Gene transcription profiling in mussels (Mytilus sp.) exposed to the model compounds Copper chloride and Benzo[a]pyrene

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Preface and acknowledgement

This master thesis were funded by the Norwegian Institute of Water Research (NIVA), and the laboratory work was carried out at NIVAs headquarter, Oslo, Norway. This thesis presents a study of a *Mytilus sp.* oligoarray evaluating transcriptional effects of Copper chloride and Benzo[a]pyrene to digestive gland of the common mussel, *Mytilus edulis,* for the purpose to use in environmental monitoring.

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Abstract

Environmental pollution is increasing in coastal areas and is responsible for adverse health effects in marine wild life. The oceans are vast and the sources of pollution are many, which make the effects of pollution difficult to assess in a reliable and valid way. Different toxic components have different mode of actions, leading to series of responses in organism. The first early responses to a toxicant, in most cases a system at the molecular level designed to target the specific toxicant, is important to identify. A series of biological marker (biomarker) analyses have thus been developed to assess the effects of exposure to single chemicals (metals and organic compounds) and complex pollution discharges. Single biomarkers are useful as indicators of the presence pollution as they reflect biochemical and physiological changes, but they may not necessary predict the effects on the whole organism. Several mussel species in the genus *Mytilus* has frequently been used to assess chemical pollution in the aquatic environment and with the availability of high-content molecular methods such as microarrays, makes this group of species highly interesting for use in toxicity assessment and environmental monitoring. The aim of this project was to evaluate the performance of a newly developed high-content *Mytilus sp.* oligonucleotide array (oligoarray) and to specifically assess the transcriptional effects in *M. edulis* digestive organ after exposure to the fundamentally different pollutants Copper chloride ($CuCl_2$) and the Polycyclic Aromatic Hydrocarbon (PAH) Benzo[a]pyrene (BaP).

The results showed that an oligoarray based on *M. edilus* and *M. galloprovinciales* could be used to study the differential expression in *M. edulis* digestive gland after exposure to Cu and BaP. There were also indentified different MoA for Cu and BaP, as well as several genes and pathways related to general stress responses of toxicity for both compounds. Further work on the development of the array includes inclusion of additional sequence information from other sequencing projects, redundancy reduction and further verification of the microarray results by Real Time quantitative Polymerase Chain Reaction (RT-qPCR) to assess the dynamic range and sequence specificity of the array.

Keywords: Pollution, Environmental monitoring, biomarkers, common mussel *(Mytilus sp.),* BaP, Cu, microarray, gene expression.

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Introduction

Coastal ecosystems are increasingly threatened by anthropogenic activities, such as industry, shipping, agriculture and oil production. These activities pollute directly via discharges or indirectly via terrestrial runoff. The major pollutants of concern are heavy metals and organic compounds because their potential toxicity and environmental persistency threatens even the most robust costal ecosystem. Coastal areas are biologically and ecologically important areas as they are the main areas of marine primary production and thus the source of nutrients for higher organisms in the marine ecosystem and food web. Pollutants adsorbed to nutrients are ingested and additionally taken up by passive diffusion and accumulated (Torres et al. 2008). Uptake and metabolism of heavy metals and organic pollutants can cause death or sub-lethal effects that disrupt the endocrine, reproductive, and immune systems and lead to reproductive failure, deformities, malfunctions and cancer (Bolognesi et al. 1999; Donkin et al. 1991).

The effects of pollutants in the marine ecosystem are difficult to study because the vastness of the oceans makes it difficult to relate observed effects to a specific episode or the concentrations of chemicals might be lower than what is possible to measure with chemical analyses. Consequently, cause-effect relationships often rely on performance of *in situ* biological monitoring with selected indicator species, such as bivalve molluscs and sentinel fish species to measure the effects of marine pollutants. Systematic monitoring with mussels was first launched with the "Mussel Watch" program in the United States in the1970s (Goldberg 1975) followed by several European research programmes (Gabrieldes 1997; Narbonne et al. 1999). These monitoring programmes use several methodological approaches, ranging from assessment of subtle alterations at the molecular level to large scale effects on the community level, with the aim to identify and predict effects and toxicity of chemical pollutants. However, there is a lack of extrapolation of results between the various levels of biological organization. Different toxic compounds have different modes of actions leading to a series of response mechanisms in an organism, and as all primary interactions between a toxic component and an organism take place at the molecular level, it is important to identify the biological processes and pathways leading to effects in higher levels of ecological relevance.

The use of genomic technology such as microarrays and other high-content transcriptional analysis as a hypothesis generating and predictive tool can offer the possibilities to assess both single as well as multiple biological responses with the use of one single analysis. The advantage and strength of the method lays in the ability to study relationships between genes and discover functional groups with potential to generate predictive hypothesis that can be linked to a level of higher ecological relevance. The present study focus on the evaluation of a newly developed *Mytilus sp.* microarray using the two well-known aquatic pollutants Cu and BaP as the initial step for use of the method in toxicity testing and environmental monitoring.

1.1 The common mussel (Mytilus sp.) as a model specie in environmental monitoring

Mytilus californianus, M. edulis, M. galloprovincialis and *M. trossulus* are four species in the genus *Mytilus* of the family Mytilidae, which are all important in aquaculture and commercial harvesting from natural grown populations around the world (FAO 2010). These mussels are sentinel marine organisms that is extensively used for environmental monitoring because they are responsive and robust to pollutants, easy to manage and study, and have a global distribution (Hopkin 1993). *Mytilus sp.* are intertidal and sub-tidal organisms with habitats in sheltered harbours or estuaries or on rocky shores of the rough coast with high tolerance for extreme and shifting conditions of temperature and salinity as well as exposure to air. These mussels are stationary filter feeders and lives of a diet that mainly consists of phytoplankton, detritus, and organic particles filtered from the surrounding water. The mussels can therefore bioaccumulate organic or inorganic (metals) pollutants directly from the surrounding waters, as well as from compounds bound to the filtered particles (Moore 1988; Seed 1976; Viarengo 1985).

The four species are morphologically similar and they have a wide plasticity of shape and size caused by variations in the environmental conditions they are living in. The distribution of the four species overlaps around the world and the different species can hybridize and produce fertile hybrids (Beaumont et al. 2007). The different species can be genetically identified by variations in satellite DNA. These findings show that the genome of *M. californianus* is the most divergent of the four species, which is

further supported by some differences in external morphology (size) and the lack of cross-hybridisation with other mussels of *Mytilus sp.* despite situated in same locations (Skurikhina et al. 2001). *Mytilus edulis* is native to the Northern Atlantic coasts, Europe and North America. *M. galloprovinciales* has its origin in the Mediterranean Sea, but are also found on the Atlantic coast of Spain as well as the southern Pacific coast of America. *M. trossulus* is found on the Pacific coast from California to Hong Kong, in the North West Atlantic Ocean, on the pacific coast of North America (North of San Francisco) and Canada, and in the Baltic Sea. *M. californianus* is mainly situated along the west coast of North America, occurring from Northern Mexico to the Aleutian Islands of Alaska (McDonald & Koehn 1988; Seed 1992)

1.2 Biological effects of environmental pollutants

In ecotoxicology the relationship between dose and response provide the basis for the assessment of risks and hazards from environmental pollution. According to Paracelsus (1493-1541) "All things are poison and nothing is without poison; only the dose makes a thing a poison".

Ecotoxicology aim to identify the effect that environmental pollutants and other stressors causes upon the environment. The result of exposure to toxic compounds could in the worst case be lethal or cause adverse sub-lethal effects. Acute toxicity with a mortal outcome is often a result of short time (episodic) exposure to a high concentration of a pollutant. Sub-lethal effects are often caused by long time exposure to low doses of pollutants (chronic exposure) where the chemical concentration can be below the analytical detection limits, or the biological effects are found at a stage where the chemical exposure has ceased but still responsible for a delayed response in the organism (Pretti & Cognetti-Varriale 2001)

The focus in traditional risk assessment of environmental pollution has been on use of chemical analyses in the field and compared to controlled laboratory toxicity tests to determine whether the chemical can cause negative or toxic effects to living organisms. The terms “effect concentration” (EC) and “lethal dose concentration”

(LD) is used, linked to single compounds. Laboratory studies are necessary to assess risk on an individual level and to establish a relationship between concentration (dose) and response of a specific compound, thereby judging its ecological relevance (link effects on the individual level to effects on a population, community and ecosystem level). However, these single compound studies are seldom applicable to real-life situations where organisms are exposed to varying levels of mixtures of toxic compounds (Mothersill et al. 2007), were some compounds can cause sub-lethal effects from chronic exposure. These long-term effects of these sub-lethal exposures remain uncertain and represent one of the main problems of marine pollution. An alternative for lethality testing in laboratories is the use of biological markers (biomarkers) and sub-lethal tests that is more related to welfare and health of an organism or a population. This has been elegantly captured in the definition presented by Peakall et al. (1994):

A biomarkers is the "biological response to a chemical or chemicals that gives a measure of exposure and sometimes, also of toxic effects".

Peakall et al. (1994)

Biomarkers are highly sensitive towards specific effects of uptake and metabolism of pollutants and thus useful to assess sub-lethal effects. Metallothionein induction, acetylcholinesterase inhibition, cytochrome P450 system induction, imposex, lysosomal enlargement and lysosomal membrane destabilisation, and peroxisome proliferation are some examples of biomarkers that can be measured by several methodological approaches (biochemistry, cytochemistry, immunochemical methods) and that are used to evaluate exposure to and effect of different contaminants (metals, organic xenobiotics and organometallic compounds (Cajaraville et al. 2000)

Unfortunately, for most biomarkers on a lower biological level there are not established a relationship between the biomarker response and ecological relevant parameters at population level such as reproduction, development and growth. This relationship may be difficult to establish with simple measures this since many biomarkers respond similarly to several environmental pollutants and there are variations in biological response between different species for the same pollutant (Guilhermino 2007). The benefit of using molecular and cellular methods to assess toxicity are that they can both detect toxicity at the sub-cellular level and it has a

potential to generate hypothesis and predict changes in higher level of biological organisation before any serious effect are observed in the individual or at population level (Cajaraville et al. 2000) as shown in Figure 1.

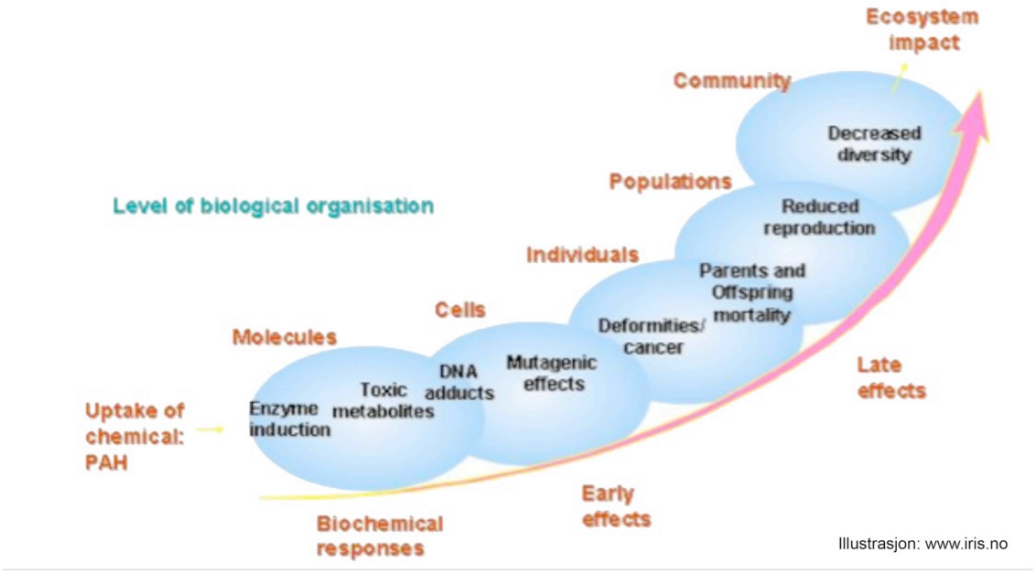


Figure 1. The illustration shows potential biological effects of pollutants at different levels of organisation. The use of molecular tools can potentially detect effects at the molecular level and further discover pathways to effects of higher ecological relevance (Illustration adapted from: http://iris.no/Internet/akva.nsf/wvDocID/C80B7ECA0C1B6F80C125771C002AFAD9).

1.3 Molecular toxicology

1.3.1 Toxicology and genomics

Understanding mechanisms of toxicity at the molecular level is an important part of the development and assessment of techniques that measure and demonstrate the harmful effects of pollutants in a complimentary manner to traditional toxicology. Toxicogenomics or ecotoxicogenomics represents the study of the interaction between the genome and adverse biological effects caused by exogenous agents (Elisavet T. Gatzidou 2007). Combining conventional toxicology endpoints with the study of effects of chemicals and stressors by monitoring of changes in gene expression gives the opportunity to identify undiscovered pathways and mechanisms affected by exposure to pollutants. This provides us with a better understanding of the interactions

between the molecular pathways in an organism and a more comprehensive assessment of molecular and biochemical effects of toxicity (Ankley et al. 2007).

The gene expression in a cell describes the current state of and reflect the activities occurring in a given cell or organism. A normal healthy cell has a baseline level of gene expression. The amount and types of expressed genes varies with stage of development, growth and reproduction as well as differentiation between different cell types (organs) and are changes related to normal homeostatic adjustments (Elisavet T. Gatzidou 2007). Other factors that affect the gene expression are environmental stress such as toxins, drugs and chemicals or changes in environmental conditions that the organism has to adapt to. An organism has the ability to handle many of these factors by its repair and detoxification mechanisms, and then return to its normal state of gene expression and baseline metabolism.

The deoxyribonucleic acid (DNA) in an organism carries all the genetic information needed for life. The genetic information is preserved in genes, small parts of the DNA that encodes for different protein molecules with structural, signalling, transport proteins, receptor, enzyme, and gene regulating functions. The gene regulating proteins are activator or repressor proteins that switch the genes on and off and control the expression of the genes and transcription of the DNA to Ribonucleid acid (RNA). The total of RNA, including the messengerRNA (mRNA) constitute the transcripts of DNA (transcriptome) and contain the recipe of how to assembly amino acids into the different types of protein molecules.

The most developed and applied analysis of gene expression is by analysing the mRNA levels (transcriptomics), although analysis of proteins (proteomics) is also used. The genes are expressed with varying efficiencies and the amount of mRNA does not always reflect the level of proteins in cells, tissue and fluids and may therefore not be directly comparable. The translation of one single RNA strand can be origin of several proteins as and post translational modification of the proteins (e.g. phosphorylation and acetylation) can alter the function of the proteins (Bruce et al. 2008; Elisavet T. Gatzidou 2007).

1.3.2 Gene expression analysis

The development of DNA and RNA sequencing started in the beginning of the 1970's. Two of the most groundbreaking discoveries in this field was Walter Fiers and his colleagues discovery of the RNA sequence of the first complete gene and the complete genome sequence of the Bacteriophage MS2 (Fiers et al. 1976; Min Jou et al. 1972). In 1977, Sanger and his colleagues introduced use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators (Sanger et al. 1977). This work led to rapid evolution of sequencing technology during the1980's and 1990's, and the technologies currently in use have significantly improved in cost efficiency, throughput and accuracy.

DNA sequencing is currently dominated by pyrosequencing and whole genome sequencing ("shot-gun sequencing"). Analyzes of RNA have developed from the single gene analysis by Northern blot and Polymerase Chain Reaction (PCR), to the use of microarray technology and Serial Analysis of Gene Expression (SAGE) that can analyze thousands of RNA transcripts in one single assay (Schirmer et al. 2010). Microarray technology have in particular the recent years gained popularity due to high-content screening capability, generation of standardised and comparable data formats and availability of commercial and custom products targeted a number of species. The oliogonucleotide array (oligoarray) technology is based on using probes based on short sequences from cDNA sequences or EST and has the possibility to analyse all known transcripts in an organism simultaneously. The limitation of this technique is the feature information that is included in the array, depending on the available genomic information on the organism you work with.

There are prepossessed microarrays available for a few model species with a fully sequenced genome (e.g. human, mice and rat). The alternative for non-model species is to make a custom microarray based on ESTs from a complimentary DNA (cDNA) library of a set of a known or a partially known genome. A microarray consists of a small membrane or glass slide that contains short sequences of target DNA. Complimentary DNA or RNA samples are hybridized to the probes (target DNA) in the array. Oligonucleotide arrays are based on short oligonucleotide sequences synthesized directly onto the surface of the array. The longer sequence, the more

specific to the individual target. Photolithographic synthesis is used on a silica substrate where light and light-sensitive masking agents build one nucleotide at a time into a sequence (Agilent and Affymetrix microarrays). In spotted microarrays, the probes are oligonucleotides, cDNA or small fragments of PCR products that correspond to mRNAs. The probes are synthesized prior to deposition on the array surface and are then "spotted" onto the glass.

To study differential expression, normalized data from the gene expression of an exposed organism is compared to a control (unexposed) sample. The gene expression profile from the microarray analyses gives a molecular signature (fingerprint) of exposure to specific classes of pollutants. The molecular signature, which reflects the biochemical and biological responses of an organism, can then be used to assess co-regulation of genes (functional groups), pathways and cell function or to predict the toxicity of unknown compounds with different modes (mechanisms) of action, MoA (Hamadeh, H. K. et al. 2002a; Hamadeh, H. K. et al. 2002b; Waring et al. 2001).

However there are several biological and environmental factors like development stage, size, sex, reproduction cycle, water temperature, pH, and salinity that can affect the general gene expression and that should be considered in the exposure study of a microarray experiment (Franzellitti et al. 2010; Hagger et al. 2010; Hamer et al. 2008; Kube et al. 2007) Another factor which should be consider is the type of tissue as different types of tissue might have differences in the expression of genes (Craft et al. 2010), which calls for the need of standardized tests.

1.3.3 Model compounds and effects on gene expression

In most microarray analyses, the expression of a small selection of genes in the array has been verified by an alternative molecular method like Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). The choice of BaP and Cu as model compounds to evaluate the gene expression analysis done in this pilot study of the first version of the *Mytilus sp.*microarray, is based on the use of compounds that is well studied and documented and the possibility to use literature to verify classic biomarker genes, general stress responses or pathways as done by Bundy et al. (2008). BaP and Copper are some of the most frequent used and studied compounds

representing the two groups of pollutants: the organic pollutants and heavy metals.

When pollutants enter an organism they will cause certain changes: directly by altering the gene expression, or indirect by binding to proteins or altering macromolecules. These changes initiate protective responses (detoxification mechanisms) or repair mechanisms. Protective responses can reduce the concentration of free pollutants in the cell to prevent or limit interaction with cellular components. This can be done by reducing the bioavailability of the pollutants by binding of the pollutants or their metabolic products to other molecules. Most responses are not specific to one compound, but related to a group of compounds or a response of general stress. Repair mechanisms restore damage to the cellular system caused by pollutants (e.g. induction of stress proteins that repair cellular damage or damage to DNA) back to the normal functional state, homeostasis.

The effects of pollution and the severity of response vary with the physico-chemical properties of the compound, the dose and time of exposure, as well as the species of the exposed organism and its stage of development. The physico-chemical properties such as the lipophilicity and size of the molecules that influence the bioavailability as well as biological processes such as uptake, distribution, metabolism and excretion of the chemical in an organism (Jones & de Voogt 1999; Walker et al. 2006).

Several studies have successfully assessed the use of *Mytilus sp.*in environmental monitoring by gene expression analysis with microarray technology based on a limited number of genes (Banni et al. 2009; Dondero et al. 2006a; Dondero et al. 2006b; Zorita et al. 2007). The recent years worldwide sequencing efforts have produced a high number of Expressed Sequence Tags (ESTs) for different *Mytilus* species and other mussels of the class of bivalva and in December 2008, the SRS browser (EMBL release 97) contained 151,292 nucleotide sequences these species (Venier et al. 2009). This is increasing the possibilities to gain more insight to knowledge on the molecular bases of biological processes of bivalves.

BaP is known of its properties as pro-mutagenic compound that can be biotransformated into a mutagen. BaP is a PAH composed of carbon and hydrogen atoms which consists of 2 or more fused benzene rings. PAHs are present naturally in fossil fuel, and as humans exploit these resources and all combustion processes

generates PAHs the levels of these compounds has increased in the environment (Hylland 2006). BaP have a short biological half-life in most organisms, but uptake and metabolism of organic pollutants are known to cause adverse sub-lethal effects that disrupt the endocrine, reproductive, and immune systems and lead to reproductive failure, deformities, malfunctions and cancer (Donkin et al. 1991). A well-known protective response to organic compounds is induction of enzymes that metabolize the organic compounds in to less toxic, water-soluble metabolites and conjugates that can easily be excreted by the organism. In some cases, as with BaP, the protective responses and metabolism of pollutants in an organism can lead to production of active metabolites that can be toxic to the cell trough a process called bioactivation. BaP is not toxic when it enters an organism, but its chemical form is changed by metabolism. BaP is hydrolyzed by the enzyme monooxygenase cytochrome P_{450} 1A (CYP 1A1), a phase I biotransformation process to a less lipophilic, but reactive epoxide, benzo[a]pyrene diol epoxide. Further, the epoxide will be detoxified by the Glutathione S-transferase (GST) in a phase II biotransformation process, or it can form adducts with other cellular components like DNA (Besselink et al. 1998; Machella et al. 2004; Woottona et al. 1996). Most DNA adducts are quickly restored to the original state by repair mechanisms, but in some cases non-repaired DNA adducts may cause mutations and cancer (Figure 1).

Copper is a trace metal that is essential in many enzymatic processes in an organism and thus it is naturally occurring, but toxic in high concentrations. Copper exists dissolved as ions and bound to particles in seawater (Balls 1985). Anthropogenic sources of copper are antifouling paint on boats and aquaculture equipment and technical installations as well as combustion, municipal wastewater and mining activities. The concentrations in coastal areas are generally below acute threshold and the sub-lethal effects of chronic Cu exposure are therefore more relevant. Some effects of chronic exposure to Cu are DNA strand breakage, damage of actin cytoskeleton, reduction of lysosomal membrane stability, and induction of Reactive Oxygen Species (ROS) leading to oxidative damage of cellular macromolecules (Bolognesi et al. 1999; Viarengo 1994) An example of a protective transcriptomic response to copper is metallothionein, or MTs, a group of metal-binding proteins, which are induced in an organism by exposure to high concentrations of metal ions.

The protein binds the metal ions, which are stored or excreted (Dondero et al. 2005; Dondero et al. 2006a; Walker et al. 2006).

The initiation of protective responses and repair mechanisms has a high energy cost for an organism. This require reorganization of resources that otherwise would be used for growth and reproduction, and may also affect the immune defence and the organisms ability of detoxification (Nicholson & Lam 2005). These responses can be recognized in gene transcription profiles as up-regulation (induction) or down-regulation (suppression) of genes related to such biological processes.

1.4 Aims

The aim of this study was to evaluate the first version of a newly developed oligoarray for *Mytilus sp*. and assess the single chemicals effect in *M. edulis* by use of the model compounds Cu and BaP. The main purpose of developing and this microarray was to asses the possibilities to use changes in gene expression of *Mytilus sp.* as a biomarker and tool in environmental monitoring and to if possibly extend the mechanistic understanding of sub lethal effects in *Mytilus sp.* The potential cross-species hybridization of the array was assessed to explore the potential of using sequence information from the four Mytilus species *M. californianus, M.edulis, M.galloprovinciales and M. trossulus* for increasing the genome coverage by cross-species hybridisation and to develop an universal Mytilus oligoarray for use with multiple species of *Mytilus* in ecotoxicological research and in environmental monitoring.

2 Materials and methods

Use of microarray technology is a complex and interdisciplinary process. It involves the design of the microarray (functional genome research), biological experiment, microarray experiment, image and statistical analysis.

Gene expression responses in the digestive gland (hepatopancreas) of *M. edulis* exposed to $CuCl_2$ and BaP for 7 days were performed by microarray analysis using an in-house synthesised Agilent 105k custom oligoarray. In summary, RNA from the digestive organ was extracted by the Guanidinium thiocyanate-phenol-chloroform method, purified by Lithium Chloride Trizol method, and the resulting RNA were prepared for microarray analysis by a standard one-colour microarray low input amplification, labelling and hybridisation protocol. The samples were then hybridised to the in-house synthesised 105k *Mytilus sp.* oligoarray, washed and scanned prior to processing of data by using appropriate biostatistics and bioinformatics to obtain biologically meaningful information.

Although tissue from *M. edulis* exposed to several concentrations were available, only tissue of *M. edulis* exposed to 320 µg/L $CuCl_2$ and 50 µg/L BaP were used in this study based on its potential to show results of sub-lethal effects.

2.1 Development and design of the *Mytilus sp.*custom array

The Microarray design was performed in cooperation with Knut Erik Tollefsen, NIVA. The procedure is outlined in Fig. 2. Approximately 62k Expressed Sequence Tags (ESTs) were obtained from the NCBI GenBank and subjected to EST pre-processing, clustering and contig assembly by using a local installation of ESTExplorer (http://estexplorer.biolinfo.org) In essence, vectors were removed, low quality sequence repeats were masked and the resulting sequences subjected to clustering and contig assembly using semi-rigid parameters (CAP3=80%, 50 base pair open reading frame). The resulting consensus sequences (contigs) and single ESTs (singlets/singletons) were subjected to blasting, mapping and annotation by a local installation of Blast2Go (http://www.blast2go.org/) using default parameters with minor modifications (Tab. 1). Further, sequences were blasted against the NCBI non-

redundant database using blastX (EV=1.0E-3, min. 20 hits) and blast results complemented with a blastX against the Swissprot protein database. Sequences with blast hits were mapped against the Blast2Go database and resulting mapped sequences annotated in a sequential manner according to decreasing stringency using cut-off values of 1) EV=1.0E-6, cut-off: 55, HSP coverage cut-off: 75; 2) EV=1.0E-6, cut-off: 55, HSP coverage cut-off: 0 and 3) EV=1.0E-6, cut-off: 60, HSP coverage cut-off: 0, Evidence code weight: ISS=1.0, IEA=1.0). Gene ontology (GO) terms were enriched by merging Interpro annotations to existing GOs and the GOs augmented by ANNEX before removal of the first level annotations. The resulting 31k sequences were subjected for probe design using subjected to 60-mer probe production by means of a Best Probe algorithm (BP probes: one probe that best represent the 3'-end sequence) and a Best Distribution algorithm (BD probes: 3 probes that are distributed along the 3'-end of the sequence) eArray (https://earray.chem.agilent.com/earray/). The resulting 105k oligo arrays were constructed by the combination of BP probes (2-3 replicates) and BD probes (no replication), where only the BP probes were used for the current work. The BD probes were included primarily to evaluate the probe production algorithms and had low value for assessing biological effects.

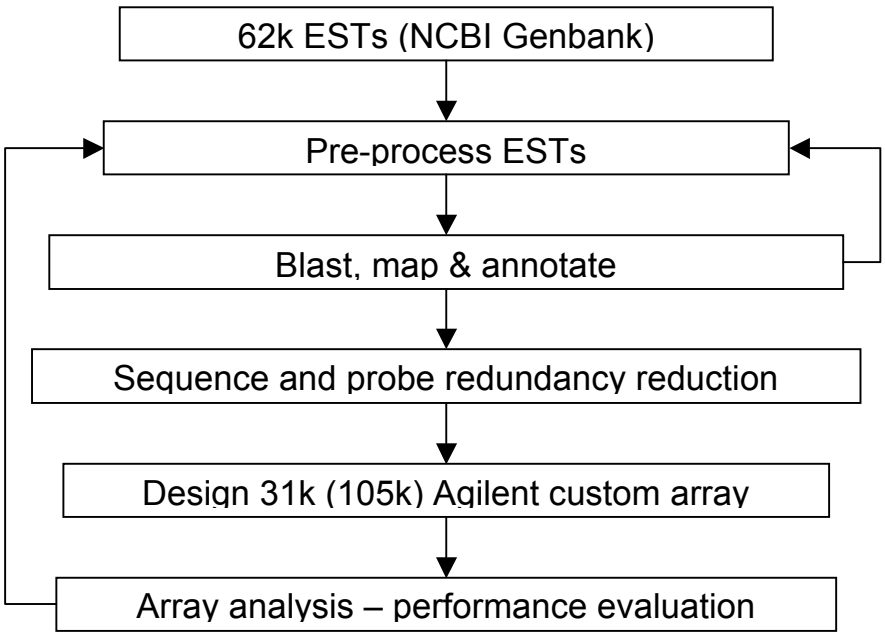


Figure 2. Overview of the microarray design and evaluation process of the 31k *Mytils sp.* microarrray

Table 1 The distribution of singletons, contigs and annotated sequences for each of the four species used for probe design on the 31k Mytilus Sp. Oligoarray.The source EST sequences from NCBI (March 2009) is also provided.

Specie	ESTs* (Genbank)	Singeltons (Array)	Contigs (Array)	Total (Array)	Annotated (Array)
M. edulis	4887	2621	450	3071	463
M. californianus	42355	14540	7439	21979	5659
M. galloprovincialis	14645	3986	1859	5845	1890
M. Trossulus	503	51	20	71	27
Total	62390	21198	9768	30966	8039

* NCBI Genbank ESTs available March 2009

2.2 Exposure studies

The exposure studies where performed in cooperation with Steven Brooks, NIVA. Mussels (*Mytilus edulis*) were collected from an intertidal population located near the NIVA Marine Research Station, Solbergstrand (NIVA-MRS, Norway). The mussels were placed in 50 L tanks of flow-through filtered (10 μm) seawater at NIVA-MRS. The seawater was taken from a 60 m depth from the outer Oslo fjord. Mussels were allowed to acclimate in the laboratory for approximately 2 weeks prior to the exposure experiments.

The exposure studies were conducted in June 2008. Groups of ten mussels were exposed for 7 days to waterborne concentrations of Benzo[a]pyrene (1, 10 and 50 μg/L) and copper chloride(10, 100 and 320 μg/L). In the case of BaP, acetone was added (0.0005% final exposure concentration) to enhance the dissolution of BaP in the seawater.

The seawater was replenished with freshly made solutions on day 3 of the 7 day exposure. The seawater was continually aerated throughout the experiment. The mussels were not fed during the exposure period. Physical parameters (temperature range:10.6 - 10.9 ^{o}C, pH: ~8.0, salinity ~34 ‰, dissolved oxygen 8.5 - 9.9 mg/L) were measured at the start and end of the exposure (Table 5, Appendix).

At the end of the 7 day exposure, the mussels were sampled and individual length and weight (after removal of excess internal water and external debris) determined. Digestive gland (following removal of the stylus) and gill tissue from five mussels

from each exposure group were sampled, the tissue was snap frozen in liquid nitrogen and stored at -80^{o}C until further processing.

2.3 Chemicals, reagents and solutions

All chemicals, reagents and solutions used in the study and analysis were of highest quality and a detailed list is provided in the Appendix, Table 1.

Benzo[a]Pyrene exposure solution:

A 10 g/L stock of Benzo[a]Pyrene (Sigma) containing acetone was diluted with filtered seawater to achieve exposure concentrations of 1, 10 and 50 µg/L. (The concentration of acetone in the exposure water was 0.0005%)

Cu exposure solution:

A 100 mg/L Cu Cl_2 (Sigma) working stock solution was prepared with distilled water and diluted with filtered seawater to achieve exposure concentrations 10, 100 and 320 µg/L.

Lithium chloride solution:

8 M lithium chloride in distilled water

Ethanol:

75% ethanol in distilled water

2.4 Sample preparation and microarray analysis

An oligonucleotide array contains probes consisting 1000's of pre-selected cDNA sequences each consisting of 60 nucleotides. For the RNA samples to be hybridized to the probes on the microarray chip it has to be synthesized to cRNA by reverse transcriptase and labelled with fluorescence. The intensity of each spot in the microarray determines the abundance of target cRNA hybridized to the cDNA probes. Fluorescent cRNA are generated by T7 RNA polymerase that simultaneously amplifies target material and incorporates cyanine3-labeled Cytidine triphosphate

(CTP) before hybridization of labelled cRNA to the microarray. Positive RNA controls was added to the samples designed to anneal to a complementary probe set on the array. Arrays were scanned and raw fluorescence data further processed to obtain meaningful biological data.

2.1.1 RNA extraction

Total RNA was isolated from *M. edulis* digestive gland tissue by a improved Guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi (1987). Frozen tissue (50-100 mg) was homogenized in 1.5 mL centrifugation tubes (Nucn, Roskilde, Denmark) containing ~8 single precellys 1.4 mm ceramic (zirconium oxide) beads (Bertin technologies, Saint Quentin en Yvelines Cedex, France) and 1 mL TRIzol® by use of a Precellys24 homogenizer (Bertin technologies). To permit complete dissociation of nucleoprotein complexes, the homogenized samples where incubated at room temperature for 15 minutes. Chloroform (0.2 mL) was added and the tubes shaken vigorously by hand (15 seconds) before incubation at room temperature for 2-3 minutes. The samples where centrifuged with a bench top centrifuge (Thermo Electron Corporation, Osterode, Germany) at 12 000g for 10 minutes at 4°C. The centrifugation separated the mixture into three phases: a lower red organic phase (protein, cell debris, phenol and chloroform), a white interphase where DNA was contained and a colourless upper aqueous phase containing RNA. The RNA-containing aqueous phase was transferred to fresh 1.5 mL eppendorf tubes. Isopropanol (0.5 mL) was added to precipitate RNA from the aqueous phase. The tubes where flicked gently and incubated at room temperature for 10 minutes before centrifugation at 12 000g and 4°C for 10 minutes. After centrifugation the precipitated RNA formed a gel like pellet at the bottom of the tube. The supernatant was discarded from the tube and the pellet washed in 1 mL 75 % ethanol. Samples were vortexed and centrifuged at 7 500g in 4°C for 5 minutes. All ethanol was discarded and the pellets were air-dried (5-10 min.) to allow complete removal of ethanol. Pellets were resuspended in 70-300 µL nuclease free water by pipetting and incubation at 70°C for 5 minutes with lids open. The resuspended RNA samples was immediately put on ice after incubation and stored at -80°C until further processing.

2.1.2 Purification of isolated RNA by LiCl

RNA was purified by a LiCl method modified from Cathala et al. (Cathala et al. 1983). Isolated RNA (minimum 1µg/mL) samples were added 0.2 volumes of 8 M lithium chloride solution (in Nuclease free water) to precipitate pure RNA. After a brief vortex and incubation at -20°C for 30 minutes, the samples where centrifuged at 16 000g for 20 minutes at 4°C. The supernatant was discarded from the tube and the pellet washed in 1 mL 75 % ethanol by vortexing the samples vigorously. All ethanol was discarded and the pellets were air-dried (5-10) to let any remaining ethanol evaporate. Pellets were re-suspended in 70-300 µL nuclease free water by pipetting up and down and incubation (minimizes risk of ethanol contamination) at 70°C for 5 minutes. Samples were immediately put on ice after incubation and stored at -80°C until further processing.

2.1.3 Column clean up of isolated RNA and RNA from enzymatic reactions

All samples were purified with Qiagen RNeasy Mini Kit (Quiagen, West Sussex, UK) with some practical modifications. Samples with a maximum of 100 µg RNA was adjusted to a volume of 100 µL with Nuclease free water and added 350 µL RTL (guanidine-thiocyanate–containing buffer and ethanol) in 1.5 mL eppendorf tubes. After addition of 250 µL ethanol the samples where mixed well by pipetting up and down and the total volume of 700 µL was transferred to RNeasy mini spin columns (Quiagen, West Sussex, UK) placed in 2 mL collection tube and centrifuged in room temperature for 30 seconds at 8000g. Flow-through from the collection tubes was discarded and 500 µL RPE buffer added to the RNeasy spin column before centrifugation at room temperature for 30 seconds at 8000g to wash the spin column membrane. Flow-through was discarded and the wash step repeated with the same collection tube. A third wash was applied by addition of 500 µL RPE to the RNeasy spin column and the samples was centrifuged at room temperature for 2 min. at 8000g to dry the spin column membrane. The old collection tube was discarded, the RNeasy spin column placed in a new 2 mL collection tube and centrifuged at room temperature for 1 minute at full speed to avoid ethanol and RPE buffer carry-over during the RNA elution. RNeasy spin column tubes lid was left open at room

temperature for 2 minutes to evaporate any residual ethanol and the columns transferred to fresh 1.5 mL eppendorf tubes. Any drops on inside edge surrounding column membrane were removed by pipetting. Nuclease-free water was preheated to 37°C and 30-50 µL added directly to the spin column membrane and incubated 1 minutes at room temperature before centrifugation at 8000g for 1 minute to elute RNA.

2.1.4 Removal of DNA contamination

Pooled samples of isolated and purified RNA was treated with DNAse enzyme by Ambion Turbo DNA-free kit according to manufacturers recommendations (Applied Biosystems, Austin TX, USA) to remove potential DNA contamination. Sample RNA (max. 10 µg RNA in 50 µL) was added 0.1 volume 10x TURBO DNase buffer and 1 µL TURBO DNase and mixed gently before incubation at 37°C for 20-30 minutes. After resuspention of DNase inactivation reagent by flicking or vortexing the tube, 0.1 volume (minimum 2 µL) was added to the DNase treated RNA and the solution and mixed well. Samples was incubated 5 minutes in room temperature whit occasionally mixing by flicking the tube before centrifugation at 10 000 x g for 1.5 minutes and supernatant containing the purified RNA was carefully transferred to sample tubes.

2.1.5 RNA/cRNA quantification and quality control

RNA quantity and purity was controlled by measuring the absorbance ratios of 260/280 and 260/230 by a Thermo Scientific Nanodrop® ND-1000 UV-Visible Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

In short, the sample loading area (pedestal) was cleaned with a laboratory lens wipe paper (Watman, Mainestone, UK) and instrument blanked and initialized by use of 1-2 µL nuclease-free water. A RNA sample (1-2 µL) was loaded onto the pedestal and measured by the instrument protocol for Nucleic acids. Loading area was cleaned between each measurement. Selected settings in Nanodrop software when measuring RNA was Nucleic Acid with sample type RNA-40 with an output of RNA in ng/ µL.

RNA is considered to be of good quality with a 260/280 ratio above 1.8 and a 260/230 ratio above 2.0 and is used as a cut-off value for sample selection. Lower ratios than this indicateed sample contamination that might influence further downstream enzymatic reactions.

2.1.6 RNA integrity control

Integrity of RNA was determined by use of Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) according to vendor's instructions. Reagents where equilibrated to room temperature in 30 minutes before use whereas RNA Samples where kept on ice until use. The RNA 6000 Nano gel matrix (550 µL) was centrifuged in a spin filter for 10 minutes at 1500g at room temperature before distributed into aliquots of 65 µL and stored at 4°C. In essence, dye concentrate was vortexed for 10 seconds and spun down and 1 µL of dye added to a 65 µL aliquot of filtered gel. A Gel-dye mix was vortexed and centrifuged at 13000g for 10 minutes at room temperature. The RNA 6000 Nano chip where loaded with x ul gel-dye mix, RNA 6000 Nano marker, RNA samples and pre-denaturated nucleic acid standards (ladder). After vortexing the chip in a Mini Shaker (IKA Works, Wilmington, USA) for 1 minute at 2400 rpm the chip were run directly in the Agilent 2100 bioanalyzer using the Agilent bioanalyzer 2100 Expert Software. The RNA integrity was determined by visual evaluation of resulting eletropherogram.

2.1.7 RNA amplification and labelling

Microarray analysis was performed by the Agilent one-colour protocol (Agilent technologies) according to manufacturers instructions. Agilent Gene Expression hybridization kit (Agilent technologies, Cedar Creek, TX, USA) was used for labelling and amplification of cRNA. One-Colour Spike-Mix were prepared by mixing One-Colour Spike-Mix by vortexing, then heating at 37°C before a second vortexing and a brief centrifuging. Agilent One-Colour Spike-Mix Stock (2 µL) was added to 38 µL of Dilution buffer before vortexing and spin down and first dilution (2 µL) was added to 48 µL of dilution buffer to make a second dilution. Second dilution

was mixed by vortexing and given a spin down before 2 µL was added 36 µL Dilution buffer and mixed by vortexing to make a Third dilution.

In short, good quality samples (n=X-X) of equal amounts of RNA were pooled in groups of 3, representing each treatment group and control groups. Synthesis of cDNA were performed by bringing total RNA (200 ng), T7 promoter primer (1,2 µL) and third dilution Spike-Mix (2 µL) to a total volume of 11,5 µL by adding nuclease-free water followed by incubation at 65°C for 5 minutes and then placed immediately on ice. Each sample was added 5X First Strand Buffer (4 µL, pre heated at 80°C for 4 min.), 0.1 M DTT (2 µL), 10 mM dNTP mix (1 µL), MMLV-RT (1 µL), RNase Out (0,5 µL). cDNA Samples was incubated for 2 hours at 40 °C followed 15 minutes at 65°C in a PCR Eppendorf Mastercycler gradient (Eppendorf, Hamburg, Germany) and moved directly to ice. To perform cRNA synthesis and Cy-3 labelling each sample where added a total volume of 60 µL Transcription Master mix and incubated at 40°C for 2 hours. Volume of reagents per reaction of mastermix: Nuclease-free water (15.3 µL), 4X Transcription buffer (20 µL), 0.1 M DTT (6 µL), NTP mix (8 µL), 50 % PEG (6.4 µL pre-warmed at 40°C for 1 min.) RNaseOUT (0.5 µL), Inorganic pyrophosphatase (0.6 µL), T7 RNA Polymerase (0.8 µL) and Cyanine 3-CTP (2.4 µL).

Purification of labelled cRNA was performed essentially as described previously (section 2.4.3) and purified cRNA was eluted in 30 µL of nuclease-free water and further quantification of labelled cRNA was performed according to section (2.4.5). Yield and Specific activity was calculated by use of Cyanine 3 dye concentration (pmol/µL), RNA absorbance ratio (260 nm/280 nm) and cRNA concentration (ng/µL). Labelled and incorporated where measured by settings: Microarray, sample type RNA-40, one colour and colour cy3, with an output of pmol/µL cy3. Specific activity of Cy3 and cRNA yield needed to be of a sufficient level to proceed to hybridization step; Yield >1.65 µg and Specific activity > 9.0 pmol Cy3 per µg cRNA. Yield and Specific activity was calculated by use of Cyanine 3 dye concentration (pmol/µL), RNA absorbance ratio (260 nm/280 nm) and cRNA concentration (ng/µL).

Yield:

(*cRNA concentration*) * 30 µL (*elution volume*) / 1000 = µg cRNA

Specific activity:

(*Concentration of Cy3*)/ (*cRNA concentration*) * 1000 = pmol Cy3 per µg cRNA

2.1.8 Hybridization

Agilent Lyophilized 10X Blocking Agent was added 500 µL nuclease-free water, mixed gently by vortexing and centrifuged for 5-10 seconds. Labelled cRNA samples (1.65 µg) were added diluted 10X Blocking Agent (25 µL), 25X Fragmentation Buffer (5 µL) and brought to a total volume of 120 µL with nuclease-free water before incubation at 60°C for 30 minutes to fragment the cRNA. The fragmentation reaction was stopped by adding 125 µL 2X GEx Hybridisation Buffer HI-RPM to the cRNA Fragmentation mix and mixed gently by pipetting before 1 minute centrifuging at room temperature at 13000 rpm. Samples was placed on ice and 120 µL of the hybridisation mix where immediately loaded on to the microarray placed in a Agilent hybridisation chambers, and hybridized in a rotor Shel Lab Microarray hybridization oven (Sheldon Manufacturing Inc., Oregon, USA) for 17 hours at 65°C.

Gene expression wash buffer 1 & 2 where added 0.005 % Triton X-102. Staining dishes, racks and stir bars where washed in Agilent stabilization and drying solution with acetonitrile and additional wash with Milli-Q water. Array-gasket sandwich was removed from the hybridization chamber after 17 hours (exact time were noted) and slides assembled in a staining dish with pre-heated (17h, 37°C) Gene Expression (GE) wash buffer 1 at room temperature. Array slide was transferred to a rack in new dish containing GE wash buffer for 1 minute with magnetic stirring (setting 4) before the slide rack was placed in dish with GE wash buffer 2 (pre-heated 17 ours, 37°C) for 1 minute with magnetic stirring (setting 4). The rack was applied in Agilent stabilization and drying solution 5 minutes. Slides were placed in Agilent slide holders and immediately scanned in an Agilent DNA microarray scanner (Agilent Technologies, Santa Clara, CA, USA) at 5 µm resolution. Image analysis (grid placement, spot intensity and ratio determination, outlier flagging and overall

scanning quality assessment) was conducted by Agilent Feature extraction (FE) version 10.2 (agilent Technologies).

2.1.9 Biostatistics and bioinformatics

Raw signal data text files of the scanned images was imported, spot normalized and outlayers flagged with the software Genespring GX 10.0.2 (Agilent technologies) using a 25 Quantile algorithm and baseline transformation to median of all samples. Quality control of the features was performed based on a statistic Quality control Metrics-Report from FE and features were filtered on expression (20-100%) and outlier flagged after importing to Genespring GX. Due to the limited sample analysis design with lack of replication, fold change was calculated for each individual features in the exposed group compared to their respective controls (Fold change = normalized intensities (exposed)/ normalized intensities (control)) in Genespring GX. A cut-off for further assessment of gene expression and bioinformatics were set to fold change >2.0. All features above the 2-fold cut off were subjected to functional enrichment analyzes by FatiScan (single gen enrichment analysis) and FatiGo (gene-set analyses) (http://www.babelomics.org/). GO terms were further analyzed and visualized by Blast2GO (http://www.blast2go.org/), a tool for functional annotation of (novel) sequences and analysis of annotation data.

3 Results

3.1 RNA quality

Purity and quantity of the RNA samples was evaluated by the 260/280 nm and 260/230 absorbance ratios obtained from NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). Ratios of all samples were above quality cut off and accepted for further use with microarray analysis. RNA integrity was analyzed by Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and visually evaluated. A sharp single peak (or one sharp band in the gel-like image) in the region expected to contain ribosomal RNA18S were observed in all samples (Fig. 3) Table containing results of RNA quality control of all samples are found in Appendix, Tab.2.

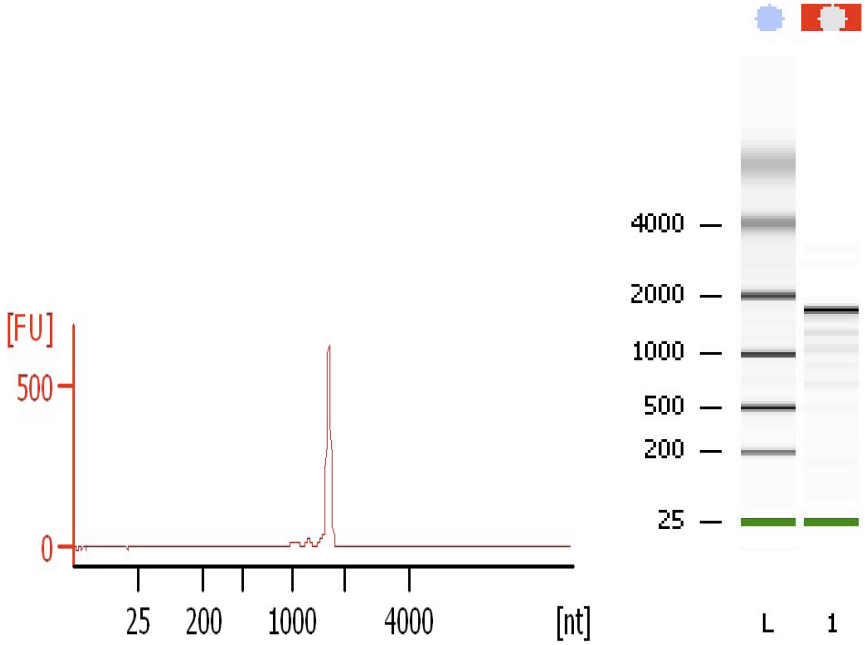


Figure 3. Bioanalyzer electropherogram (left) and gel image (right) of RNA from one representative sample. One large peak in the electropherogram and one distinctive band (number 2 from right) in the gel image indicate RNA of good quality.

Quality control of cRNA and incorporation of Cy3 were performed by Nanodrop, results shown in Appendix, Tab.3. All samples were within recommended quality cut-off (cRNA Yield of 1500 ng and an incorporation of Cy3 > 9 mol/µg cRNA) and accepted for further use with microarray analysis.

3.2 Exposure studies

The exposure study was designed by NIVA and was conducted at NIVA-MFS January 2009. There were two parallel experiment were groups of 10 mussels of the specie *M. edulis* were exposed to waterborne solutions of BaP and $CuCl_2$ for 7 days. The mussels seemed healthy and did not show any signs of reduced or different filtration activity in the different exposure tanks. Corresponding observation were observed when sampling the mussels, all animals closing their shells (valves) exposed to movement. There was no significant difference in Shell width or length (Fig. 4). Only 5 of the 10 mussels of each group were sampled for this experiment (Tab. 6, Appendix).

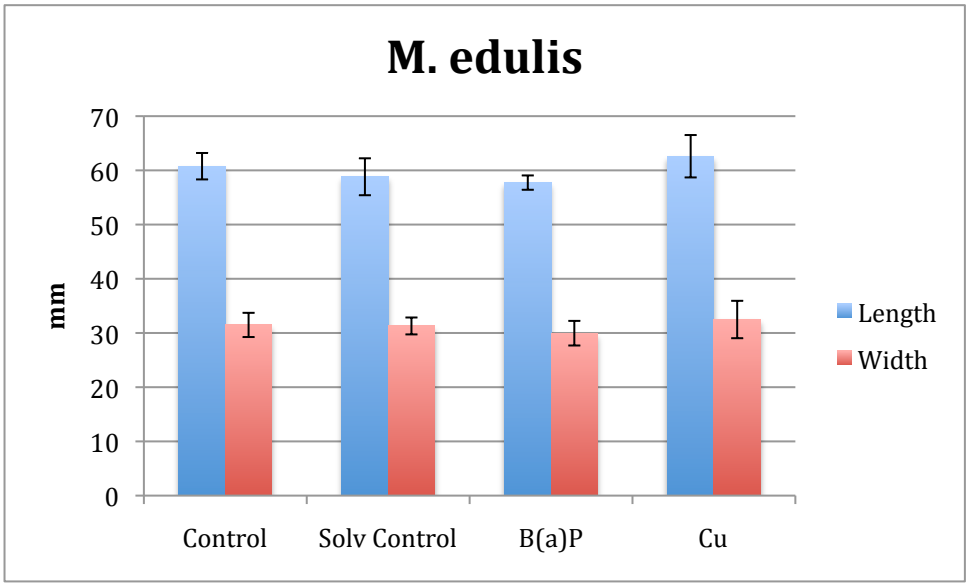


Figure 4. Size of mussels in exposure study. Shown is Average±std of length and width of mussels after 7 days of exposure to BaP, $CuCl_2$ and control groups. There were 5 mussels in each group.

3.3 Global gene expression

Due to the amount of data generated by microarray analyses, all microarray data from this project are available in the database of Norwegian Institute of Water Research, Oslo, Norway. Project number 27279, Geniom.

After 7 days of exposure to copper and BaP, changes in the gene expression between exposed mussels and control groups were evaluated. Of totally 30966 features on the

Mytilus sp. Arrays, 54 % of all features where found to be of sufficient quality for being subjected to further bioinformatics analysis after normalisation and filtering for filtration on outlayers (flags) expression (Table 4, Appendix). Control normalised gene expression in the digestive gland was found to vary considerable with fold changes ranging from no change to over 100 000 times difference between exposed and control animals. Only 3 % to 4 % of the features were modulated more than a 10-fold compared to the control groups, thus suggesting that the majority of genes were not affected or only moderate affected by the treatments. Application of a fold cut-off of 2, assumed to discriminate genes being affected from those being marginally affected by the treatments, led to reduction of number of "relevant" features to 6305 for BaP (3174 being up-regulated and 3130 being down-regulated) and 5009 for Cu (2500 being up-regulated and 2509 being down-regulated), respectively.

Cross-hybridisation potential

The hybridization success (percentage of features passing the filtration and normalisation steps) of the cDNA from *M. edulis* to the probes originating from the different *Mytilus* species seemed to vary considerably (Fig. 5 and Fig. 6). The hybridisation success to probes from *M. edulis* and *M. galloprovinciales* was 70 % (Cu) and 85 % (BaP), whereas *M. californianus* probes apparently displayed a lower hybridisation with 44 % (Cu) and 43% (BaP) success. *M. trossulus* probes had an intermediate hybridisation with 56 % (Cu) and 58 % (BaP) success. The pattern of hybridization success of features assumed to be biologically relevant (e.g. 2-fold regulated features) showed the same trend, although with a lower absolute hybridization success, as expected (Fig. 3.2 and Fig. 3.3).

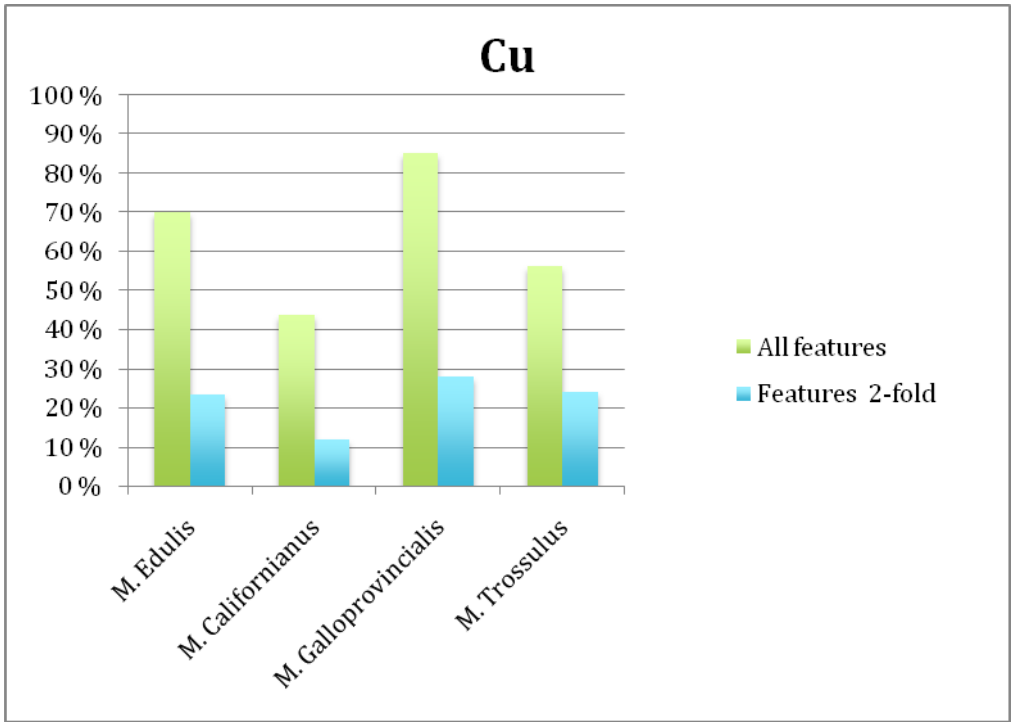


Figure 5. Hybridization success of Cu exposed samples. Shown are the percentage of all features and 2-fold regulated features with successful hybridization to the microarray distributed on the four *Mytilus* species represented in the array.

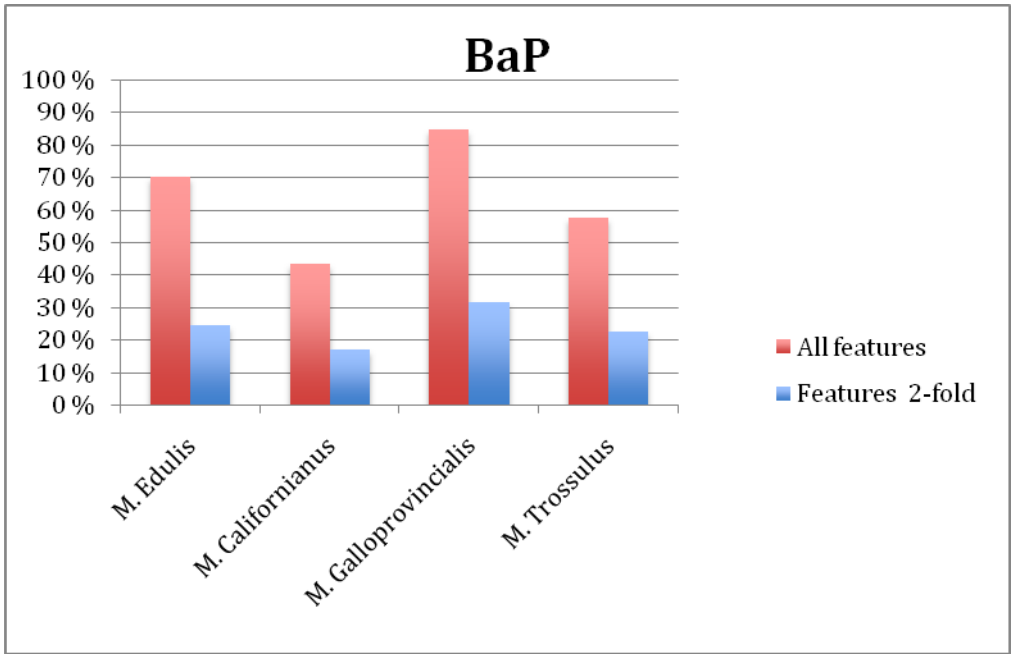


Figure 6. Hybridization success of BaP exposed samples. Shown are the percentages of all features and 2-fold regulated features with successful hybridization to the microarray distributed on the four *Mytilus* species represented in the array.

Although no statistics were applied due to the lack of biological replication, the results suggested that the array worked better with probes from *M. edulis* and *M. galloprovinciales* than with *M. californianus.* Although probes from *M. trossulus* seemed to have a intermediate hybridisation success, the limited number of features (71 sequences) being available for this specie limit the value for inclusion in the data treatment and was therefore discarded from further processing.

Analyses of the microarray data using feature data originating from *M. edulis and M. galloprovinciales* probes showed 2353 (Cu) and 2594 (BaP) features could be considered "biological relevant" (2-fold regulation). Of these features, 1737 was solely regulated by Cu while 1978 by BaP, and 616 of the features caused a change in regulation by both compounds (Fig. 7). About 36 % of the features whose expression were modulated by Cu were also modulated by the BaP exposed group, while 31 % of the features whose expression was modulated by the BaP exposed group were also modulated Cu exposure.

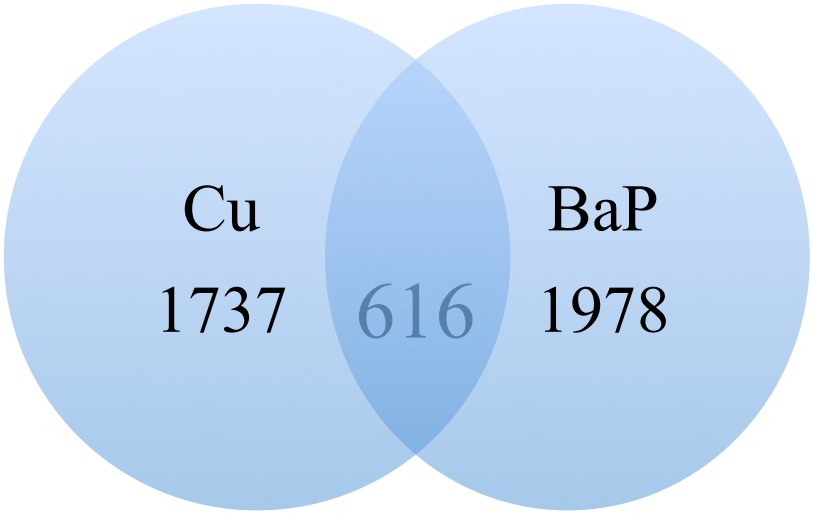


Figure 7. Venn diagram representation of gene expression patterns of *M. edulis* and *M. galloprovincialis* After 7 days of exposure to BaP and Cu. Diagram based on 2-fold regulated features.

Of the biological relevant features that were modulated after the 7 days of copper exposure, 1144 features were up-regulated and 1209 were down-regulated compared to the control group. The fold change of up-regulated features after exposure to Cu was in a range from 2.0 to 5420 times higher than the control while the features being suppressed by the treatment ranged from 2.0 to 7643 times lower gene expression

than that determined in the control group. In a similar way, 1142 features were up-regulated while 1452 were down-regulated in the digestive organ of mussels exposed to BaP compared to the control group. The fold change of up-regulated features after exposure to Cu was in a range from 2.0 to 1370 times higher than the control while the features being suppressed by the treatment ranged from 2.0 to 636 times lower gene expression than that determined in the control.

3.3.1 Functional enrichment analysis

Of all *M. edulis* and *M. galloprovinciales* features being more than 2-fold regulated, 44 % and 41 % of the features modulated by Cu and BaP, respectively, had been assigned a blast hit of predicted protein function (gene name) and were related to a annotated gene. Of this, 695 of Cu and 767 of of the BaP modulated features were assigned one or more GO-terms, which were the basis of further enrichment analysis with FatiGO and Fatiscan.

No features were to found to be significant regulated by FatiGO.
Functional gene set enrichment analysis with Fatiscan ($p < 0.05$) revealed 15 significant enriched GO-terms related to 4 features up regulated in Cu exposed mussels (Tab.2).

Tabel 2. Gene ontology (GO) and genes of significantly enriched GO-terms being more than 2-fold up regulated in the digestive gland of common mussel (*M. edulis)* after 7 days of exposure to 320 µg/L $CuCl_2$.

GO-term	Gene Ontology (GO)		Sequence ID	Adjusted p-value
	Biological process	Molecular Function		
GO:0030246		carbohydrate binding	MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	2.64E-02
GO:0030247		polysaccharide binding	MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	6.43E-03
GO:0008061		chitin binding	MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	2.92E-03
GO:0001871		pattern binding	MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	6.43E-03
GO:0006066	alcohol metabolic process		MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	2.99E-02
GO:0006040	amino sugar metabolic process		MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	2.92E-03
GO:0006041	glucosamine metabolic process		MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	2.92E-03
GO:0006044	N-acetylglucosamine metabolic process		MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	2.92E-03
GO:0006030	chitin metabolic process		MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	2.92E-03
GO:0006022	aminoglycan metabolic process		MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	2.92E-03
GO:0044262	cellular carbohydrate metabolic process		MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	2.64E-02
GO:0005976	polysaccharide metabolic process		MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	8.88E-03
GO:0005975	carbohydrate metabolic process		MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	4.59E-02
GO:0005996	monosaccharide metabolic process		MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563	1.70E-02
GO:0000272	polysaccharide catabolic process		MG_Contig996,MG_Contig995, MG_Contig1145,MG_58306563,	2.77E-02

The results showed an overrepresentation of up-regulated features related to metabolic possesses of chitin and carbohydrates, identifying pathways ranging from general levels of cellular, primary and nitrogen compound metabolic processes down to a more specific processes such asN-acetylgucosamine and chitin metabolism as well as catabolism of polysaccharides (Fig. 8). Interestingly, 4 features were identified as being responsible for this functional GO enrichment and these were all strongly regulated) The were 4 features present in all the significant enriched GO-terms, all 4 features were coding for proteins that binds chitin and all heavily up-regulated (fold change in parentheses): Chitinase 1,MG_Contig996 (1287,6) and MG_Contig 995

(346,7) representing the gene coding for Chitinase 1, MG_1145 (1025,7), and MG_58306563 (869,7) representing the genes coding for eosinophil chemotactic cytokine and chitin binding peritrophin-a domain containing protein, respectively. Chitin consists of polysaccharides, thus the function of binding carbohydrates and polysaccharide as shown in Fig 8.

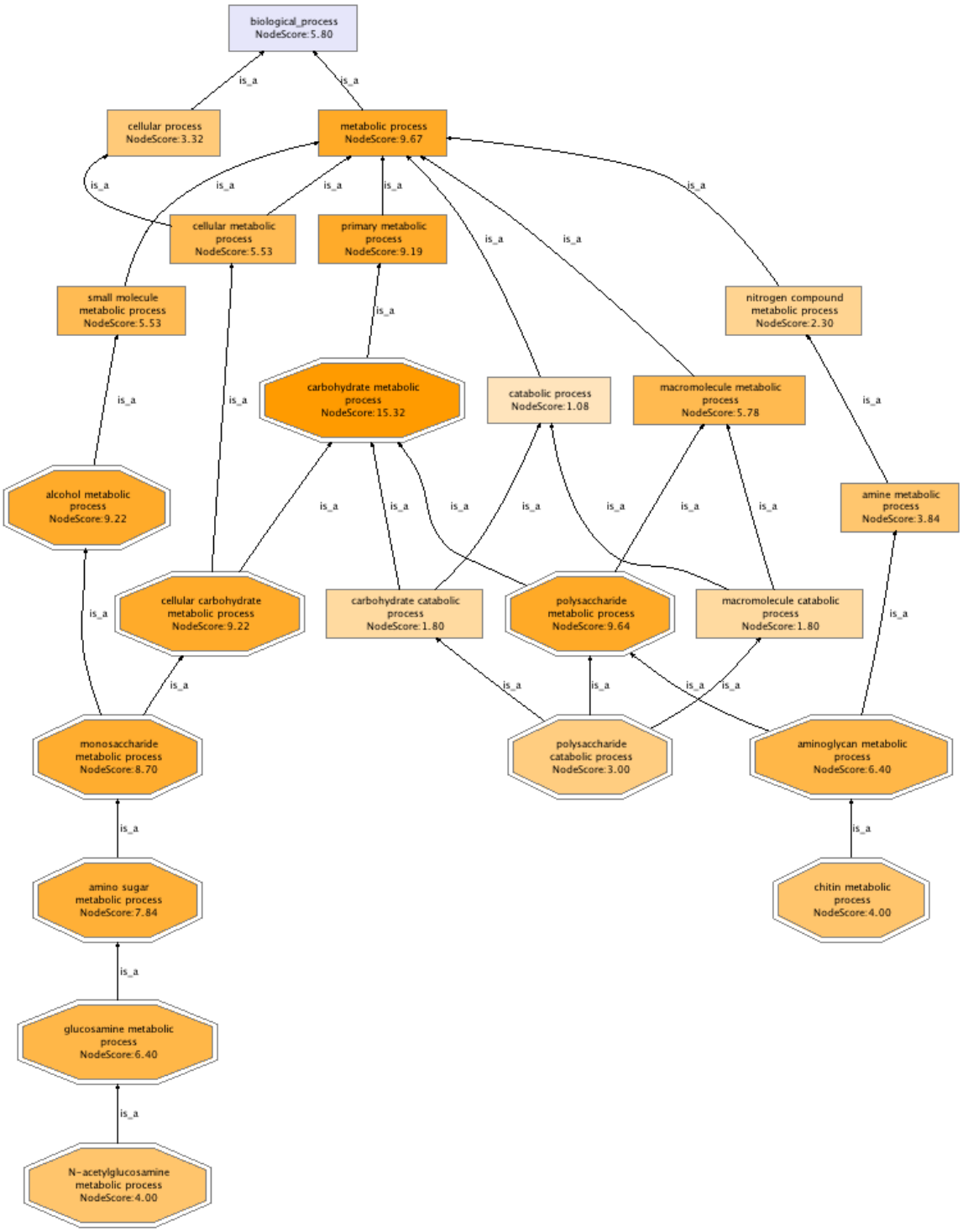


Figure 8. Functional enriched GO-terms of up-regulated biological processes in *M. edulis* digestion gland after 7 days exposure to 320 µg/L $CuCl_2$. Changes in regulation are relative to control group. The node score is based on no. Of sequences and distance to the node where each sequence was annotated.

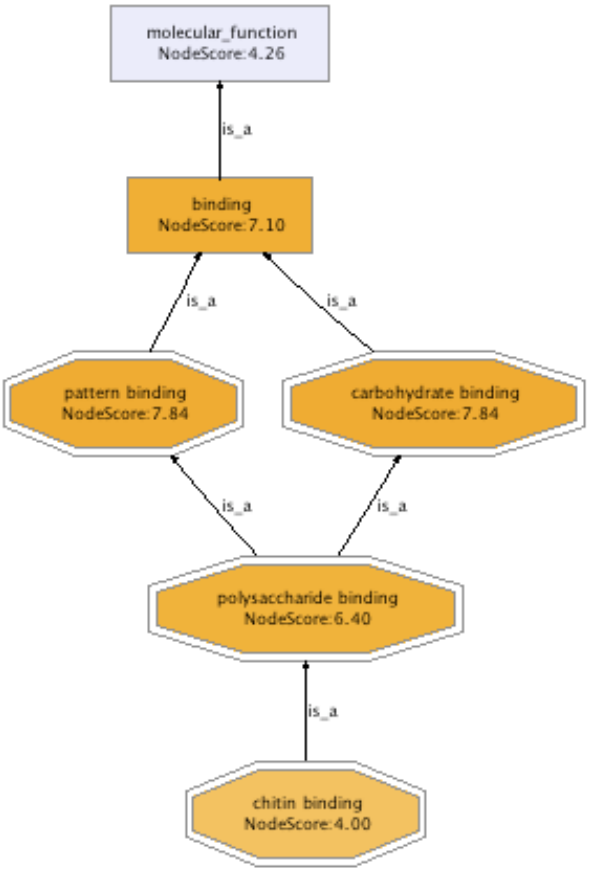


Figure 9. Molecular functions of significantly (0.05) enriched GO-terms for up-regulated in *M. edulis* digestion gland after 7 days exposure to 320 µg/L $CuCl_2$. Changes in regulation are relative to control group. The node score is based on number of sequences converging at one node and penalties by the distance to the node where each sequence was annotated.

No significant enriched GO-terms were identified among the down-regulated genes after Cu exposure, nor any of the genes in both up and down-regulated genes after exposure to BaP.

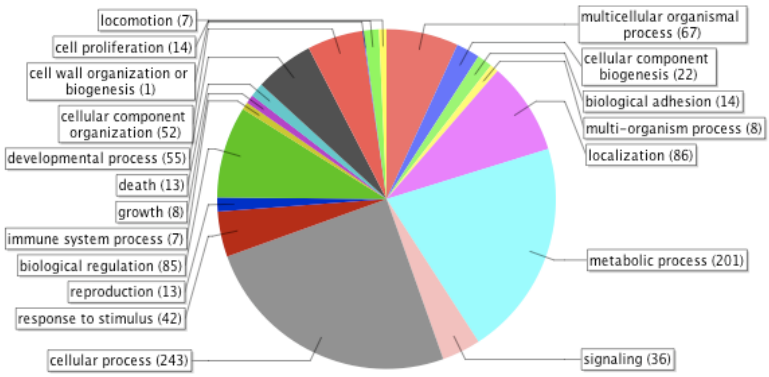
3.3.2 Single genes responses to Cu and BaP

Due to limited direct ecotoxicological information being provided by GO annotations, further assessment of the biological relevance of gene expression in *M. edulis* after Cu and BaP exposure were performed on selections of genes assumed to be of ecotoxicological relevance (Tab. 3). Although not being statistically tested, these genes (features) were all passing the biological relevance criteria (e.g. 2 Fold cut off)

and have previously been associated with toxicological mechanisms in mussels or other organisms.

To broadly illustrate the effects of Cu and BaP on the biological processes in the exposed mussels, genomic analyses of GO-terms associated to biological processes and molecular function of annotated genes being more than 2-fold regulated relative to control groups were applied (Fig 9).

Biological processes modulated by Cu



Biological processes modulated by BaP

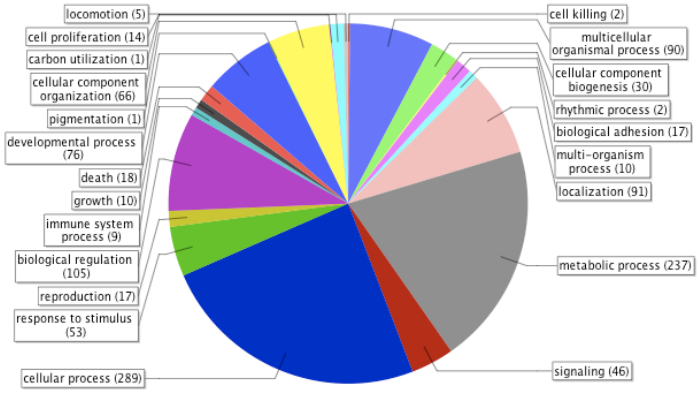


Figure 9. Functional genomics analysis. Shown are GO-terms of biological processes at a general level (level 2) associated to features modulated >2-fold by Cu and BaP exposure. The figures are based on annotated features of *M. edulis* and *M. galloprovinciales*.

The genomic analysis shows that mainly the same biological processes are affected at a general level in the animals exposed both to Cu and BaP, however this analysis does not tell anything about the suppression or induction of the related features. There are some differences in the number of features related to the different biological processes; some of these differences are related to the number of annotated features modulated by the different compounds. There are only in on group of the biological process, locomotion, were Cu exposed mussels are presented with a higher number of modulated features than of the BaP exposed mussels. The major groups of biological processes modulated by both compounds are related to cellular and metabolic process followed by biological regulation, localization and multicellular organismal process. The main differences are the biological processes solely modulated by Cu; cell wall organization, and by BaP; which is cell killing, rhythmic process, carbon utilization and pigmentation.

Tabel 3. A selection of single gene responses being more than 2-fold up regulated in the digestive gland of common mussel (*M. edulis)* after 7 days of exposure to 320 µg/L $CuCl_2$. The corresponding array sequence Id and the Accession number are added to assist identifying the relevant array probes and the Genbank ESTs. Absent Genbank accession nr. is available at NIVA, Oslo, Norway, Project nr.: 27279, Geniom.

Seq. name	Seqence Description	Fold regulation		GenBank Accession
		BaP	Cu	
MG_58305869	abcb p-glyco protein	-7.4	-8.1	AJ623399
ME_Contig 288	Baculoviral iap repeat-containing	4.1	-15.2	AM880313
MG_Contig960	calmodulin	<2	-2.2	-
MG_37650420	calmodulin	-3.9	<2	AJ516842
MG_58306563	chitin binding peritrophin-a domain containing protein	54.9	869.7	AJ624093
MG_Contig995	chitinase 1	17.2	1287.6	AJ625051
ME_Contig308	copper radical oxidase	<2	32.8	AM880297
ME_Contig306	collectin sub-family member 12	<2	- 6.7	-
ME_Contig312	cytochrome b	7.3	-5.7	AM880783
ME_Contig315	cytochrome c oxidase subunit ii	3.2	-22.0	EH663406
ME_164592541	cytochrome oxidase subunit 3	13.8	-36.2	AM881478
ME_164592504	cytochrome p450	-2.4	<2	AM878907
MG_Contig1082	defensin a	328.7	-42.4	EH662768
MG_58307352	ferritin	<2	3.8	AJ624882
MG_223028087	glutathione s-transferase	5.3	-8.5	FL494071
MG_223023156	heat shock 70kda protein 12a	7.0	-2.6	FL490944
MG_Contig1326	lysozyme	<2	6.8	-
ME_Contig362	metallothionein	2.0	<2	-
ME_164593616	monooxygenase dbh-like 2	-9.6	13.4	AM878983
MG_154349819	myticin c precursor	<2	108.7	EH663368
MG_Contig1404	myticin c precursor	9.9	<2	EH662822
MG_154348989	mytilin d precursor	-2.3	-8.7	EH662538
ME_164566584	nadh dehydrogenase subunit 1	18.8	-11.5	AM881784
MG_58307396	small heat shock protein p26	<2	5.2	AJ624926
MG_Contig1739	superoxide dismutase	-7.6	2.7	-
ME_Contig444	tyrosine-protein kinase src64b	3.9	- 13.1	AM879528
MG_Contig1835	vitelline envelope zona pellucida domain 6	-6.3	4.4	-

As earlier presented in Fig. 8 more than 30 % of >2-fold modulated features were affected in both Cu and BaP exposed mussels. This is reflected both in Fig. 8 were the majority of GO-terms were modulated in a similar manner by both compounds, and in Tab. 3 representing the selection of features coding for genes of ecotoxicological relevance. Being selected by their ecotoxicological relevance, the features in the list are related to biological processes involved in metabolism immune system, response to stimulus, development and reproduction (Related GO-terms available in data depository, NIVA, Oslo, Norway. Project nr.: 27279 Geniom).

The majority of the features in Tab. 3 are both modulated in mussels exposed to Cu and BaP, suggesting that most of these genes are related to general stress or defence reactions to toxicity. Of totally 27 features, 12 features were found to be involved in responses related to metabolic processes, Four of these features were related to mitochondrial electron transport and energy generation: Cytochrome c oxidase subunit ii, cytochrome oxidase subunit 3, cytochrome b, and nadh dehydrogenase subunit 1, all found to be down regulated in Cu exposed mussels and up regulated in BaP exposed mussels, indicating a general change in energy production in different directions related to the exposure of different compounds. Further there were 5 features related oxidorecutase activity: Superoxide dismutase that was related to metabolism of ROS, cytochrome p450 which known of phase I metabolism of xenobiotics, while ferritin, metallothionein and monooxygenase dbh-like 2 were related to metal binding. Both Superoxid dismutase and cytochrome p450 was down regulated by BaP, while Superoxid dismutase were down regulated by Cu and cytochrome p450 was absent from >2-fold features modulated by Cu. Ferretin and monooxygenase were found being down regulated by Cu, while monooxygenase dbh-like 2 was down regulated by BaP and ferretin was absent in >2-fold regulated features modulated by BaP. Metallothionein were slightly up-regulated by BaP, while absent in Cu exposed mussels. Further, Glutathion s-transferase, a phase II enzyme of xenobiotics, were up-regulated by BaP and down regulated by Cu. Features related to chitin and carbohydrate metabolism showed the strongest response amongst listed features, represented by chitinbinding peritrophi-a domain containing protein and chitinase 1 both heavily up regulated in both Cu and BaP exposed animals.

Of totally 10 features involved in immune system and response to stimulus, 4 of these features were absent in BaP exposed mussels. Of defensin a precursor, mytilin d precursor, myticin c precursor and lysosyme, being microbial peptides in the humoral immune system, defensin a and mytilin d were down-regulated by Cu exposure, while the later were up-regulated. Defensin were heavily up-regulated in BaP exposed mussels, as well as an more moderate up-regulation of myticin c, while mytilin d were slightly down-regulated. Further, Calmodulin, tyrosine-protein kinase src64b, collectin sub-family member 12, Caculoviral iap repeat-containing, all involved in activation and regulation of the humoral immune system, were all suppressed by Cu exposure, while baculoviral iap repeat-containing and tyrosin-protein kinase src64b were induced by BaP.

Small heat shock protein p26 and Heat shock 70dka protein 12 a, related to stress response, were both modulated by Cu, 5.2 and -2.6 fold, respectively, while only Heat Small heat shock protein p26 a (5.2 fold) were regulated by BaP. Abcb p-glycoprotein related to multidrug resistance were suppressed by both Cu and Bap, while Vitelline envelope zona pellucida domain 6, a protein involved in reproduction, were up-regulated in BaP exposed animals and down regulated in Cu exposed animals.

4 Discussion

Natural contamination and anthropogenic pollution of marine environment are likely to have major impact on function and survival of marine wildlife. The marine mollusc and key specie *Mytilus sp.* have been extensively used to monitor marine pollution. It has both evolutionary adapted and genetically programmed response to toxic chemicals, but the research of and the understanding of these mechanisms is deficient especially when it comes to sub lethal effects. This prompts the need for developing of biomarkers of exposure and effect along with a general understanding of the molecular and physiological effects of marine contamination and pollution. High-content screening approaches such as provided by microarray analysis is used both for genetic and genomic purposes, but the advantage and strength of the method lays in the ability to study relationships between features and discover functional groups, hence the focus on functional pathways in this study. This focus gives a major advance considering that expression of individual genes may vary from case to case and that alterations in pathways where these features are involved usually are consistent.

The aim of this project was to evaluate the performance of a newly developed high-content *Mytilus sp.* oligonucleotide array (oligoarray) and to assess the transcriptional effects in *M. edulis* digestive organ after exposure to the pollutants Cu and BaP. The results showed that an oligoarray based on *M. edilus* and *M. galloprovinciales* could be used to study the differential expression in *M. edulis* digestive gland after exposure to Cu and BaP. There were also indentified different MoA for Cu and BaP, as well as several genes and pathways related to general stress responses of toxicity for both compounds.

As this experiment was a pilot study with restricted finances and with the purpose to develop a first generation *Mytilus sp.* mussel microarray there were no biological replicates in the experiment and thus no significance analyses were run. However, the model compounds used in this experiment have many known effect mechanisms described and verified by available literature comparable to this experiment. The use of pooled samples can reduce statistical power, but it gives less effect of possible outliers (Cooper et al. 2007)

4.1 Microarray analysis

Features of four species of *Mytilus sp*. were implemented in the array design to provide broad gene coverage and to evaluate the cross-species hybridization potential between the *M. edulis* and the three other species. The microarray design was based on a total of 30966 EST and Contigs distributed on all four *Mytilus* species. Overall, 74 % of the nucleotide sequences in the array showed no or poor identity with publicly available sequences and are undiscovered transcripts of *Mytilus sp.* genes with possibility for future genomic research and functional studies. However we realise that some of these ESTs and contigs are short and might interference with the 3'-untranslated regions of the transcripts and therefore it is not possible to reveal their identity by sequence similarity comparison as observed in similar studies of molluscs (Tanguy et al. 2007; Venier et al. 2009). The variation in number of annotated sequences amongst the *Mytilus* species was apparent, reflecting the genomic research efforts of the species: *M. edulis* 15 %, *M. californianus* 26 %*, and M. galloprovinciales* 32 %. For *M. trossilus* over 38 % of the sequences were annotated, but there were only 71 sequences available for inclusion on the array. These results indicate that there is need of a redundancy reduction and further evaluation of the probes to increase the quality of the array.

The global hybridization success of the array was 54 %, which suggests that the differences in hybridization quality between the arrays were minimal. However, the hybridisation success of the total number of probes on the array seemed to be highly influenced by differences in hybridization quality between the cRNA from *M. edulis* digestive gland and the probes of the four species on the arrays, showing a variation from 43 % for *M. californianus* and 85 % for *M. galloprovincialis.* The results suggested that the array worked well and the hybridization success were good between *M. edulis and M. galloprovinciales*. This result is supported by studies of mitochondrial DNA in *Mytilus sp.* by hoeh *et al.* (1997) revealing that these two species were most similar amongst the four *Mytilus* species. Further the results indicated that the hybridisation between *M. edulis cDNA* and probes originating from *M. Californianus* EST sequences were probably too low to be suitable for use in analysis of M. edulis expression analysis. This is consistent with findings that *M.*

Californianus mitochondrial DNA and 18S rRNA exhibit the most divergent genome of the four *Mytilus* species. The fact that *M. californianus* were not suitable for hybridization with *M. edilus* seem to have had major influence of the total hybridization success of the mircorarrays. *M. californianus* represented 71 % of the probes and the low hybridisation success of *M. californianus* features highly influences the overall score of 54 % probes above quality analysis threshold. This can be illustrated by comparing *M. edulis and M. galloprovinciales* with a total hybridisation success of 80 %. Even though *M. trossulus* dispayed comparable cross-hybridization potential as *M.edulis* and *M. galloprovinciales*, supported by genetic studies indicating similarities between these species (Borsa et al. 1999; Riginos & Cunningham 2005), the availability of only 71 singletons and contigs did not provide sufficient grounds for including them in the data treatment.

The pattern of hybridization success showed the same trend for the 2-fold regulated features for both Cu and BaP exposed mussels, although with lower absolute hybridization rate as expected.

Further analyses of differently regulated features of *M. edulis* and *M. galloprovinciales* shows that Copper and BaP have selective MoA, clearly illustrated by being only 23 % of total 2353 (Cu) and 28 % of totally 2594 (BaP) differentially regulated features (more than 2-fold). This is in agreement with studies of transcriptomic and metabolic of the earthworm *Lumbricus rubellus* response to Cu, where a 2-fold cut of was used to provide a more complete representation of functional group effects (Bundy et al. 2007). In addition MoA of BaP and Cu were clearly difference by only 30 % common genes being regulated by both treatments.

4.1.1 Functional enrichment analysis

Functional enrichment analysis with FatiGO did not give any significant over-representation of single genes. FatiGO analyses the significant over-representation of functional annotations by single gene enrichment analysis ranging two lists of genes against eachother. The lack of assigned GO-terms to the features is the reasons of this result and is discussed later in this section.

Functional enrichment analysis revealed 15 significant enriched ($p<0.05$) GO-terms by the *M. edulis* and *M. galloprovinciales* features up regulated in mussels exposed to Cu. The enriched GO-terms were related to biological processes of chitin and N-acetylglucosamine metabolism as well as catabolism of polysaccharides, and their molecular functions of carbohydrate and chitin binding. Chitin, which is a polysaccharide consisting of monomers identified as N-Acetyl-glucosamine, is a key structural component in mollusc shell formation. The previous data suggest that Cu affect chitin and carbohydrate metabolism and have previously been linked to exposure to metals in mussels and other invertebrates (Dondero et al. 2010; Poynton et al. 2006). Regulation of the same group of features gave rise to functional enrichment of in all GO-terms, pointing out the genes chitinase1, eosinophil chemotactic cytokine and chitin binding periotrophin-a domain containing protein as major target for the toxic effects of Cu in *M. edulis.* Chitinase, which is vital in the digestion of endogenous cytoskeleton chitin (Furuhashi et al. 2009) as well as in exogenous feed from certain types of zooplankton, unicellular fungi and algae (Birkbeck & McHenery 1984). The pathways of the biological processes and molecular functions involved, indicates that Cu is bound to chitin or to polysaccharides in the cytoskeleton, which is degenerated by chitinase enzymes and removed. This will further increase the availability of carbohydrates to synthesise new chitin for cytoskeleton purposes or increase the availability of carbohydrates for energy. There could also be a possibility that there are an induction of chitinase enzymes in the digestive gland to increase the digestion rate and thus the availability of carbohydrates to compensate for use of energy by induction of stress and detoxification mechanisms. This should be further evaluated by assessing the effect of other features involved in the same and adjacent biological processes.

There was unfortunately no significant enrichment of GO-terms assigned to features down regulated by Cu or the features modulated by BaP, nor any significant enrichment of single genes by the FatiGO analysis mentioned earlier in this section. The genomes of the *Mytilus* species are only partially known, the total amount of nucleotide sequences of *M. edulis* and *M. galloprovincialis* available at NCBI Genbank ESTs available March were 19,532, including a high rate of redundancy and

only 12 % (2353) being annotated. In contrast the fully sequenced genome of the fruit fly *Drosophila melanogaster* encodes for more that13,600 genes (Adams et al. 2000; Myers et al. 2000) and is a well developed model for studying both biological processes and evolutionary genetic. The lack of thoroughly annotated genome information has been a challenge in use of microarray technology for most species (non-model organisms). The later development and use of EST information has certainly contributed to an essential increase in the available genome information and to the development of new genetic markers for environmental studies. However, the limitation of this technique is the feature information that is included in the array, depending on the available genomic information on the organism you work with, and the genetic information available for *Mytilus sp.* still seems to be too inadequate to take advantage of the new genomic tools available, such as enrichment analysis.

4.1.2 Single gene response to Cu and BaP

Copper is an excellent model compound for demonstrating an integrative ecotoxicogenomic approach, as it is an essential element that is also highly toxic to mussels at in high concentrations. As a result of exposure, it was expected that general toxic-response pathways would be induced as well as specific biological mechanisms that are essential for copper-handling and homeostasis (Steinberg et al. 2008).

The data did not reveal classic exposure biomarker features for heavy metal as the metal sequestering protein Metallothionein that are involved in transport and detoxification of metals. There are though previous reports that indicate that Cu is not a good inducer of MT in the digestion gland of some species, being absent or giving very variable results (Bundy et al. 2008; Dondero et al. 2005). Interestingly, MT was slightly induced by BaP (2.0), in contrary to the Cu exposed mussels. It should be mentioned that there are many isoforms of metallothionein which may be more or less specific to different types of metal and also that the quality of the sequences used un the present array may not be optimal for sensitively detection alterations in gene expression (Zorita et al. 2007). Optimal hybridisation is normally obtained with probes made within the 1000 base pairs from the 3'-end of mRNA, and probe source

sequences outside this region may lead to loss of target detection (Agilent Technologies: personal communication).

Many of the negative effect responses of Cu are reported due to the reactivity of Cu with H_2O_2 and its potential to undergo (Fenton-like) redox reactions to form ROS, which also happens as a result of exposure to exenobitoics as BaP. This results in cellular damage such as peroxidation of membranes and DNA damage and the ability of the system to detoxify is critical to the outcome of damage or reparation (Craig et al. 2007; Dondero et al. 2006a; Steinberg et al. 2008). There were several ROS scavenging and protecting enzymes being differently regulated after exposure to Cu, amongst superoxide dismutases (SOD) and gluthationes-s transferase (GST). These enzymes takes part in oxidoreductase activity that might have a cytoprotective action and play an role in cell homeostasis by reduction of oxidative stress by limiting levels of free copper ions or other reactive oxygen species (Jaiswal 1994).

Superoxide dismutase as MT, protects cells from oxidative damage and is an important intracellular antioxidant involved in cellular processes as redox balance, cell signalling and apoptosis (Bundy et al. 2008). Superoxid dismutase as well as another cellular antioxidase, monooxygenase dbh-like 2, was slightly up-regulated after Cu exposure and clearly suggesting that that Cu had caused oxidative stress in the musslel. The monooxygenase dbh-like 2 involved in oxireductase activity acting on copper radicals as an acceptor (Hahn et al. 2007).

Both GST and abcb-p glyco protein in the Cu exposed animals, which may be expected on basis of GST's role in phase II metabolism of xcenobiotics (including PAHs) and the abcb p-glycoprotein as a transporter protein involved in phase III system of detoxification and excretion of phase II metabolites. The suppression might suggest that the priority of energy is focused to metal detoxification processes. Mason & Jenkins (1995) suggested that GST is less important in detoxification of metals as MT maintains this role.

Further indication of stress are the induction of the small heat shock protein p26 involved in modulation of actin microfilament dynamics, Heat shock proteins are proteins that reacts to different of environmental stress, not only heat, but also to xenobiotics, hypoxia, inflammation or other stressors (Cashikar et al. 2005). Another heat shock protein, 70dka protein 12a were down regulated in Cu exposed mussels,

which is consistent with previous findings in studies related to both Cu and Cd (Brulle et al. 2006; Dondero et al. 2006a). The HSPs have different functions and studies of stress responses in *Mytilus sp.* and other bivalves have shown that Hsp-70 induction mainly occurs by organic contaminants (Cruz-RodrÌguez & Chu 2002; Snyder et al. 2001), this supports the induction of the heat shock 70kda protein 12 in the mussels exposed to BaP.

Induction of several features associated with mitochondrial electron transport and oxidative phosphorylation and suggested that the copper exposure lead to a disruption of energy metabolism, represented by cytochrome B, cytochrome c oxidase subunit 3 and nadh dehydrogenase subunit 1. This is consistent with previous observations in other organisms were exposure to Cu have led to an mitochondrial dysfunction and further mobilisation of genes involved in carbohydrate metabolism (Bundy et al. 2008; Heron et al. 2001; Levenson et al. 1999; Totaro et al. 1986). A reduction in production of ATP by oxidative phosporylation and aerobic respiration would potentially result in redistribution of energy production trough anaerobic processes and thus a mobilisation of genes involved in sugar mobilisation and energy production, in this case present by an upregulation of chitinase 1 and chitin binding peritrophin-a domain containg protein, digestive enzymes of chitin (Weiss et al. 2006). A further indication of this is the significant enrichment of chitinase and N-acetyglucosamine metabolic processes in functional enrichment analysis.

Copper caused a clear induction of metal ion binding proteins as ferritin and Copper radical oxidase. Ferritin involves in storage and scavenging of Iron may also regulated as a consequence of oxidative stress, which can indirectly caused by heavy metals in invertebrates exposed to metals (Poynton et al. 2006) in *Daphina magna* exposed to Cu, Cd and Zn. Copper oxidase, which was up-regulated 32.8 fold after exposure to 320 ug/L Cu in the mussels, have previously been induced at concentrations as low as 6 ug/L (1/10 of LC_{50}) Cu in *Daphina magna.* The clear induction of copper oxidase may suggest a central role of this gene in cellular homeostasis of metals and oxidative stress, and warrants further studies to properly assess potential use as a biomarker for Cu exposure and effects. Although the MoA may not be entirely understood, copper oxidase may potentially play an important role in detoxification metals.

The Cu exposure also resulted in modulation of genes related to the humoral immune system (cell mediated immune system), represented by the microbial peptide encoding genes: Mycticin c, mytilin d, and defensin a, and the lyzosym which is known of being effective accumulator of metals (Dondero et al. 2006a). Lysozym was induced by the Cu exposure as expected, while the microbal peptide encoding genes, being expressed in haemocytes, did not exhibiting any clear trend in terms of behaviour, myticin c being heavily induced (108-fold) while mytilin d and myticin c were suppressed. There are no obvious reasons that can explain this, although the mussels seemed healthy when sampled, there might have been microbial activity causing this, or it could be due to the probe quality. The trend of genes involved in activation of the humoral immune system showed a clear trend in suppression their function. In bivalve molluscs, cell-mediated immunity is carried out by haemocytes, representing their primary defence against bacterial infection (Canesi et al. 2002). Haemocytes are equivalent with monocytes/macrophages in vertebrates and kills microbes through phagocytosis and various cytotoxic reactions. Tyrosine protein kinase, which was suppressed more than 13-fold after exposure to Cu in mussels, play an important role in activation of mussel haemocytes by cytokines (signal molecules) acting trough tyrosine kinase mediated transduction pathways (Canesi et al. 2003). Collectins, which are known to induce the production of pro-inflammatory molecules like cytocines as well as playing a role in host defence through their ability to bind to carbohydrate antigens of microorganisms (Ohtani et al. 2001), was suppressed more than 6-fold in mussel exposed to Cu. Binding of collectins to microorganisms can ease microbial removal through aggregation, complement activation, opsonisation and activation of phagocytosis, and inhibition of microbial growth (Wetering et al. 2004). These down regulation of this genes are similar to effect observed in corresponding studies of other species (Poynton et al. 2006; Steinberg et al. 2008) and might indicate that the exposure suppresses pathogen resistance. The immune defence and haemocytes are also affected by the down regulation of calmodulin, a calcium binding protein involved in cellular calsium homeostase which influences the Ca^{2+}-ATPase activity that regulates the phenoloxidase activity of haemocytes (Tujula et al. 2001). An increase in intracellular calmodulin may suppress innate immune functions as inflammatory mediator generation and the phagocytosis and killing of microbes (Serezani et al. 2008).

Both Cu and BaP have been shown interfere with endocrine regulated processes in flounder hepatocytes when present at sufficiently high concentrations (Leaver et al. 2010) The observed induction of vitelline envelope zona pellucida domain 6 in mussels after exposure to Cu suggest that Cu may also interact with oocyte development also in mussels. Although the mechanism of Cu in endocrine disruption has not been properly determined, investigation of whether Cu affects the reproduction of mussels may be justified.

Benzo[a]pyrene is one of many PAHs that have been identified as major risk factors for developing cancer, and it's one of the most frequently used model compound for this class of xenobiotics, although not much investigation have been done to assess effects of this compounds in mussels on a genetic level. The BaP exposure revealed modulation of features encoding classic biomarkers as the phase I enzyme cytochrome P-450s (CYPs) and phase II enzyme GST related to metabolism of xenobiotic, as well as general biomarkers of toxicity and environmental stress. Interestingly, the phase I enzyme Cytochrome P-450 were slightly down regulated (-2.4) by the BaP exposure, which contradict that BaP has led to up-regulation of this gene in other species (Castorena-Torres et al. 2008; Lee et al. 2006). This contradictive result might be due to that are many iso types of this enzyme, and there is suggested that that the general CYPs used present in other species as CYP 1A1 and CYP 1B1 lacks a homolog in mussels (Cajaraville et al. 2000; Dondero et al. 2006a). As expected glutathione GST were induced in the BaP exposed mussels, but then a gain, features encoding the ATP-binding cassette transporters (ABC transporter protein) abcb p glyco protein involved in phase III system of detoxification and excretion of xenobiotic metabolites and oxidative stress, were down regulated. This is not commonly seen by exposure to BaP (Dondero et al. 2006b; Lee et al. 2006) However, both SOD and monooxygenase dbh-like 2, important cellular antioxidants as mentioned earlier in this section, were both down regulated, in contradiction to observed in other studies of mussels exposed to BaP (Winston et al. 1996) (Dondero et al. 2005). This may suggest to assess regulation of this genes by qPCR to evaluate whether this is an analytical artefact or biologically relevant.

Many of the processes induced or suppressed in an organism by copper exposure are general processes that are reactions to environmental stress or toxicity, affecting biological processes as metabolism, immune system and reproduction. However, the

effect of these processes varies due to the toxicity and modes of action of the compound, and it is apparent that upon toxic effects of exposure to Cu and BaP there are differences in these strategies. This is especially present by the up regulation of the features involved in the mussel's hummoral immune system and energy generation. As most features related to immune defence and response to stimuli was suppressed in the Cu exposed mussels, 5 of these 10 features were absent in mussels exposed to BaP and most of the features present were up-regulated. Especially defensin a, related to stress response and oxadive phosphorylation were heavily induced (328.7) indicating that the immune system were activated by inflammatory responses. Similar modulation is observed in studies of Pyrene exposure of juvenile rainbow trout (Krasnov et al. 2005). Further, features related to chitin metabolism, and energy generation were up-regulated These processes are mainly general biological processes affected by stress that might be induced to provide energy to other processes as detoxification of the organism. The single genes modulated by BaP use in this array needs further evaluation by comparing to studies by BaP exposure in other organisms to provide more insight to the biological processes affected.

Analysis of the microarray data identified several genes and pathway related to general stress responses of toxicity for both Cu and BaP. As a pilot study, it was limited to the use of one time-point and one exposure concentration, which gives a narrowly picture of the effect of toxicity on the gene-expression. However, the purpose was to produce sub-lethal effects, therefore also the choice of using tissue of *M. edulis* exposed to 320 µg/L $CuCl_2$ and 50 µg/L BaP although tissue from *M. edulis* exposed to several concentrations were available.

As the organism have a system that can adapt to environmental stress by its repair and detoxification mechanisms, a future microarray study would after evaluation and redundancy reduction benefit from using multiple exposure times and concentrations to gain a more complete picture of the gene expression response and be able to strengthen the potential use of this array as a tool in environmental monitoring.

5 Conclusion

The results showed that an oligoarray based on *M. edilus* and *M. galloprovinciales* could be used to study the differential expression in *M. edulis* digestive gland after exposure to Cu and BaP. The largest cross-hybridization potential were observed between the *M. edulis* cDNA and the probes from *M. edulis* and *M. galloprovinciales* by 80 % hybridization success, and that these species are most likely to provide good quality expression data. Of totally 8898 features of *M. edulis* and *M. galloprovinciales* in the array, only 26 % of these features were annotated and assigned GO-terms, leading to poor results of the functional enrichment analysis. The genetic information available for *M. edulis* and *M. galloprovincialis* seems to be too inadequate to take advantage of the genomic tools available at this time.

The different MoA of the model compounds were clearly illustrated by 23 % (Cu) and 28 % (BaP) of the differently regulated features being modulated by the exposure. In addition MoA of Cu and BaP were clearly difference by only 30 % common genes being regulated by both treatments. Further analysis identified several genes and pathways related to general stress responses of toxicity for both Cu and BaP. In wide terms, Copper exposure caused suppression of biological processes related to immune response, metabolic and oxidative processes (included xenobiotic metabolism), while biological processes related to ROS scavenging, Endocrine responses and carbohydrate metabolism were induced. This shows an antioxidant response and effects of re-establishment of cell homeostasis caused by the exposure. BaP on the other hand, showed an induction of biological processes related to immune response, carbohydrate metabolism as well as other metabolic and oxidative processes, although only some features involved in detoxification of xenobiotics and ROS scavenging. Biological processes related to ROS and Endocrine responses were suppressed. These results clearly show that there is possible to detect trends of the MoA of Cu and BaP,

Further work on the development of a new improved version of the array includes inclusion of additional sequence information from other sequencing projects, redundancy reduction and further verification of the microarray results by RT-qPCR asses the dynamic range and sequence specificity of the array.

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Appendix:

Due to the amount of data generated by microarray analyses, all data are available in the database of Norwegian Institute of Water Research, Oslo, Norway. Project number 27279, Geniom.

Table 1. Reagents and kits used in microarray experiment.

Reagents/Kit	Producer	Product number
2-Mercaptoethanol (molecular biology grade)	Merck	444203
Acetonitrile, HPLC grade	Sigma Aldrich	
Agilent RNA 6000 Reagents kit	Agilent Technologies	5188-5902
Benzo[a]pyrene	Sigma Aldrich	
Bioanalyzer RNA 6000 Nano Kit	Agilent	5067-1511
Chloroform (Trichloromethane) analytic grade	Merck	
Copper sulfate	Fluka (Sigma Aldrich)	35185
Copper(II) chloride 0.1 M solution	Fluka (Sigma Aldrich)	18865
Dithiothreiotol (DTT)	Sigma Aldrich	432815
Ethanol (Rectified spirit)	Kemetyl	
Gene Expression hybridization kit	Agilent Technologies	5188-5242
Gene Expression Wash Buffer 1	Agilent Technologies	5188-5325
Gene Expression Wash Buffer 2	Agilent Technologies	5188-5326
Guanidine hydrochloride 98 %	Alfa Aesar	A13543
Isopropanol		
Lithium chloride (analytic grade)	Merck	438002
Low RNA Linear Amplification kit	Agilent Technologies	5988-9667
Nuclease free water (molecular biology grade)	5Prime	2500020
One-Color RNA Spike-in Kit	Agilent Technologies	5188-5282
Quick Amp Labeling Kit, one-color	Agilent Technologies	5190-2307
Rnase Away	Molecular Bio Products	7003
Rneasy Mini Kit	Qiagen	74104
Sodium chloride	Merck	567441
Stabilization and drying solution	Agilent	5185-5979
Trizol	Invitrogen	15596-018
Turbo DNA-free kit	Ambion	AM1907

Table 2. Quality control of *M. edulis* RNA samples.

Sample ID	ng/ul	A260/ A280	A260/ A230	Tissue Weight (mg)	Volume (µl)	Yield µg RNA	RNA quality	Used for MA
BaP1 - 1	644.44	1.94	2.40	21.50	100	64.44	Ok	
BaP1 - 2	754.90	1.94	2.58	25.40	100	75.49	Ok	
BaP1 - 3	1271.49	2.00	2.52	31.30	100	127.15	Ok	
BaP1 - 4	1042.44	2.00	2.51	31.00	100	104.24	Ok	
BaP1 - 5	925.83	1.98	2.44	23.80	100	92.58	Some degradation	
BaP10 - 1	1223.57	2.04	2.50	25.10	100	122.36	Excellent	
BaP10 - 2	1344.19	2.02	2.47	35.70	100	134.42	Some degradation	
BaP10 - 3	1193.27	2.03	2.39	36.40	100	119.33	Excellent	
BaP10 - 4	1337.97	2.00	2.53	38.20	100	133.80	Excellent	
BaP10 - 5	953.96	2.01	2.38	31.40	100	95.40	Excellent	
BaP50 - 1	1030.63	1.99	2.49	35.80	100	103.06	Excellent	x
BaP50 - 2	653.65	1.95	2.55	26.40	100	65.37	Excellent	
BaP50 - 3	692.75	1.94	2.50	21.70	100	69.28	Some degradation	
BaP50 - 4	1176.93	1.99	2.46	27.00	100	117.69	Excellent	x
BaP50 - 5	1049.62	1.99	2.42	27.20	100	104.96	Excellent	x
Control - 1	513.40	1.89	2.55	20.10	100	51.34	Excellent	
Control - 2	728.49	1.98	2.36	23.30	100	72.85	Ok	
Control - 3	811.13	1.93	2.59	22.10	100	81.11	Excellent	x
Control - 4	605.91	1.94	2.48	23.90	100	60.59	Excellent	x
Control - 5	563.43	1.92	2.43	21.00	100	56.34	Excellent	x
Cu100 - 1	586.20	1.94	2.55	19.60	100	58.62	Excellent	
Cu100 - 2	1194.80	1.99	2.54	28.40	100	119.48	Excellent	
Cu100 - 3	878.01	1.98	2.47	35.00	100	87.80	Ok	
Cu100 - 4	756.55	1.97	2.45	26.70	100	75.66	Ok	
Cu100 - 5	1107.38	1.96	2.45	23.30	100	110.74	Excellent	
Cu32 - 1	922.22	1.97	2.54	18.90	100	92.22	Excellent	
Cu32 - 2	1383.73	2.00	2.49	32.90	100	138.37	Ok	
Cu32 - 3	837.13	1.97	2.52	26.40	100	83.71	Ok	
Cu32 - 4	896.57	1.98	2.52	27.40	100	89.66	Excellent	
Cu32 - 5	1464.91	2.00	2.51	24.80	100	146.49	Excellent	
Cu320 - 1	983.08	2.01	2.52	30.50	100	98.31	Degradation	
Cu320 - 2	1148.88	1.98	2.47	28.80	100	114.89	Excellent	x
Cu320 - 3	425.34	1.90	2.49	20.90	100	42.53	Ok	x
Cu320 - 4	970.93	2.00	2.51	35.30	100	97.09	Some degradation	
Cu320 - 5	829.65	1.96	2.49	29.00	100	82.97	Excellent	x
Solvent - 1	922.65	1.96	2.54	25.40	100	92.27	Excellent	x
Solvent - 2	646.14	1.94	2.53	20.00	100	64.61	Some degradation	
Solvent - 3	1032.82	1.97	2.55	25.00	100	103.28	Some degradation	
Solvent - 4	1362.00	2.02	2.43	28.70	100	136.20	Excellent	x
Solvent - 5	923.50	2.04	2.47	28.30	100	92.35	Ok	x

Table 3. Quality of cRNA, pooled samples.

Code	Sample ID	cRNA ng/ul	cRNA Yield ug	Cy3 pmol/ul	Incorp. Rate pmol/ug
Mussel Solvent	M1	155.41	4.66	1.71	11.00
Mussel Cu320	M2	149.11	4.47	1.63	10.93
Mussel Control	M3	144.81	4.34	1.56	10.77
Mussel BaP50	M4	139.50	4.19	1.42	10.18

Table 4. *Mytilus sp.*features on array after filtration, distributed by species.

Specie	Features on array	Features post filtration Cu vs. Control	Features of sufficient quality 2-fold
M. Edulis	3071	2155	719
M. Californianus	21979	9585	2638
M. Galloprovincialis	5827	4970	1634
M. Trossulus	71	40	17
Total	30948	16750	5008
Specie	Features on array	Features post filtration BaP vs. solvent	Features of sufficient quality 2-fold
M. Edulis	3071	2159	755
M. Californianus	21979	9496	3694
M. Galloprovincialis	5827	4938	1839
M. Trossulus	71	41	16
Total	30948	16634	6304

Table 5. Physicochemical concentrations of the seawater from selected exposure tanks at the start and end of the 7-day exposure.

Tank	Salinity (‰)		Dissolved oxygen (mg/L)		pH		Temperature (°C)	
	Start	End	Start	End	Start	End	Start	End
Control	34.1	34	9.8	9.0	8.1	8.3	10.6	10.8
50 B(a)P	34.1	34.1	8.6	8.5	7.9	8.2	10.6	10.9
320 Cu	34.1	34	9.9	9.4	8.2	8.4	10.6	10.9

Table 6. *M. edulis* sample size at end of exposure

Treatment	Mussel	Length	Width
		cm	cm
Control	1	57.48	35.14
	2	62.71	31.63
	3	63.51	30.43
	4	60.66	31.03
	5	59.48	29.20
Solv Control	1	60.25	28.83
	2	57.48	31.44
	3	58.63	32.82
	4	63.48	32.40
	5	54.26	31.00
B(a)P	1	56.99	30.82
(50µg/L)	2	58.14	29.47
	3	56.02	31.38
	4	58.02	26.24
	5	59.54	31.90
Cu	1	66.31	35.54
(320µg/L)	2	59.77	28.66
	3	67.23	36.66
	4	58.64	30.36
	5	61.09	31.21