUTION* 146 (1): 9-18.

Mattson MP (2008). Hormesis defined. *AGEING RESEARCH REVIEWS* 7: 1-7.

McCarty LS, Borgert CJ (2006). Review of the toxicity of chemical mixtures: Theory, policy, and regulatory practice. *REGULATORY TOXICOLOGY AND PHARMACOLOGY* 45 (2): 119-143.

McClellan-Green P, Romano J, Oberdorster E (2007). Does gender really matter in contaminant exposure? A case study using invertebrate models. *ENVIRONMENTAL RESEARCH* 104 (1): 183-191.

McDonagh B, Tyther R, Sheehan D (2005). Carbonylation and glutathionylation of proteins in the blue mussel *Mytilus edulis* detected by proteomic analysis and Western blotting: Actin as a target for oxidative stress. *AQUATIC TOXICOLOGY* 73 (3):315-326.

McDonagh B, Sheehan D (2007). Effect of oxidative stress on protein thiols in the blue mussel *Mytilus edulis*: Proteomic identification of target proteins. *PROTEOMICS* 7 (18): 3395-3403.

McDonald TA (2002). A perspective on the potential health risks of PBDEs. *CHEMOSPHERE* 46: 45–755.

McDonald TA (2005). Polybrominated diphenylether levels among United States residents: daily intake and risk of harm to the developing brain and reproductive organs. *INTEGR ENVIRON ASSESS MANAG* 1: 343–354.

McKillen DJ, Chen YA, Chen CM, et al. (2005). Marine Genomics: A clearing-house for genomic and transcriptomic data of marine organisms. *BMC GENOMICS* 6: Article 34.

McLachlan JA (2001). Environmental signalling: what embryos and evolution teach us about endocrine disrupting chemicals? *ENDOCRINE REVIEWS* 22: 319–341.

Medesani DA, Susana L, Greco L, et al. (2004). Interference of cadmium and copper with the endocrinecontrol of ovarian growth, in the estuarine crab *Chasmagnathus granulate*. *AQUATIC TOXICOLOGY* 69 (2): 165-174.

Meerts IATM, Letcher RJ, Hoving S, et al. (2001). In vitro estrogenicity of polybrominated diphenyl ethers, hydroxylated PBDEs, and polybrominated bisphenol A compounds. *ENVIRONMENTAL HEALTH PERSPECTIVES* 109 (4): 399-407.

Mehendale HM, Thakore KN, Rao CV (1994). Autoprotection - stimulated tissue-repair permits recovery from injury. *JOURNAL OF BIOCHEMICAL TOXICOLOGY* 9 (3): 131-139.

Mehendale HM (1995). Toxicodynamics of low level toxicant interactions of biological significance: Inhibition of tissue repair. *TOXICOLOGY* 105 (2-3): 251-266.

Meier S, Andersen TE, Norberg B, et al. (2007). Effects of alkylphenols on the reproductive system of Atlantic cod (*Gadus morhua*). *AQUATIC TOXICOLOGY* 81 (2): 207-218.

Merchant M, Weinberger SR (2000). Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry. *ELECTROPHORESIS* 21 (6): 1164-1177.

Meyer E, Haag AL, Von Dippe P, et al. (2003). Physiological genomics and the identification of genes involved in differential growth rates of bivalve larvae. *INTEGRATIVE AND COMPARATIVE BIOLOGY* 43 (6): 880-880.

Meyer HE and Stuhler K (2007). High-performance proteomics as a tool in biomarker-discovery. *PRACTICAL PROTEOMICS* 2 (1): 18-26.

Mi J, Apraiz I, Cristobal S (2007). Peroxisomal proteomic approach for protein profiling in blue mussels (*Mytilus edulis*) exposed to crude oil. *BIOMARKERS* 12 (1): 47-60.

Mikhailov AT, Torrado M, Mendez J (1995). Sexual-Differentiation of reproductive tissue in bivalve molluscs – Identificaiton of male associated polypeptide in the mantle of mytilus-galloprovincialis LMK. *INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY.* 39(3) 545-548.

Minier C, Forget-Leray J, Bjornstad A, Camus L (2008). Multixenobiotic resistance, acetyl-choline esterase activity and total oxyradical scavenging capacity of the Arctic spider crab, *Hyas araneus*, following exposure to Bisphenol A, Tetra Bromo Diphenyl Ether and Diallyl phtalate. *MARINE POLLUTION BULLETIN* 56 (8): 1410-1415.

Miura Y, Kano M, Yamada M, et al. (2007). Proteomic study on X-irradiation-responsive proteins and ageing: Search for responsible proteins for radiation adaptive response. *JOURNAL OF BIOCHEMISTRY* 142: 145-155.

Monsinjon T, Andersen OK, Leboulenger F, et al. (2006). Data processing and classification analysis of proteomic changes: a case study of oil pollution in the mussel, *Mytilus edulis*. *PROTEOME SCIENCE* 4(17): 1-13.

Monsinjon T, Knigge T (2007). Proteomics applications in ecotoxicology. *PROTEOMICS* 7(16): 2997-3009.

Moore MN (2002). Biocomplexity: the post-genome challenge in ecotoxicology *AQUATIC TOXICOLOGY* 59(1-2): 1-15.

Moore MN, Depledge MH, Readman JW, et al. (2004). An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. *MUTATION RESEARCH-FUNDAMENTAL AND MOLECULAR MECHANISMS OF MUTAGENESIS* 552 (1-2): 247-268.

Morel NM, Holland JM, van der Greef J, et al.(2004). Primer on medical genomics part XIV: Introduction to systems biology - A new approach to understanding disease and treatment. *MAYO CLINIC PROCEEDINGS* 79(5): 651-658.

Morelle W, Canis K, Chirat F, et al. (2006). The use of mass spectrometry for the proteomic analysis of glycosylation. *PROTEOMICS* 6 (14): 3993-4015.

Morgan KT, Ni H, Brown HR, Yoon L, Qualls CW et al. (2002). Application of cDNA microarray technology to in vitro toxicology and the selection of genes for a real-time RT-PCR-based screen for oxidative stress in Hep-G2 cells. *TOXICOLOGIC PATHOLOGY* 30 (4): 435-451.

Mulchandani A, Chen W, Mulchandani P, et al. (2001). Biosensors for direct determination of organophosphate pesticides. *BIOSENSORS & BIOELECTRONICS* 16(4-5): 225-230.

Murado MA, Vazquez JA (2007). The notion of hormesis and the dose-response theory: A unified approach. *JOURNAL OF THEORETICAL BIOLOGY* 224(3): 489-499.

Mushak P (2007). Hormesis and its place in nonmonotonic dose-response relationships: Some scientific reality checks. *ENVIRONMENTAL HEALTH PERSPECTIVES* 115 (4): 500-506.

Narbonne JF, Daubeze M, Clerandeau C, et al. (1999). Scale of classification based on biochemical markers in mussels: application to pollution monitoring in European coasts. *BIOMARKERS* 4: 415-424.

Narvia M, Rantamaki P (1997). Aminotransferases in the bivalve mollusc *Mytilus edulis* L and short term effects of crude oil in brackish water *BIOMARKERS* 2 (4): 253-258.

Nawaz M, Manzl C, Lacher V, et al. (2006). Copper-induced stimulation of extracellular signal-regulated kinase in trout hepatocytes: The role of reactive oxygen species, Ca2+, and cell energetics and the impact of extracellular signal-regulated kinase signaling on apoptosis and necrosis. *TOXICOLOGICAL SCIENCES* 92 (2): 464-475.

Neff JM (2002). Bioaccumulation in marine organisms, Effect of contaminants from oil well produced water, *ELSEVIER SCIENCE LTD.*, Amsterdam p. 452.

Neff JM, Johnsen S, Frost TK, et al. (2006). Oil well produced water discharges to the North Sea. Part II: Comparison of deployed mussels (*Mytilus edulis*) and the

DREAM model to predict ecological risk. *MARINE ENVIRONMENTAL RESEARCH* 62 (3): 224-246.

Neumann NF, Galvez F (2002). DNA microarrays and toxicogenomics: applications for ecotoxicology? *BIOTECHNOLOGY ADVANCES* 20 (5-6): 391-419.

Nicholson JK, Lindon JC, Holmes E (1999). 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data *XENOBIOTICA* 29 (11): 1181-1189.

Nicholson JK, Connelly J, Lindon JC, Holmes E (2002). Metabonomics: a platform for studying drug toxicity and gene function. *NATURE REVIEWS DRUG DISCOVERY 1* (2): 153-161.

Nimrod AC, Benson WH (1996). Environmental estrogenic effects of alkylphenol ethoxylates. *CRITICAL REVIEWS IN TOXICOLOGY* 26 (3): 335-364.

Nuwaysir EF, Bittner M, Trent J, Barrett JC, Afshari CA (1999). Microarrays and toxicology: The advent of toxicogenomics. *MOLECULAR CARCINOGENESIS* 24 (3): 153-159.

Oberdorster E, Brouwer M, Hoexum-Brouwer T, Manning S, McLachlan JA (2000). Long-term pyrene exposure of grass shrimp, *Palaemonetes pugio*, affects molting and reproduction of exposed males and offspring of exposed females. *ENVIRONMENTAL HEALTH PERSPECTIVE*.108 (7): 641- 646.

Oberdorster E, Cheek AO (2001). Gender benders at the beach: Endocrine disruption in marine and estuarine organisms. *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY* 20 (1) 23-36.

OECD (1997) Draft Detailed Review Paper: Appraisal of TestMethods for Sex-Hormone Disrupting Chemicals, Paris: Organization for Economic Cooperation and Development, April.

Oehlmann J, Schulte-Oehlmann U (2003). Endocrine disruption in invertebrates. *PURE AND APPLIED CHEMISTRY* 75 (11-12) 2207-2218.

Oehlmann J, Schulte-Ohelmann U, Bachmann J, Oetken M, Lutz I, Kloas K, Ternes TA (2006). Bisphenol A induces superfeminisation in the ramshorn snail *Marisa cornuarietis* (Gastropoda: prosobranchia) at environmental relevant concentrations. *ENVIRONMENTAL HEALTH PERSPECTIVE* 114: 127–133.

Oehlmann J, Di Benedetto P, Tillmann M, et al.(2007). Endocrine disruption in prosobranch molluscs: evidence and ecological relevance. *ECOTOXICOLOGY* 16 (1) 29-43.

Oetken M, Bachmann J, Schulte-Oehlmann U et al. (2004). Evidence for endocrine disruption in invertebrates. *INTERNATIONAL REVIEW OF CYTOLOGY - A SURVEY OF CELL BIOLOGY* 236: 1-44.

O'Farrell PH (1975). High resolution two-dimensional electrophoresis of proteins. *JOURNAL OF BIOLOGY AND CHEMISTRY* 250: 4007-4021.

Olsen GH, Sva E, Carroll J, et al. (2007). Alterations in the energy budget of Arctic benthic species exposed to oil-related compounds. *AQUATIC TOXICOLOGY* 83 (2): 85-92.

Olsson B, Bradley BP, Gilek M, Reimer O, Shepard JL, Tedengren M (2004). Physiological and proteomic responses in *Mytilus edulis* exposed to PCBs and PAHs extracted from Baltic Sea sediments. *HYDROBIOLOGIA* 514 (1-3): 15-27.

Ong SE, Kratchmarova I, Mann M (2003). Properties of C-13-substituted arginine in stable isotope labeling by amino acids in cell culture (SILAC). *JOURNAL OF PROTEOME RESEARCH* 2 (2): 173-181.

Orlando EF, Guillette LJ (2007). Sexual dimorphic responses in wildlife exposed to endocrine disrupting chemicals. *ENVIRONMENTAL RESEARCH* 104 (1): 163-173.

Osenberg CW, Schmitt RJ, Holbrook SJ, Canestro D (1992). Spatial scale of ecological effects associated with an open coast discharge of produced water. In: Ray JP, Engelhardt FR (eds) Produced water: Technological/environmental isssues and solutions. Plenum Press, New York, pp. 387-402.

OSPAR (1986). PARCOM recommendation 86/1 of a 40 mg/L emmision standard for plattforms.

OSPAR (2006). OSPAR recommendation 2006/4 amending OSPAR recommendation 2001/1 for the management of produced water from offshore installations.

Owen R, Galloway TS, Hagger JA, et al. (2008). Biomarkers and environmental risk assessment: Guiding principles from the human health field. *MARINE POLLUTION BULLETIN* 56 (4): 613-619.

Pait AS, Nelson JO (2003). Vitellogenesis in male *Fundulus heteroclitus* (killifish) induced by selected estrogenic compounds, *AQUATIC TOXICOLOGY* 64 (3): 331–342.

Pandey A, Mann M (2000). Proteomics to study genes and genomes. *NATURE* 405 (6788): 837-846.

Paradela A, Albar JP (2008). Advances in the analysis of protein phosphorylation. *JOURNAL OF PROTEOME RESEARCH* 7 (5): 1809-1818.

Park RA, Clough JS, Wellman MC (2008). AQUATOX: Modeling environmental fate and ecological effects in aquatic ecosystems. *ECOLOGICAL MODELLING* 213 (1): 1-15.

Patterson SD, Aebersold RH (2003). Protemics the first decade and beyond. *NATURE GENETICS* 33: 311-323 Suppl. S.

Patterson SD (2004). How much of the proteome do we see with discovery-based proteomics methods and how much do we need to see? *CURRENT PROTEOMICS* (1): 3-12.

Paweletz CP, Gillespie JW, Ornstein DK, Simone NL, Brown MR, Cole KA, Wang QH, Huang J, Hu N, Yip TT, Rich WE, Kohn EC, Linehan WM, Weber T, Taylor P, Emmert-Buck MR, Liotta LA, Petricoin EF (2000). Rapid protein display profiling of cancer progression directly from human tissue using a protein biochip. *DRUG DEVELOPMENT RESEARCH* 49 (1): 34-42.

Paweletz CP, Trock B, Pennanen M, Tsangaris T, Magnant C, Liotta LA, Petricoin EF (2001). Proteomic patterns of nipple aspirate fluids obtained by SELDI-TOF: Potential for new biomarkers to aid in the diagnosis of breast cancer. *DISEASE MARKERS* 17 (4): 301-307.

Peakall D, Burger J (2003). Methodologies for assessing exposure to metals: speciation, bioavailability of metals, and ecological host factors. *ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY* 56 (1): 110-121.

Peakall DB ed. (1992). Animal Biomarkers as Pollution Indicators. *ECOTOX SER 1* Chapman & Hall.

Peck LS, Clark MS, Clarke A, et al. (2005). Genomics: applications to Antarctic ecosystems. *POLAR BIOLOGY* 28 (5): 351-365:

Pelzer AE, Feuerstein I, Fuchsberger C, et al. (2007) Influence of blood sampling on protein profiling and pattern analysis using matrix-assisted laser desorption/ionisation mass spectrometry. *BJU INTERNATIONAL* 99 (3): 658-662.

Pennie WD, Tugwood JD, Oliver GJA, et al. (2000). The principles and practice of toxicogenomics Applications and opportunities. *TOXICOLOGICAL SCIENCES* 54 (2): 277-283.

Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC, Liotta LA (2002). Use of proteomic patterns in serum to identify ovarian cancer. *LANCET.* 359 (9306): 572-577.

Petricoin EF, Liotta LA (2004a). SELDI-TOF-based serum proteomic pattern diagnostics for early detection of cancer. *CURRENT OPINION IN BIOTECHNOLOGY* 15 (1): 24-30.

Petricoin EF, Liotta LA (2004b). Proteomic approaches in cancer risk and response assessment. *TRENDS IN MOLECULAR MEDICINE* 10 (2): 59-64.

Plumb RS, Stumpf CL, Gorenstein MV, et al.(2002), Metabonomics: the use of electrospray mass spectrometry coupled to reversed-phase liquid chromatography shows potential for the screening of rat urine in drug development. *RAPID COMMUNICATIONS IN MASS SPECTROMETRY* 16 (20): 1991-1996.

Plumb RS, Stumpf CL, Granger JH, et al. (2003). Use of liquid chromatography /time-of-flight mass spectrometry and multivariate statistical analysis shows promise for the detection of drug metabolites in biological fluids. *RAPID COMMUNICATIONS IN MASS SPECTROMETRY* 17 (23): 2632-2638.

Podrabsky JE, Lopez JP, Fan TWM, et al. (2007). Extreme anoxia tolerance in embryos of the annual killifish *Austrofundulus limnaeus*: insights from a metabolomics analysis. *JOURNAL OF EXPERIMENTAL BIOLOGY* 210 (13): 2253-2266.

Pojana G, Gomiero A, Jonkers N, et al. (2007). Natural and synthetic endocrine disrupting compounds (EDCs) in water, sediment and biota of a coastal lagoon. *ENVIRONMENT INTERNATIONAL* 33: 929-936.

Poon TCW (2007). Opportunities and limitations of SELDI-TOF-MS in biomedical research: practical advices. *EXPERT REVIEW OF PROTEOMICS* 4 (1): 51-65.

Porte C, Janer G, Lorusso LC, et al. (2006). Endocrine disruptors in marine organisms: Approaches and perspectives. COMPARATIVE *BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY* 143 (3): 303-315.

Provan F, Bjornstad A, Pampanin DM, et al. (2006). Mass spectrometric profiling - A diagnostic tool in fish? *MARINE ENVIRONMENTAL RESEARCH* 62 105-108 Suppl. S.

Pytharopoulou S, Kouvelaa EC, Sazakli E, et al. (2006). Evaluation of the global protein synthesis in *Mytilus galloprovincialis* in marine pollution monitoring: Seasonal variability and correlations with other biomarkers. *AQUATIC TOXICOLOGY* 80(1): 33-41.

Qi XM, Li PJ, Liu W, et al.(2006). Multiple biomarkers response in maize (*Zea mays* L.) during exposure to copper. *JOURNAL OF ENVIRONMENTAL SCIENCES-CHINA* 18 (6): 1182-1188.

Quackenbush J (2007). Extracting biology from high-dimensional biological data. *JOURNAL OF EXPERIMENTAL BIOLOGY* 210 (9): 1507-1517.

Raamsdonk LM, Teusink B, Broadhurst D, Zhang NS, Hayes A, Walsh MC, Berden JA, Brindle KM, Kell DB, Rowland JJ, Westerhoff HV, van Dam K, Oliver SG

(2001). A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. *NATURE BIOTECHNOLOGY* 19 (1): 45-50.

Rabilloud T (1998). Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis. *ELECTROPHORESIS* 19 (5): 758-760.

Rabilloud T (2002). Two-dimensional gel electrophoresis in proteomics: Old, old fashioned, but it still climbs up the mountains. *PROTEOMICS* 2 (1): 3-10.

Reynolds T (2002). For proteomics research, a new race has begun. *JOURNAL OF THE NATIONAL CANCER INSTITUTE* 94 (8): 552-554.

Ringwood AH, Hameedi MJ, Lee RF, et al. (1999). Bivalve Biomarker Workshop: overview and discussion group summaries. *BIOMARKERS* 4(6): 391-399.

Robosky LC, Robertson DG, Baker JD, Rane S, Reily MD (2002). In vivo toxicity screening programs using metabonomics. *COMBINATORIAL CHEMISTRY & HIGH THROUGHPUT SCREENING* 5 (8): 651-662.

Rodriguez EM, Medesani DA, Fingerman M (2007). Endocrine disruption in crustaceans due to pollutants: A review. *COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY A-MOLECULAR & INTEGRATIVE PHYSIOLOGY* 146 (4): 661-671.

Rodriguez-Mozaz S, Marco MP, de Alda MJL, et al. (2004). Biosensors for environmental monitoring of endocrine disruptors: a review article. *ANALYTICAL AND BIOANALYTICAL CHEMISTRY* 378 (3): 588-598.

Rodriguez-Ortega MJ, Grøsvik BE, Rodriguez-Ariza A, Goksoyr A, Lopez-Barea J (2003). Changes in protein expression profiles in bivalve molluscs (*Chamaelea gallina*) exposed to four model environmental pollutants. *PROTEOMICS* 3 (8): 1535-1543.

Roepke TA, M.J. Snyder MJ, G.N. Cherr GN (2005). Estradiol and endocrine disrupting compounds adversely affect development of sea urchin embryos at environmentally relevant concentrations. *AQUATIC TOXICOLOGY* 71 (2): 155–173.

Roesijadi G (1992).Metallothioneins in metal regulation and toxicity in aquatic animals. *AQUATIC TOXICOLOGY* 22 (2): 81-114.

Rogers MA, Clarke P, Noble J, Munro NP, Paul A, Selby PJ, Banks RE (2003). Proteomic profiling of urinary proteins in renal cancer by surface enhanced laser desorption ionization and neural-network analysis: Identification of key issues affecting potential clinical utility. *CANCER RESEARCH* 63 (20): 6971-6983.

Ross PL, Huang YLN, Marchese JN, et al.(2004). Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *MOLECULAR & CELLULAR PROTEOMICS* 3 (12): 1154-1169.

Rotchell JM, Ostrander GK (2003). Molecular markers of endocrine disruption in aquatic organisms. *JOURNAL OF TOXICOLOGY AND ENVIRONMENTAL HEALTH-PART B-CRITICAL REVIEWS* 6 (5): 453-495.

Roux MM, Townley IK, Raisch M, et al. (2006). A functional genomic and proteomic perspective of sea urchin calcium signaling and egg activation. *DEVELOPMENTAL BIOLOGY* 300 (1):416-433.

Roux MM, Radeke MJ, Goel M, et al. (2008). 2DE identification of proteins exhibiting turnover and phosphorylation dynamics during sea urchin egg activation. *DEVELOPMENTAL BIOLOGY* 313 (2): 630-647.

Saavedra C, Bachere E, (2006). Bivalve genomics. *AQUACULTURE* 256 (1-4): 1-14.

Samanta MP, Tongprasit W, Istrail S, et al.(2006). Report - The transcriptome of the sea urchin embryo. *SCIENCE* 314 (5801): 960-962.

Samuelsson LM, Forlin L, Karlsson G, et al. (2006). Using NMR metabolomics to identify responses of an environmental estrogen in blood plasma of fish. *AQUATIC TOXICOLOGY* 78 (4): 341-349.

Sanchez JC, Guillaume E, Lescuyer P, Allard L, Carrette O, Scherl A, Burgess J, Corthals GL, Burkhard PR, Hochstrasser DF (2004). Cystatin C as a potential cerebrospinal fluid marker for the diagnosis of Creutzfeldt-Jakob disease. *PROTEOMICS* 4 (8): 2229-2233.

Sanni S, Øysæd KB, Høivangli V, Gaudebert B (1998). A continuous flow system (CPS) for chronic exposure of aquatic organisms. *MARINE ENVIRONMENTAL RESEARCH* 46 (1-5): 97-101.

Sapsford KE, Bradburne C, Detehanty JB, et al.(2008). Sensors for detecting biological agents. *MATERIALS TODAY* 11 (3): 38-49.

Satoh K, Nagai F, Aoki N (2001).Several environmental pollutants have binding affinities for both androgen receptor and estrogen receptor alpha. *JOURNAL OF HEALTH SCIENCE* 47 (5): 495-501.

Scantz SL, Widholm JJ (2001). Cognitive effects of endocrine-disrupting chemicals in animals. *ENVIRONMENTAL HEALTH PERSPECTIVES* 109 (12):1197-1206.

Schaefer J, Nierhaus KH, Lohff B, et al. (1998). Mechanisms of autoprotection and the role of stress-proteins in natural defenses, autoprotection, and salutogenesis. *MEDICAL HYPOTHESES* 51 (2): 153-163.

Schena M, Shalon D, Davis RW, et al. (1995). Quantitative monitoring of gene-expression patterns with a complementary-DNA Microarray. *SCIENCE* 270 (5235): 467-470.

Schena M, Heller RA, Theriault TP, et al. (1998). Microarrays: biotechnology's discovery platform for functional genomics. *TRENDS IN BIOTECHNOLOGY* 16 (7): 301-306.

Schlenk D (1999). Necessity of defining biomarkers for use in ecological risk assessments *MARINE POLLUTION BULLETIN* 39 (1-12): 48-53.

Schmidt A, Kellermann J, Lottspeich F (2005). A novel strategy for quantitative proteomics using isotope-coded protein labels. *PROTEOMICS* 5 (1): 4-15.

Scigelova M, Makarov A (2006). Orbitrap mass analyzer - Overview and applications in proteomics. *PROTEOMICS* Pages: 16-21 Suppl: S

Seed R (1969). Ecology of *Mytilus edulis* L. (Lamellibranchiata) on exposed rocky shores: I. Breeding and settlement, *OECOLOGIA* 3 p. 277

Segner H. (2003). Need for establishing integrated programs to monitor endocrine active compounds. *PURE AND APPLIED CHEMISTRY* 75 (11-12): 2435-2444.

SFT (Norwegian Pollution Control Authority), 1999. Miljøovervåking av petroleumsvirksomheten på norsk sektor. SFT Guidelines 99-01. SFT, Oslo, Norway. ISBN 82-7655-164-5.

Sharpe and Shakkebaek, (1993). R.M. Sharpe and N.E. Shakkebaek, Are estrogens involved in falling sperm counts and disorder of the male reproductive tract? *THE LANCET* 341: 1392–1395.

Shen RQ, Milowsky MI, Ozaki N, Navarro D, Sumitomo M, Xu Y, Nanus DM (2002). Detection of the p110 beta subunit of phosphatidylinositol 3-kinase complexed with neutral endopeptidase. *ANTICANCER RESEARCH* 22 (5): 2533-2538.

Shepard JL, Bradley BP (2000). Protein expression signatures and lysosomal stability in *Mytilus edulis* exposed to graded copper concentrations. *MARINE ENVIRONMENTAL RESEARCH* 50 (1-5): 457-463.

Shiu WY, Ma KC, Varhanickova D, Mackay D (1994). Chlorophenols and alkylphenols - a review and correlation of environmentally relevant properties and fate in an evaluative environment. *CHEMOSPHERE* 29 (6): 1155-1224.

Shrader EA, Henry TR, Greeley MS, Bradley BP (2003). Proteomics in zebrafish exposed to endocrine disrupting chemicals. *ECOTOXICOLOGY* 12 (6): 485-488.

Shriver Z, Raguram S, Sasisekharan R (2004). Glycomics: A pathway to a class of new and improved therapeutics. *NATURE REVIEWS DRUG DISCOVERY* 3 (10): 863-873.

Silvestre F, Dierick JF, Dumont V, et al. (2006). Differential protein expression profiles in anterior gills of *Eriocheir sinensis* during acclimation to cadmium. *AQUATIC TOXICOLOGY* 76 (1): 46-58.

Singer CF, Hudelist G, Galid A, et al. (2003). Pharmacological modulation of local feedback mechanisms as a therapeutic approach in breast cancer treatment. *DRUGS OF TODAY* 39 (12): 917-926.

Snape JR, Maund SJ, Pickford DB, Hutchinson TH (2004). Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *AQUATIC TOXICOLOGY* 67 (2): 143-154.

Sodergren E, Weinstock GM, Davidson EH, et al.(2006). Research article - The genome of the sea urchin *Strongylocentrotus purpuratus*. *SCIENCE* 314 (5801): 941-952.

Sonnenschein C, Soto AM (1998). An updated review of environmental estrogen and androgen mimics and antagonists. *JOURNAL OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY* 65 (1-6): 143-150.

Soto A, Kortenkamp A, Skakkebaek NE, et al. (2008). Low dose mixture effects of endocrine disrupters: implications for risk assessment and epidemiology - Panel discussion. *INTERNATIONAL JOURNAL OF ANDROLOGY* 31: 238-240.

Spelsberg and Riggs (1987). T.C. Spelsberg and B.L. Riggs, Evidence of estrogen receptors in normal human osteoblast-like cells. *SCIENCE* 241 (1987): 84–86.

Staples A, Adams WJ, Parkerton TF, et al. (1997). Aquatic toxicity of eighteen phthalate esters. *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY* 16 (5): 875-891.

Stebbing A (1982). Hormesis – The Stimulation of growth by low-levels of inhibitors. *SCIENCE OF THE TOTAL ENVIRONMENT* 22: 213+.

Stegeman H (1970). Proteinfraktionierungen in Polyacrylamid und die Anwendung afu die genetishe Analyse bei Pflanzen. *ANGEW. CHEM* 82: 640.

Stentiford GD, Viant MR, Ward DG, et al. (2005). Liver tumors in wild flatfish: A histopathological, proteomic, and metabolomic study. *OMICS-A JOURNAL OF INTEGRATIVE BIOLOGY* 9 (3): 281-299.

Stohs SJ, Bagchi D (1995). Oxidative mechanisms in the toxicity of metal-ions *FREE RADICAL BIOLOGY AND MEDICINE* 18 (2): 321-336.

Storey and Storey (2004). Metabolic rate depression in animals: transcriptional and translational controls. *BIOLOGICAL REVIEWS* 79 (1): 207-233.

Stoughton RB (2005). Applications of DNA microarrays in biology. *ANNUAL REVIEW OF BIOCHEMISTRY* 74: 53-82.

Sturve J, Hasselberg L, Falth H, et al. (2006). Effects of North Sea oil and alkylphenols on biomarker responses in juvenile Atlantic cod (*Gadus morhua*). *AQUATIC TOXICOLOGY* 78: 73-78, Suppl. 1.

Sugni M, Mozzi D, Barbaglio A, et al.Endocrine disrupting compounds and echinoderms: new ecotoxicological sentinels for the marine ecosystem. *ECOTOXICOLOGY* 16 (1): 95-108.

Sumpter JP, Jobling S (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *ENVIRONMENTAL HEALTH PERSPECTIVES* 103: 173-178, Suppl. 7.

Sumpter JP (2005). Endocrine disrupters in the aquatic environment: An overview. *ACTA HYDROCHIMICA ET HYDROBIOLOGICA* 33 (1): 9-16.

Sumpter JP, Johnson AC (2005). Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. *ENVIRONMENTAL SCIENCE & TECHNOLOGY* 39 (12):4321-4332.

Sundt RC, Baussant T (2003). Uptake, tissue distribution and elimination of C4-C7 alkylated phenols in cod. Dietary vs. waterborne exposure. Stavanger, Norway, RF-Akvamiljø / RF-Akvamiljø Report AM 2003/001 27 pp.

Sundt RC, Pampanin DM, Larsen BK, Brede C, Herzke D, Bjornstad A, Andersen OK. (2006). The BEEP Stavanger Workshop: Mesocosm exposures. *AQUATIC TOXICOLOGY* 78: 5-12.

Taban IC Bechmann RK, Torgrimsen S, Sundt R, Larsen BK, Bjornstad A, Andersen OK. (2003). DNA damage in mussels following exposure to three potential endocrine disruptors using the comet assay and alkaline unwinding. BEEP project meeting, Barcelona, Spain (4th – 6th December 2003).

Taban IC, Bechmann RK, Torgrimsen S, Baussant T, Sanni S (2004). Detection of DNA damage in mussels and sea urchins exposed to crude oil using comet assay. *MARINE ENVIRONMENTAL RESEARCH* 58 (2-5): 701-705.

Taenzler V, Bruns E, Dorgerloh M, et al. (2007). Chironomids: suitable test organisms for risk assessment investigations on the potential endocrine disrupting properties of pesticides. *ECOTOXICOLOGY* 16 (1): 221-230.

Tang N, Tornatore P, Weinberger SR (2004). Current developments in SELDI affinity technology. *MASS SPECTROMETRY REVIEWS* 23 (1): 34-44.

Tassi E, Al-Attar A, Aigner A, Swift MR, McDonnell K, Karavanov A, Wellstein A (2001). Enhancement of fibroblast growth factor (FGF) activity by an FGF-binding protein. *JOURNAL OF BIOLOGICAL CHEMISTRY* 276 (43): 40247-40253.

Tatarazako N, Oda S (2007). The water flea *Daphnia magna* (Crustacea, Cladocera) as a test species for screening and evaluation of chemicals with endocrine disrupting effects on crustaceans. *ECOTOXICOLOGY* 16 (1): 197-203.

Thayer KA, Melnick R, Burns K, et al. (2005). Fundamental flaws of hormesis for public health decisions. *ENVIRONMENTAL HEALTH PERSPECTIVES* 113 (10): 1271-1276.

Thulasiraman V, McCutchen-Maloney SL, Motin VL, Garcia E (2001). Detection and identification of virulence factors in *Yersinia pestis* using SELDI ProteinChip (R) system. *BIOTECHNIQUES* 30 (2): 428-432.

Traum AZ, Wells MP, Aivado M, et al. (2006). SELDI-TOF MS of quadruplicate urine and serum samples to evaluate changes related to storage conditions. *PROTEOMICS* 6 (5): 1676-1680.

Truhaut, R. (1977). Eco-Toxicology - Objectives, Principles and Perspectives, *ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY* 1 (2): 151-173.

Tyers M, Mann M (2003). From genomics to proteomics. *NATURE* 422 (6928): 193-197.

Tyler CR, Jobling S, Sumpter JP (1998). Endocrine disruption in wildlife: A critical review of the evidence. *CRITICAL REVIEWS IN TOXICOLOGY* 28 (4): 319-361.

Unlu M, Morgan ME, Minden JS (1997). Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. *ELECTROPHORESIS* 18 (11): 2071-2077.

Urfer W, Grzegorczyk M, Jung K (2006). Statistics for proteomics: A review of tools for analyzing experimental data. *PROTEOMICS* 48-55, Suppl: S.

Utvik TIR 1999: Chemical characterisation of produced water from four offshore oil production platforms in the North Sea. *CHEMOSPHERE* 39(15): 2593-2606.

Utvik TIR, Durell GS, Johnsen, S (1999). Determining produced water originating polycyclic aromatic hydrocarbons in North Sea waters: Comparison of sampling techniques. *MARINE POLLUTION BULLETIN* 38 (11): 977-989.

Vahter M, Akesson A, Liden C, et al. (2007). Gender differences in the disposition and toxicity of metals. *ENVIRONMENTAL RESEARCH* 104 (1): 85-95.

Van der Greef J, Stroobant P and Van der Heijden R (2004). The role of analytical sciences in medical systems biology. *CURRENT OPINION IN CHEMICAL BIOLOGY* 8: 559-565.

Van der Woude H, Alink GM, Rietjens IMCM (2005). The definition of hormesis and its implications for in vitro to in vivo extrapolation and risk assessment. *CRITICAL REVIEWS IN TOXICOLOGY* 35 (6): 603+.

Van 't Veer LJ, Bernards R (2008). Enabling personalised cancer medicine through analysis of gene-expression patterns. *NATURE* 452 (7187): 564-570.

Velculescu VE, Vogelstein B, Kinzler KW (2000). Analysing uncharted transcriptomes with SAGE. *TRENDS IN GENETICS* 16 (10): 423-425.

✓

Venter JC, Adams MD, Myers EW, et al. (2001). The sequence of the human genome. *SCIENCE* 291 (5507): 1304 – 1351.

Venter JC, Remington K, Heidelberg JF, et al. (2004). Environmental genome shotgun sequencing of the Sargasso Sea. *SCIENCE* 304 (5667): 66-74.

Verlecar XN, Jena KB, Chainy GBN (2008). Seasonal variation of oxidative biomarkers in gills and digestive gland of green-lipped mussel *Perna viridis* from Arabian Sea. *ESTUARINE COASTAL AND SHELF SCIENCE* 76: 745-752.

Verslycke T, Ghekiere A, Raimondo S, et al. (2007). Mysid crustaceans as standard models for the screening and testing of endocrine-disrupting chemicals. *ECOTOXICOLOGY* 16 (1): 205-219.

Viant MR, Rosenblum ES, Tjeerdema RS (2003). NMR-based metabolomics: A powerful approach for characterizing the effects of environmental stressors on organism health. *ENVIRONMENTAL SCIENCE & TECHNOLOGY* 37 (21): 4982-4989.

Viarengo A. (1985). Biochemical effects of trace metals. *MARINE POLLUTION BULLETIN* 16 (4): 153-158.

Viarengo A, Burlando B, Ceratto N, Panfoli I (2000). Antioxidant role of metallothioneins: A comparative overview. *CELLULAR AND MOLECULAR BIOLOGY* 46 (2): 407-417.

Vos JG, Becher G, van den Berg M, et al.(2003). Brominated flame retardants and endocrine disruption. *PURE AND APPLIED CHEMISTRY* 75 (11-12): 2039-2046.

Walker CC, Salinas KA, Harris PS, et al. (2007). A proteomic (SELDI-TOF-MS) approach to estrogen agoinst screening. *TOXICOLOGICAL SCIENCES* 95 (1): 74-81.

Ward DG, Wei W, Cheng Y, et al. (2006). Plasma proteome analysis reveals the geographical origin and liver tumor status of dab (*Limanda limanda*) from UK marine waters. *ENVIRONMENTAL SCIENCE & TECHNOLOGY* 40 (12): 4031-4036.

Weltje L, Schulte-Oehlmann U (2007). The seven year itch - progress in research on endocrine disruption in aquatic invertebrates since 1999. *ECOTOXICOLOGY* 16 (1): 1-3.

Wenk MR (2005). The emerging field of lipidomocs. *NATURE REVIEWS DRUG DISCOVERY* 4 (7): 594-610.

Werner I, Nagel R (1997). Stress proteins hsp60 and hsp70 in three species of amphipods exposed to cadmium, diazinon, dieldrin and fluoranthene. *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY* 16 (11): 2393-2403.

Werner I, Hinton DE (1999). Field validation of hsp70 stress proteins as biomarkers in Asian clam (*Potamocorbula amurensis*): is downregulation an indicator of stress? *BIOMARKERS* 4 (6) 473-484.

Westermeister R, Naven T (2002). Proteomics in practice - a laboratory manual of proteome analysis. *Wiley-VCH* Verlag-GmbH Weinheim.

Wetherill YB, Akingbemi BT, Kanno J, et al. (2007). In vitro molecular mechanisms of bisphenol A action. *REPRODUCTIVE TOXICOLOGY*. 24: 178-198.

White R, Jobling S, Hoare SA, Sumpter JP, Parker MG (1994). Environmentally persistent alkylphenolic compounds are estrogenic. *ENDOCRINOLOGY* 135 (1): 175-182.

WHO (1994). Brominated diphenyl ethers. Environ. Health Criteria 162, World Health Organization, Geneva .

Wiechert W, Schweissgut O, Takanaga H, et al. (2007). Fluxomics mass spectrometry versus quantitative imaging. *CURRENT OPINION IN PLANT BIOLOGY* 10 (3): 323-330.

Wiemer JC, Prokudin A. (2004). Bioinformatics in proteomics: application, terminology, and pitfalls. *PATHOLOGY RESEARCH AND PRACTICE* 200 (2): 173-178.

Wilkins MR, Sanchez JC, Gooley AA, et al. (1996). Progress with proteome projects: Why all proteins expressed by a genome should be identified and how to do it. *BIOTECHNOLOGY AND GENETIC ENGINEERING REVIEWS* 13: 19-50.

Xiao Z, Jiang X, Beckett ML, Wright GL (2000). Generation of a baculovirus recombinant prostate-specific membrane antigen and its use in the development of a novel protein biochip quantitative immunoassay. *PROTEIN EXPRESSION AND PURIFICATION* 19 (1): 12-21.

Xiao Z, Adam BL, Cazares LH, Clements MA, Davis JW, Schellhammer PF, Dalmasso EA, Wright GL (2001). Quantitation of serum prostate-specific membrane antigen by a novel protein biochip immunoassay discriminates benign from malignant prostate disease. *CANCER RESEARCH* 61 (16): 6029-6033.

Yokota H, Tsuruda Y, Maeda M, Oshima Y, Tadokoro H, Nakazono A, Honjo T, and Kobayashi K (2000). Effect of bisphenol A on the early life stage in Japanese medaka (*Oryzias latipes*). *ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY* 19:1925–1930.

Zachariassen KE, Aunaas T, Børseth JF, Einarson S, Nordtug T, Olsen A, Skjervo G (1991). Physiological-parameters in ecotoxicology. *COMPARATIVE*

BIOCHEMISTRY AND PHYSIOLOGY C-PHARMACOLOGY TOXICOLOGY & ENDOCRINOLOGY 100 (1-2): 77-79.

Zhang LQ, Yu WJ, He T, Yu J, Caffrey RE, Dalmasso EA, Fu SY, Pham T, Mei JF, Ho JJ, Zhang WY, Lopez P, Ho DD (2002). Contribution of human alpha-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. *SCIENCE* 298 (5595): 995-1000.

Zhao YJ, Li SQ, Davidson A, et al. (2007). A MEMS viscometric sensor for continuous glucose monitoring. *JOURNAL OF MICROMECHANICS AND MICROENGINEERING* 17: 2528-2537.

Zorita I, Ortiz-Zarragoitia M, Soto M, et al. (2006). Biomarkers in mussels from a copper site gradient (Visnes, Norway): An integrated biochemical, histochemical and histological study. *AQUATIC TOXICOLOGY* 78: 109-116. Suppl. 1.

Zouros E (2000). The exceptional mitochondrial DNA system of the mussel family Mytilidae. *GENES & GENETIC SYSTEMS* 74 (6): 313-318.

URL's:

http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/

http://ec.europa.eu/environment/endocrine/strategy/substances_en.htm#top

http://www.ourstolenfuture.org/Basics/chemlist.htm.

http://www.wwf.org.uk/filelibrary/pdf/causeforconcern01.pdf).

http://www.emc.maricopa.edu/faculty/farabee/BIOBK/BioBookENDOCR.html

http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/index.html

http://en.wikipedia.org/wiki/Biomarker

http://apps.isiknowledge.com

http://www.bio-rad.com/proteinchip/

http://www.showcaves.com/english/no/mines/Visnes.html

www.eurofins.no

http://norgeibilder.no/.

http://www.iris.no/Internet/akva.nsf/wvDocID/D9265337DF6A70E4C1256EB90069A

BC1

http://www.iris.no/Internet/student.nsf/5CD78704522281FBC12567F60051ECEE/431C

334F2973BFEAC12572570057F7DD?opendocument

http://en.wikipedia.org/wiki/List_of_omics_topics_in_biology)

http://www.medterms.com

http://www.marinegenomics.org

http://www.marine-genomics-europe.org/

http://www.marlin.ac.uk/species/Hyasaraneus.htm;

http://www.seawater.no/fauna/Leddyr/sandpyntekrabbe.htm

http://www.marlin.ac.uk/species/strongylocentrotusdroebachiensis.htm

http://www.racerocks.com/racerock/eco/taxalab/ensy02/aldoc.htm

Publication 1

ELSEVIER

Aquatic Toxicology 78S (2006) S5–S12

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

The BEEP Stavanger Workshop: Mesocosm exposures

Rolf C. Sundt[a,*], Daniela M. Pampanin[a], Bodil K. Larsen[a], Cato Brede[a], Dorte Herzke[b], Anne Bjørnstad[a], Odd Ketil Andersen[a]

[a] *IRIS – International Research Institute of Stavanger AS, Mekjarvik 12, N-4070 Randaberg, Norway*
[b] *Norwegian Institute for Air Research, Hjalmar Johansens gt 14, 9296 Tromsø, Norway*

Abstract

Within the BEEP project (Biological Effects of Environmental Pollution in Marine Ecosystems) the Work Package 1 was addressed to the development of new and more sensitive biomarkers of exposure in several sentinel organisms. Within this framework, common mesocosm exposures of organic pollutants relevant for marine ecosystems were conducted in the facilities of Akvamiljø a/s (Stavanger, Norway). In the first experiment, Atlantic cod (*Gadus morhua*), turbot (*Scophthalmus maximus*) and shore crab (*Carcinus maenas*) were exposed to nonylphenol, North Sea crude oil and a combination of crude oil and alkylated phenols. Mussels (*Mytilus edulis*) were exposed to North Sea crude oil and a combination of crude oil, alkylated phenols and PAHs. In the second experiment, Atlantic cod, turbot, mussel and spider crab (*Hyas araneus*) were exposed to the plasticizers bisphenol A and diallyl phatalate and the brominated flame retardant BDE-47. The main purpose of the present study was to provide the 30 participating Institutes with samples which had been exposed to defined contaminant concentrations in a controlled laboratory exposure for 3 weeks. This paper describes the mesocosm experimental design, the transplantation and treatment of the organisms, and the contaminant exposures.

© 2006 Elsevier B.V. All rights reserved.

Keywords: Atlantic cod; Turbot; Shore crab; Spider crab; Mussel; Crude oil; PAH; Bisphenol A; Diallyl phatalate; PBDE; Plasticizer; Biomarkers; Marine ecosystem; Mesocosm

1. Introduction

Within the EU funded BEEP project (Biological Effects of Environmental Pollution in Marine Ecosystems) the main objective of Work Package 1 was to develop new and more sensitive biomarkers of exposure in several sentinel organisms. In particular, new technologies, such as genomics and proteomics, have been applied to ecotoxicology; a special effort was devoted to the implementation of these new molecular approaches. Moreover, due to the importance of linking the biological effects of pollutants to their consequences at the population level, the activities had been developed aiming to identify biomarkers capable of reflecting the effects to toxic chemicals on the reproductive performance of the organisms. A major challenge was to improve and develop knowledge of biomarkers in marine organisms commonly utilized in monitoring programs exposed in laboratory to various classes of pollutants.

The main purpose of the present exposure study was to provide the 30 different participating institutes with samples which had been exposed to defined contaminant concentrations in a controlled laboratory exposure for 3 weeks. This paper describes the mesocosm experimental design, the transplantation and treatment of the organisms, and the contaminant exposures.

Adequate laboratory and support facilities have been necessary to perform this task. It was considered important to use a large-scale experimental facility ("mesocosm"), since exposures to known levels of contaminants under controlled conditions was an important issue. Therefore, all the biomarkers, developed in the different laboratories, were validated in comparison with well-known biomarkers (core biomarkers) in two common exposure experiments performed by IRIS (International Research Institute of Stavanger AS) researchers at Akvamiljø a/s (Stavanger, Norway). The indoor mesocosm facility consists of 400 and 600 L glass fibre tanks and exposure dosing systems. A detailed description of the continuous flow system used for oil dispersions is given by Sanni et al. (1998) and a diagram of dosing system is shown in Fig. 1.

Several species representing different phylogenies and feeding types were included. Selection was based on availability together with ecological and economical relevance. The Atlantic cod (*Gadus morhua*) has considerable ecological and

---

[*] Corresponding author. Tel.: +47 51875500; fax: +47 51875540.
E-mail address: rolf.sundt@irisresearch.no (R.C. Sundt).

0166-445X/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2006.02.012

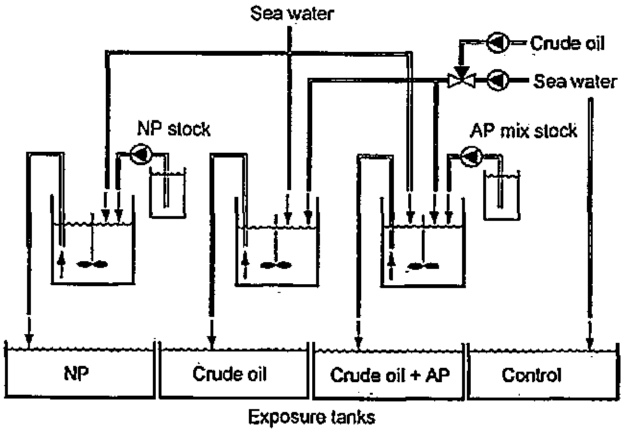


Fig. 1. Diagram of the crude oil and alkyl phenol (AP) dosing system used in the September/October 2002 experiment (NP = nonylphenol).

economical importance and can be both pelagic and associated with bottom. Turbot (*Scophthalmus maximus*) represents bottom dwelling fish that stays in close contact with the bottom sediment. Both species can be purchased from the fish farming industry. Mussel (*Mytilus edulis*) and shore crab (*Carcinus maenas*) are found mainly in the littoral zone on rocky bottom while the spider crab (*Hyas araneus*) has a deeper distribution. These three invertebrate species have wide geographical distribution and are available for catch either by scraping or by baited traps. Historically, invertebrates have been used as models for testing toxic chemicals routinely already (de Fur, 2004).

The compounds selected for the mesocosm exposures were selected on their ecological relevance. Environmental pollution related to petroleum exploitation and transport in the aquatic environment is a general problem worldwide. Accidental oil spills and vessel accidents have occurred in several countries (Pastor et al., 2001; Le Hir and Hily, 2002; Alvarez-Salgado et al., in press; Garcia de Oteyza and Grimalt, in press; Papadimitrakis et al., 2006). Therefore, crude oil from a North Sea field (Statfjord B) was used; content of polycyclic aromatic hydrocarbons (PAHs) represented about 1.5% of the total weight (Table 1).

A mixture of alkyl phenols (APs) and PAHs was designed to simulate the exposure of marine organisms to produced water from offshore oil installations. Produced water, a by-product from oil production, is a highly complex mixture of water and trace amount of oil. PAHs and APs are some of the compounds present and aromatic compounds give the most important contribution to toxicity (Utvik, 1999). Various effects due to oil and produced water exposures have been reported in marine organisms, including effects on reproduction (Krause, 1994; Giesy et al., 2000; Aarab et al., 2004), development (Baldwin et al., 1992), genotoxicity (Harvey et al., 1999; Aas et al., 2000; Taban et al., 2004), metabolism (Narvia and Rantamaki, 1997; Jonsson

Table 1
PAH measurements of the crude oil and water in the mussel exposure November–December 2002

Day of exposure: Exposure dose: Compound	 Crud oil mg/kg	7 Control µg/kg	7 1 ppm µg/kg	7 1 ppm + spike µg/kg	7 FW 1 ppm µg/kg	24 Control µg/kg	24 1 ppm µg/kg	24 1 ppm + spike µg/kg
Naphthalene	1147	0.009	1.17	25.6	1.12	0.006	0.795	19.9
C1-naphthalene	3787	0.017	3.57	57.0	3.41	0.009	2.56	46.3
C2-naphthalene	5289	0.014	4.11	51.2	3.62	0.011	3.22	45.3
C3-naphthalene	3830	0.005	2.47	16.4	1.95	0.007	2.03	15.2
Acenaphthylene	10	<0.005	0.005	<0.005	<0.005	<0.005	0.005	<0.005
Acenaphthene	10	<0.005	<0.005	0.010	0.007	<0.005	<0.005	0.005
Fluorene	136	<0.005	0.088	2.572	0.088	<0.005	0.067	2.29
Phenanthrene	253	<0.005	0.179	2.075	0.155	<0.005	0.135	1.96
C1-phen/anthr	460	<0.005	0.292	2.579	0.253	<0.005	0.261	2.60
C2-phen/anthr	439	<0.005	0.291	0.940	0.149	<0.005	0.270	0.97
Dibenzothiophene	92	<0.005	0.056	0.555	0.064	<0.005	0.049	0.54
C1-dibenzothiophene	196	<0.005	0.152	0.893	0.116	<0.005	0.138	0.92
C2-dibenzothiophene	233	<0.005	0.183	0.609	0.074	<0.005	0.164	0.64
Fluoranthene	3	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Pyrene	9	<0.005	0.007	0.007	<0.005	<0.005	0.007	0.007
Benzo(*a*)anthracene	3	<0.005	<0.005	<0.005	<0.005	<0.005	0.005	<0.005
Chrysene	24	<0.005	0.016	0.017	0.005	<0.005	0.016	0.017
C1-chrysene	38	<0.005	0.030	0.031	<0.005	<0.005	0.028	0.030
C2-chrysene	41	<0.005	0.037	0.039	<0.005	<0.005	0.034	0.036
Benzo(*b*)fluoranthene	8	<0.005	<0.005	0.005	<0.005	<0.005	0.006	<0.005
Benzo(*k*)fluoranthene	0	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Benzo(*b* + *k*)fluoranthene	7	<0.005	<0.005	<0.005	<0.005	<0.005	0.008	<0.005
Benzo(*a*)pyrene	5	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Indeno(1,2,3-cd)pyrene	0	<0.005	<0.005	<0.005	<0.005	<0.005	0.005	<0.005
Benzo(*g*,*h*,*i*)perylene	2	<0.005	<0.005	<0.005	<0.005	<0.005	0.006	<0.005
Dibenzo(*a*,*h*)anthracene	0	<0.005	<0.005	<0.005	<0.005	<0.005	0.005	<0.005

Samples were collected in header tanks that were further diluted 1:1 before entering the exposure tanks. Quantification limit 0.005 µg/kg for single components. FW: filtered water.

et al., 2004) and alterations in protein expression (Gimeno et al., 1998; Hasselberg et al., 2004; Bjørnstad et al., 2006).

Toxicity of APs to different species has been reported in literature for marine organisms, such as fish (Hall and Kier, 1984; Lipnick et al., 1985) and shrimp (McLeese et al., 1979, 1981). In the first mesocosm exposure, 4-nonylphenol (NP) was used as a positive control for endocrine disruption effects. NP is used in the production of nonylphenol ethoxylates (NPEs), NP phosphates and aminocarbar insecticides (Maguire, 1999). NPEs are a large group of non-ionic surfactants employed in lubricating oils, plastics, household and industrial detergents, paper and textile industries; NP phosphates are commonly used as stabilizer and antioxidant agents in both rubber and plastic industries (Lee, 1999). In the aquatic environment, NPEs are biodegraded to deethoxylated intermediates, of which NP is the final product. NP has a low solubility in water, therefore it is more persistent than NPEs (Heinis et al., 1999). Moreover it is highly lipophilic and may consequently be accumulated by aquatic organisms (Ekelund et al., 1990). NP is known to alter the hormonal system of several organisms. Previous studies demonstrated that NP can mimic the action of endogenous estrogens by binding estrogen receptors in fish (Sonnenschein and Soto, 1998; Ying et al., 2002).

In general, estrogens and estrogen mimics that enter the environment are known to present a serious threat to the development and reproduction of vertebrates by disrupting their normal endocrine function. Some compounds are under consideration for their potential effects on the endocrine system, accurate evaluations are suggested to identify these negative effects in key marine organisms.

Bisphenol A (BPA; 4,4-isopropylidene diphenol) is a chemical intermediate used primarily in the production of epoxy resins and polycarbonate products. BPA is a monomer component used in numerous consumer products, including food-contact plastics. BPA has been identified in surface waters and, hence, has been the subject of considerable research into its potential effects on aquatic organisms. Negative effects on reproduction have been demonstrated in various organisms (Yokota et al., 2000; Kang et al., 2002; Pait and Nelson, 2003; Honkanen et al., 2004; Roepke et al., 2005).

The plasticizer diallyl phtalate (DAP) have been suggested to function as xenoestrogen as well (Harris et al., 1997). The phthalates represent a class of chemicals used widely and diversely in industry in the production of polyvinyl chloride to make it flexible and workable and, to a lesser degree, in paints, lacquers and cosmetics (Harris et al., 1997). They are released into the environment during manufacturing processes and during the life time of the manufactured products as well as through wastewater discharge and have been detected in sediment, water and air (Fatoki and Vernon, 1990).

Polybrominated diphenyl ethers (PBDEs) are ubiquitous chemicals with different bromination degree characterised by a substantial industrial use; they are used as flame retardant in plastics and in textile coating (WHO, 1994). Commercial production consists predominantly of decabromodiphenyl ether, after the phase out of the production of penta- and octamixture in 2004. The annual market demand in 2003 has been estimated as 56,000 tonnes (Thomsen, 2002). PBDEs are accumulated and biomagnified in the environment and comparatively high levels are found often in marine biota (de Wit, 2002). Leakage of the compound from dump sites into to the environment has been demonstrated (Öberg et al., 2002). They are lipophilic compounds, which are easy removed from the aqueous environment and are predicted to be absorbed onto sediments and particulate matter or to fatty tissues, aiding their distribution throughout the environment up the food chain (Vos et al., 2003). Their presence in biota is reported since the 1980s (Christensen et al., 2002), PBDEs have been measured in more than 50 species at different trophic levels in Europe, Asia, North America and the Arctic environment. PBDEs are structurally comparable to PCBs and DDT and, therefore, their chemical properties, persistence and distribution in the environment follow similar patterns (Gustafsson et al., 1999; Helleday et al., 1999). They are a growing problem in the environment and concern over their fate and effects is warranted (Rahman et al., 2001; Damerud, 2003). In particular, effects by disrupting the endocrine system is suggested (Legler and Brouwer, 2003; Vos et al., 2003). We selected BDE-47 for this study based on presence of the component in biota and deleterious effects after exposure to this compound reported by Eriksson et al. (2001).

2. Exposure system

The first experiment was carried out over to 3 weeks periods from October to December 2002. The exposure was performed using a continuous flow system (CFS), designed for performing studies of chronic exposures of marine organisms to mixtures of poorly water soluble substances. Oil dispersions were made mechanically by passing oil and seawater through a high pressure mixing valve. The compositions of the AP and PAH mixtures used in the spike, as well as the composition of PAHs in the crude oil are listed in Tables 1–3.

The spike was made by mixing equal amounts of the two solutions. Acetone (grade >99.5%) was used as carrier to enhance the dissolution of compounds in water. High precision peristaltic pumps were used to ensure the correct doses in the exposure tanks. The spike was added to the oil emulsion via a 5 L flask

Table 2
Composition of PAH mix stock used to spike oil

Compound	g/L
Naphthalene	1.863
C1-Naphthalenes	3.360
C2-Naphthalenes	3.513
C3-Naphthalenes	1.868
Fluorene	0.211
Phenanthrene	0.190
C1-Phenanthrenes	0.308
C2-Phenanthrenes	0.265
Dibenzothiophene	0.041
C1-Dibenzothiophene	0.088
C2-Dibenzothiophene	0.084
Σ PAHs	11.708

Table 3

Composition of alkyl phenols (APs) in mix and amounts of APs in water from header tank (average of three GC/MS measurements in the September–October 2002 experiment)

AP compound	g/L in mix	No. of C	AP in water (ppb)
P-cresol	33.800	C_1	89.567 ± 14.01
M-ethylphenol	6.540	C_2	36.167 ± 5.050
3,5-Dimethylphenol	6.540		
2,4,6-Trimethylphenol	3.800	C_3	16.400 ± 1.819
2-tert-butylphenol	0.236	C_4	0.120 ± 0.069
3-tert-butylphenol	0.236		
4-Butylphenol	0.236		
4-Pentylphenol	1.620	C_5	–
ΣC_{1-5} APs		C_{1-5}	142.205 ± 20.774
Nonylphenol		C_9	43.923 ± 25.818

with spin-bar mixing. Sea water for the experiment was taken from 80 m depth (below the thermocline) and filtered through a sand filter, salinity was 34‰ and temperature 11 ± 1 °C.

3. Transplantation and maintenance of organisms

Juvenile Atlantic cod and turbot were purchased from Grieg Marine Farms A/S, Nedstrand and Stolt Sea Farm, Øye, respectively. Both species were fed with dry palletized fish fodder (Dana feed marine, 14% fat) daily the first 2 weeks of exposure. To secure sufficient amount of bile, fishes were starved during the last week before the sampling.

Adult specimens of shore crab and spider crab were collected by baited traps from a clean site at Karmøy Island, western Norway. Crabs were fed throughout the entire experiment with raw shrimps (*Pandalus borealis*) collected from a clean site. Mussels (7 ± 1 cm) were collected below the low water mark in a clean site in Førlandsfjorden (western Norway). Mussels were fed once daily with a dense solution of *Isocrysis galbana* (2 L of algae solution per tank). All organisms were held 2 weeks in quarantine at the experiment facility prior each exposure experiment.

4. First mesocosm exposure: oil, AP and NP

During the period September–October 2002, cod, turbot, shore crab and mussel were exposed for 3 weeks to: (a) 0.5 ppm of North Sea crude oil (Statfjord), (b) 0.5 ppm of North Sea crude oil spiked with 0.1 ppm AP mix, (c) 30 ppb NP (nominal concentrations) and a control (Table 4).

Due to uncertainty around the pathogenic condition of the mussels from this first experiment, an additional experiment was conducted with this species in November–December 2002. Mussels were then exposed for 3 weeks to: (a) 0.5 ppm of North Sea crude oil, (b) 0.5 ppm of North Sea crude oil spiked with 0.1 ppm AP mix and 0.1 ppm PAH mix (nominal concentrations) and (c) a control. The crude oil contains approximately 1.5% PAHs of weight and distribution of compounds is given in Table 1.

The spike was prepared by mixing equal amounts of the AP and PAH solutions. The final acetone stock contained 32.36 g hydrocarbons per litre and this spike mixture was dosed at 0.2 mg total hydrocarbons/litre seawater. Nominal concentrations of spikes were 0.0915 μg/L for total PAH mix and 0.1 μg/L for total AP mix.

The AP mix was manufactured by CHIRON a/s and the amounts of the different components are given in Table 3. There was no lethality of organisms during the experiment.

4.1. Exposure monitoring

In the September–October 2002 experiment the size distribution and quantity of oil droplets was measured by a Coulter Multisizer® equipped with a 70 mm aperture tube. Samples were collected from the inflow in order to avoid particles other than oil in the seawater (algae, feces, micro-organisms) and 2 × 500 μL water was analysed. Data is shown in Table 5. The method was

Table 4

Nominal concentrations of contaminants (ppm) and methods employed for exposure monitoring

Species and exposure period	NP	Crude oil	Crude oil/APs	Crude oil/PAHs/APs	Bisphenol A	BDE-47	DAP
Atlantic cod September/October 2002	0.03[a]	0.5[b,c]	0.5/0.1[a]	–	–	–	–
Turbot September/October 2002	0.03[a]	0.5[b,c]	0.5/0.1[a]	–	–	–	–
Shore crab September/October 2002	0.03[a]	0.5[b]	0.5/0.1[a]	–	–	–	–
Mussel September/October 2002	–	0.5[b]	0.5/0.1[a]	–	–	–	–
Mussel November/December 2002	–	0.5[d]	–	0.5/0.1/0.1[d]	–	–	–
Atlantic cod March–April 2003	–	–	–	–	0.05[e]	0.005[f,g]	0.05[h]
Turbot March–April 2003	–	–	–	–	0.05[e]	0.005[f,g]	0.05[b]
Spider crab March–April 2003	–	–	–	–	0.05[e]	0.005[f]	0.05[h]
Mussel March–April 2003	–	–	–	–	0.05[e]	0.005[f,g]	0.05[h]

[a] APs in water from header tank by GCMS.

[b] Oil droplets in water by multi sizer.

[c] PAH metabolites in bile by GCMS.

[d] PAH in water from header tank by GCMS.

[e] Bisphenol A in tank water by GCMS.

[f] PBDE in tank water by GCMS.

[g] PBDE in tissue by GCMS.

[h] DAP in tank water by GCMS.

Table 5
Average estimates of oil concentration in water from the September–October 2002 experiment, based on 15 multi seizer measurements on water from inflow

Species	Crude oil (ppm)	Crude oil and APs (ppm)
Atlantic cod and turbot	0.515 ± 0.218	0.484 ± 0.114
Shore crab and mussel	0.560 ± 0.196	0.609 ± 0.262
All tanks combined	0.535 ± 0.201	0.546 ± 0.194

Table 7
Exposure conditions for the exposure in March–April 2003

Compound	Nominal concentrations (μg/L)	Mean measured concentrations (μg/L)	Number of measurements
Bisphenol A (Diphenylopropane)	50	59.4 ± 10.659	7
BDE-47 (2,2′,4,4′ tetra bromo diphenyl ether)	5	0.23 ± 0.189	8
Diallyl phthalate	50	38.3 ± 9.693	6

Water samples were measured by GC/MS.

validated with fluorescence measurements. In order to confirm uptake of PAHs in fish, PAH metabolites in bile were measured with gas chromatography/mass spectrometry (GC/MS). Method for extraction and analysis is described by Jonsson et al. (2004) and data are shown in Table 6.

Water samples for determination of APs were collected 7th, 10th and 14th of October. In order to avoid analytical interference with the PAHs, samples were taken from the header tanks. APs were derivatized in the water phase under basic conditions (pH > 8) to methyl phenyl carbonates, followed by extraction with cyclohexane (Grahl-Nielsen and Landgren-Skjellerudsven, 1982). Qualitative and quantitative determinations of alkylated phenols in the extracted samples were done by GC/MS in the selected ion monitoring (SIM) mode. Data are shown in Table 3.

In the repeated mussel experiment (November–December 2002) concentrations of PAHs in water were quantified by GC/MS. Water samples were extracted prior to analysis by liquid/liquid extraction with cyclohexane. The 16 PAH compounds which are included in the US Environmental Protection Agency (EPA) list of priority pollutants were analysed including benzo($b+k$)fluoanthene and the alkylated congeners of naphthalene (C_1–C_3), phenanthrene/anthracene (C_1–C_2) and chrysene (C_1–C_2). In addition, both the parental and C_1–C_2 congeners of dibenzothiophene were determined. In order to get a measure of the amount of PAHs that had left the oil droplets into the water, a sample of filtered water was analysed. Oil droplets were collected on a 0.45 μm Whatman GF/F 55 mm diameter filter from a 2 L oil emulsion sample.

The C_{1-5} APs, C_1 phenols (*p*-cresol), C_2 phenols (*m*-ethylphenol and 3,5-dimethylphenol), C_3 phenols (2,4,6-trimethylphenol), C_4 phenols (2-(1,1-dimethyl)ethylphenol, 3-(1,1-dimethyl)ethylphenol, 4-butylphenol), C_5 phenols (pentylphenol), and C_9 (nonylphenol) in water were measured by GC/MS. Methode is described by Grahl-Nielsen and Landgren-Skjellerudsven (1982).

5. Second mesocosm exposure: BPA, DAP and BDE-47

During the period March–April 2003, cod, turbot, spider crab and mussel were exposed for 3 weeks to: (a) 50 ppb of BPA (Merck, EC No. 201-245-8, purity >97%), (b) 50 ppb of DAP (Fluka EC No. 2050163, purity >98%) and (c) 5 ppb of BDE-47 (Chiron Product no 1688.12, purity >95.6%) nominal concentrations. Exposure concentrations were selected based on previously reported LC_{50} values that were divided with a factor 100. Acetone (grade >99.5) was used as carrier for all components; the concentration in the exposure units was kept lower than 2 ppb (PNEC 25 ppb). No lethality of organisms was recorded during the experiment.

5.1. Exposure monitoring

Water samples for analysis of BPA and DAP were collected from the tanks and preserved with HCl. Compounds were extracted by solid phase extraction onto Strata-X adsorbent (Phenomenex, USA) and eluted with acetone. Analysis of samples were performed by a gas chromatograph (GC; 5890, Hewlett-Packard, USA) connected to a mass spectrometer (MS; 5972, Hewlett-Packard). The GC was equipped with a 30 m Zebron ZB-5 capillary column with 0.25 mm i.d. and 0.25 μm film thickness (Phenomenex). Average water concentrations of BPA and DAP are shown in Table 7.

Water samples for BDE-47 analysis were collected from the tanks, preserved with HCl and stored at 2–4 °C prior analyses.

Table 6
PAH metabolites (mg/L, measured by GCMS) in fish bile from the September/October 2002 experiment

Compound	Cod control $n=3$	Cod 0.5 ppm oil $n=6$	Turbot control $n=2$	Turbot 0.5 ppm oil $n=6$
1-OH-naphthalene	nd	0.091 ± 0.068	nd	0.222 ± 0.045
2-OH-naphthalene	0.021 ± 0.036	0.174 ± 0.142	0.008 ± 0.006	0.068 ± 0.013
C_1-OH-naphthalene	0.975 ± 0.331	4.145 ± 1.781	0.594 ± 0.101	7.306 ± 1.332
C_2-OH-naphthalene	0.389 ± 0.150	16.076 ± 7.508	0.368 ± 0.024	52.875 ± 15.648
C_3-OH-naphthalene	0.762 ± 0.213	17.264 ± 4.097	0.926 ± 0.404	27.993 ± 7.767
1-OH-phenanthrene	nd	0.532 ± 0.073	nd	1.090 ± 0.224
C_1-OH-phenanthrene	0.362 ± 0.113	11.639 ± 1.799	0.174 ± 0.022	11.145 ± 3.029
C_2-OH-phenanthrene	0.408 ± 0.163	6.270 ± 0.895	0.156 ± 0.050	6.236 ± 1.052
1-OH-pyrene	0.170 ± 0.026	0.952 ± 0.078	0.120 ± 0.023	1.239 ± 0.302

Quantification limit 0.005 μg/L for single components. nd, not detected.

Two hundred milliliter were first extracted with 50 mL hexane and second with 50 mL toluene. Both extracts were combined, dried over Na_2SO_4 and volume reduced to 50 μL.

In order to confirm uptake of BDE-47 to the organisms, liver from cod and turbot as well as whole soft tissue of mussel were analysed. The biota samples were collected and stored at −20 °C in glass containers. Prior to analysis samples were extracted and prepared as described previously (Herzke et al., 2002). Briefly, tissue samples were homogenized, subsequently dried in a 10-fold amount of dry sodium sulfate, and extracted. The amount of extractable organic material was determined gravimetrically. Lipid removal was performed on a gel permeation chromatography (GPC) system. An additional fractionation was carried out on a florisil column. A recovery standard (octachloronaphthalene, 10 μL of a 1 ng/μL solution in isooctane) was added prior to quantification.

For quantification, crystalline reference material was obtained from Cambridge Isotope Laboratories (Woburn, MA, USA). Solvents of pesticide grade were employed (E. Merck, Darmstadt, Germany). ^{13}C-isotope labeled BDE-77 purchased from Cambridge Isotope Laboratories (Woburn, MA, USA) was used as internal standard. Analysis was performed by a 8560 Mega gas chromatograph (CE Instruments, Milan, Italy) was equipped with a 30 m DB5-MS column (0.25 mm i.d. and 0.25 μm film thickness; J&W, Folsom, USA), a guard column (0.53 mm i.d., 2.5 m length deactivated, J&W) and a restriction capillary (0.18 mm i.d., 1.5 m length deactivated, J&W). Helium (6.0 quality, Hydrogas, Porsgrunn, Norway) was used as carrier gas at a flow rate of 1 mL/min. Two microliter of the sample extract were injected on-column with an AS800 automatic injection system, (CE Instruments). The following temperature program was used: 70 °C (2 min), then 15 °C/min to 180 °C and 5 °C/min to 280 °C (10 min isothermal). Quantification was carried out by low resolution mass spectrometry (LRMS) using a MD 800 mass spectrometer (Finnigan, San Jose, CA, USA) with an ionisation energy of 70 eV. The transfer line temperature was held at 280 °C and the source temperature was set to 220 °C. Quantification of metabolites was performed using a Varian 1200 mass spectrometer. The MS was operated in the EI mode. Samples were analysed for a set of 9 PBDEs used for standard environmental monitoring (28, 47, 71, 77, 99, 100, 138, 153 and 154) in order to detect possible debromination products and impurities of the used standard. The samples were screened for possible neutral and phenolic metabolisation products of BDE-47 as well by using the method described by Berger et al. (2004).

Table 8
Measured concentrations of BDE-47 in tissue by GC/MS, lipid contents in tissue and lipid normalized values

Species (tissue)	Number of measurements	Tissue (μg/g)	Lipid (%)	μg/g Tissue lipid normalized
Cod (liver)	2	49.3 ± 2.7	63.1	81.8
Turbot (liver)	2	15.1 ± 0.7	7.4	209.0
Mussel (soft tissues)	1 pool of 2	2.9	1.3	225.1

Lipid content was analyzed according to Folch et al. (1957).

Lipid analyses were done according to Folch et al. (1957). Measured concentrations in water and tissue are shown in Tables 7 and 8, respectively.

6. Discussion and conclusions

In large-scale exposure experiments, attainment of acceptable accordance between nominal and measured concentration of the contaminants is a recurring challenge. Due to several chemical, physical and biological factors, concentrations in the water may differ from the nominal concentration. Uneven partitioning in the exposure unit, e.g. coating on surfaces and particulate matter is expected to be the major cause. In the present study we conclude that real exposure concentrations are within acceptable limits to cover the purpose.

When data from all tanks are combined multi sizer based oil concentration data from the 0.5 ppm crude oil groups from the September–October 2002 experiment is 0.535 ± 0.201 ppm for the oil groups and 0.546 ± 0.194 ppm for the oil/AP groups. Measurements from the first mesocosm exposure show that most of the smaller PAHs, especially the naphthalene's leave the oil droplets for the water. Whilst the most alkylated phenanthrenes and dibenzothiophenes still partly remain in the oil droplets. Bioavailability of PAHs is confirmed by levels of metabolites present in bile from cod and turbot. Different levels of PAH metabolites (highest concentrations in turbot) and different ratio between 1-OH-naphtalene and 2-OH-naphtalene between the two species were observed. This indicates that the two fish species have different uptake and capacity to metabolise the compounds. Presence of smaller amounts of light PAHs in control tanks indicate some airborne carryover of volatile compounds from tanks receiving oil and PAH mix. GC/MS measurements adjusted for dilution showed that the average C_{1-5} AP concentration was 0.142 ± 0.021 ppm (142% of nominal concentration). Average measured concentration of NP was 146% of the nominal input.

In the second mesocosm exposure average measured concentration of BPA and DAP was 119 and 77%, respectively. Measured BDE-47 concentration in water was in average only 4.6% of nominal concentration, with increasing tendency throughout the experiment, because of remobilisation events. Despite the low concentration considerable amounts of the compound have been taken up by the organisms (Table 8). The high concentrations measured in both cod and turbot as well as the low nominal concentration in the water could be explained by the fact that the pure BDE-47 was likely adsorbed to particles, e.g. food reminders and faeces, or the container walls, and by this very easy accessible for the animals leading to direct intake of the substance. Analysis revealed the tribrominated BDE-28 in water and biota as well. The concentration in water was 11.7% of the BDE-47 (19.9 ± 8.4 pg/mL). It is not considered likely that the presence of BDE-28 in biota is a result of metabolic modification of BDE-47, but rather due to incomplete purification of the congener in the synthesis process and relative higher concentrations (compared with the purity of 95% of the used BDE-47) in the water are due to higher water solubility of BDE-28. Possible contribution to effects is not clarified, but based on relative amounts and expected toxic potential (Tittlemier et al., 2002) significant toxic contribution from BDE-28 is expected to be low. Hydroxylated metabolisation products could not be detected in water or in biological samples.

Acknowledgements

This research was funded by Norwegian Research Council project no. 133724/420 and European Commission contract: BEEP (EVK3-2000-00543). The authors would like to thank Kjell Birger Øysæd, Atle Nævdal, Grete Jonsson for help with chemical analysis and colleagues from IRIS - International Research Institute of Stavanger AS for their support through the BEEP project. Additional funding have been provided by Akvamiljø a/s, through the "Biomarker Validations" project (project no. P695008). The authors also thank Statoil for providing crude oil.

References

Aarab, N., Minier, C., Lemaire, S., Unruh, E., Hansen, P.D., Larsen, B.K., Andersen, O.K., Narbonne, J.F., 2004. Biochemical and histological responses in mussel (*Mytilus edulis*) exposed to North Sea oil and to a mixture of North Sea oil and alkylphenols. Mar. Environ. Res. 58 (2–5), 437–441.

Aas, A., Baussant, T., Balk, L., Liewenborg, B., Andersen, O.K., 2000. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. Aquat. Toxicol. 51 (2), 241–258.

Alvarez-Salgado, X.A., Herrera, J.L., Gago, J., Otero, P., Soriano, J.A., Pola, C.G., García-Soto, C., in press. Influence of the oceanographic conditions during spring 2003 on the transport of the Prestige tanker fuel oil to the Galician coast. Mar. Poll. Bull., corrected proof available online from October, 2005.

Baldwin, J.D., Pillai, M.C., Cherr, G.N., 1992. Response of embryos of the Sea-Urchin Strongylocentrotus-Purpuratus to aqueous petroleum waste includes the expression of a high-molecular-weight glycoprotein. Mar. Biol. 114 (1), 21–30.

Berger, U., Herzke, D., Sandanger, T.M., 2004. Two trace analytical methods for determination of hydroxylated PCBs and other halogenated phenolic compounds in eggs from Norwegian birds of prey. Anal. Chem. 76 (2), 441–452.

Bjørnstad, A., Larsen, B.K., Skadsheim, A., Jones, M.B., Andersen, O.K., 2006. The potential of ecotoxicoproteomics in environmental monitoring: Biomarker profiling in mussel plasma using proteinchip array technology. J. Toxicol. Env. Health A 69 (1), 77–96.

Christensen, J.H., Glasius, M., Pécsel, M., Platz, J., Pritzl, G., 2002. Polybrominated diphenyl ethers (PBDEs) in marine fish and blue mussels from southern Greenland. Chemosphere 47, 631–638.

Darnerud, P.O., 2003. Toxic effects of brominated flame retardants in man and wildlife. Environ. Int. 29, 841–853.

de Fur, P.L., 2004. Use and role of invertebrate models in endocrine disruptor research and testing. ILAR J. 45 (4), 484–493.

de Wit, C.A., 2002. An overview of brominated flame retardants in the environment. Chemosphere 46, 583–624.

Ekelund, R., Bergman, Å., Granmo, Å., Berggren, M., 1990. Bioaccumulation of 4-nonylphenol in marine animals—A re-evaluation. Environ. Poll. 64 (2), 107–120.

Eriksson, P., Jakobsson, E., Fredriksson, A., 2001. A novel class of developmental neurotoxicants in our environment? Environ. Health Perspect 109, 903–908.

Fatoki, O.S., Vernon, F., 1990. Phthalate esters in river of the Greater Manchester area, UK. Sci. Total Environ. 95, 227–232.

Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipid from animal tissue. J. Biol. Chem. 226, 497–509.

Garcia de Oteyza, T., Grimalt, J.O. GC and GC–MS characterization of crude oil transformation in sediments and microbial mat samples after the 1991 oil spill in the Saudi Arabian Gulf coast. Environ. Poll., in press.

Giesy, J.P., Pierens, S.L., Snyder, E.M., Miles Richardson, S., Kramer, V.J., Snyder, S.A., Nichols, K.M., Villeneuve, D.A., 2000. Effects of 4-nonylphenol on fecundity and biomarkers of estrogenicity in fathead minnows (*Pimephales promelas*). Environ. Toxicol. Chem. 19 (5), 1368–1377.

Gimeno, S., Komen, H., Jobling, S., Sumpter, J., Bowmer, T., 1998. Demasculinisation of sexually mature male common carp, Cyprinus carpio, exposed to 4-tert-pentylphenol during spermatogenesis. Aquat. Toxicol. 43 (2–3), 93–109.

Grahl-Nielsen, O., Landgren-Skjellerudsven, B., 1982. A New Method for Analysis of Phenols in Water. Report of the Workshop of the Working Party 8. Commision of the European Communities. Dübendorf, Schwitzerland, pp. 64–65.

Gustafsson, K., Bjork, M., Burreau, S., Gilek, M., 1999. Bioaccumulation kinetics of brominated flame retardants (polybrominated diphenyl ethers) in blue mussels (*Mytilus edulis*). Environ. Toxicol. Chem. 18, 1218–1224.

Hall, L.H., Kier, L.B., 1984. Molecular connectivity of phenols and their toxicity to fish. Bull. Environ. Contam. Toxicol. 32, 354–362.

Harris, C.A., Henttu, P., Paker, M.G., Sumpter, J.P., 1997. The estrogenic activity of phthalate esters in vitro. Environ. Health Perspectives 105, 802–810.

Harvey, J.S., Lyons, B.P., Page, T.S., Stewart, C., Parry, J.M., 1999. An assessment of the genotoxic impact of the Sea Empress oil spill by the measurement of DNA adduct levels in selected invertebrate and vertebrate species. Mut. Res. Genetic Toxicol. Environ. Mut. 441 (1), 103–114.

Hasselberg, L., Meier, S., Svardal, A., Hegelun, T., Celander, M.C., 2004. Effects of alkylphenols on CYP1A and CYP3A expression in first spawning Atlantic cod (*Gadus morhua*). Aquat. Toxicol. 67 (4), 303–313.

Heinis, L.J., Knuth, M.L., Liber, K., Sheedy, B.R., Tunell, R.L., Ankley, G.T., 1999. Persistence and distribution of 4-nonylphenol following repeated application to littoral enclosures. Environ. Toxicol. Chem. 18, 363–375.

Helleday, T., Tuominen, K.L., Bergman, Å., Jenssen, D., 1999. Brominated flame retardants induce intragenic recombination in mammal cells. Mutat. Res. 439, 137–147.

Herzke, D., Kallenborn, R., Nygård, T., 2002. Organochlorines in egg samples from Norwegian birds of prey: Congener-, isomer- and enantiomer specific considerations. Sci. Total Environ. 291 (1–3), 59–71.

Honkanen, J.O., Holopainen, I.J., Kukkonen, J.V.K., 2004. Bisphenol A induces yolk-sac oedema and other adverse effects in landlocked salmon (Salmo salar m. sebago) yolk-sac fry. Chemosphere 55 (2), 187–196.

Kang, I.J., Yokota, H., Oshima, Y., Tsuruda, Y., Imada, N., Tadokoro, H., Honjo, T., 2002. Effect of bisphenol A on the reproduction of Japanese medaka (*Oryzias latipes*). Environ. Toxicol. Chem. 21, 2394–2400.

Jonsson, G., Bechmann, R.K., Bamber, S.D., Baussant, T., 2004. Bioconcentration, biotransformation, and elimination of polycyclic aromatic hydrocarbons in sheepshead minnows (*Cyprinodon variegatus*) exposed to contaminated seawater. Environ. Toxicol. Chem. 23, 1538–1548.

Krause, P.R., 1994. Effects of an oil production effluent on gametogenesis and gamete performance in the purple sea urchin (*Strongylocentrotus purpuratus stimpson*). Environ. Toxicol. Chem. 13 (7), 1153–1161.

Le Hir, M., Hily, C., 2002. First observations in a high rocky-shore community after the Erika oil spill (December 1999, Brittany, France). Mar. Poll. Bull. 44 (11), 1243–1252.

Lee, H.B., 1999. Review of analytical methods for the determination on nonylphenol and related compounds in environmental samples. Water Qual. Res. J. Canada 34, 3–35.

Legler, J., Brouwer, A., 2003. Are brominated flame retardants endocrine disruptors? Environ. Int. 29, 879–885.

Lipnick, R.L., Bickings, C.K., Johnson, D.E., Eastmond, D.A., 1985. In: Bahner, R.C., Hansen, D.J. (Eds.), Comparison of QSAR Predictions with Fish to Toxicity Screening Data for 110 Phenols, Aquatic Toxicology and Hazard Assessment: Eight Symposium, ASTM STP 891. American Society for Testing and Material, Philadelphia, pp. 153–176.

Maguire, R.J., 1999. Review of the persistence of nonylphenol and nonylphenol ethoxylates in aquatic environments. Water Qual. Res. J. Canada 34, 37–78.

McLeese, D.W., Zitko, V., Peterson, M.R., 1979. Structure-lethality relationships for phenols, anilines and other aromatic compounds in shrimp and clams. Chemosphere 2, 53–57.

McLeese, D.W., Zitko, V., Sergeant, D.B., Burridge, L., Mtcalfe, C.D., 1981. Lethality and accumulation of alkylphenols in aquatic fauna. Chemosphere 10 (7), 723–730.

Narvia, M., Rantamaki, P., 1997. Aminotransferases in the bivalve mollusc *Mytilus edulis* L. and short term effects of crude oil in brackish water. Biomarkers 2 (4), 253–258.

Öberg, K., Warman, K., Öberg, T., 2002. Distribution and levels of brominated flame retardants. Chemosphere 48, 805–809.

Pait, A.S., Nelson, J.O., 2003. Vitellogenesis in male Fundulus heteroclitus (killifish) induced by selected estrogenic compounds. Aquat. Toxicol. 64 (3), 331–342.

Papadimitrakis, J., Psaltaki, M., Christolis, M., Markatos, N.C., 2006. Simulating the fate of an oil spill near coastal zones: the case of a spill (from a power plant) at the Greek Island of Lesvos. Environ. Modell. Software 21 (2), 170–177.

Pastor, D., Sanchez, J., Porte, C., Albaigés, C., 2001. The Aegean Sea Oil Spill in the Galicia Coast (NW Spain). I. Distribution and fate of the crude oil and combustion products in Subtidal sediments. Mar. Poll. Bull. 42 (10), 895–904.

Rahman, F., Langford, K.H., Scrimshaw, M.D., Lester, J.N., 2001. Polybrominated diphenyl ether (PBDE) flame retardants. Sci. Total Environ. 275, 1–17.

Roepke, T.A., Snyder, M.J., Cherr, G.N., 2005. Estradiol and endocrine disrupting compounds adversely affect development of sea urchin embryos at environmentally relevant concentrations. Aquat. Toxicol. 71 (2), 155–173.

Sanni, S., Øysæd, K.B., Høivangli, V., Gaudebert, B., 1998. A Continuous Flow System (CFS) for chronic exposure of aquatic organisms. Mar. Environ. Res. 46 (1), 97–101.

Sonnenschein, C., Soto, A.M., 1998. An updated review of environmental estrogen and androgen mimics and antagonists. J. Steroid Biochem. Mol. Biol. 65 (1–6), 143–150.

Taban, I.C., Bechmann, R.K., Torgrimsen, S., Baussant, T., Sanni, S., 2004. Detection of DNA damage in mussels and sea urchins exposed to crude oil using comet assay. Mar. Environ. Res. 58, 701– 705.

Thomsen, C., 2002. Brominated flame retardants-development and application of analytical methods for characterization of human exposure. PhD Thesis. University of Oslo.

Tittlemier, S.A., Hallsorson, T., Stern, G.A., Tomy, G.T., 2002. Vapor pressures, aqueous solubilities, and Henry's law constants of some brominated flame retardants. Environ. Toxicol. Chem. 21, 1804–1810.

Utvik, T.I.R., 1999. Chemical characterisation of produced water from four offshore oil production platforms in the North Sea. Chemosphere 39 (15), 2539–2606.

Vos, J.G., Becher, M., van de Berg, J., de Boer, P.E.G., Leonards, 2003. Brominated flame retardants and endocrine disruption. Pure Appl. Chem. 75 (11–12), 2039–2046.

WHO, 1994. Broominated diphenyl ethers, Environ. Health Criteria 162. World Health Organization, Geneva.

Ying, G.G., Williams, B., Kookana, R., 2002. Environmental fate of alkylphenols and alkylphenol ethoxylates–a review. Environ. Int. 28 (3), 215–226.

Yokota, H., Tsuruda, Y., Maeda, M., Oshima, Y., Tadokoro, H., Nakazono, A., Honjo, T., Kobayashi, K., 2000. Effect of bisphenol A on the early life stage in Japanese medaka (*Oryzias latipes*). Environ. Toxicol. Chem. 19, 1925–1930.

Publication 2

Journal of Toxicology and Environmental Health, Part A, 69:77–96, 2006
Copyright© Taylor & Francis Inc.
ISSN: 1528–7394 print / 1087–2620 online
DOI: 10.1080/15287390500259277

Taylor & Francis
Taylor & Francis Group

THE POTENTIAL OF ECOTOXICOPROTEOMICS IN ENVIRONMENTAL MONITORING: BIOMARKER PROFILING IN MUSSEL PLASMA USING PROTEINCHIP ARRAY TECHNOLOGY

A. Bjørnstad[1,2], B. K. Larsen[1], A. Skadsheim[1], M. B. Jones[2], O. K. Andersen[1]

[1]RF-Akvamiljø, Randaberg, Norway, and
[2]School of Biological Sciences, University of Plymouth, Devon, United Kingdom

*New global technologies, allowing simultaneous analysis of thousands of genes, proteins, and metabolites (so-called "omics" technologies), are being adopted rapidly by industry, academia, and regulatory agencies. This study evaluated the potential of proteomics in ecotoxicological research (i.e., ecotoxicoproteomics). Filter-feeding mussels (*Mytilus edulis*) were exposed continuously for 3 wk to oil, or oil spiked with alkylphenols and extra polycyclic aromatic hydrocarbons. The influence of chronic exposure on mussel plasma protein expression was investigated utilizing ProteinChip array technology in combination with surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI TOF MS). Results indicated that exposure to spiked oil had a more significant effect on protein expression in mussels than oil alone. In total, 83 mass peaks (intact or modified proteins/peptides) were significantly altered by spiked oil, while 49 were altered by oil. In exposed organisms, the majority of peaks were upregulated compared to controls (i.e., 69% in oil and 71% in spiked oil). Some peaks (32 in total) were affected by both treatments; however, the degree of response was higher in the spiked oil group for 25 of the 32 commonly affected features. Additionally, certain peaks revealed exposure- or gender-specific responses. Multivariate analysis with regression tree-based methods detected protein patterns associated with exposure that correctly classified masked samples with 90–95% accuracy. Similarly, 92% of females and 85% of males were correctly classified (independent of exposure). Results indicate that proteomics have the potential to make a valuable contribution to environmental monitoring and risk assessment.*

Keywords: ecotoxicology, *Mytilus edulis*, oil, produced water, Proteomics, SELDI TOF MS

Increasing numbers of chemicals released into the environment provide greater potential for impact on terrestrial and aquatic wildlife. Since the late 1960s, significant effort has been directed to develop the best possible tools, at different levels of biological organization, for assessing how pollutants affect

---

The authors thank the Norwegian Research Council (project 133724/420) and the European Commission [contract BEEP (EVK3-2000-00543)] for funding, colleagues at Akvamiljø for help with sampling, Emily Lyng for proofreading the manuscript, and Ciphergen Biosystems AS (Copenhagen) for technical and scientific support.

Address correspondence to Anne Bjørnstad, RF-Akvamiljø, Mekjarvik 12, N-4070, Randaberg, Norway. E-mail: anne.bjornstad@rf.no

ecosystems and the organisms constituting them. In the early phase of environmental monitoring, the most common approach was to measure physical and chemical variables with the occasional implementation of biological variables (Lam & Gray, 2003). With the realization that some environmental pollutants, such as herbicides and insecticides, also produced deleterious effects, attention moved away from contaminant monitoring to measuring biological effects. Biomarkers representing molecular, cellular, and physiological changes in an organism, following exposure to various types of pollutants (Peakall, 1992), emerged as promising and useful monitoring tools in the mid 1980s. New biomarkers are being developed constantly; however, it has become clear that no single biomarker will serve to indicate the full effect of environmental pollutants (Galloway et al., 2004a, 2004b). Analogous to diagnosis in human medicine, it is recognized that most pollutant effects depend on the determination of suites of responses, rather than any pollutant-specific or disease-specific response.

A new trend in ecotoxicology and biomedical research is the application of so-called "omics" technologies. These are methods that have the potential to monitor complete classes of cellular molecules such as messenger RNAs, proteins, and intermediary metabolites in a single analysis (Morgan et al., 2002; Nicholson et al., 2002; Lau et al., 2003; Botstein & Risch, 2003; Clish et al., 2004; Petricoin & Liotta, 2004a), compared to traditional analyses that rely on only one endpoint. By allowing simultaneous analysis of thousands of genes, proteins, and metabolites, these new global technologies have enabled a wider approach to biological questions, since toxicity generally involves not only changes in a single gene but rather a cascade of gene interactions (Nuwaysir et al., 1999; Aardema & MacGregor, 2002). To date, few ecotoxicological studies have utilized "omics" technologies. Snape et al. (2004) proposed the term "ecotoxicogenomics" to describe the integration of genomics (transcriptomics, proteomics, and metabolomics) into ecotoxicology, and defined it as "the study of gene and protein expression in non-target organisms that is important in responses to environmental toxicant exposures." The authors emphasized the need for ecotoxicology to move toward a more holistic approach, which integrates high-throughput "omics" technologies. Identification of endpoints and responses from such an approach could potentially improve risk assessment through a clearer insight into mechanisms of actions gained by an increased level of information obtained at the molecular level. Improved knowledge regarding cellular control and defense mechanisms will allow a more robust extrapolation between model species and target species (MacGregor 2003), as well as reducing uncertainties involved in predicting threshold levels of various types of toxicity. It has also been suggested that genetic variation is the major cause for variation in susceptibility to disease and toxicant exposure variants (Aardema & MacGregor, 2002; Ashton et al., 2002; Botstein & Risch, 2003), indicating that a certain set of genes or proteins could be used to discover sensitive species and (sub)populations.

The aim of the present study was to evaluate the potential of proteomics as a tool for biomarker discovery and monitoring in ecotoxicological research (i.e., the potential for ecotoxicoproteomics). A case study was carried out where suspension feeding mussels (*Mytilus edulis*) were exposed to environmentally relevant concentrations of oil alone, or oil spiked with short-chain alkylphenols (APs) and extra polycyclic aromatic hydrocarbons (PAHs). The spike was made to reflect the composition of APs and PAHs found in water produced from offshore platforms and installations in the North Sea oil fields. Produced water, a by-product from oil and gas production, is a highly complex mixture of water, dispersed oil (microdroplets of oil in water), and chemicals. Trace amounts of oil, PAHs, and APs are only some of the compounds present; however, the aromatic compounds are assumed to be the most important contributors to toxicity (Utvik, 1999). Various effects of oil and produced water compounds were reported in marine organisms, including adverse effects on reproduction (Krause, 1994; Giesy et al., 2000), development (Baldwin et al., 1992), genotoxicity (Harvey et al., 1999; Aas et al., 2000; Taban et al., 2004), metabolism (Narvia & Rantamaki, 1997; Jonsson et al., 2004), and alterations in protein expression (Gimeno et al., 1998; Hasselberg et al., 2004). In spite of much effort, knowledge of the fate and effects of effluents related to oil and gas production is limited, indicating a need for continued research, as well as an evaluation of appropriate assessment tools. In the present study, the influence of exposure to oil and spiked oil was investigated on plasma protein expression in mussels, searching for particular protein forms being either induced or suppressed following exposure to oil alone or to oil in combination with APs and PAHs.

MATERIAL AND METHODS

Collection and Maintenance of Animals

Blue mussels were colleted in late October 2002 at 0.5–1 m depths in Førlandsfjorden (Norway), a fjord classified as clean according to criteria given by Norwegian pollution control authorities (SFT). They were transported back to the laboratory on ice, transferred immediately to clean running sea water (salinity of 34), and kept for 2 wk. The mussels were fed algae (*Isochrysis* sp.) throughout the acclimation and exposure periods.

Exposures

Exposures were performed using a continuous flow system (CFS), designed for performing studies of chronic exposure of marine organisms to mixtures of poorly water-soluble substances (described by Sanni et al., 1998). Mussels were exposed for 3 wk to either 0.5 ppm dispersed North Sea crude oil (i.e., Statfjord B oil) or 0.5 ppm oil spiked with a mixture of 0.2 ppm APs and 0.1 ppm extra PAHs (nominal concentrations); control mussels received only filtered seawater (salinity of 34, 10–12°C). Oil dispersions were made mechanically by passing oil

TABLE 1. PAH Composition Recorded in Statfjord B Crude Oil and Some Characteristics of the PAH Molecules

Compound / quantity	Statfjord (µg/g oil)	Mass (m/z)	Log K_{ow}
Naphthalene	1147.0	128.2	3.34
C1-naphthalene	3787.3	142.2	3.88
C2-naphthalene	5288.7	156.2	4.37
C3-naphthalene	3830.3	170.2	4.86
Acenaphthylene	10.0	152.2	4.1
Acenaphthene	9.7	154.2	3.95
Fluorene	135.9	166.2	4.21
Phenanthrene	252.9	178.2	4.57
Anthracene	0.0	178.2	4.58
C1-phen/anthr	460.2	192.2	5.1
C2-phen/anthr	439.4	206.2	
Dibenzothiophene	91.9	184.2	4.38
C1-dibenzothiophene	196.5	198.2	
C2-dibenzothiophene	232.9	212.2	
Fluoranthene	2.6	202.2	5.1
Pyrene	8.6	202.2	5.1
Benzo[a]anthracene	3.3	228.2	5.67
Chrysene	23.9	228.2	5.71
C1-chrysene	37.6	242.2	
C2-chrysene	41.5	256.3	
Benzo[b]fluoranthene	7.7	252.3	6.4
Benzo[k]fluoranthene	0.0	252.3	6.5
Benzo[b+k]fluoranthen	6.9	—	
Benzo[a]pyrene	4.7	252.3	6.3
Indeno[1,2,3-cd]pyrene	0.0	276.3	6.92
Benzo[g,h,i]perylene	1.7	276.3	7
Dibenzo[a,h]anthracene	0.0	278.3	6.71
Sum PAH	16020.9		
Sum PAH in 1 ppm dose (µg)	16.0209		

Note. Log K_{ow} is the logarithm of the octanol to water coefficient K_{ow}. Log K_{ow} values on alkylated PAHs are hard to find. A thumb rule is to add from 0.3 to 0.5 log units per methyl group added; see also http://logkow.cisti.nrc.ca. A 1 ppm oil dosage would equal a quantity of 1 mg oil/kg seawater.

and seawater through a high pressure mixing valve. The composition of the AP and PAH mixtures used in the spike, as well as the composition of PAHs in Statfjord B oil, are listed in Tables 1 and 2. The spike was made by mixing equal amounts of the two solutions. Acetone (grade >99.5) was used as a carrier, and high-precision peristaltic pumps (model 33, Harvard Apparatus, USA, purchased from B&K Universal, Nittedal, Norway) were used to ensure correct doses in the exposure tanks. The spike was added to the oil emulsion via a 5-L mixing flask with strong spin-bar mixing. This flask fed a diluted spike solution into the oil emulsion. The acetone carrier enhanced the dissolution of APs in water.

TABLE 2. Composition of APs and PAHs Used in the Spike

PAH mix		AP mix	
Compound	g/L acetone	Compound	g/L acetone
Naphthalene	7.649	*p*-Cresol	33.8
C1-Naphthalenes	13.792	*m*-Ethylphenol	6.54
C2-Naphthalenes	14.42	3,5-Dimethylphenol	6.54
C3-Naphthalenes	7.669	2,4,6-Trimethylphenol	3.8
Fluorene	0.867	2-*tert*-Butylphenol	0.236
Phenanthrene	0.779	3-*tert*-Butylphenol	0.236
C1-Phenanthrenes	1.264	4-*n*-Butylphenol	0.236
C2-Phenanthrenes	1.089	4-Pentylphenol	1.62
Dibenzothiophene	0.167		
C1-Dibenzothiophene	0.363		
C2-Dibenzothiophene	0.347		
Sum	48.405	Sum	53.01

Exposure Monitoring

Exposures were monitored by frequently measuring the average oil droplet size by a Coulter II particle size analyzer equipped with a 70-mm aperture tube. Oil concentrations were calculated from the estimated particle size and number of particles in the water. Semiquantitative fluorescence analysis of total hydrocarbon concentration (THC) in the water was measured (as described in Aas et al., 2000) five times during the exposure period. Additionally, THC in water was measured twice by gas chromatography (GC; HP5890, Hewlett Packard, USA) connected to a mass spectrometer (MS; Finnigan SSQ7000, USA) and analyzed in selected ion mode (GC/MS-SIM) as described in Baussant et al. (2001).

Sample Collection

Hemolymph was withdrawn by needle aspiration from the posterior adductor muscle of individual mussels and centrifuged for 10 min at 3000 × g and 4°C. The supernatant (plasma) was harvested, and a protease inhibitor cocktail (P2714, Sigma-Aldrich) was added (2.5 mg/ml), snap frozen, and stored at −80°C until analysis. A gonad smear from each mussel was examined under a light microscope for gender determination. Seventy mussels (shell size 7.5 ± 0.7 cm) were sampled and analyzed per treatment.

Sample Preparation on ProteinChip Arrays

ProteinChip arrays (from Ciphergen Biosystems, Palo Alto, CA) with various chemical or biochemical surfaces (and hence varying chromatographic properties) were tested prior to analysis. The principle of these surfaces is to selectively retain a subset of proteins of common properties, allowing others to be washed away to reduce sample complexity. Each array has eight

chromatographic "spots," allowing eight samples or replicates to be analyzed per array (detailed description of the ProteinChip array technology is given in Merchant & Weinberger, 2000; Issaq et al., 2003; Tang et al., 2004). ProteinChip arrays coated with carboxylate groups, providing weak cation-exchange (WCX) properties, in combination with a binding buffer containing 50 m*M* sodium acetate (NaAc) and 0.05% Triton X-100 at pH 4.5, were found to bind mussel plasma proteins most efficiently. Bioprocessors (Ciphergen Biosystems), each holding 12 arrays, and hence allowing 96 samples to be processed in parallel, were used for sample processing and incubation. The bioprocessors form separate wells above each spot on the arrays, enabling loading of volumes up to 500 μl per well.

All chromatographic spots were preactivated with 100 μl of 10 m*M* HCl for 5 min, rinsed quickly with ultrapure water (3 × 100 μl), and incubated with 100 μl of binding buffer for 5 min, after which plasma samples, diluted 1:10 in binding buffer, were applied and incubated (100 μl per spot) overnight at 4°C with vigorous agitation. Following incubation, the arrays were washed 3 times for 10 min with 50 m*M* NaAc, pH 4.5, and rinsed quickly with ultrapure water twice to remove weakly bound proteins. The arrays were removed from the bioprocessor and air dried. A matrix solution (saturated sinapinic acid (Ciphergen Biosystems), resolved in 50% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid), was applied to each spot of the ProteinChip arrays twice (2 × 0.6 μl per spot), allowing the applied solution to dry between applications.

SELDI TOF MS Analysis

The arrays were analyzed immediately on a PBS-IIc time of flight mass spectrometer using ProteinChip Software version 3.1 (Ciphergen Biosystems). Mass spectra were recorded on the following settings: 91 laser shots/spot surface in a positive ionization mode (65 of the shots were collected, starting at position 20 and ending at position 80 of the spot), laser intensity 214, detector sensitivity 8, detector voltage 2900 V, data acquisition from 0 to 180,000 Da, and optimum mass range focus from 2500 to 15,000 Da. Given the time of flight and the known length of the tube and voltage applied, the mass-to-charge ratio (*m/z* value) for each mass peak is estimated automatically. A typical SELDI mass spectrum consists of the sequentially recorded number of ions arriving at the detector (the mass peak height or relative intensity) coupled with the corresponding *m/z* value. The PBS-IIc instrument was externally calibrated with bovine insulin (5733.58 Da) and bovine immunoglobulin (Ig) G (147,300.0 Da) mass standards (Ciphergen Biosystems).

Data Handling and Statistics

Data handling is one of the most challenging parts of proteome analysis, and numerous bioinformatics tools are being developed around current proteomic platforms to help handle, process, and meaningfully interpret the large body of data that is emerging from such analysis. A similar approach to that

developed successfully in medical SELDI-TOF MS studies was chosen for our data: for example, preliminary data processing and interpretation using the Biomarker Wizard feature of the ProteinChip Software (version 3.1) followed by multivariate analysis with regression tree-based methods (Breiman et al., 1984) embodied in Biomarker Pattern software (version 4.0.1).

Briefly, in Biomarker Wizard, mass spectra from all treatment groups were imported into one experimental file. Baseline subtraction was performed and the spectra were mass normalized using 3 mass peaks (intact or modified proteins/peptides) prominent in all spectra (*m/z* 4036 Da, *m/z* 12,470 Da, and *m/z* 27,402 Da), and further normalized to the total ion content for the amplitudes of all peaks between 2500 and 180,000 Da in the spectra compared. Background noise was subtracted from the same *m/z* region prior to data collection. Protein/peptide peaks with similar *m/z* values (peak closeness 0.5% of mass) were automatically grouped across all the spectra together into peak clusters, if present in a minimum of 50% of the samples from one treatment group. The peaks used to generate these clusters had to meet a minimum signal-to-noise ratio (S/N). Being aware of the risk of loosing diagnostic information, only peaks with $S/N \geq 5$ that were present in the *m/z* range 2500–180,000 Da were collected and evaluated in this study. The data were tested for differences between treatment groups using the Mann–Whitney–Wilcoxon test; differences at $p < .05$ were considered significant.

Biomarker Discovery

After peak detection and preliminary statistical analysis, raw data were exported from Biomarker Wizard to Microsoft Excel to determine the presence/absence/fold changes of peaks between treatment groups, and examine the data for potential exposure or gender-specific alterations of proteins. The data were exported simultaneously to Biomarker Pattern software for identification of potential multivariate patterns classifying exposed and control mussels. The algorithm in this software examines each peak cluster present in the spectra and assesses its quality as a classifier (described in Fung & Enderwick, 2002). Based on the selected classifiers, the software generates and tests different classification models, using a cross-validation method that randomly picks 10% of the samples. The models (classification trees) with the best prediction success and lowest error costs were chosen for further testing. Mass spectra from unknown samples were classified subsequently by likeness to the pattern found in the plasma mass spectra used to create the classification model. Fifty samples from each treatment group were used to build the classification models, while 20 samples from each of the 3 treatment groups were kept for blind testing of the models.

RESULTS AND DISCUSSION

Profiling of mussel plasma on WCX ProteinChip arrays revealed 2287 distinct peaks and corresponding *m/z* values when data were collected at the

highest sensitivity. In addition to real peptide and protein peaks, the unfiltered mass spectra contained electronic noise as well as chemical noise due to the ionization matrix used. Further analysis of data was restricted to those *m/z* values with a signal-to-noise ratio greater than or equal to 5, present within the mass range of 2500 to 180,000 Da. Analysis of 70 samples (35 males and 35 females) from each treatment yielded 149 peaks with *m/z* values between 2716 and 159,764 Da that met the mentioned criteria. Among the 149 qualifying peaks, 90 had *m/z* values between 2500 and 10,000 Da, 30 were between 10,000 and 20,000 Da, and 31 peaks had *m/z* values greater than 20,000 Da. The dominance of low-molecular-weight protein forms is in accordance with what has been reported for medical studies utilizing the same proteomic technology (Petricoin et al., 2002; Li et al., 2002; Rogers et al., 2003; Conrads et al., 2004). Information regarding the difference between exposed mussels and controls, however, was present throughout the entire *m/z* region studied.

General Response

In general, results indicated that exposure to spiked oil had a greater effect on protein expression in mussels than oil alone. While 83 of the detected proteins were significantly altered by spiked oil, 49 were altered by oil. The ratio of abundance of proteins common to groups elucidated proteins whose expression was significantly altered. Both exposure regimes had a predominantly upregulating effect on protein expression in mussel plasma; 69% of all mass peaks detected in mussels exposed to oil and 71% in the spiked oil group were upregulated compared to controls. When only significantly altered peaks were considered, 68% were upregulated in both exposure groups. Females exposed to oil had 20 significantly upregulated and 5 downregulated peaks; oil-exposed males had 16 upregulated and 12 downregulated peaks. In the spiked oil group, females had 29 upregulated and 16 downregulated, while 33 peaks were upregulated and 13 downregulated in males. Average changes in protein expression (as the ratio exposed/control) were less than twofold for mussels exposed to both oil (1.6-fold) and spiked oil (1.9-fold) when all detected peaks were compared.

Examination of differences between exposed organisms and controls revealed a complex response pattern. Each treatment group was divided into females and males for comparisons of responses, and only one peak (*m/z* 46,134 Da) was significantly altered in all four groups (oil females, oil males, spiked oil females, and spiked oil males). Eight peaks were affected (six upregulated and two downregulated) in three of the exposure groups—however, not all of them in the same three groups. While certain protein features were affected by only one exposure condition, others responded to both exposures, where the response pattern could be either similar or opposite with regard to induction or suppression. Some protein forms were affected in only one gender, and some revealed opposite responses in males and females. Treatment- and gender-specific responses are described in the following sections. Examples of the different responses are given in Table 3.

TABLE 3. Examples of Complex Response Patterns in Mussels Exposed to Oil and Spiked Oil (OAP)

m/*z*(Da)	Oil—♀	Oil—♂	OAP—♀	OAP—♂	Significant response in
46,134	↑	↑	↑	↑	All groups
44,988	↑	ns	↑	↑	3 Groups
78,669	ns	↓	↓	↓	
10,834	↓	↓	↓	ns	
3842	↑	ns	ns	ns	Only oil exposed (females, males or both)
4359	ns	↓	ns	ns	
6302	↓	↑	ns	ns	
23,390	ns	ns	ns	↑	Only OAP exposed (females, males or both)
27,119	ns	ns	↓	ns	
135,202	ns	ns	↓	↓	
4047	ns	↑	ns	↑	Males only
5653	ns	↓	ns	↓	
10,132	ns	↓	ns	↑	
5481	↑	ns	↑	ns	Females only
7048	↓	ns	↓	ns	
3952	↓	↑	ns	ns	Females and males; opposite response
23,690	ns	ns	↓	↑	

Note. ↑ = upregulation, ↓ = downregulation, ♀ = females, ♂ = males, ns = not significant response.

Treatment-Specific Response

Pollutants with different chemical features are likely to affect distinct physiological or biochemical processes through binding to endogenous structures. Depending on which molecules or mechanisms are affected, the impact will, theoretically, give rise to patterns of changes that are specific to the various pollutants. Zachariassen et al. (1991) reported that a range of physiological parameters responded differently in mussels according to which pollutant the mussels were exposed to and suggested that the combinational use of these physiological parameters might serve as a pollutant-specific fingerprint in environmental monitoring. Results from the present study support this hypothesis, as mussels exposed to oil alone revealed a plasma protein expression pattern totally different from mussels exposed to oil spiked with APs and extra PAHs. For example, 17 mass peaks were significantly altered only in samples from oil-exposed mussels, while 51 peaks showed specific response to spiked oil. Although common responses were also observed in the 2 treatment groups, the degree of response was markedly higher in the spiked oil group for 25 out of 32 peaks that were significantly altered by both treatments. Pollutant-specific changes in protein expression have also been observed in mussel gills (Shepard & Bradley, 2000), in mussel digestive gland (Rodriguez-Ortega et al., 2003; Knigge et al., 2004), in fish liver (Shrader et al., 2003), and in fish gills (Högstrand et al., 2002).

Since the pollutants used in this study were complex mixtures, it is not easy to determine which components contributed most to the observed effects. However, if robust protein patterns are identified for specific chemicals and mixtures, these patterns may be used to identify a pollutant in cases where its identity is not known. To test this hypothesis, our data (the 149 mass peaks and corresponding *m/z* values) were exported to Biomarker Pattern software for identification of potential multivariate patterns classifying exposed from control mussels. Two comparisons were made, control versus oil and control versus spiked oil. All mass spectra were divided into training sets (to create prediction models) and test sets (for subsequent blind testing of the models). For comparison of control versus oil, nine distinct models were generated. The best performing model (Table 4), utilizing a combination of 11 mass peaks, classified randomized blind samples with 90% sensitivity (correct classification of exposed) and 90% specificity (correct classification of controls). The best prediction model for comparison of control versus spiked oil needed 10 peaks to predict controls with 95% accuracy, and individuals from the spiked oil treatment with 90% accuracy. Tests were also performed to investigate whether it was possible to generate good prediction models that could distinguish controls from exposed individuals, as well as discriminate individuals from the two exposure groups. Fifteen distinct models were generated, of which only one was able to classify all 3 groups with >80% accuracy (i.e., control = 90%, oil = 80%, and spiked oil = 85%).

TABLE 4. Prediction Models for Comparison of Controls Versus Exposed

Groups compared	Prediction success (%), model: *n* = 100		Prediction success (%), test of model: *n* = 40		Classifiers, *m/z* *(Da)*
	Control	Exposed	Control	Exposed	
Control vs. oil	100	98	90	90	6302 (↑) 3973 (↑) 5423 (↑) 6815 (↑) 11,301 (↓) 4061 (↑) 5333 (↑) 3887 (↑) 159,763 (↓) 8958 (↑) 9091 (↑)
Control vs. OAP	100	100	95	90	69,213 (↓) 3692 (↑) 3810 (↑) 3770 (↑) 4213 (↑) 4185 (↑) 13,057 (↓) 4021 (↑) 4300 (↑) 10,544 (↑)

Note. Importance of classifiers is decreasing from top to bottom (i.e., *m/z* 6302 Da and *m/z* 69,213 Da being the most important peaks for the classification of mussels exposed to oil or OAP, respectively). OAP = spiked oil, ↑ = upregulated, ↓ = downregulated.

Although the prediction success for the masked test samples in the present study was satisfactory, it is not certain that another set of samples from mussels exposed to oil and spiked oil collected at, for example, a different time of the year would perform equally well if tested on the same prediction model. It is important to bear in mind the dynamic nature of the proteome. For example, different biomarker profiles are found at the onset of a disease versus the late stage where symptoms and indirect effects are prominent (Van der Greef et al., 2004). Similarly, factors like seasonal changes, reproductive cycles, nutrient availability, age, and so on are likely to affect protein expression in an organism. Thus, an ideal protein biomarker profile for environmental pollutant monitoring should consist of a subset of proteins that are robust against external factors other than the one under investigation.

Gender-Specific Response

Protein expression was altered differently in males and females following exposure. For example, *m/z* 3952 Da, 6302 Da, 7017 Da, 23,690 Da, and 69,213 Da were downregulated in females and upregulated in males, while *m/z* 4155 Da was upregulated in females and downregulated in males. Another 40 peaks were significantly altered only in males, 9 of which showed similar response to both treatments, 9 that responded only to oil exposure, and 22 that responded only to the spiked oil treatment. Similarly, 36 peaks were affected only in females, 8 by both exposures, 8 by oil, and 20.by spiked oil. Common response in males and females were observed for 20 peaks.

An attempt was made to create a prediction model for classification of mussels from six different treatment groups (i.e., mussels from the three treatment groups divided into males and females). The best model included 31 *m/z* values as classifiers and managed to predict blind samples of control males and females, oil-exposed males, and females exposed to spiked oil with 80% accuracy. While 70% of the oil-exposed females were classified correctly, 30% were misclassified as control males. Only 60% of males exposed to spiked oil were correctly classified. The remaining 40% were misclassified as control females.

Without knowledge of the protein identity and function, it is difficult to interpret the functional relevance of these findings. For example, are gender-specific responses an indication of endocrine disruption? Oil-production-related effluents, PAHs and particularly APs, have been associated with endocrine disruption, and the estrogenic activity of APs in fish has been well established both in vitro and in vivo (White et al., 1994; Nimrod & Benson, 1996; Arukwe et al., 2000, 2001). Modulated steroid metabolism in echinoderms (Den Besten et al., 1993), as well as effects on moulting and reproduction in male grass shrimps, and offspring of exposed mothers (Oberdörster et al., 2000), has been observed following exposure to PAHs. Krause et al. (1994) found that the reproduction of caged sea urchins was impaired up to 100 m from a produced water outfall. Similarly, the reproductive performances and growth rate of

caged mussels were disrupted between 100 and 1000 m from an active produced water discharge (Osenberg et al., 1992). Negative effects of gonadal development in mussels from the present exposure study, as well as an induction of vitellogeninlike proteins in both males and females, were reported by Aarab et al. (2004). However, even though both oil and compounds in the spiked oil mixtures clearly have the potential to interfere with endocrine functions, the explanation for gender-specific responses may be other than endocrine disruption. Alternatively, males and females may have different susceptibility to one or several of the different toxicities potentially induced by oil, or oil in combination with APs and PAHs; this remains to be investigated.

Finding the function and full identity of the discriminating protein and peptide features was beyond the scope of this study. Nevertheless, it is important to link key molecules and their functions to provide essential mechanistic information of the underlying toxicity, as well as to provide a basis for antibody production of a set of biomarkers as robust alternatives or complements to single endpoint biomarkers. Despite the precision of the mass information yielded by the SELDI technique, the analysis does not directly provide a sequence-based identification. Furthermore, information regarding how post-translation modifications might have altered the protein size is not provided. Additional effort is therefore necessary to reveal the true identity of the protein(s) of interest. Several biomedical studies have identified key proteins discovered utilizing ProteinChip array technology in combination with SELDI TOF MS (Thulasiraman et al., 2001; Zhang et al., 2002; Diamond et al., 2003; Sanchez et al., 2004). A detailed description of methodology for protein purification and characterization that is applicable to ProteinChip arrays is described in Caputo et al. (2003).

Gender-Specific Protein Patterns

Grouping all 210 samples by gender, independent of exposure, identified 64 distinct mass peaks differing between the 2 genders with a *p* value less than .05. The number of female-specific and male-specific peaks was identical (i.e., 32). These peaks were predominant in one of the genders in all treatment groups. Examples of gender-specific peaks are shown in Figure 1. For example, *m/z* 5866 Da revealed a 25-fold higher expression in males when samples were compared as either males or females independent of exposure. Another peak, *m/z* 5970 Da, was primarily expressed in females; however, it was also induced in males exposed to spiked oil. Classification tree analysis showed that no single protein or peptide peak was able to completely separate the two genders; however, using a combination of only 2 peaks at *m/z* 5866 Da and 7853 Da, respectively, 92% of the females and 85% of the males were correctly classified from the blind randomized set of test samples. As the differences between males and females seem to be robust against exposures, and protein expression (as opposed to gene expression) can be determined in body fluids, these gender-specific protein profiles may be useful in noninvasive gender determination in, for instance, aquaculture.

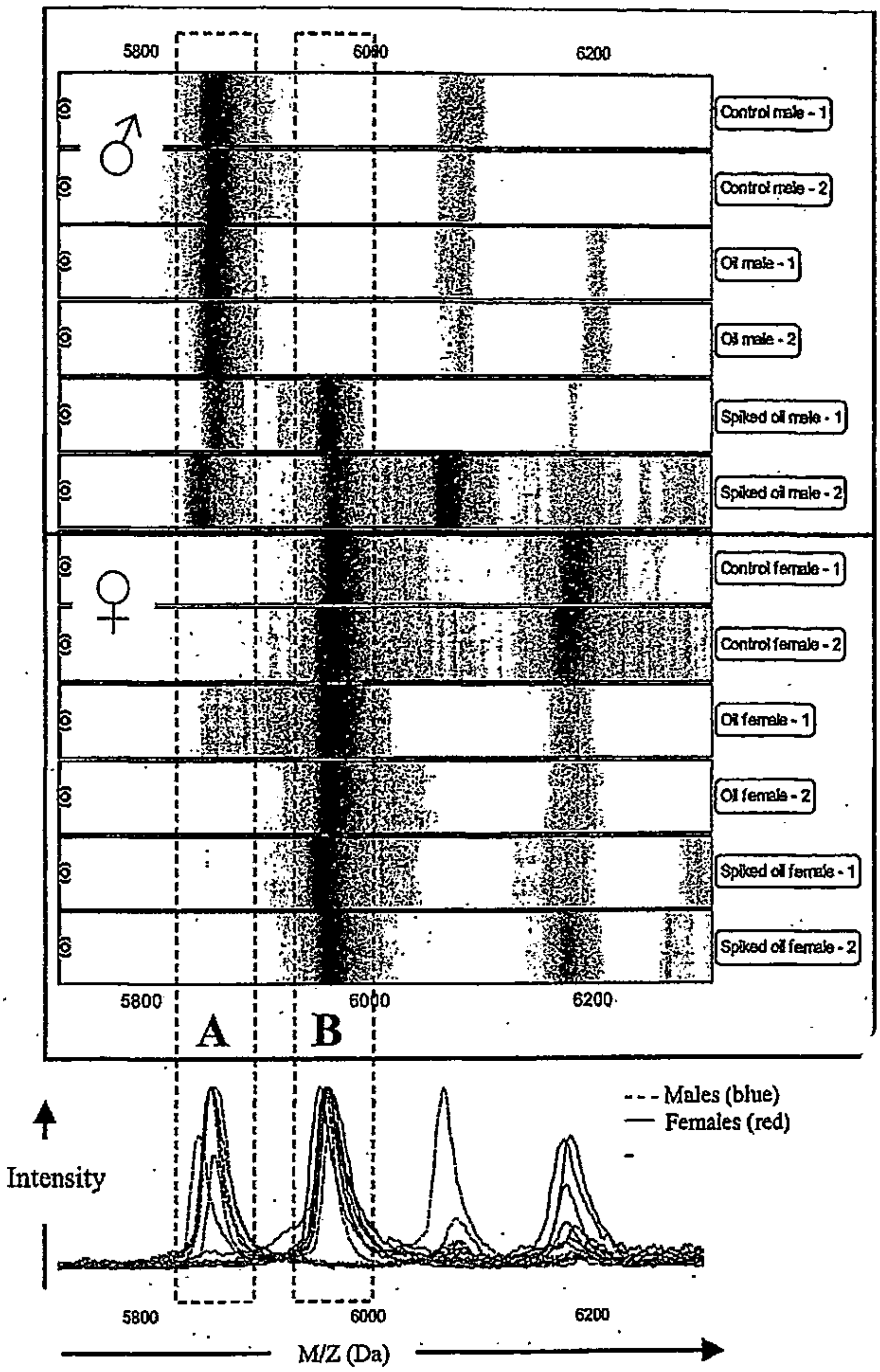


FIGURE 1. Gender-specific protein forms. Partial protein expression profile showing ex in peaks primarily expressed in either females or males, visualized as "gel view" (top) and " (bottom). (A): *m/z* 5866 Da, 25-fold upregulated in males when males and females are cc dent of exposure. (B): *m/z* 5970 Da, expressed in females (3.5-fold upregulated in a exposed to spiked oil.

Reproducibility

A great challenge with any new methods is to ensure that it is reproducible and, hence, reliable. To monitor the reproducibility between ProteinChip arrays in the present study, two control samples were divided into three replicates that were incubated and analyzed on three different arrays (Figure 2). The coefficient of variance (CV) calculated for 149 m/z values (between 2.5 and 180 kD) in the 2 samples varied between 0.6 and 7.9% in sample 1 and between 0.7 and 13.3% in sample 2. CV calculations were performed before mass and intensity normalization of spectra. While the "between-array" reproducibility was satisfactory, the individual variability for plasma samples within one treatment group was high (Figure 2, comparison of "gel views" from control sample 1 and 2).

Chemistry

The average oil concentration in water based on 15 multisizer measurements was 0.56 ± 0.19 ppm in the tanks receiving only oil, and 0.61 ± 0.26 ppm in the tanks with spiked oil. Semiquantitative fluorescence analysis of THC concentrations in the water showed that water from the oil treatment had an average ($n = 5$) THC of 0.25 ± 0.04 ppm, while the average concentration in water from the spiked oil treatment was 0.36 ± 0.07 ppm. Total PAH by GC/MS-SIM was only measured for the oil treatment, and average ($n = 2$) PAH concentration, converted to THC, was 0.38 ± 0.07 ppm.

No method for measuring concentrations of APs in water or biotawas available. Sundt and Baussant (2003) cited the following log K_{ow} (partitioning coefficient) for some of the substances: 4-*tert*-butylphenol (3.04–3.31), 4*n*-pentylphenol (unknown), 4*n*-hexylphenol (3.60), 4*n*-heptylphenol (4.00), while Shiu et al. (1994) reported log K_{ow} for *p*-cresol to be 1.62–2.06. The APs will thus have a water solubility ranging from somewhat more than naphthalene to somewhat less than C1-naphthalene, and a liability for uptake in the mussels ranging from somewhat less than naphthalene to somewhat more than C1-naphthalene; see Table 1 for comparisons. A careful indication of waterborne exposure would therefore be that approximately 80% to possibly 95% of the APs were available for uptake from the spike based on how the quantities of naphthalene and C1-naphthalene in the water were measured (Skadsheim, personal observations). It is therefore suggested that for sum alkylphenols, 0.16–0.19 mg total alkylphenols/L seawater constituted the spike exposure.

Use of (Prote)"Omic" Data in Risk Assessment

An effective environmental management strategy is dependent on accurate risk assessment to minimize potential harmful consequences of manmade and natural environmental impacts on ecosystem and human health. There is general agreement that current procedures, including determination of physical and chemical variables or measurements of whole-organism

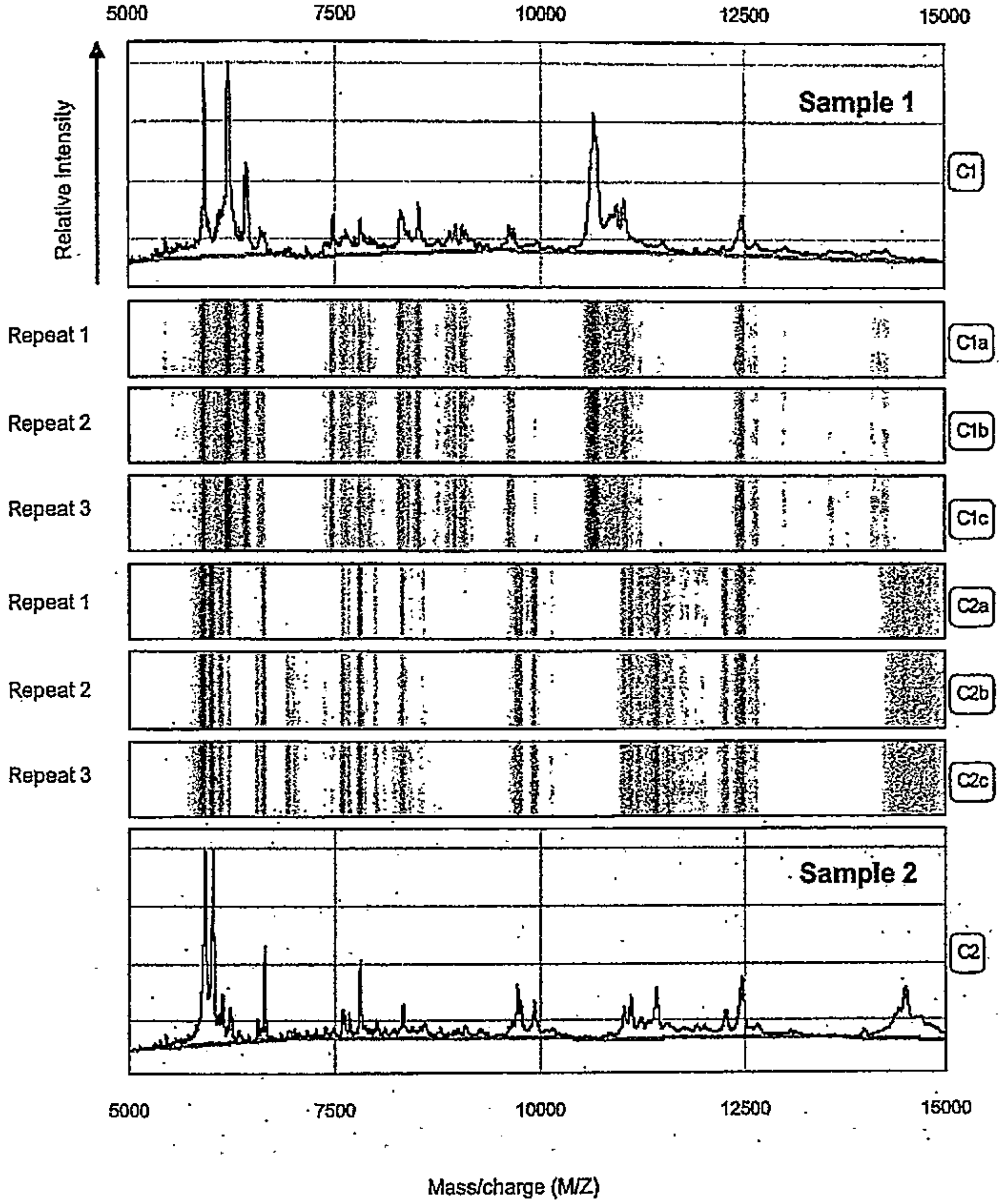


FIGURE 2. Example of array-to-array reproducibility. Two plasma samples from control mussels (sample 1 and 2) were applied on three different ProteinChip arrays to check the reproducibility between arrays. The figure shows partial mass spectra and gel views obtained from SELDI TOF MS analysis of the samples. The coefficient of variance (CV) for *m/z* values between 2.5 and 180 kD ($n = 149$) was 0.6–7.9% for sample 1 and 0.7–13.3% for sample 2. CV was calculated before normalization of spectra.

responses (e.g., mortality, growth, reproduction) of generally sensitive indicator species, have limited ability to predict the likely adverse effects of anthropogenic pollutants or activities on complex ecosystems and their components (Peakall, 1992; Galloway et al., 2004a, 2004b; Moore et al., 2004; Snape et al.,

2004). Although such approaches are useful for identifying chemicals of potential concern, selected endpoints should also include those capable of evaluating the sublethal toxicity, chemical mode(s) of action, critical "exposure windows," threshold levels of various types of toxicity (e.g., immunotoxicity, genotoxicity, endocrine disruption), variation in response and susceptibility, and effect of various factors like genetics, gender, age, diet, reproductive cycles, and so on (Schlenk, 1999; Gibb et al., 2002; MacGregor, 2003; Moore et al., 2004).

Currently, there is no method available that covers all the factors identified in the previous section. A multidisciplinary approach is therefore necessary to provide a more holistic understanding of the fate and effects of chemicals, and hence a more accurate prediction of potential risks. Including proteomics and other "omics" technologies in existing risk assessment approaches has many advantages. It would, for example, allow high-throughput screening of potential changes in thousands of cellular molecules simultaneously, and hence provide a more rapid evaluation of a chemical's toxic potential. As changes in cellular molecules are thought to precede toxic outcomes, the response patterns could be used to predict adverse responses at an early stage, for a wide variety of species. Cunningham et al. (2003) suggested two possible approaches to incorporate "omic" data in (eco)toxicology: (1) a target approach in which one assesses the expression levels of key biochemical pathways identified a priori, and (2) a "shotgun" approach in which gene (protein and metabolite) expression profiling, coupled with bioinformatics techniques, is used to identify those key pathways.

Proteomic approaches are now actively used, for instance, in cancer risk and response assessment in human medicine, as the technology allows detection of cancers at their earliest stages, even in the premalignant state, and ultimately translates into a higher cure rate (Petricoin & Liotta, 2004b). Additionally, the information obtained by these global analyses is used to identify high-risk patients, cause or consequence of disease processes, and how each individual patient responds to therapy.

In spite of the potential of "omic" data, regulatory bodies will need to gain confidence in the accuracy, reproducibility, sensitivity, and robustness of these new methods in order to fully integrate "omic" information into risk and safety assessment.

CONCLUSIONS

The hypothesis tested in this study was that exposing mussels to oil alone, or oil spiked with APs and extra PAHs, would result in unique combinations of expressed protein species that could be indicative of either exposure or effect. The results revealed specific protein expression profiles that could be used to distinguish (1) controls from exposed organisms, (2) type of exposure, (3) degree of response, and (4) males from females independent of exposure. Additionally, males and females responded differently to exposure, in the sense that

exposure affected different protein forms in the two genders. The latter indicates the necessity of knowing the gender of the test organisms, as well as having a balanced distribution of males and females in the different exposure groups. The potential mechanisms of actions behind the complex response patterns have not been investigated in this study; however, certain trends were observed (e.g., females exposed to oil showed similar protein expression patterns as control males, while males exposed to spiked oil revealed patterns similar to control females). Results from the present study clearly indicate that the proteome contains information about both specific stressors and their effects, and it is believed that an ecotoxicoproteomic approach has the potential to make a valuable contribution to environmental research, as well as environmental risk assessment, that complements existing methodologies. However, for ecotoxicoproteomics to fulfill its potential, national and international collaboration will be essential in order to obtain necessary baseline information, genomic sequence information (particularly for nonmodel species), as well as a standardization of methods to ensure both interexperiment and interlaboratory reproducibility.

REFERENCES

Aarab, N., Minier, C., Lemaire, S., Unruh, E., Hansen, P. D., Larsen, B. K., Andersen, O. K., and Narbonne, J. F. 2004. Biochemical and histological responses in mussel (*Mytilus edulis*) exposed to North Sea oil and to a mixture of North Sea oil and alkylphenols. *Marine Environ. Res.* 58:437–441.

Aardema, M. J., and MacGregor, J. T. 2002. Toxicology and genetic toxicology in the new era of "toxicogenomics": Impact of "-omics" technologies. *Mutat. Res. Fundam. Mol. Mech. Mutagen.* 499:13–25.

Aas, E., Baussant, T., Balk, L., Liewenborg, B., and Andersen, O. K. 2000. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: A laboratory experiment with Atlantic cod. *Aquat. Toxicol.* 51:241–258.

Arukwe, A., Thibaut, R., Ingebrigtsen, K., Celius, T., Goksøyr, A., and Cravedi, J. P. 2000. In vivo and in vitro metabolism and organ distribution of nonylphenol in Atlantic salmon (*Salmo salar*). *Aquat. Toxicol.* 49:289–304.

Arukwe, A., Kullman, S. W., and Hinton, D. E. 2001. Differential biomarker gene and protein expressions in nonylphenol and estradiol-17 beta treated juvenile rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 129:1–10.

Ashton, G. H. S., McGrath, J. A., and South, A. P. 2002. Strategies to identify disease genes. *Drugs Today* 38:235–244.

Baldwin, J. D., Pillai, M. C., and Cherr, G. N. 1992. Response of embryos of the sea-urchin *Strongylocentrotus purpuratus* to aqueous petroleum waste includes the expression of a high-molecular-weight glycoprotein. *Mar. Biol.* 114:21–30.

Baussant, T., Sanni, S., Skadsheim, A., Jonsson, G., Børseth, J. F., and Gaudebert, B. 2001. Bioaccumulation of polycyclic aromatic compounds: 2. Modelling, bioaccumulation in marine organisms chronically exposed to dispersed oil. *Environ. Toxicol. Chem.* 20:1185–1195.

Botstein, D., and Risch, N. 2003. Discovering genotypes underlying human phenotypes: Past successes for mendelian disease, future approaches for complex disease. *Nat. Genet.* 33:228–237.

Breiman, L., Friedman, J., Olshen, R., and Stone, C. 1984. *Classification and regression trees.* Pacific Grove, CA: Wadsworth.

Caputo, E., Moharram, R., and Martin, B. M. 2003. Methods for on-chip protein analysis. *Anal. Biochem.* 321:116–124.

Clish, C. B., Davidov, E., Oresic, M., Plasterer, T. N., Lavine, G., Londo, T., Meys, M., Snell, P., Stochaj, W., Adourian, A., Zhang, X., Morel, N., Neumann, E., Verheij, E., Vogels, J. T. W. E., Havekes, L. M., Afeyan, N., Regnier, F., Van Der Greef, J., and Naylor, S. 2004. Integrative biological analysis of the APOE*3-Leiden transgenic mouse. *OMICS- Integrative Biol.* 8:3–13.

Conrads, T. P., Fusaro, V. A., Ross, S., Johann, D., Rajapakse, V., Hitt, B. A., Steinberg, S. M., Kohn, E. C., Fishman, D. A., Whiteley, G., Barrett, J. C., Liotta, L. A., Petricoin, E. F., and Veenstra, T. D. 2004. High-resolution serum proteomic features for ovarian cancer detection. *Endocrine-Related Cancer* 11:163–178.

Cunningham, M. L., Bogdanffy, M. S., Zacharewski, T. R., and Hines, R. N. 2003. Workshop overview: Use of genomic data in risk assessment. *Toxicol. Sci.* 73:209–215.

Den Besten, P. J., Lemaire, P., Livingstone, D. R., Woodin, B., Stegeman, J. J., Herwig, H. J., and Seinen, W. 1993. Time-course and dose-response of the apparent induction of the cytochrome-P450 monooxygenase system of pyloric ceca microsomes of the female sea star *Asterias rubens L* by benzo[a]pyrene and polychlorinated biphenyls. *Aquat. Toxicol.* 26:23–39.

Diamond, D. L., Zhang, Y. N., Gaiger, A., Smithgall, M., Vedvick, T. S., and Carter, D. 2003. Use of ProteinChip (TM) array surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) to identify thymosin beta-4, a differentially secreted protein from lymphoblastoid cell lines. *Am. Soc. Mass Spectrom.* 14:760–765.

Fung, E. T., and Enderwick, C. 2002. ProteinChip (R) clinical proteomics: Computational challenges and solutions. *Biotechniques* 34: (suppl. S.).

Galloway, T. S., Brown, R. J., Browne, M. A., Dissanayake, A., Lowe, D., Jones, M. B., and Depledge, M. H. 2004a. A multibiomarker approach to environmental assessment. *Environ. Sci. Technol.* 38:1723–1731.

Galloway, T. S., Brown, R. J., Browne, M. A., Dissanayake, A., Lowe, D., Jones, M. B., and Depledge, M. H. 2004b. Ecosystem management bioindicators: The ECOMAN project—A multi-biomarker approach to ecosystem management. *Mar. Environ. Res.* 58:233–237.

Gibb, H. J., Checkoway, H., and Stayner, L. 2002. Improving risk assessment: Priorities for epidemiologic research. *Hum. Ecol. Risk Assess.* 8:1397–1404.

Giesy, J. P., Pierens, S. L., Snyder, E. M., Miles-Richardson, S., Kramer, V. J., Snyder, S. A., Nichols, K. M., and Villeneuve, D. A. 2000. Effects of 4-nonylphenol on fecundity and biomarkers of estrogenicity in fathead minnows (*Pimephales promelas*). *Environ. Toxicol. Chem.* 19:1368–1377.

Gimeno, S., Komen, H., Gerritsen, A. G. M., and Bowmer, T. 1998. Feminisation of young males of the common carp, *Cyprinus carpio*, exposed to 4-*tert*-pentylphenol during genderual differentiation. *Aquat. Toxicol.* 43:77–92.

Harvey, J. S., Lyons, B. P., Page, T. S., Stewart, C. J., and Parry, M. 1999. An assessment of the genotoxic impact of the Sea Empress oil spill by the measurement of DNA adduct levels in selected invertebrate and vertebrate species. *Mutat. Res.* 441:103–114.

Hasselberg, L., Meier, S., Svardal, A., Hegelund, T., and Celander, M. C. 2004. Effects of alkylphenols on CYP1A and CYP3A expression in first spawning Atlantic cod (*Gadus morhua*). *Aquat. Toxicol.* 67:303–313.

Högstrand, C., Balesaria, S., and Glover, C. N. 2002. Application of genomics and proteomics for study of the integrated response to zinc exposure in a non-model fish species, the rainbow trout. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 133:523–535.

Issaq, H. J., Veenstra, T. D., Conrads, T. P., and Felschow, D. 2003. The SELDI-TOF MS approach to proteomics: Protein profiling and biomarker identification. *Biochem. Biophys. Res. Commun.* 292:587–592.

Jonsson, G., Bechmann, R. K., Bamber, S. D., and Baussant, T. 2004. Bioconcentration, biotransformation, and elimination of polycyclic aromatic hydrocarbons in sheepshead minnows (*Cyprinodon variegatus*) exposed to contaminated seawater. *Environ. Toxicol. Chem.* 23:1538–1548.

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

Krause, P. R. 1994. Effects of an oil production effluent on gametogenesis and gamete performance in the purple sea-urchin (*Stronglyocentrotus purpuratus stimpson*). *Environ. Toxicol. Chem.* 13:1153–1161.

Lam, P. K. S., and Gray, J. S. 2003. The use of biomarkers in environmental monitoring programmes. *Mar. Pollut. Bull.* 46:182–186.

Lau, A. T. Y., He, Q. Y., and Chiu, J. F. 2003. Proteomic technology and its biomedical applications. *Acta Biochim. Biophys. Sin.* 35:965–975.

Li, J. N., Zhang, Z., Rosenzweig, J., Wang, Y. Y., and Chan, D. W. 2002. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. *Clin. Chem.* 48:1296–1304.

MacGregor, J. T. 2003. The future of regulatory toxicology: Impact of the biotechnology revolution. *Toxicol. Sci.* 75:236–248.

Merchant, M., and Weinberger, S. R. 2000. Recent advancements in surface-enhanced laser desorption/ionization–time of flight–mass spectrometry. *Electrophoresis* 21:1164–1177.

Moore, M. N., Depledge, M. H., Readman, J. W., and Leonard, D. R. P. 2004. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. *Mutat. Res. Fundam. Mol. Mech. Mutagen.* 552:247–268.

Morgan, K. T., Ni, H., Brown, H. R., Yoon, L., Qualls, C. W., Crosby, L. M., Reynolds, R., Gaskill, B., Anderson, S. P., Kepler, T. B., Brainard, T., Liv, N., Easton, M., Merrill, C., Creech, D., Sprenger, D., Conner, G. , Johnson, P. R., Fox, T., Sartor, M., Richard, E., Kuruvilla, S., Casey, W., and Benavides, G. 2002. Application of cDNA microarray technology to in vitro toxicology and the selection of genes for a real-time RT-PCR-based screen for oxidative stress in Hep-G2 cells. *Toxicol. Pathol.* 30:435–451.

Narvia, M., and Rantamaki, P. 1997. Aminotransferases in the bivalve mollusc *Mytilusedulis L* and short term effects of crude oil in brackish water. *Biomarkers* 2:253–258.

Nicholson, J. K., Connelly, J., Lindon, J. C., and Holmes, E. 2002. Metabonomics: a platform for studying drug toxicity and gene function. *Nat. Rev. Drug Discov.* 1:153–161.

Nimrod, A. C., and Benson, W. H. 1996. Environmental estrogenic effects of alkylphenol ethoxylates. *Crit. Rev. Toxicol.* 26:335–364.

Nuwaysir, E. F., Bittner, M., Trent, J., Barrett, J. C., and Afshari, C. A. 1999. Microarrays and toxicology: The advent of toxicogenomics. *Mol. Carcinogen.* 24:153–159.

Oberdörster, E., Brouwer, M., Hoexum-Brouwer, T., Manning, S., and McLachlan, J. A. 2000. Long-term pyrene exposure of grass shrimp, *Palaemonetes pugio*, affects molting and reproduction of exposed males and offspring of exposed females. *Environ. Health Perspect.* 108:641–646.

Osenberg, C. W., Schmitt, R. J., Holbrook, S. J., and Canestro, D. 1992. Spatial scale of ecological effects associated with an open coast discharge of produced water. In *Produced water: Technological/environmental issues and solutions*, eds. J. P. Ray, and F. R. Engelhardt, pp. 387–402. New York: Plenum Press.

Peakall D. B., ed. 1992 *Animal biomarkers as pollution indicators. Ecotoxicology Ser. 1.* Chapman & Hall.

Petricoin, E. F., and Liotta, L. A. 2004a. SELDI-TOF-based serum proteomic pattern diagnostics for early detection of cancer. *Curr. Opin. Biotechnol.* 15:24–30.

Petricoin, E. F., and Liotta, L. A. 2004b. Proteomic approaches in cancer risk and response assessment. *Trends Mol. Med.* 10:59–64.

Petricoin, E. F., Ardekani, A. M., Hitt, B. A., Levine, P. J., Fusaro, V. A., Steinberg, S. M., Mills, G. B., Simone, C., Fishman, D. A., Kohn, E. C., and Liotta, L. A. 2002. Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 359:572–577.

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

Rogers, M. A., Clarke, P., Noble, J., Munro, N. P., Paul, A., Selby, P. J., and Banks, R. E. 2003. Proteomic profiling of urinary proteins in renal cancer by surface enhanced laser desorption ionization and neural-network analysis: Identification of key issues affecting potential clinical utility. *Cancer Res* 63:6971–6983.

Sanchez, J. C., Guillaume, E., Lescuyer, P., Allard, L., Carrette, O., Scherl, A., Burgess, J., Corthals, G. L., Burkhard, P. R., and Hochstrasser, D. F. 2004. Cystatin C as a potential cerebrospinal fluid marker for the diagnosis of Creutzfeldt–Jakob disease. *Proteomics* 4:2229–2233.

Sanni, S., Øysæd, K. B., Høivangli, V., and Gaudebert, B. 1998. A continuous flow system (CPS) for chronic exposure of aquatic organisms. *Mar. Environ. Res.* 46:97–101.

Schlenk, D. 1999. Necessity of defining biomarkers for use in ecological risk assessments. *Mar. Pollut. Bull.* 39:48–53.

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

Shiu, W. Y., Ma, K. C., Varhanickova, D., and Mackay, D. 1994. Chlorophenols and alkylphenols—A review and correlation of environmentally relevant properties and fate in an evaluative environment. *Chemosphere* 29:1155–1224.

Shrader, E. A., Henry, T. R., Greeley, M. S., and Bradley, B. P. 2003. Proteomics in zebrafish exposed to endocrine disrupting chemicals. *Ecotoxicology* 12:485–488.

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

Sundt, R., and Baussant, T. 2003. *Uptake, tissue distribution and elimination of C4–C7 alkylated phenols in cod. Dietary vs. waterborne exposure*. Stavanger, Norway, RF-Akvamiljø/RF-Akvamiljø Report AM 2003/001.

Taban, I. C., Bechmann, R. K., Torgrimsen, S., Baussant, T., and Sanni, S. 2004. Detection of DNA damage in mussels and sea urchins exposed to crude oil using comet assay. *Mar. Environ. Res.* 58:701–705.

Tang, N., Tornatore, P., and Weinberger, S. R. 2004. Current developments in SELDI affinity technology. *Mass Spectrom. Rev.* 23:34–44.

Thulasiraman, V., McCutchen-Maloney, S. L., Motin, V. L., and Garcia, E. 2001. Detection and identification of virulence factors in *Yersinia pestis* using SELDI ProteinChip (R) system. *Biotechniques* 30:428–432.

Utvik, T. I. R. 1999. Chemical characterization of produced water from four offshore oil production platforms in the North Sea. *Chemosphere* 39:2593–2606.

Publications *in preparation*

- **A. Bjørnstad,** O. K. Andersen, and M. B. Jones (2009). Ecotoxicology moves into the omics era. Review article to be *submitted* to *Ecotoxicology*
- **A. Bjørnstad,** B. K. Larsen, R. C. Sundt, J. Barsiene, M. B. Jones, and O. K. Andersen (2009). Species and gender specific responses to endocrine disrupting chemicals - a proteomics approach. Article to be *submitted* to *J. Toxicol. Environ. Health.*
- **A. Bjørnstad,** R.K Bechmann, T. Baussant, O. K. Andersen, and M. B. Jones (2009). Dose-response relationships of oil on protein expression in molluscs and echinoderms, assessed by ProteinChip SELDI technology. Articles to be *submitted* to *Aquatic Toxicology.*
- **A. Bjørnstad,** B. K. Larsen, J. Barsiene, M. B. Jones, and O. K. Andersen (2009). Protein expression in Mytilus edulis deployed along a fieldgradient in the vicinity of a copper mine. Article to be *submitted* to *Environmental Health Perspectives.*