Mussel (*Mytilus edulis*) hemocytes for *in vitro* testing

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Abstract

Mytilus edulis *is one of the most abundant and widely distributed benthic invertebrate species found in the North Atlantic. The aim of this study was to test the applicability of hemocytes from* Mytilus edulis *for* in vitro *toxicity testing, and quantify the cytotoxic response following exposure of the hemocytes to selected PAHs (naphthalene, phenanthrene, pyrene, benzo(*a*)pyrene, 2,6-dimethylnaphthalene, 1,5-/1,7-dimethylphenanthrene). Cell viability was assessed with three methods; metabolic activity (measured as alamar blue reduction capacity), membrane integrity (measured as 5-carboxyfluorescein diacetate, acetoxymethyl ester reduction capacity), and intracellular glutathione level (measured as monochlorobimane binding capacity). The use of multiple cytotoxicity endpoints has the advantage of giving possible insight into the mechanism of toxicity.*

In order to identify an optimal medium to maintain the viability of hemocytes in vitro*, four media were assessed (Leibovitz (L-15), L-15 supplemented with antibiotics, RPMI 1640 and phosphate-buffered saline (PBS)). It was decided to culture the cells in L-15 supplemented with antibiotics to ensure the aseptic conditions. The hemocytes cultured in the different media maintained high viability after 24 hr, 48 hr, and 96 hr, as opposed with those of a 192-hr incubation period.*

In this study, DMSO was used as a vehicle for PAHs; therefore the cytotoxicity of three DMSO concentrations was examined (0.2%, 0.8%, and 3.2%), in order to avoid its cytotoxic effects. The data revealed cytotoxic effect in the 3.2% DMSO treatment, while no cytotoxic effect was observed after 0.2% and 0.8% DMSO treatment.

A general tendency of increasing toxicity with the exposure period was observed by the EC_{50} values determined for the tested PAHs. Comparable EC_{50} values for each time point (24 hr and 48 hr) were observed from the fluorescent indicator dyes. However, in some cases variable EC_{50} values were determined.

*The possible toxic mechanism discussed for each PAH included the following; 1) the general membrane disruption (cell membrane and mitochondrial membrane) and loss of organelle due to the lipophilic properties of the compounds as in case of naphthalene, phenanthrene, and pyrene, 2) the biotransformation role in toxicity and formation of toxic metabolites and the lipophilicity were potential mechanisms proposed for benzo(*a*)pyrene, 3) the structure-toxicity relationship was conducted to the subsided PAHs mechanism. By ranking the*

PAHs cyototoxicity it was found that pyrene and benzo(a)*pyrene are the most toxic compounds. This study established responses in the cell viability of the blue mussel hemocytes exposed to different PAHs.*

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Abbreviations

AB	Alamar blue™
BaP	Benzo(a)pyrene
CF	5-Carboxyfluorescein
CFDA-AM	5-Carboxyfluorescein diacetate, acetoxymethyl ester
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC_{50}	Half maximal effective concentration
E.P.A.	Environmental protection agency
GSH	Glutathione
GST	Glutathione S-transferase
KH_2PO_4	Monopotassium phosphate
Log K_{ow}	Log octanol-water partition coefficient
L-15	Leibovitz
L-15(+)	Leibovitz supplemented with antibiotics
LC_{50}	50% lethal concentration
mBCI	Monochlorobimane
M.edulis	*Mytilus edulis*
Na_2HPO_4	Disodium hydrogen phosphate
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffer saline
ROS	Reactive oxygen species
RPMI	Royal park memorial institute
THC	Total hemocyte count

1   Introduction

1.1   Bivalve molluscs

Bivalve molluscs are common sentinels widely used in invertebrate toxicology studies. Their worldwide distribution combined with their sedentary nature and ability to bioaccumulate pollutants as filter-feeders; make them an ideal species for investigation. Most of the studies to date have been concerned with the widespread species, the blue mussel, *Mytilus edulis* (Wootton *et al.*, 2003). A range of contaminants including polycyclic aromatic hydrocarbons (Grundy *et al.*, 1996) and heavy metals (Coles *et al.*, 1995) have been investigated for their affect on hemocytes, using *M. edulis* as a model organism. In fact, cellular toxicology gives insight and understanding of the ecotoxicological processes by elucidation of the mechanisms of action and toxicological effects.

The function of bivalve immune system is to maintain an efficient protection against various invaders, however, its efficiency may be weakened by the presence of chemical contaminants (Auffret and Oubella, 1997); specifically with the presence of (PAHs) (Gómez-Mendikute *et al*., 2002). The bivalve immune system is integrated with other physiological systems. Unlike a closed system where lymph and blood function separately, the circulatory system in bivalves is an open circulation system with hemolymph containing hemocytes (Reiber and McGaw, 2009). Hemolymph, the circulatory fluid of bivalves, transports nutrients, enzymes, metabolic wastes, respiratory gases, and toxicants throughout the body. Hence, it can provide information on the health assessment of animals or populations (Gustafson *et al.*, 2005). It is used *in vitro* in assessing biomarker responses to environmental stressors, and it is usually extracted either via the posterior or anterior adductor muscles (Al-Subiai *et al.*, 2009). The hemocytes are found circulating within the hemolymph, and have a vital role in immune system such as phagocytosis, as well as their role in cytotoxicity through the production of reactive oxygen intermediates. They are a sensitive target for environmental contaminants. Therefore, they are frequently used in *in vitro* studies to assess toxicants interfering with hemocyte functions (Grundy *et al.*, 1996; Gagnaire *et al.*, 2006).

In vitro studies are well-suited for the rapid screening of many pollutants, and for providing a significant amount of information about mechanisms of toxicity. Besides, they are

inexpensive to carry out. Furthermore, though there is mostly coherence between *in vivo* and *in vitro* response, the *in vitro* studies were used as an alternative to the experimentation on animals. Thus, they helped limit unnecessary suffering (Devlin *et al.*, 2005). In that respect, it is interesting to use a sensitive *in vitro* laboratory bioassay to measure the response of biomarkers. The use of reliable bioassay provides increase in understanding the sensitivity of the cells by quantifying the cytotoxic response of the contaminants.

1.2   Cellular methods

Measuring the cell viability by the fluorometric assays has advantages over other classical existing assays (e.g. staining with fluorescent deoxyribonucleic acid (DNA)-specific dyes or reduction of tetrazolium compounds). The fluorometric assays can be adapted for a large scale *in vitro* screening and no radioactive or toxic materials are used (Nakayama *et al.*, 1997). In this study, cell viability was measured with three fluorescent dyes; alamarBlue^{TM} (AB), 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) and monochlorobimane (mBCI).

1.2.1   Alamar Blue

The water-soluble dye alamarBlue^{TM} (AB) has previously been used to quantify *in vitro* viability of various cells (Ahmed *et al.*, 1994; Schreer *et al.*, 2005). AB is a redox indicator with both visible color and fluorescence changes in response to the chemical reduction of the medium (Shahan *et al.*, 1994). Resazurin, the non-fluorescent oxidized form, is the functional dye in the commercial reagent AB. In living cells, resazurin reduces into the fluorescent form resorufin and changes its color from blue into pink (Montejano *et al.*, 2005). The conversion is catalyzed by reductases, which are found in the mitochondrial membranes, as well as in the cytosol of the living cells. The fluorescence reading would therefore be expected to some extent to reflect the metabolic activity of cells (Schreer *et al.*, 2005). There are several advantages for using the AB assay. AB is added directly to the cells after the incubation period, it is non-toxic to the cells and the user. However, it should also be mentioned that the AB fluorescence intensity is sensitive to temperature (Nakayama *et al.*, 1997).

1.2.2   5-Carboxyfluorescein diacetate acetoxymethyl ester

The non-fluorescent dye, 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), is an esterase substrate that can enter living cells and be hydrolyzed by intracellular non-specific esterase, to form the highly fluorescent 5-carboxyfluorescein (CF). The substrate diffuses rapidly into the cells and the product slowly comes out. The metabolized CFDA-AM can be read fluorometrically. The fluorescence reading would reflect the cells membrane integrity. Cells with intact plasma membranes would maintain a cytoplasmic environment that supports the activity of esterase. An advantage of this assay, is both dyes, the AB and the CFDA-AM, can be used alongside each other without interference in the fluorescent measurements because the two probes are measured at different emission wavelengths (Schirmer *et al.*, 1997; Schreer *et al.*, 2005).

1.2.3   Monochlorobimane

Glutathione (GSH) is the main non-protein thiol in the cells and plays a critical role in defense and detoxification. All of the main biological events that entail the thiol-redox state contain GSH interactions. Some examples include DNA synthesis, signal transduction and gene transcription. A low GSH level in the hemolymph indicates signs of oxidative stress (Esteban-Pretel and Pilar López-García, 2006).

In order to bind free thiols, monochlorobimane is used. It is a cell permeable non-fluorescent dye. It forms a fluorescent adduct with GSH in a reaction catalyzed by GSH *S*-transferase. Monochlorobimane fluorescence is used as an indirect indicator of GSH concentration (Fernández-Checa and Kaplowitz, 1990).

1.3   Polycyclic aromatic hydrocarbons (PAHs)

All over the world, the marine environment is continuously polluted by oil spillage from tanker accidents, natural seepage, and releases from offshore production (Saeed and Al-Mutairi, 2000). Therefore, a notable environmental issue is coastal pollution by chemical contaminants such as hydrocarbons. *M. edulis* is usually used as an indicator of habitat exposure to organic contaminants in coastal waters. It assesses the status of chemical contaminants (Chase *et al.*, 2001). As one example, Bocquené *et al*. (2004) carried out a 3-year survey *in situ* using various

biological markers in *M.edulis* exposed to oil that came ashore after the French "Erika" oil spill in December 1999.

Crude oil is a complex mixture of PAHs, phenols, and heterocyclic compounds containing sulphur and nitrogen (Saeed and Al-Mutairi, 2000). PAHs compounds contribute most to the toxicity of most oils (Koyama and Kakuno, 2004). PAHs are a family of hydrocarbons containing two or more fused aromatic ring structures (benzene), in linear angular or cluster arrangement (Brown *et al.*, 1996). The PAHs range from the simple two-fused benzene ring like naphthalene ($C_{10}H_8$), to complex hydrocarbons such as the seven-fused ring, coronene ($C_{24}H_{12}$) (Manoli and Samara, 1999).

There are natural and anthropogenic sources of PAHs, originating from multiple processes such as biogenic, petrogenic, and pyrogenic processes (Spink *et al.*, 2008). The natural sources arise from biogenic precursors that are common constituents of terrestrial higher plants in the digenetic process (Magi *et al.*, 2002). The anthropogenic sources originated from either petrogenic or pyrogenic processes. PAHs from petrogenic sources derive from petroleum products. While in the case of pyrogenic process, they are derived from incomplete combustion processes in industrial operations, power plants, and so forth (e.g. petroleum, vehicle emissions, wood, coal, *etc.*) (Saha *et al.*, 2009). Direct sources of PAHs in the marine environment comprise of oil spills, sewage, and runoff from roads.

Many propose the term 'baseline toxicity' to assess the process of chemicals that elicit their toxicity by interfering with membrane fluidity. Seventy percent of all commercial chemicals have this property including the PAHs (Engraff *et al.*, 2011). PAHs are also a group of organic compounds which are of major concern due to the documented carcinogenicity in many experiments of several of its members (Manoli and Samara, 1999).

The US Environmental Protection Agency (E.P.A.) has identified 16 unsubstituted PAHs as priority pollutants (Manoli and Samara, 1999). In the current study, the cytotoxicity of four PAHs, naphthalene, phenanthrene, pyrene, and benzo(*a*)pyrene, that are selected by the E.P.A. and two substituted PAHs, 2,6-dimethylnaphthalene and 1,5-/1,7-dimethylphenanthrene, were measured in the hemocytes of blue mussel.

1.4   *Objective*

The overall aim of the study was to optimize the use of isolated hemocytes from blue mussels (*Mytilus edulis)* for *in vitro* testing and to quantify the cytotoxic response following exposure to selected PAHs.

The following questions were investigated:

1. What are the optimal cell medium and density for the hemocytes of blue mussels to be used for *in vitro* testing?
2. Is DMSO toxic to hemocytes at concentrations used in *in vitro* tests?
3. What is the cytotoxicity of selected PAHs (naphthalene, phenanthrene, pyrene, benzo(*a*)pyrene, 2,6-dimethylnaphtalene, and 1,5-/1,7-dimethylphenantrene) to blue mussel hemocytes measured as metabolic activity, membrane stability and intracellular glutathione level?

2 Materials and Methods

2.1 Collection of animals and sampling area

Blue mussels are found along the entire coast of Norway. The sample was collected from the University's marine biological station of Drøbak. The city lies 40 kilometers south of Oslo, Norway, located in the outer Oslofjord.

Mussels were collected of both sexes and similar size (shell length 6 ± 1.5 cm). After collection, they were immediately transported to Oslo University in plastic bags. The mussels were placed in each of two 6 liter plastic tanks, which were continuously aerated. The water was changed every day before feeding, to ensure water quality parameters. The mussels were fed regularly, around once per week, with Shellfish-diet 1800 (*Isochrysis, Pavlova, Tetraselmis, Thalassiosira weissflogii*) and kept at a constant temperature of 10ºC until extraction of hemolymph.

2.2 Buffers, solutions and media

2.2.1 Culture media

Phosphate Buffered Saline (PBS) was prepared by dissolving the following: 23.2 g of NaCl, 1.48 g of Na_2HPO_4, 0.43 g of KH_2PO_4 in 1 liter Milli-Q water. 1 M NaOH was added to the mixture and the pH was fixed at 7.4.

Leibovitz' medium was purchased from Biowhittaker. The antibiotic that consists of penicillin (100 Units/mL), streptomycin (100 µg/L) and amphotericin (0.25 µg/L) was added to L-15 medium.

2.2.2 Preparation of the cytotoxicity probes and buffer

Tris buffer (50 mM, pH 7.5) is a dye working solution containing 11.6 µL CFDA-AM stock solution, 579 µL AB, and 11 µL cytotoxic buffer. The solution was every time prepared every time immediately prior to use.

The cytotoxic buffer was prepared by adding 0.97 g of trizma-base and 6.61 g trizma-HCL in 1 liter of distilled water. The pH was adjusted to 7.5. CFDA-AM stock solution consists of 5 mg

CFDA-AM and 2.35 mL DMSO. While the mBCI stock solution consisted of 25 mg mBCI and 1.82 mL DMSO. CFDA-AM and mBCI were kept frozen until use. Alamar blue was purchased as a ready-to-use solution and kept refrigerated.

2.3   *Preparation of hemocytes*

2.3.1   Extraction of the hemolymph

The hemolymph was withdrawn from the posterior adductor muscle of the mussels. To access the posterior adductor muscle and to prevent the bivalves from completely closing their two shells, a solid scalpel was inserted between the two shells and with gentle prising, the shell was opened. Seawater was drained from the mantle cavity to ensure that there was no possibility of accidentally drawing up seawater. During this process, the mussel was held with the posterior upwards, and with the anterior resting on a bed of paper towel. The protocol for hemolymph extraction was adapted from (Coray *et al.*, 2007) with minor changes: The hemolymph was extracted using a sterile 1 mL syringe, with a 23-gauge needle, which was pretreated with cold phosphate buffered saline (PBS). Most collections yielded 0.5 mL hemolymph from each mussel. The colorless hemolymph was then immediately transferred to cooled sterile tissue culture tubes (Falcon), containing 0.l mL PBS. It was aliquotted and mixed 50:50 (hemolymph/PBS with 10 mM EDTA) and vortexed for about 3 seconds. All the work was maintained on ice to avoid hemocyte clumping.

2.3.2   Quantification of hemocytes – viability assay

The viability of the freshly collected hemocytes was quantified microscopically by the use of trypan blue exclusion (Pappenheimer, 1917). In eppendorf vials, two parts of trypan blue were mixed with one part of the cells suspension (20 µL + 10 µL). Live cells will exclude the dye due to their intact membranes, while oppositely dead cells do, appearing blue under the microscope. Trypan blue requires manual cell counting by using the embedded grid of a hemocytometer. Therefore, cells were located using a microscope with a lens magnification of 40x, and hemocyte counts were recorded using the Bürker-Türk (BT) haemocytometer.

The hemocytes were further diluted with Leibovitz's medium supplemented with antibiotics (L-15(+)) (see Section 2.2.1) to hold a concentration of 200 000 cells/mL. The cells

were then seeded out into 96-well Costar® cell culture plates (Corning labware, NY, USA), 200 µL of the suspensions to each well. The microtiter plates were put in an incubation chamber at 10ºC. The different plates were incubated for 24 hr, 48 hr, 96 hr and 192 hr, respectively.

2.4   In vitro *exposure of hemocytes*

Prior to assessment of cytotoxicity (Section 2.5) within 24 hr, half of the culture medium (100 µL) was replaced with the exposure solution (Ellesat *et al.*, 2010), to give a total volume of 200 µL per well. The exposures consisted of varying concentrations of the test compounds dimethyl sulfoxide (DMSO) and PAHs.

2.5   *Cytotoxicity assays*

The cytotoxicity assay was carried out using the exposed cells. The assay was applied both during establishment of cell culture conditions and during the toxicity test studies. The procedure was the same for all experiments. The protocol was adapted from (Schirmer *et al.*, 1998).
To assess the cytotoxicity, all culture media were removed from each well and 100 µL of Tris buffer (Section 2.2.2) was added. The microtiter plates were incubated at room temperature on an orbital table shaker at 100 rpm for 30 minutes. Due to the dyes' sensitivity to light, every step of this protocol was carried out in the dark. The fluorescence plate reader was set to excitation and emission wavelength pairs of 540-590 nm (AB), 485-530 (CFDA-AM). The fluorescence was then set to excitation and emission wavelength pairs of 360-460 for the third measurement (mBCI). After conducting readings, the average of the blank (culture media) was calculated and subtracted from the data.

2.6   *Reference toxicity*

As a reference for toxicity, copper as $CuSO_4$ x $5H_2O$ was used in this study. Thus, a 2-fold serial dilution was performed from the stock solution of 5 mg/mL copper to obtain the following concentrations: 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, and 0.019 mg/mL. Stock solutions were prepared in the culture medium L-15(+).

2.7   Optimization of cell culture density and medium

2.7.1   Optimization of cell density

Seeding out the different cell concentrations, 100 000, 200 000, and 400 000 cells/mL assessed the effect of cell-seeding density on hemocyte viability. The cells were seeded out into 96-well Costar® cell culture plates.

Four replicates were used, each of which consisted of hemolymph from five mussels. The hemolymph extraction and the viability assay were carried out as mentioned above (Sections 2.3.1 and 2.3.2). Nevertheless there were minor changes in the viability assay: Rather than diluting the hemocytes to hold a concentration of only 200 000 cells/mL of medium, two other hemocyte concentrations were screened in parallel. Those were 100 000 and 400 000 cells/mL of medium; to determine which hemocyte concentration would perform best. The microtiter plates were placed under the microscope and pictures were taken.

2.7.2   Optimization of culture media

Four media were assessed for their ability to maintain viability of hemocytes *in vitro*: L-15, L-15(+), RPMI, and PBS. Autoclaving was used for sterilization of media. This experiment was not only conducted to screen multiple media, but also to function as a platform for the following two experiments.

Five replicates were used, each of which consisted of hemolymph from five mussels. The hemolymph extraction and the viability assay were carried out as mentioned above (Sections 2.3.1 and 2.3.2), although with minor changes in the viability assay: Instead of diluting hemocytes with one medium, four different media were screened in parallel to determine which medium would perform best with the hemocytes. The hemocytes were incubated for 24 hr, 48 hr 96 hr, and 192 hr prior the cytotoxicity measurement. Effects of the media on hemocytes count and viability were analyzed by the cytotoxicity assays (Section 2.5).

2.8   Cytotoxicity of DMSO

It was of great importance to test the optimal DMSO concentration, to avoid its cytotoxic side effects. This was achieved by determining the maximal concentration usable for the cell viability. Various DMSO concentration test samples were prepared by slowly adding 5, 20, and

80 µL of DMSO to 2495, 2480, 2420 µL of L-15(+) media, respectively, to reach a final volume of 2500 µL and DMSO concentrations of 0.2%, 0.8%, and 3.2%, respectively.

Five replicates were used, each of which consisted of hemolymph from five mussels. The hemolymph extraction and the viability assay were carried out as mentioned above (Sections 2.3.1 and 2.3.2), and *in vitro* exposure of hemocytes were executed as mentioned (Section 2.4). The incubation periods were 24 hr, 48 hr, and 96 hr. Prior to assessment of cytotoxicity within 24 hr, half of the media and cells were removed, and 100 µL of the three different DMSO concentrations (0.2%, 0.8%, and 3.2%) were added to the microtiter plates. Effects of DMSO exposure on cell viability and hemocyte count were assessed by the cytotoxicity assays (Section 2.5). Copper was used as a reference for toxicity (Section 2.6).

2.9   Cytotoxicity of PAHs

The cytotoxicity assays were performed to determine the cytotoxic effect of six PAHs compounds on the hemocytes. Based on 50% lethal concentration (LC_{50}) for fish (ATSDR, 2009), 8 serial four-fold dilutions of each compound were prepared in 0.2% DMSO diluted in L-15(+) medium. The experiment protocol was carried out based on the outcome of the cell density and culture media optimization (Sections 2.7.1 and 2.7.2), as well as the best DMSO concentration performance (Section 2.8).

Three replicates were used, each of which consisted of hemolymph from five mussels. The hemolymph extraction, viability assay, and *in vitro* exposure of hemocytes were conducted as mentioned above (Sections 2.3.1, 2.3.2, and 2.4). The incubation lasted for 24 hr and 48 hr. Prior to assessment of cytotoxicity within 24 hr, half of the media and cells were removed and 100 µL of the eight different PAHs concentrations were added to the 96-well microtiter plates. Effects of PAHs exposure on cell viability and hemocyte count were analyzed by the cytotoxicity assays (Section 2.5). Copper was used as a reference for toxicity (Section 2.6).

2.10   Statistical procedures

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Kruskal–Wallis test followed by Dunns test was used for evaluating differences

between groups whenever variances between groups were not homogenous. The Wilcoxon test was used to determine which treatment groups differed from the control. Bonferroni correction was used wherever applicable (repeated tests with the same group, e.g. control against treatments). Wherever the data allowed, a four-parameter logistic regression was used to fit a line to toxicity data for each PAH at the two time points. The significance level was set to $p \leq 0.05$ for rejection of H_0 in all analyses.

3   Results

3.1   Cell density optimization

Microscope examination was completed immediately after extracting and mixing the hemocytes with L-15(+) medium to reach the different cell concentrations (100 000, 200 000, and 400 000 cells/mL). The examination revealed a variety of single and aggregated cells, which exhibited different sizes due to various cell concentrations in the media. This is displayed below in Figure 1. At the concentration of 100 000 cells/mL the total number of hemocytes was low and no aggregation was obtained (Figure 1*a*). At concentration 200 000 cells/mL more and separated hemocytes were observed (Figure 1*b*). On the contrary, hemocytes at concentration 400 000 cells/mL had a roughly circular spontaneous aggregation (Figure 1*c*). Hence, the cell density of 200 000 cells/mL was chosen for the following cytotoxicity measurements.
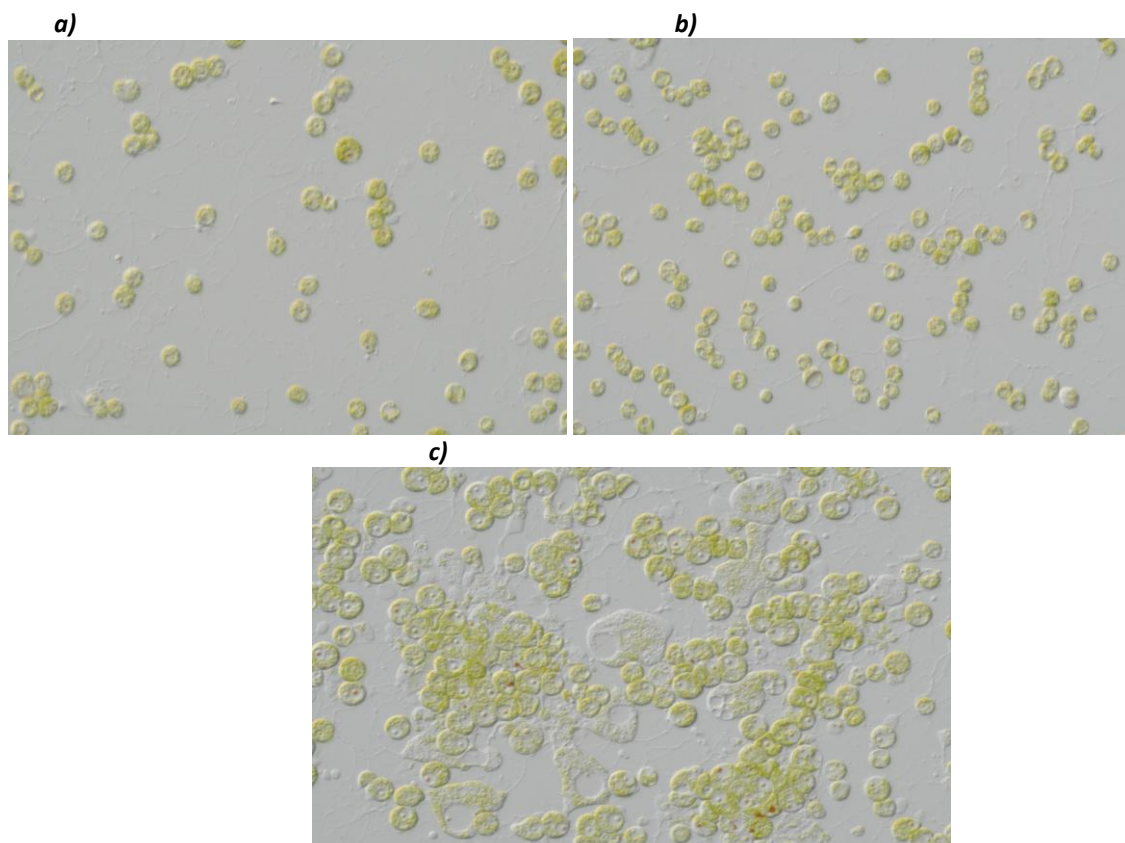
Figure 1. Representative micrographs of hemocytes from *Mytilus edulis* seeded out at a concentrations of (*a*) 100 000, (*b*) 200 000 and (*b*) 400 000 cells/mL. The hemocytes were mixed with L-15(+) as a cell culture (n=5).

3.2   Cell medium

3.2.1   Metabolic activity

The optimization of the cell medium was performed by testing L-15, L-15(+), RPMI, and PBS as a culture media for the hemocytes. Between the hemocytes cultured in the assorted media, a variance in fluorescence was observed after the incubation periods of 48 hr (p=0.004, Kruskal-Wallis) and 96 hr (p=0.0001, Kruskal-Wallis). Comparing this differentiation in rank sum, for each two media, highlighted the difference. The hemocytes cultured in L-15 had higher metabolic activity compared with those cultured in L-15(+), RPMI and PBS media after 48 hrs and 96 hrs incubation (p<0.005, Dunn's test) (Figure 2).

There was a correlation between increasing the incubation period and decreasing the metabolic activity of cells in each medium. The difference was observed between cultures of 24 hr and 192 hr of incubation. The 24 hr incubation had the highest metabolic activity compared to that of 192 hr for all the media (p<0.005, Dunn's test). There was no significant difference observed between the viability data after 24 hr, 48 hr, and 96 hr incubation periods (Figure 2).
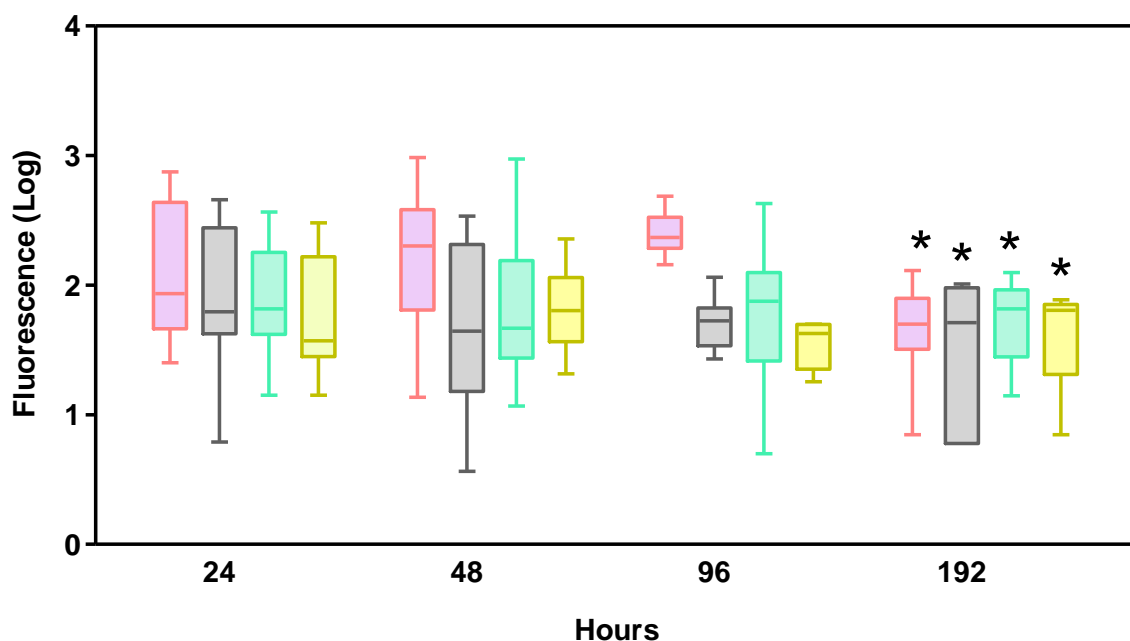
Figure 2. Metabolic activity of hemocytes held in L-15 (pink), L-15(+) (grey), RPMI (blue) or PBS (yellow). Cytotoxicity was measured following 24 hr, 48 hr, 96 hr, and 192 hr incubation periods. Cell viability decreased with increasing the incubation periods. The lowest cell viability was observed after 192 hr (*) incubation period (n=5).

3.2.2   Membrane integrity

There were differences in fluorescence between the media data following incubation periods of 48 hr (p=0.0003, Kruskal-Wallis) and 96 hr (p=0.0006, Kruskal-Wallis) (Figure 3). The hemocytes cultured in the L-15 had higher membrane integrity than those cultured in the other three media (p<0.005, Dunn's test).

The length of exposure (24 hr, 48 hr, 96 hr and 192 hr) caused significantly different effects on the cell viability for each medium (L-15 (p<0.0001, Kruskal-Wallis), L-15(+) (p<0.0001, Kruskal-Wallis), RPMI (p<0.0001, Kruskal-Wallis), and PBS (p<0.0001, Kruskal-Wallis)). When comparing the incubation periods, the 24 hr media and cells incubation had the highest membrane integrity compared to that of 192 hr (L-15, L-15(+), RPMI, and PBS (p<0.005, Dunn's test)) (Figure 3).
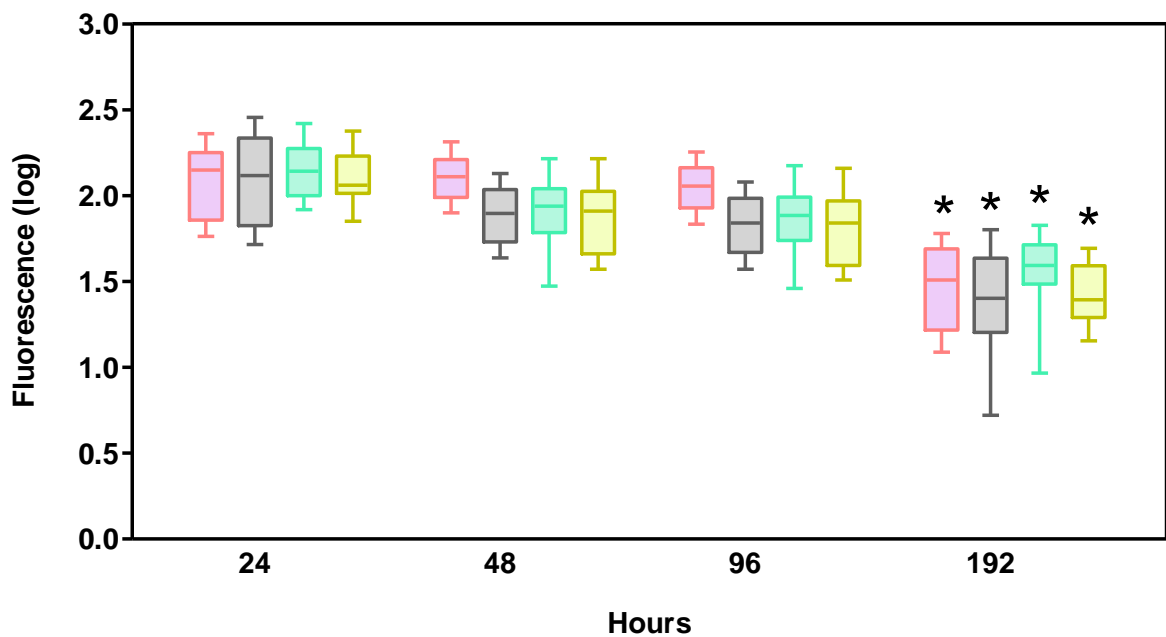
Figure 3. Membrane integrity of hemocytes held in L-15 (pink), L-15(+) (grey), RPMI (blue) or PBS (yellow). Cytotoxicity was measured following 24 hr, 48 hr, 96 hr, and 192 hr incubation periods. Cell viability decreased with increasing the incubation periods. The lowest cell viability was observed after 192 hr (*) incubation period (n=5).

3.2.3 Glutathione concentration

There were differences in the hemocyte glutathione concentration between the media following the incubation periods: 48 hr (p=0.004, Kruskal-Wallis), 96 hr (p<0.0001, Kruskal-Wallis), and 192 hr (p=0.0002, Kruskal-Wallis) (Figure 4). The highest glutathione concentration was found in hemocytes cultured in L-15 medium after 48 hr and 192 hr (p<0.005, Dunn's test).

There was a correlation between increasing the incubation periods and decreasing the glutathione content of hemocytes that cultured in each medium (L-15 (p<0.0001, Kruskal-Wallis), L-15(+) (p<0.0001, Kruskal-Wallis), RPMI (p<0.0001, Kruskal-Wallis), and PBS (p<0.0001, Kruskal-Wallis)). The 24 hr incubation had the highest glutathione concentration compared to 48 hr, 96 hr, and 192 hr incubation (p<0.005, Dunn's test). While, the results for 192 hr incubation were the lowest compared to all the other incubation peroids (p<0.005, Dunn's test) (Figure 4).
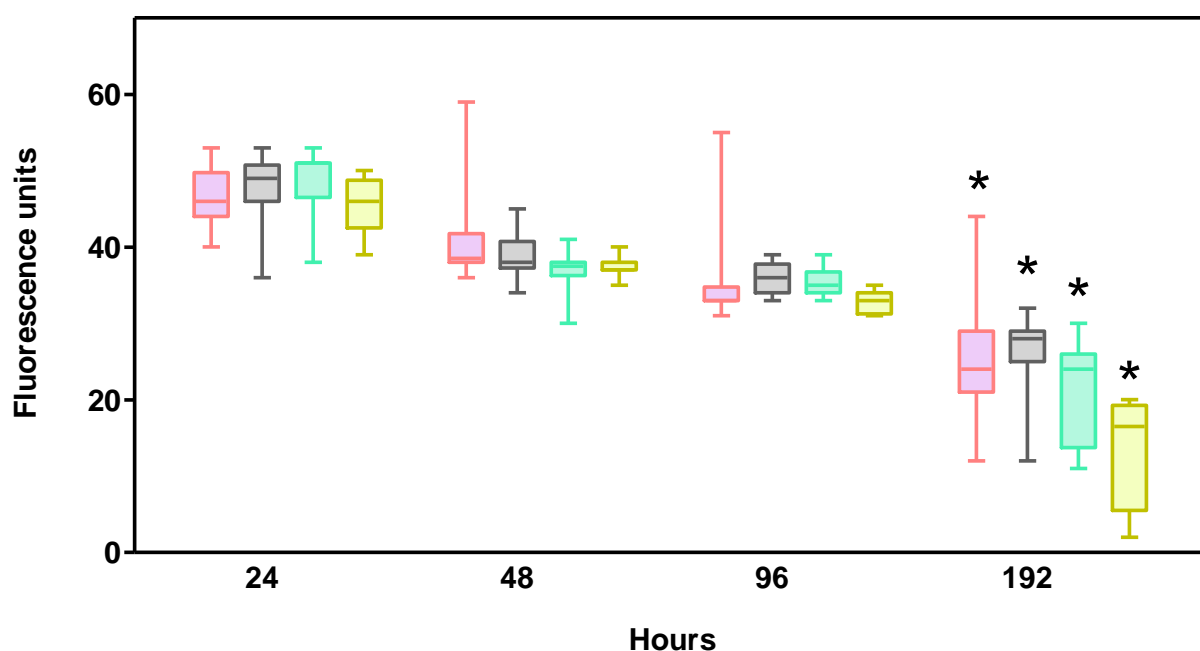
Figure 4. Glutathione content of hemocytes held in L-15 (pink), L-15(+) (grey), RPMI (blue) or PBS (yellow). Cytotoxicity was measured following 24 hr, 48 hr, 96 hr, and 192 hr incubation periods. Cell viability decreased with increasing the incubation periods. The lowest cell viability was observed after 192 hr (*) incubation period (n=5).

3.3 DMSO concentrations

3.3.1 Metabolic activity

As seen in Figure 5, the metabolic activity of the hemocytes seemed be unaffected after treatment with 0.2% and 0.8% DMSO concentrations at all of the incubation periods (24 hr, 48 hr, and 96 hr) compared to the control. The only observed effect was from the exposure to 3.2% DMSO concentration, which decreased the metabolic activity after 24 hr, 48 hr, and 96 hr (p*=0.001, p*=0.0001, and p*=0.0001, respectively, Wilcoxon test) compared to the same incubation periods of the control.

Increasing the incubation period caused decreasing in the metabolic activity. There was a difference between hemocytes exposed to 0.2%, 0.8%, and 3.2% DMSO after 24 hr (p=0.003, Kruskal-Wallis), 48 hr (p=0.0003, Kruskal-Wallis), and 96 hr (p=0.001, Kruskal-Wallis) of incubation.
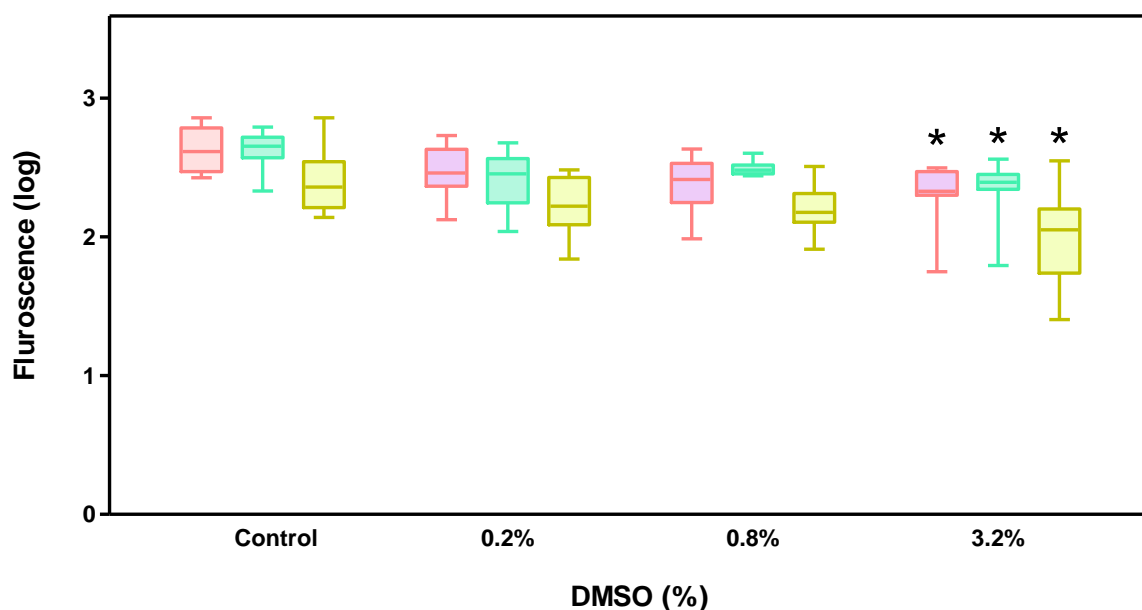
Figure 5. The effect of (0.2%, 0.8%, 3.2%) DMSO on the metabolic activity of the hemocytes. The hemocytes were incubated for 24 hr (pink), 48 hr (blue), and 96 hr (yellow) before the cytotoxicity assessment. The cells were maintained for 24 hr before they were exposed to the different DMSO concentrations (n=5).

3.3.2 Membrane integrity

The membrane integrity of the hemocytes appeared to be unaffected at 0.2% and 0.8%, DMSO concentrations compared to the control. The only observed effect was from the exposure to 3.2% DMSO concentration, which decreased the membrane integrity after 24 hr, 48 hr, and 96 hr (p*=0.0002, p*=0.0001, and p*=0.0001, respectively, Wilcoxon test) compared to the same incubation periods of control (Figure 6).

The fluorescence of hemocytes incubated with 0.2%, 0.8%, and 3.2% DMSO varied after 24 hr (p=0.0008, Kruskal-Wallis), 48 hr (p=0.0005, Kruskal-Wallis), and 96 hr (p=0.0009, Kruskal-Wallis) of incubation.
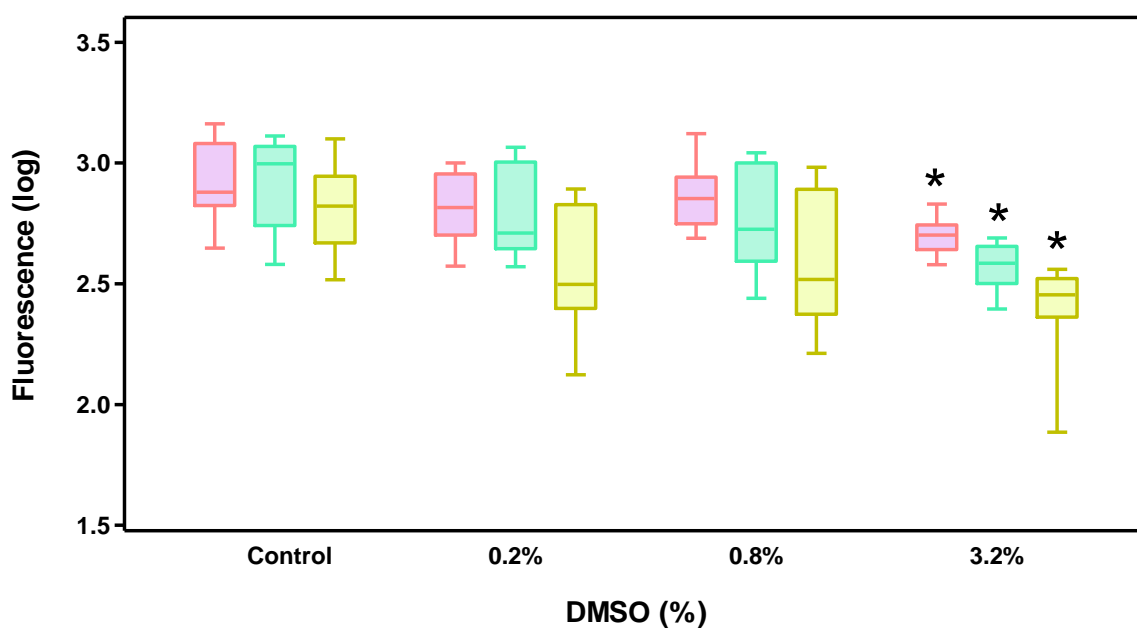
Figure 6. The effect of (0.2%, 0.8%, 3.2%) DMSO on the membrane integrity of the hemocytes. The hemocytes were incubated for 24 hr (pink), 48 hr (blue), and 96 hr (yellow) before the cytotoxicity assessment. The cells were maintained for 24 hr before they were exposed to the different DMSO concentrations (n=5).

3.3.3   Glutathione concentration

The data of glutathione concentration did not show any significant difference at the tested concentrations of DMSO (Figure 7).

However, a difference occurred in data of hemocyte glutathione content exposed to 0.2%, 0.8%, and 3.2% DMSO after 48 hr (p=0.02, Kruskal-Wallis) and 96 hr (p<0.0001, Kruskal-Wallis) of incubation.
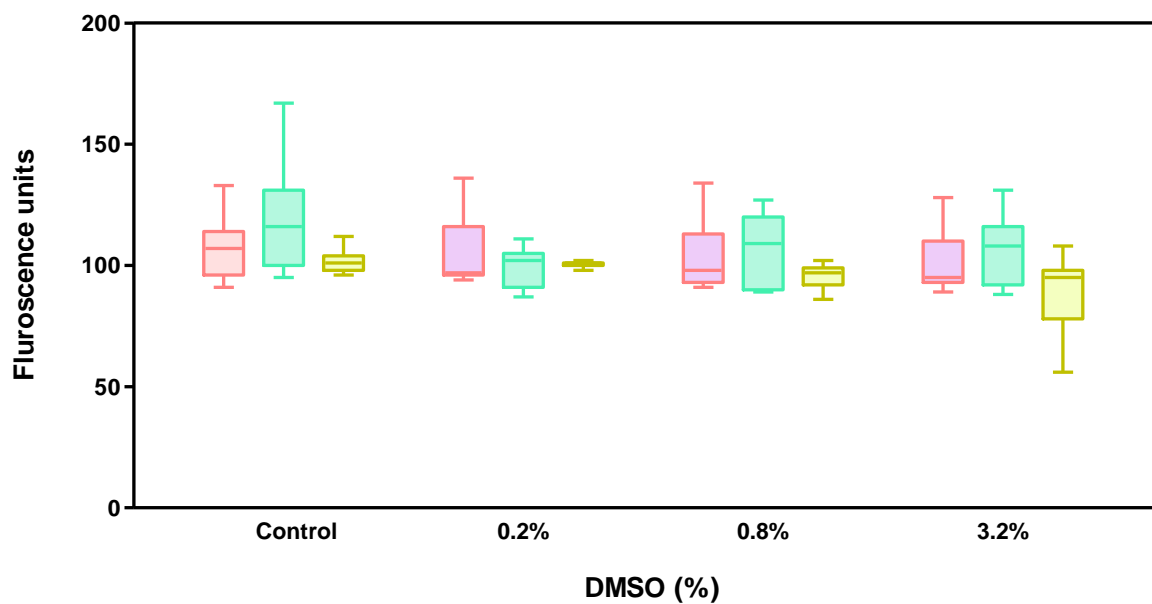
Figure 7. The effect of (0.2%, 0.8%, 3.2%) DMSO on the glutathione concentration of the hemocytes. The hemocytes were incubated for 24 hr (pink), 48 hr (blue), and 96 hr (yellow) before the cytotoxicity assessment. The cells were maintained for 24 hr before they were exposed to the different DMSO concentrations (n=5).

3.4 PAH cytotoxicity

3.4.1 Naphthalene cytotoxicity

Cells exposed to different naphthalene concentrations had a decrease in cell viability in a dose dependent manner (Figure 8). The 24 hr EC_{50} (half maximal effective concentration of naphthalene) for metabolic activity was higher (7.9×10^{-5} mg/L) than that of 48 hr (1.9×10^{-5} mg/L). An increase in 24 hr EC_{50} (9.9×10^{-1} mg/L) for membrane integrity was observed comparing to the EC_{50} for the other probes. The 24 hr EC_{50} for glutathione content was (3.1×10^{-3} mg/L) (Table 1).
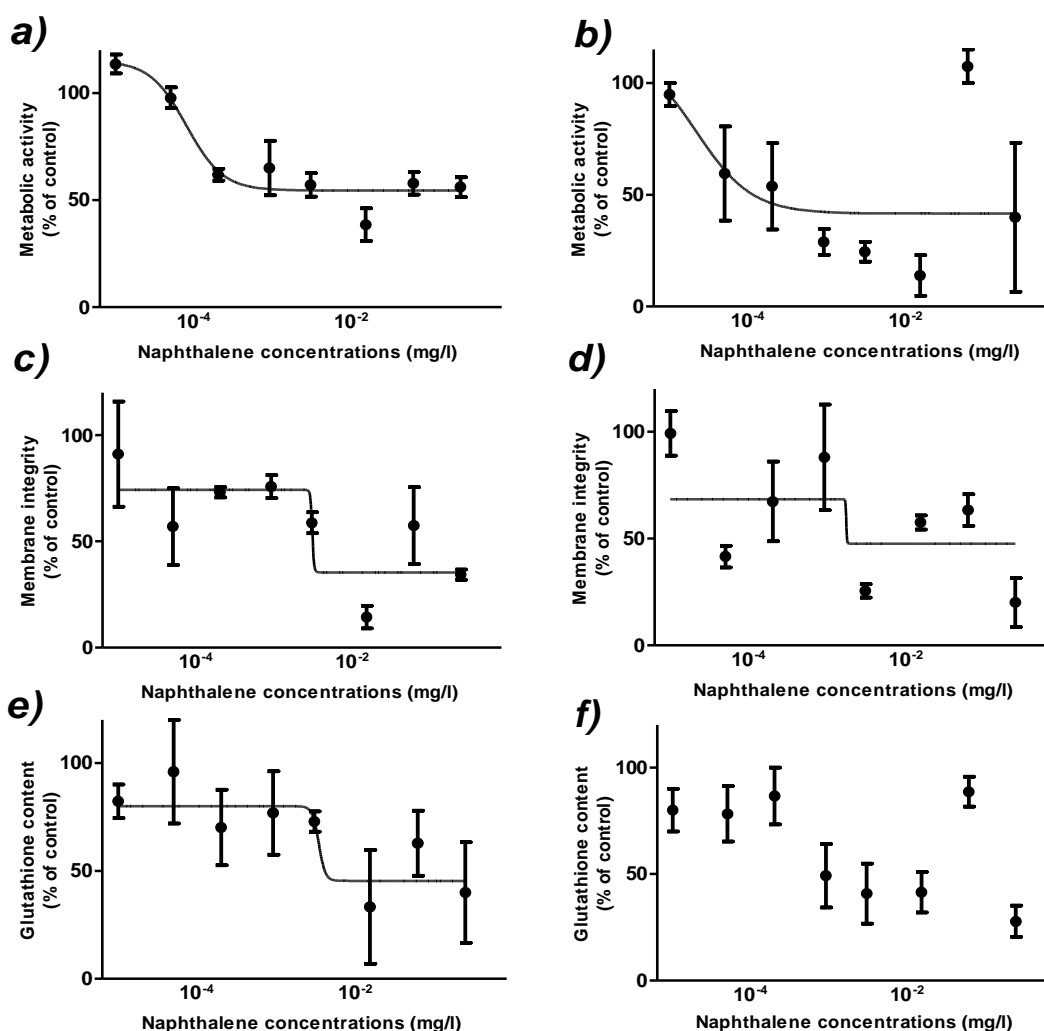
Figure 8. Metabolic activity (*a, b*), membrane integrity (*c, d*), and glutathione content (*e, f*) in the hemocytes following exposure to naphthalene. The cells were exposed for 24 hr (*a, c, e*) and 48 hr (*b, d, f*) prior to assessment of cytotoxicity (mean ± SEM, n = 3). R^2= 0.8, 0.26 (AB), 0.38, 0.23 (CFDA-AM), 0.27, not computed (mBCI), at 24 hrs and 48 hrs, respectively.

3.4.2 Phenanthrene cytotoxicity

The data showed that phenanthrene caused a dose dependent cytotoxicity (Figure 9). For all the probes, the 48 hr EC_{50} was lower than that of 24 hr exposure. The 24 hr EC_{50} values for metabolic activity and membrane integrity were (6.3×10^{-4} and 7.9×10^{-4} mg/L). At the same time point the EC_{50} for glutathione content was (1.0×10^{-5} mg/L). After 48 hr, the EC_{50} values for membrane integrity and glutathione content were lower (1.9×10^{-5} and 2.5×10^{-6} mg/L), respectively (Table 1).
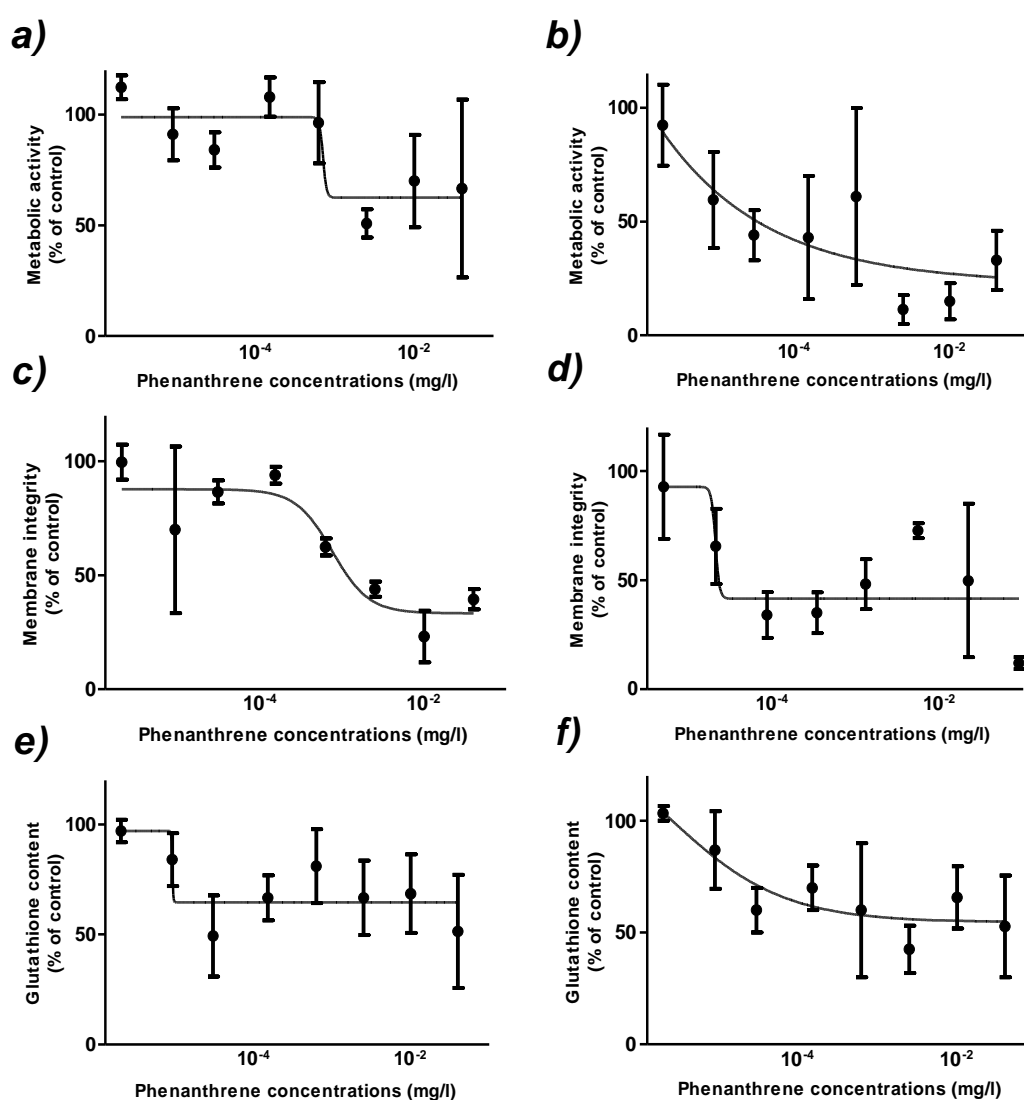
Figure 9. Metabolic activity (*a, b*), membrane integrity (*c, d*), and glutathione content (*e, f*) in the hemocytes following exposure to phenanthrene. The cells were exposed for 24 hr (*a, c, e*) and 48 hr (*b, d, f*) prior to assessment of cytotoxicity (mean ± SEM, n = 3). R^2= 0.28, not computed (AB), 0.54, 0.32 (CFDA-AM), and 0.21, 0.37 (mBCI), at 24 hr and 48 hr, respectively.

3.4.3 2,6-Dimethylnaphthalene

There was a concentration dependent decrease of cell viability following exposure to increasing concentrations of 2,6-dimethylnaphthalene (Figure 10). The 24 hr EC_{50} values were higher than 48 hr EC_{50} for the probes measured. With a 24 hr exposure, EC_{50} value of (3.0×10^{-2} mg/L) was obtained for metabolic activity and membrane integrity. After 48 hr exposure the EC_{50} were lower for metabolic activity and membrane integrity (3.9×10^{-5} and 6.3×10^{-5} mg/L) (Table 1).
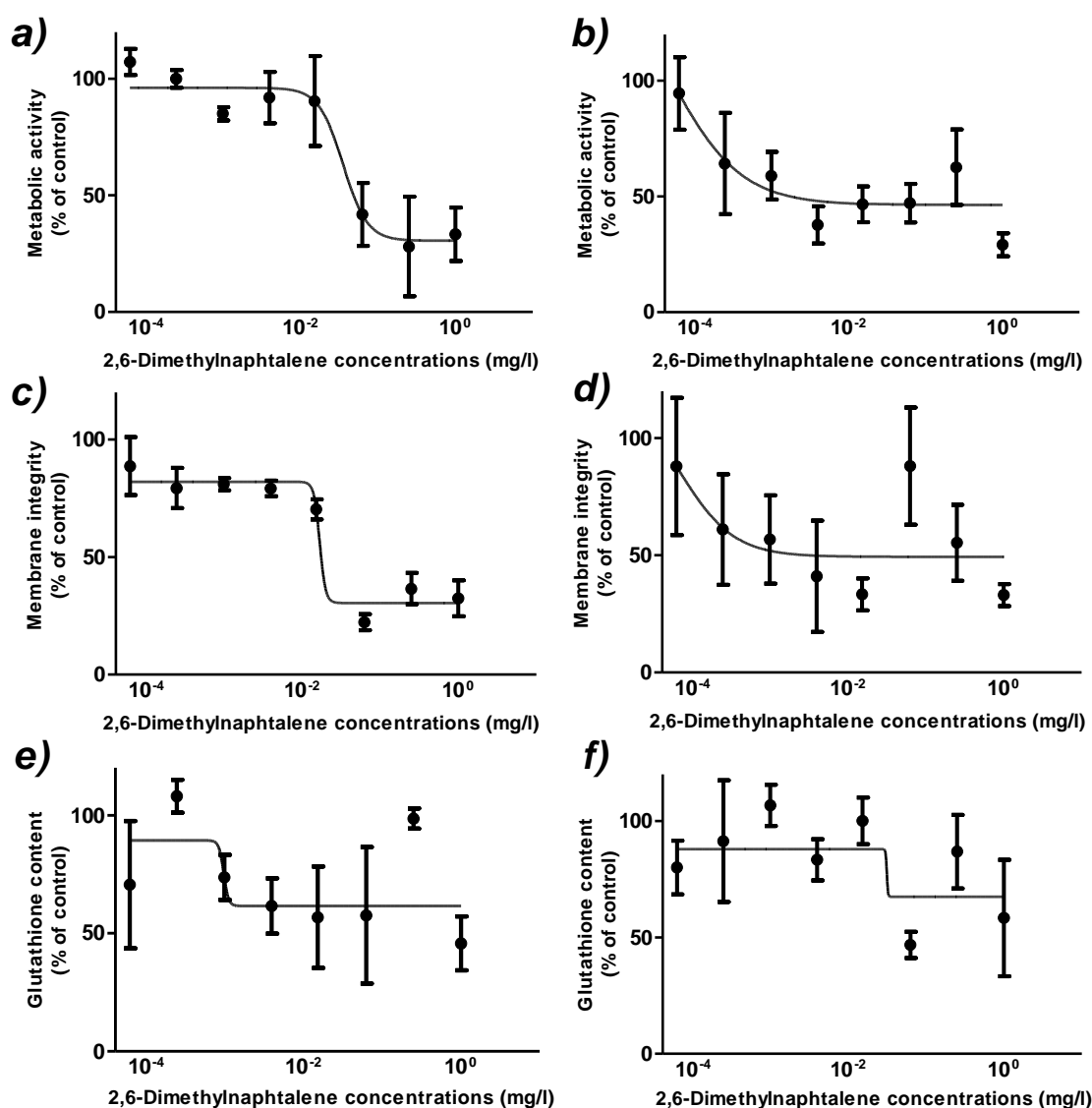
Figure 10. Metabolic activity (*a, b*), membrane integrity (*c, d*), and glutathione content (*e, f*) in the hemocytes following exposure to 2,6-dimethylnaphthalene. The cells were exposed for 24 hr (*a, c, e*) and 48 hr (*b, d, f*) prior to assessment of cytotoxicity (mean ± SEM, n = 3). R^2 = 0.70, 0.38 (AB), 0.70, 0.13 (CFDA-AM), and 0.26, 0.26 (mBCl), at 24 hr and 48 hr, respectively.

3.4.4   1,5-/1,7-Dimethylphenanthrene

A cell viability reduction was observed after exposure the hemocytes to 1,5-/1,7-dimethylphenanthrene (Figure 11). In general, the 24 hr EC_{50} values were higher than those for the 48 hr exposure. The EC_{50} obtained after 24 hr were 1.0×10^{-3}, 1.9×10^{-2} and 1.0×10^{-2} mg/L for metabolic activity, membrane integrity and glutathione content, respectively, with lower values after a 48 hr exposure period (Table 1).
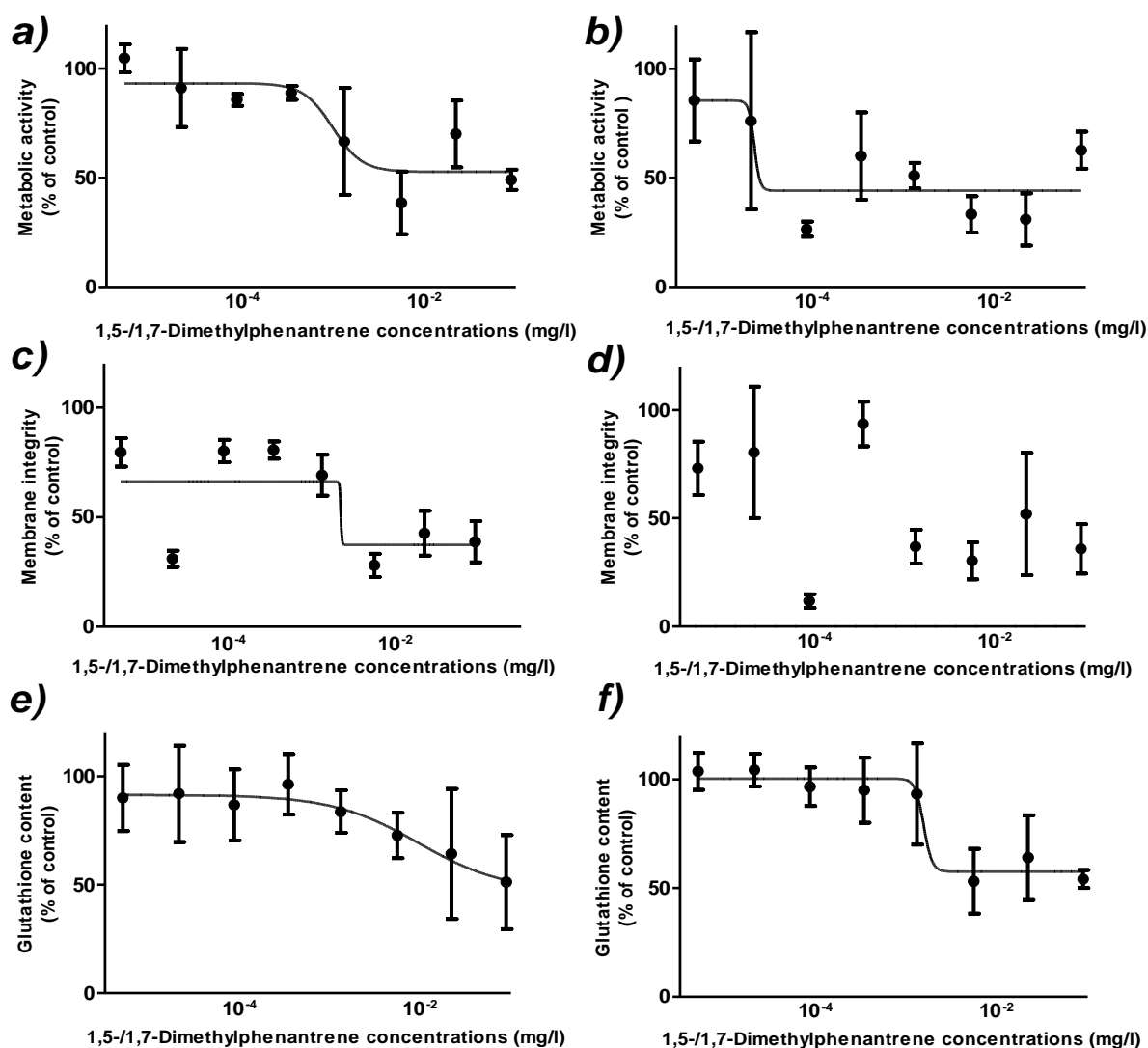
Figure 11. Metabolic activity (*a, b*), membrane integrity (*c, d*), and glutathione content (*e, f*) in the hemocytes following exposure to 1,5-/1,7-dimethylphenanthrene. The cells were exposed for 24 hr (*a, c, e*) and 48 hr (*b, d, f*) prior to assessment of cytotoxicity (mean ± SEM, n = 3). R^2= 0.44, 0.25 (AB), 0.39, not computed (CFDA-AM), and 0.26, 0.50 (mBCl), at 24 hr and 48 hr, respectively.

3.4.5   Pyrene

The results showed that the cell viability was reduced in a dose responsive manner after exposure the hemocytes to pyrene (Figure 12). The 48 hr EC_{50} values were lower than those of 24 hr for each of the fluorescent indicator dyes. The same 24 hr EC_{50} (5.0×10^{-5} mg/L) was observed for the metabolic activity and membrane integrity. The 48 hr EC_{50} values determined for membrane integrity and intracellular GSH concentration were (6.3×10^{-7} and 1.5×10^{-6}). Apparent metabolic activity appeared to decrease from 24 (5.0×10^{-5} mg/L) to 48 hr (1.9×10^{-13} mg/L).
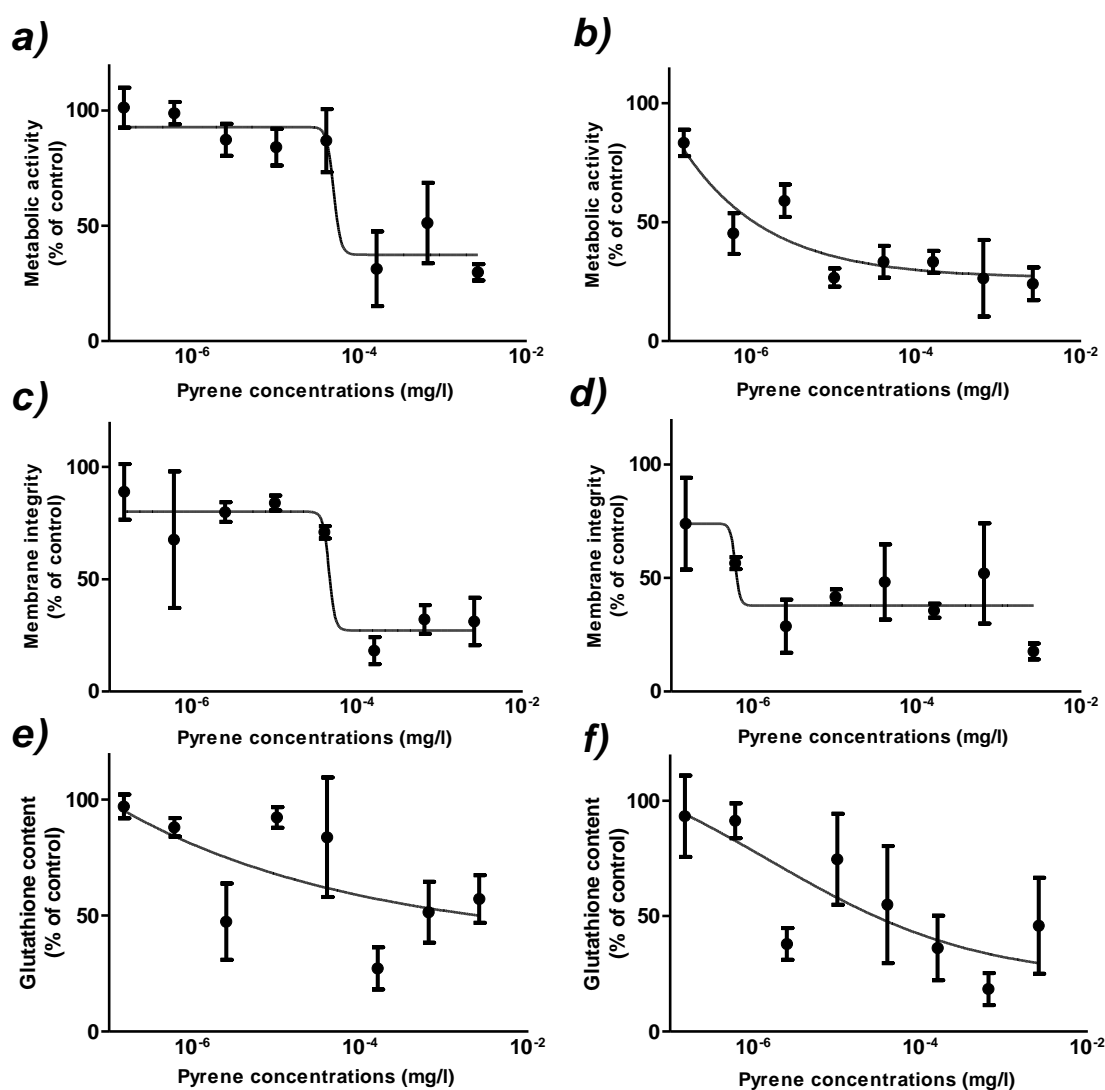
Figure 12. Metabolic activity (*a, b*), membrane integrity (*c, d*), and glutathione content (*e, f*) in the hemocytes following exposure to pyrene. The cells were exposed for 24 hr (*a, c, e*) and 48 hr (*b, d, f*) prior to assessment of cytotoxicity (mean ± SEM, n = 3). R^2 = 0.69, 0.61 (AB), 0.62, 0.26 (CFDA-AM), and not computed, 0.46 (mBCl), at 24 hr and 48 hr, respectively.

3.4.6 Benzo(*a*)pyrene

The data showed that benzo(*a*)pyrene caused a dose dependent cellular cytotoxicity (Figure 13). Comparable results for each time point were observed for the three probes. The 24 hr EC_{50} was higher than that for 48 hr for all the probes measured. The EC_{50} values for metabolic activity and membrane integrity were (1.0×10^{-5} and 1.2×10^{-5} mg/L), respectively after 24 hr incubation. At the second time point the EC_{50} values for metabolic activity and glutathione content were (5.0×10^{-7} and 1.9×10^{-7} mg/L), respectively (Table 1).
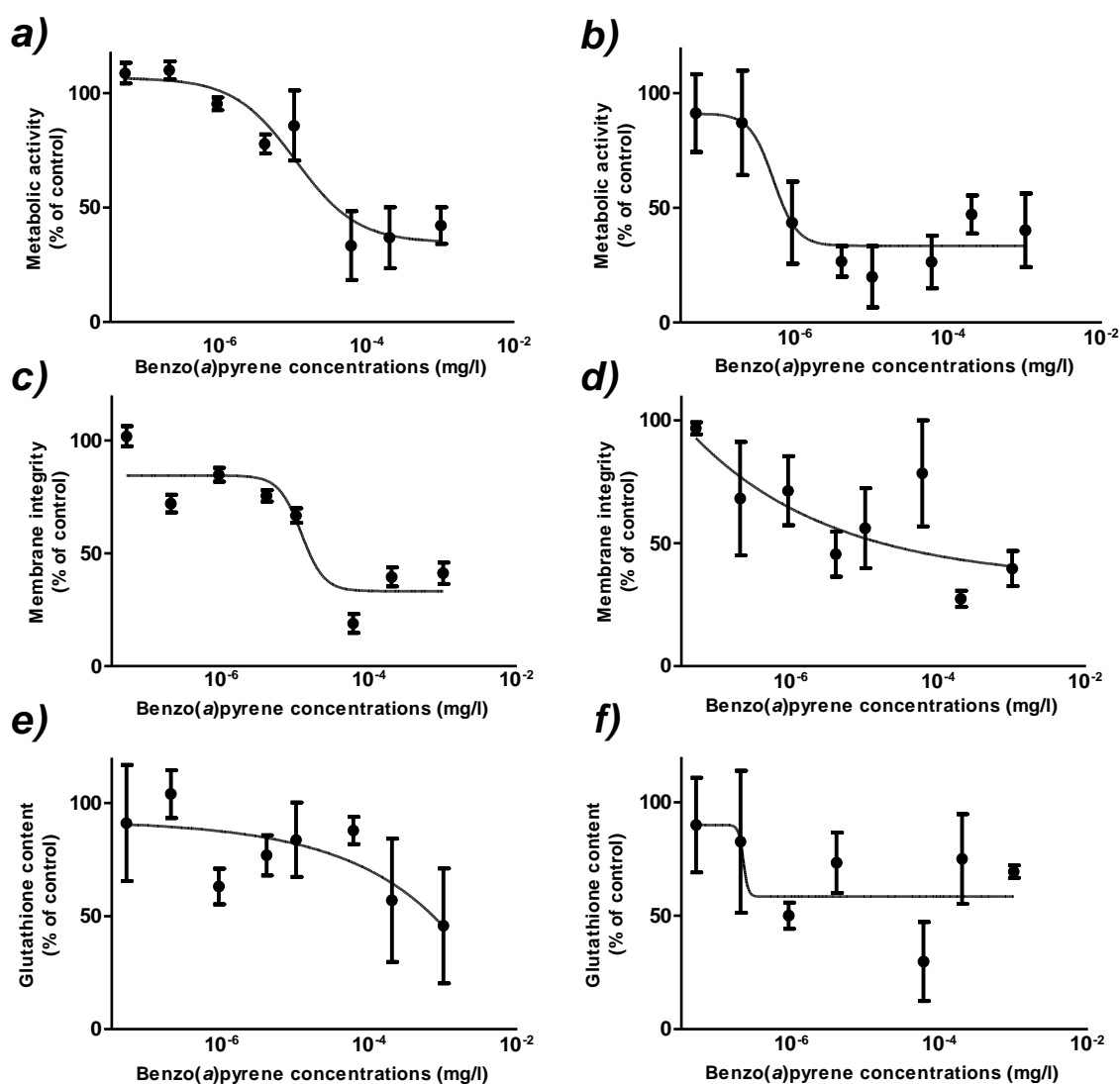
Figure 13. Metabolic activity (*a, b*), membrane integrity (*c, d*), and glutathione content (*e, f*) in the hemocytes following exposure to benzo(*a*)pyrene. The cells were exposed for 24 hr (*a, c, e*) and 48 hr (*b, d, f*) prior to assessment of cytotoxicity (mean ± SEM, n = 3). R^2= 0.76, 0.53 (AB), 0.80, not computed (CFDA-AM), and not computed, 0.17 (mBCI), at 24 hr and 48 hr, respectively.

Table 1. Summary of PAHs EC$_{50}$

Probe PAH	*AB* 24 hr (EC$_{50}$)	*CFDA-AM* 24 hr (EC$_{50}$)	*mBCI* 24 hr (EC$_{50}$)	*AB* 48 hr (EC$_{50}$)	*CFDA-AM* 48hr (EC$_{50}$)	*mBCI* 48hr (EC$_{50}$)
Naphthalene	7.9×10^{-5}	9.9×10^{-1}	3.1×10^{-3}	1.9×10^{-5}	1.5×10^{-3}	*
Phenanthrene	6.3×10^{-4}	7.9×10^{-4}	1.0×10^{-5}	*	1.9×10^{-5}	2.5×10^{-6}
2,6-Dimethylnaphthalene	3.0×10^{-2}	3.0×10^{-2}	2.0×10^{-2}	3.9×10^{-5}	6.3×10^{-5}	3.0×10^{-2}
1,5-/1,7-Dimethylphenanthrene	1.0×10^{-3}	1.9×10^{-2}	1.0×10^{-2}	2.5×10^{-5}	*	1.5×10^{-3}
Pyrene	5.0×10^{-5}	5.0×10^{-5}	*	1.9×10^{-13}	6.3×10^{-7}	1.5×10^{-6}
Benzo(*a*)pyrene	1.0×10^{-5}	1.2×10^{-5}	*	5.0×10^{-7}	*	1.9×10^{-7}

The EC$_{50}$ (mg/L) of the cytotoxic activity of PAHs determined after 24 hr and 48 hr exposure of hemocytes. (* = not computed).

4   Discussion

4.1   Cell density optimization

Cell aggregation is a term used to describe hemocytes as they become sticky and attach to one another and foreign surfaces. It is a spontaneous event that could occur either with the isolation of hemolymph from mussels or immediately afterwards. This phenomenon was studied previously *in vitro* by Chen and Bayne (1995) using *Mytilus californianus* hemocytes. In the present study, cell aggregation was observed at a concentration of 400 000 cells/mL, whereas cells remained dispersed at lower cell densities (100 000 and 200 000 cells/mL).

Cell density of 400 000 cells/mL was not the optimal cell density in this study because of the potential adverse effects that might occur from the resulting cell aggregation. For instance, cell aggregation could provoke stress and thus reduce the cell survival rate. The coagulation of hemocytes reduces stimulation after exposure to the stressors is another likelihood. Additionally, it's possible that the high cell density results in the inability of cells to attach themselves onto the microtiter plates. Thus, they are removed by the addition of the probes. Therefore, measuring cell viability at a density of 400 000 cells/mL was not applicable. On the other hand, testing with a cell density of 100 000 cells/mL, yielded a lower cell count. This density was not recommended either, because it might lead to lower response. Hence, the density of 200 000 cells/mL is the most practical one in this study.

4.2   Cell medium optimization

Four culture media (L-15, L-15(+), RPMI, and PBS) were tested for their ability to maintain the viability of hemocytes *in vitro*. The four media gave almost comparable results. Cell viability in terms of metabolic activity, membrane integrity and glutathione content decreased with increasing the incubation periods (24 hr, 48 hr, 96 hr and 192 hr).

Pervious study reported that the isolated cells from the zebra mussel maintained in L-15 medium survived better than those in RPMI (Quinn *et al.*, 2009). In fact, L-15 culture medium, containing salts, galactose, and pyruvate (Leibovitz, 1963), has been suggested to be the most appropriate medium for *M.edulis* hemocytes cell culture (Hartl *et al.*, 2010). Nevertheless, it was

decided to culture the cells in L-15(+). This medium provided satisfactory results in the cytotoxicity assays. Since hemocytes proliferate upon infection (Arala-Chaves and Sequeira, 2000), adding antibiotics to the medium ensures the aseptic conditions by minimizing the potential bacterial contamination (Wenli and Shields, 2007). However, the amphotericin B added to the L-15(+) medium could affect hemocytes negatively by decreasing their viability (Cao *et al.*, 2003). On the other hand, PBS medium was not recommended as cell culture. This may be due to the isoosmotic detrimental effect of PBS medium that could occur.

The hemocytes cultured in the different media tested maintained high viability after 24 hr, 48 hr, and 96 hr, as opposed with those of a 192-hr incubation period. This is in accordance with the findings of Li and Shields (2007), who cultured the hemocytes of lobster in L-15, RPMI, and other media. Those tests suggest that the hemocytes survived, but did not proliferate in any of the used culture media. Based on this assumption, several attempts have been made to establish permanent and proliferated cell cultures from marine invertebrates, yet they have been unsuccessful (Rinkevich, 1999).

In summary, to maintain the cell culture and ensure the aseptic conditions, the use of L-15(+) medium seemed to be a more suitable medium for hemocytes of the blue mussel in this study, with an incubation period up to four days.

4.3   DMSO cytotoxicity

DMSO is a carrier solvent, which represents a reliable approach to dissolve PAHs. The latter are poorly soluble in media since they are hydrophobic compounds. In this case and in absence of other alternatives, DMSO as a carrier solvent is used. In this study, PAHs were diluted in DMSO. Therefore, a DMSO cytotoxicity test had to be conducted, to ensure a safe concentration to dissolve the PAHs.

In this study, cell viability measurements of hemocytes cultured in 0.2% and 0.8% DMSO concentrations were not significantly different from those of the control. When cultured in 3.2% DMSO concentration, cell viability was lower than control.

The results indicated that a concentration of 0.2% DMSO was the most suitable to be used as a vehicle for PAHs. Cultures treated with 0.8% DMSO appeared to have a cytotoxic tendency (although not significant) and those treated with 3.2% DMSO had lower number of cells compared to control. These results are in accordance with a study on mammalian cells, showing

cytotoxic effects of DMSO at concentrations between 0.5% and 6% with little or no damage at concentrations up to 0.1% (Qi *et al.*, 2008). This may indicate that the blue mussel hemocytes are almost as sensitive as mammalian cells. Conversely, several studies on fish use up to 1% DMSO concentration without any reported deleterious effects (Bell *et al.*, 1981; Kammann *et al.*, 2001).

Kontir *et al*. (1986) performed an evaluation of different vehicles (such as DMSO, acetone, methanol and ethanol), measuring the effect of B*a*P metabolism in rabbit lung microsomes. DMSO caused a less harmful effect on B*a*P metabolism. In fact, the toxicity of DMSO may be related to its action on the stability of cell membranes (Yu and Quinn, 1994).

Taken together, to avoid the potential cytotoxic side effects of DMSO the suitable DMSO concentration usable in this study appeared to be 0.2%, which agrees with a previous study by Chen and Bayne (1995) on *Mytilus* sp. hemocytes.

4.4 PAHs cytotoxicity

4.4.1 Naphthalene cytotoxicity

By definition the EC_{50} is '' the time-period over which 50% of the maximum effect is measured should be stated explicitly''(Mouton *et al.*, 2002). The EC_{50} values after 24 hr incubation were lower than those after 48 hr for metabolic activity, membrane integrity and glutathione content. A possible mechanism for naphthalene was reported previously on a rainbow trout study in which the cells exposed to concentrations up to 7.8×10^{-4} mg/L naphthalene (Schirmer *et al.*, 1998). The EC_{50} values obtained from the latter study were; (4.21×10^{-4} mg/L) for AB and (4.29×10^{-4} mg/L) for CFDA-AM immediately after exposure. After 24 hr lower EC_{50} concentration was observed. The EC_{50} was (3.9×10^{-4} mg/L) for AB and (3.8×10^{-4} mg/L) for CFDA-AM. It was concluded that the decrease was affected by perturbation of cell membrane in both terms of viability (metabolic activity and membrane integrity). On the contrary of the latter study, this study showed lower effect on the membrane integrity compared to the metabolic activity and this was determined from the EC_{50} values (Table 1). A possible explanation for the lower effect could be the alternations in membrane properties (leakage of esterase were incomplete). This explanation was suggested by Dayeh *et al.*, (2004) who also found an unexpected increase in the CF fluorescence after exposure the cells to Triton X-100. In this

study, membrane interrupter might have caused decrease in metabolic activity and GSH content. This occurs if the cell contents were dismissed when removing the toxicant-containing medium and adding Tris buffer. On the other hand, hormetic effect over 100% in comparison to control was observed at the higher metabolic activity concentration (Figure 8-*a*). This effect could be due to hormesis causing stimulatory responses at low doses of chemicals (Calabrese, 1999).

An alternative explanation for the decreased cell viability could be impairment of the mitochondria by the cytotoxic effects of naphthalene. By using isolated avian and mammalian mitochondria, Harmon and Sanbor (1982) observed inhibition of the mitochondrial respiration due to the cytotoxic effect of naphthalene. In this study, a decrease in metabolic activity might make it difficult for the cell to maintain its normal activity, with the consequence of a decrease in membrane stability and GSH level.

Another potential reason for decreased viability may be related to the findings of a study on Sprague-Dawley rats exposed to naphthalene. In that study, Vuchetich *et al.* (1996) proposed that the toxicity of naphthalene was due to its metabolic conversion to metabolites that resulted a in cell damage and glutathione depletion. However, the hypothesis regarding the naphthalene biotransformation would be less relevant in relation to this study. Invertebrates generally have a lower metabolic capacity than vertebrates, and blue mussels particularly have a low ability to metabolize PAHs (Hylland, 2006). Therefore, naphthalene cytotoxicity might be due its lipophilic nature. More specifically, it has an ability to integrate into the biological membranes and cause impairment effects (Vijayavel and Balasubramanian, 2006).

4.4.2 Phenanthrene cytotoxicity

The cell viability data showed that phenanthrene caused a dose dependent cytotoxicity after a 24 hr and 48 hr exposure. The decrease in viability was observed in terms of metabolic activity, membrane integrity, and glutathione content. The 48 hr EC_{50} values were lower than those of 24 hr (Table 1). The 24 hr EC_{50} values for metabolic activity and membrane integrity were comparable. A similar results from the two time points (2 hr and 24 hr) was observed by Schirmer *et al.* (1998) on a rainbow trout fish. The EC_{50} was found to be (9.9×10^{-4} mg/L) after 24 hr exposure to phenanthrene. It was proposed that the cytotoxic effect was due cell membrane damage. Similarly, Hannam *et al.* (2010) noted that scallops (*Pecten maximus*) exposed to phenanthrene different concentrations (5×10^{-2}, 1×10^{-2}, and 2×10^{-2} mg/L) resulted in significant reductions in cell membrane stability. In this study, the highest phenanthrene concentration used

was 0.04 mg/L, which is similar to the latter study. On the contrary, at the same concentrations of the latter study, no significant change in percentage of hemocytes containing non-specific esterase activity (interpreted as membrane stability) was observed on *M. edulis* (Wootton *et al.*, 2003).

Another possible mode of phenanthrene action was proposed by Grundy *et al.* (1996). He argued that phenanthrene impairs the lysosomal membrane integrity and immune function of *M. edulis* hemocytes, resulting in the leakage of enzymes. In this study, the consequences of the aforementioned effect might be a more general response that includes a loss of integrity of the cell membrane.

There is evidence that PAHs induced effects on the cytochrome P450 in molluscs (Moore *et al.*, 1987). However, for this reason, because invertebrates have a rate-limiting cytochrome P450 (Livingstone, 1998) and due to the lipophilic properties of phenanthrene. It is more reasonable that phenanthrene penetrate the lipid bilayer and cause impairment of cell membrane (Grundy *et al.*, 1996). In this study a similar EC_{50} values for the fluorescent assays were obsreved. Therefore a possibility of membrane damage and consequence decrease in metabolic activity and GSH content could be considered.

4.4.3 2,6-Dimethylnaphthalene and 1,5-/1,7-dimethylphenanthrene cytotoxicity

Assessing the cytotoxicity of alkyl PAHs provides more information about structure-toxicity relationships. In this study, the degree of cell viability reduction after exposing the hemocytes to 2,6-dimethylnaphtalene and 1,5-/1,7-dimethylphenanthrene appeared to be dose-responsive.

As mentioned previously, the general mechanism behind the cytotoxicity of naphthalene and phenanthrene appeared to be membrane impairment. In case of alkylated naphthalene and phenanthrene the toxic effect would probably be related to the presence of alkyl chain. In general, acute toxicity rises with increased alkyl carbon chain length (Dyer *et al.*, 2000). Increased alkyl substitution increases lipophilicity (Liu and Sylvester, 1994), strengths the impairment effect (Schultz and Cajina-Quezada, 1982) and thereby increases the toxicity of the compound tested. For instance, methyl isopropyl phenanthrene is almost ten times more toxic than phenanthrene (Turcotte *et al.*, 2011). However in this study, the EC_{50} values of alkylated compounds were found to be almost similar to those of the non-alkylated. A feasible explanation for these results might be that the hemocytes exposed to the alkylated PAHs have higher

membrane stability than those exposed to the non-alkylated PAHs. The high membrane stability may be due to the elevated levels of vitamin E (Lucy, 1972).

Another possible mechanism was reported in metabolic study of 1,6-dimethylnaphthalene in rats. Kilanowicz *et al.* (2002) suggested that the bioactivation of GSH S-conjugates was a possibility by which GSH will be associated with toxicity. However, since vertebrates have higher biotransformation capacity than invertebrates, this hypothesis is less supported (Livingstone, 1998).

4.4.4   Pyrene cytotoxicity

The data shows that pyrene caused a dose dependent cytotoxicity. The 24 hr EC_{50} were higher than those of 48 hr. This indicates that the long exposure period caused more cytotoxic effect. A possible mechanism for pyrene may explain from its properties as one of the highly lipophilic compounds (Ng *et al.*, 1992). In general, cytotoxicity increased with increasing hydrophobicity (Van der Meer *et al.*, 1991). Therefore, a possible mechanism for pyrene might be the highly penetration and the consequence effect of compromising the cell membrane. In this study, comparable EC_{50} values after 24 hr was observed from the metabolic activity and membrane integrity (Table 1). The loss of viability may be due to the disruption of mitochondrial and cell membrane integrity because of the high lipophilicity of pyrene. On the other hand, after 48 hr there was more decrease in the metabolic activity compared to the other viability assays at this time point (Table 1). A possibility for such decrease might be that the cytotoxic effect of pyrene on metabolic activity occurred before any alternations in membrane integrity. Therefore any decrease in the viability from the other two assays was a consequence of metabolic activity impairment. Another explanation might be that the hemocytes undergo apoptosis. Apoptosis in hemocytes are suggested due to inhibition of ATPase and/or mitochondrial ADP/ATP or substrate transport (Sokolova *et al.*, 2004).

On the contrary, no decrease in the non-specific esterase activity in oyster hemocytes was observed after exposure to pyrene (200 μmol/L) (Gagnaire *et al.*, 2006). Pyrene was not consistently toxic to *M. edulis in vivo,* but showed complicated behavior depending on temperature and salinity (Okay *et al.*, 2006). In sum, a possible mode of action for pyrene may be general impairment of cell membrane or still might be due to the biotransformation role in toxicity.

4.4.5   Benzo(*a*)pyrene cytotoxicity

The results indicate that hemocytes exposure to benzo(*a*)pyrene (B*a*P) reduced the cell viability in a dose responsive manner. The EC_{50} values calculated at each time point were similar, with higher EC_{50} values of the 24 hr exposure than 48 hr. This might indicate that the decrease in viability was due to one of the mechanisms mentioned below.

For B*a*P, another mode of toxicity could be proposed than previously suggested. Unlike naphthalene, B*a*P was suggested to be metabolized by *M. edulis* (Livingstone *et al.*, 1990). The antioxidant defense (glutathione) depletion could indicate enhanced ROS production caused by the metabolization of B*a*P in mussels (Livingstone *et al.*, 1990), with a subsequent deleterious effect of the ROS on the cell as general. Similarly, Gómez-Mendikute *et al.* (2002) reported deleterious effects on the immune system of *Mytilus* sp., which he suggests is due to the increased production of superoxide anion during B*a*P metabolism. Another study on the arctic scallop, observed depletion in total oxyradical scavenging capacity, this indicates an antioxidant defense (glutathione) and impairment of cell membrane stability, after exposure to B*a*P (0.0, 74.0, and 90.6 mg/L). The former had higher concentrations than this study due to difference in exposures and durations in the producers. It was also suggested that the depletion in antioxidant defense was due to ROS production, enhanced by the metabolization of B*a*P (Camus *et al.*, 2002).

Another explanation for the low cell viability could be that the mitochondria was the cellular target for B*a*P, with quinine-induced cytotoxicity, thus resulting in direct disruption of energy metabolism (Miller and Ramos, 2001). Therefore this led to the reduction in the cell viability observed from the other assays.

A third explanation could be that B*a*P evoked its toxicity by binding on lipophilic molecules, thus compromising the basic functions of cells (Camus *et al.*, 2002). The 24 hrs EC_{50} values were similar when measuring the metabolic activity and the integrity of cell. At the second time point lower, EC_{50} values were observed with comparable values of the fluorescent indicator dyes (Table 1). Thus, this might indicate a toxic mechanism with consequence cell damage.

In sum, although organic xenobiotics such as PAHs have greater potential for biotransformation in fish compared with the aquatic invertebrates (Livingstone, 1998), one could still conclude that the toxicity of B*a*P might be caused by either; metabolic formation of toxic

metabolites due to biotransformation ; or through the impairment of the cell membranes due to the high lipophilicity of BaP.

4.5   Comparison between PAHs cytotoxicity

The EC$_{50}$ of the dose response curve represented the concentration of the PAHs where 50% of the concentration showed a response. A lower EC$_{50}$ indicates higher toxicity. The EC$_{50}$ values from the three cytotoxicity assays were used to rank these compounds. BaP and pyrene were found to be the most toxic, judged by their lower EC$_{50}$ at each time point. The EC$_{50}$ values (g/mol) at the first time point (24 hr) were: 2.47×10^{-7}, 3.90×10^{-8} (AB), 2.47×10^{-7}, 4.98×10^{-8} (CFDA-AM), and not computed (mBCI). At the second time point (48 hr): 9.80×10^{-16}, 1.90×10^{-9} (AB), 3.11×10^{-9}, not computed (CFDA-AM), and 7.8×10^{-9}, 7.9×10^{-10} (mBCI) of pyrene and Bap, respectively.

As mentioned previously, BaP might evoke its toxicity by biotransformation and formation of ROS, and through other modes of action. BaP and pyrene have a log octanol-water partition coefficient (log K$_{ow}$) of 6.04 and 5.18, respectively, which is higher than the log K$_{ow}$ of naphthalene and phenanthrene (3.33 and 4.57), respectively (Hylland, 2006). Hence, it is expected that naphthalene and phenanthrene have less toxic effect. Naphthalene and phenanthrene EC$_{50}$ values were higher than pyrene and BaP. The EC$_{50}$ values (g/mol) at the first time point were: 6.19×10^{-7}, 3.50×10^{-6} (AB), 7.7×10^{-3}, 4.45×10^{-6} (CFDA-AM), and 2.46×10^{-5}, 5.60×10^{-8} (mBCI). At the second time point: 1.56×10^{-7}, not computed (AB), 1.23×10^{-5}, 1.11×10^{-7} (CFDA-AM), and not computed, 1.40×10^{-8} (mBCI) of naphthalene and phenanthrene, respectively.

2,6-Dimethylnaphtalene and 1,5-/1,7-dimethylphenanthrene EC$_{50}$ values were similar or slightly lower than the non-alkylated, which as proposed was to their higher membrane stability. The EC$_{50}$ values (g/mol) at the first time point: 2.02×10^{-4}, 6.40×10^{-6} (AB), 2.02×10^{-4}, 1.27×10^{-5} (CFDA-AM), and 2.02×10^{-4}, 6.40×10^{-5} (mBCI). At the second time point were: 2.54×10^{-7}, 1.60×10^{-7} (AB), 4.03×10^{-7}, not computed (CFDA-AM), and 2.02×10^{-4}, 1.01×10^{-5} (mBCI) of 2,6-dimethylnaphthalene and 1,5-/1,7-dimethylphenanthrene, respectively.

5   Conclusions

A cell density of 200 000 cells per mL proved to be the appropriate density. L-15(+) was found to be the most appropriate medium of choice to maintain the viability of hemocytes and to provide aseptic conditions. 0.2% DMSO concentration is the least toxic choice to the hemocytes.

This study demonstrated that *M.edulis* hemocytes exposed to the selected PAHs (naphthalene, phenanthrene, pyrene, benzo(*a*)pyrene, 2,6-dimethylnaphtalene, 1,5-/1,7-dimethyl-phenantrene) had decreased the cell viability in terms of metabolic activity, membrane integrity and glutathione content. A dose-dependent decrease was observed in hemocytes after exposure to each compound. The EC_{50} values determined for the tested PAHs indicated a general tendency of increasing toxicity with the exposure periods (24 hr and 48 hr).

Different mechanisms were proposed for each PAH. The lipophilic character of naphthalene appears to be the reason for its toxic action. This was by perturbation of cell membrane or through a specific action on mitochondria. An increased 24 hr EC_{50} value for membrane integrity was observed, which might be due alternations in membrane properties. Phenanthrene might evoke its toxicity with the similar mode of action as naphthalene but with more cytotoxic effect. For the substituted PAHs, the EC_{50} values were similar or slightly lower than the non-alkylated PAHs, which might be due to the higher membrane stability of hemocytes exposed to the alkylated PAHs. The increased toxicity of pyrene than the other PAHs might be linked to its high lipophilicity (log K_{ow} 5.18). The EC_{50} values of the assays measured were close at each time point, but with more decrease of the 48 hr EC_{50} value for metabolic activity. This might be due to apoptosis or pyrene effect on the metabolic activity. B*a*P may express its toxicity by metabolism and biotransformation or due its high lipophilicity. The two time points EC_{50} values obtained from the three assays were similar. In conclusion, this study shows the possibility that hemocytes can be successfully used as biomarkers with cell viability as endpoints.

5.1 *Future perspectives*

a) Comparative investigation into the effects of polycyclic aromatic hydrocarbon on the mussel hemocytes and other sensitive cells should be assessed. This may help to obtain insight into the variations in sensitivity and understand more about the mechanisms of PAHs-induced cell injury.

b) Marine invertebrate hemocytes under *in vitro* conditions are important tools in a variety of scientific disciplines. However, the establishment of cell cultures has been encountered with obstacles. Hence, there are considerable requirements for further testing of protocols with respect to cell culture improvements.

c) Scientists often need to count cells prior to cell culture with an accurate number of input cells. An automated cell counter is needed to obtain an accurate cell counts and to accelerate the cell work.

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7   Appendix

7.1   Appendix A: Chemicals and equipments

Beckman CoulterTM

MultisizerTM 3, Coulter counter®

BioSource Europe S.A., Nivelles, Belgium

Biowhittaker Inc., Walkerswille, Maryland, USA

Leibowitz (L-15) medium

Cambrex East Rutherford, NJ, USA

Pencicillin

Streptomycin

Amphotenzin

Chiron AS,Trondheim, Norway and Sigma-Aldrich, St. Louis, MO

Polycyclic aromatic hydrocarbons compounds

Merck, Whitehouse Station, New Jersey, USA

Monopotassium phosphate (KH_2PO_4)

Sodium chloride (NaCl)

Sodium Hydroxide (NaOH)

Molecular Probes, Paisley, United Kingdom

Alamar BlueTM (AB)

5-Carboxyfluorescein diacetate, actetoxymethyl ester (CFDA-AM)

Monochlorobimane (mBCl)

Sigma–Aldrich Norway AS, Oslo, Norway

Ethylene diamine tetraacetic acid (EDTA)

Royal Park Memorial Institute (RPMI)

Disodium hydrogen phosphate (Na_2HPO_4)

Trypan blue

Dimethyl sulfoxide (DMSO)

7.2   *Appendix B: Raw data*

Media optimization -data

AB-24 hr

L-15	L-15(+)	RPMI	PBS
1439	987	814	994
1263	1009	818	771
1314	985	769	758
1435	1148	1059	779
956	769	725	949
756	744	776	729
738	700	752	706
717	941	961	729
993	733	731	917
697	754	748	689
743	734	710	720
724	969	906	714
993	712	721	931
697	736	763	683
743	732	751	726
724	949	918	723
938	698	750	838
723	737	706	716
778	734	741	725
772	807	889	720

AB-48 hr

L-15	L-15(+)	RPMI	PBS
1592	904	740	871
1402	900	733	730
1522	902	747	718
1627	1003	941	710
880	676	691	817
757	710	705	751
746	703	708	714
758	820	868	726
896	666	646	890
757	684	576	739
720	682	686	674
715	858	843	699
847	636	685	877
712	621	674	695
682	676	710	727
676	790	895	683
1075	678	680	777
915	676	700	713
937	678	704	691
954	783	809	684

AB-96 hr

L-15	L-15(+)	RPMI	PBS
479	308	388	310
604	294	353	241
490	326	380	222
405	376	218	209
448	232	261	261
491	233	255	11
674	224	266	16
748	298	385	24
540	230	293	311
460	223	283	234
602	237	257	220
547	328	370	239
451	233	306	297
410	231	285	241
534	217	319	226
575	320	440	234
441	236	246	279
498	214	250	230
679	231	275	224
480	288	326	226

AB-192 hr

L-15	L-15(+)	RPMI	PBS
195	167	19	62
105	158	130	41
127	58	146	24
115	158	171	61
144	75	82	72
66	14	125	23
101	28	190	16
144	60	131	62
165	60	145	142
32	13	99	41
72	48	23	50
147	61	59	24
40	71	161	25
85	59	188	99
73	27	100	29
97	71	131	30
108	64	91	19
124	61	81	129
10	3	79	130
13	59	71	50

CFDA-AM-24 hr

L-15	L-15(+)	RPMI	PBS
228	297	162	213
187	240	190	136
191	244	140	160
220	397	263	155
131	118	151	182
72	70	152	113
108	64	130	115
70	91	211	126
148	67	95	92
75	100	142	83
83	75	112	125
73	127	200	127
180	161	110	209
87	159	180	115
158	187	146	123
165	277	230	84
241	192	240	250
141	344	103	196
162	306	234	183
200	346	279	171

CFDA-AM-48 hr

L-15	L-15(+)	RPMI	PBS
204	90	137	131
215	139	98	105
172	113	102	174
188	136	152	118
141	53	52	47
103	57	45	8
97	77	45	7
95	113	72	7
153	55	72	57
108	54	70	49
89	60	78	52
107	74	114	54
176	89	95	101
145	88	82	75
171	91	94	75
129	135	149	83
136	84	116	132
141	81	118	102
133	120	120	91
122	144	174	113

CFDA-AM-96 hr

L-15	L-15(+)	RPMI	PBS
181	83	125	118
189	127	88	95
152	101	94	154
166	124	138	105
125	47	48	42
91	51	40	7
88	69	41	7
85	101	66	7
136	50	66	50
96	49	64	43
78	53	70	47
94	67	104	48
157	79	85	90
130	79	74	67
156	82	85	67
113	121	134	73
123	75	106	119
124	72	107	92
117	108	108	79
108	130	159	101

CFDA-AM-192 hr

L-15	L-15(+)	RPMI	PBS
70	73	54	54
60	69	62	35
60	67	47	43
67	115	88	41
35	34	50	46
22	23	51	29
32	22	45	31
23	29	72	34
43	22	31	27
24	31	48	24
26	25	40	32
24	39	68	30
51	46	36	49
27	47	52	29
45	55	48	31
48	80	73	25
66	57	77	59
40	86	41	51
41	88	80	49
55	105	91	48

mBCI-24 hr

L-15	L-15(+)	RPMI	PBS
37	39	35	35
35	39	34	32
38	39	33	32
37	38	37	31
32	36	34	34
32	33	35	35
34	34	36	34
34	34	36	34
33	34	34	31
33	37	35	31
33	36	34	35
32	37	38	33
34	36	36	33
33	34	39	33
55	35	37	33
34	38	37	33
31	34	34	31
33	35	33	32
33	35	35	33
33	37	35	31

mBCI-48 hr

L-15	L-15(+)	RPMI	PBS
42	42	37	38
41	42	37	37
43	41	37	37
43	42	41	37
36	38	37	35
38	38	38	37
37	37	38	37
38	40	38	38
51	38	35	38
37	38	30	37
38	39	39	39
38	39	35	39
40	36	36	40
38	34	38	36
39	36	38	38
39	45	39	39
37	37	37	36
38	38	38	35
59	39	38	37
40	38	36	37

mBCI-96 hr

L-15	L-15(+)	RPMI	PBS
52	36	43	44
44	45	46	41
43	53	49	50
42	50	53	42
45	40	38	49
41	47	41	39
44	46	45	42
40	48	48	40
50	43	51	48
44	46	51	46
44	48	51	46
46	50	53	49
50	53	50	46
47	50	51	48
46	52	51	46
50	53	51	49
46	51	51	44
48	50	52	46
49	48	52	46
53	50	49	50

mBCI-192 hr

L-15	L-15(+)	RPMI	PBS
120	128	121	92
116	124	112	96
124	93	120	116
121	85	107	96
120	93	115	93
119	90	120	94
112	127	119	95
138	125	89	94
118	124	122	92
120	108	109	112
96	113	108	98
125	124	122	96
92	122	126	91
123	101	123	92
117	123	124	94
130	128	34	95
108	124	102	94
122	93	98	93
111	85	97	95
90	93	108	94

DMSO **AB**

<table>
<tr><td colspan="3">CONTROL</td><td colspan="3">0.2%</td></tr>
<tr><td>24 hr</td><td>48 hr</td><td>96 hr</td><td>24 hr</td><td>48 hr</td><td>96 hr</td></tr>
<tr><td>622,3333</td><td>523,25</td><td>585,3333</td><td>321</td><td>398,25</td><td>269,3333</td></tr>
<tr><td>608,3</td><td>492,25</td><td>184,3333</td><td>324</td><td>156,25</td><td>161,3333</td></tr>
<tr><td>267,3</td><td>371,25</td><td>138,3333</td><td>289</td><td>284,25</td><td>293,3333</td></tr>
<tr><td>643,3</td><td>487,25</td><td>205,3333</td><td>257</td><td>216,25</td><td>167,3333</td></tr>
<tr><td>333,3</td><td>348,25</td><td>228,3333</td><td>431</td><td>334,25</td><td>224,3333</td></tr>
<tr><td>320,3</td><td>430,25</td><td>582,3333</td><td>537</td><td>122,25</td><td>70,33333</td></tr>
<tr><td>383,3</td><td>507,25</td><td>241,3333</td><td>231</td><td>318,25</td><td>94,33333</td></tr>
<tr><td>275,3</td><td>214,25</td><td>149,3333</td><td>269</td><td>303,25</td><td>131,3333</td></tr>
<tr><td>286,3</td><td>554,25</td><td>192,3333</td><td>259</td><td>261,25</td><td>69,33333</td></tr>
<tr><td>414,3</td><td>325,25</td><td>300,3333</td><td>427</td><td>195,25</td><td>303,3333</td></tr>
<tr><td>721,3</td><td>447,25</td><td>720,3333</td><td>521</td><td>109,25</td><td>267,3333</td></tr>
<tr><td>458,3</td><td>618,25</td><td>347,3333</td><td>133</td><td>476,25</td><td>150,3333</td></tr>
<tr><td>437,3</td><td>389,25</td><td>151,3333</td><td>218</td><td>463,25</td><td>191,3333</td></tr>
<tr><td>295,3</td><td>615,25</td><td>162,3333</td><td>232</td><td></td><td>165,3333</td></tr>
<tr><td>412,3</td><td>449,25</td><td>308,3333</td><td>352</td><td></td><td></td></tr>
</table>

<table>
<tr><td colspan="3">0.8%</td><td colspan="3">3.2%</td></tr>
<tr><td>24 hr</td><td>48 hr</td><td>96 hr</td><td>24 hr</td><td>48 hr</td><td>96 hr</td></tr>
<tr><td>254</td><td>291,25</td><td>130,3333</td><td>205</td><td>212,25</td><td>101,3333</td></tr>
<tr><td>97</td><td>292,25</td><td>152,3333</td><td>292</td><td>25,33333</td><td>57,33333</td></tr>
<tr><td>367</td><td>345,25</td><td>137,3333</td><td>210</td><td>67,33333</td><td>42,33333</td></tr>
<tr><td>429</td><td>401,25</td><td>321,3333</td><td>107</td><td>244,25</td><td>47,33333</td></tr>
<tr><td>211</td><td>277,25</td><td>175,3333</td><td>194</td><td>362,25</td><td>153,3333</td></tr>
<tr><td>184</td><td>281,25</td><td>89,33333</td><td>210</td><td>72,33333</td><td>138,3333</td></tr>
<tr><td>169</td><td>275,25</td><td>135,3333</td><td>213</td><td>62,25</td><td>146,3333</td></tr>
<tr><td>423</td><td>320,25</td><td>150,3333</td><td>184</td><td>269,25</td><td>123,3333</td></tr>
<tr><td>127</td><td>297,25</td><td>271,3333</td><td>56</td><td>260,25</td><td>239,3333</td></tr>
<tr><td>263</td><td>315,25</td><td>165,3333</td><td>314</td><td>113,25</td><td>135,3333</td></tr>
<tr><td>261</td><td>308,25</td><td>81,33333</td><td>262</td><td>62,25</td><td>236,25</td></tr>
<tr><td>260</td><td>286,25</td><td>125,3333</td><td>299</td><td>272,25</td><td>250,25</td></tr>
<tr><td>311</td><td>324,25</td><td>235,3333</td><td>275</td><td>241,25</td><td>141,25</td></tr>
<tr><td></td><td>381,25</td><td></td><td></td><td>223,25</td><td>196,3333</td></tr>
</table>

DMSO **CFDA-AM**

<table>
<tr><td colspan="3">CONTROL</td><td colspan="3">0.2%</td></tr>
<tr><td>24 hr</td><td>48 hr</td><td>96 hr</td><td>24 hr</td><td>48 hr</td><td>96 hr</td></tr>
<tr><td>1205,6</td><td>1172,5</td><td>1259,9</td><td>862,6</td><td>1051,5</td><td>153,9</td></tr>
<tr><td>1413,6</td><td>389,5</td><td>1231,9</td><td>438,6</td><td>1008,5</td><td>241,9</td></tr>
<tr><td>1119,6</td><td>1252,5</td><td>766,9</td><td>850,6</td><td>441,5</td><td>712,9</td></tr>
<tr><td>811,6</td><td>992,5</td><td>881,9</td><td>540,6</td><td>445,5</td><td>663,9</td></tr>
<tr><td>1432,6</td><td>570,5</td><td>475,9</td><td>521,6</td><td>513,5</td><td>296,9</td></tr>
<tr><td>810,6</td><td>1143,5</td><td>345,9</td><td>982,6</td><td>976,5</td><td>281,9</td></tr>
<tr><td>443,6</td><td>380,5</td><td>328,9</td><td>374,6</td><td>917,5</td><td>132,9</td></tr>
<tr><td>1453,6</td><td>1010,5</td><td>802,9</td><td>687,6</td><td>504,5</td><td>612,9</td></tr>
<tr><td>627,6</td><td>1295,5</td><td>507,9</td><td>590,6</td><td>443,5</td><td>779,9</td></tr>
<tr><td>616,6</td><td>629,5</td><td>480,9</td><td>620,6</td><td>411,5</td><td>329,9</td></tr>
<tr><td>684,6</td><td>551,5</td><td>466,9</td><td>881,6</td><td>1162,5</td><td>252,9</td></tr>
<tr><td>666,6</td><td>446,5</td><td>1214,9</td><td>448,6</td><td>1067,5</td><td>596,9</td></tr>
<tr><td>721,6</td><td>1279,5</td><td>663,9</td><td>963,6</td><td>602,5</td><td>699,9</td></tr>
<tr><td>678,6</td><td>996,5</td><td>672,9</td><td>1000,6</td><td>372,5</td><td>299,9</td></tr>
<tr><td>757,6</td><td>731,5</td><td>414,9</td><td></td><td>431,5</td><td></td></tr>
</table>

DMSO CFDA-AM

0.8%

24 hr	48 hr	96 hr
492,6	368,5	337,9
583,6	341,5	329,9
850,6	1102,5	777,9
809,6	880,5	958,9
1323,6	681,5	264,9
1126,6	392,5	253,9
616,6	398,5	162,9
627,6	1003,5	814,9
502,6	1040,5	807,9
711,6	531,5	189,9
973,6	403,5	225,9
487,6	275,5	236,9
724,6	999,5	676,9
874,6	960,5	701,9
559,6	466,5	257,9

3.2%

24 hr	48 hr	96 hr
379,6	319,5	234,9
518,6	289,5	76,9
617,6	248,5	192,9
473,6	413,5	325,9
410,6	451,5	284,9
675,6	309,5	358,9
526,6	355,5	89,9
581,6	443,5	276,9
507,6	453,5	362,9
461,6	377,5	347,9
414,6	384,5	260,9
496,6	486,5	229,9
503,6	384,5	331,9
	489,5	310,9
		299,9

DMSO mBCI

CONTROL

24 hr	48 hr	96 hr
1205,6	1172,5	1259,9
1413,6	389,5	1231,9
1119,6	1252,5	766,9
811,6	992,5	881,9
1432,6	570,5	475,9
810,6	1143,5	345,9
443,6	380,5	328,9
1453,6	1010,5	802,9
627,6	1295,5	507,9
616,6	629,5	480,9
684,6	551,5	466,9
666,6	446,5	1214,9
721,6	1279,5	663,9
678,6	996,5	672,9
757,6	731,5	414,9

0.2%

24 hr	48 hr	96 hr
862,6	372,5	153,9
438,6	431,5	241,9
850,6	1051,5	712,9
540,6	1008,5	663,9
521,6	441,5	296,9
982,6	445,5	281,9
374,6	513,5	132,9
687,6	976,5	612,9
590,6	917,5	779,9
620,6	504,5	329,9
881,6	443,5	252,9
448,6	411,5	596,9
963,6	1162,5	699,9
1000,6	1067,5	299,9
	602,5	

0.8%

24 hr	48 hr	96 hr
850,6	1102,5	777,9
809,6	880,5	958,9
1323,6	681,5	264,9
1126,6	392,5	253,9
616,6	398,5	162,9
627,6	1003,5	814,9
502,6	1040,5	807,9
711,6	531,5	189,9
973,6	403,5	225,9
487,6	275,5	236,9
492,6	368,5	337,9
583,6	341,5	329,9
724,6	999,5	676,9
874,6	960,5	701,9
559,6	466,5	257,9

3.2%

24 hr	48 hr	96 hr
410,6	319,5	234,9
675,6	289,5	76,9
526,6	248,5	192,9
581,6	413,5	325,9
507,6	451,5	284,9
461,6	309,5	358,9
414,6	355,5	89,9
496,6	443,5	276,9
503,6	453,5	362,9
379,6	377,5	347,9
518,6	384,5	260,9
617,6	486,5	229,9
473,6	384,5	331,9
	489,5	310,9
		299,9

PAHs   AB-24 hr exposure

Naphthalene

37	33	33
27	28	33
32	40	39
34	33	36
33	35	33
31	31	33
26	38	37
36	20	31

Phenanthrene

38	40	43
36	44	32
40	37	40
48	49	43
36	38	34
42	39	38
40	38	33
40	31	34

Pyrene

33	38	41
37	42	40
40	42	45
40	45	40
37	43	47
32	42	34
44	45	42
37	35	35

*Benzo(*a*)pyrene*

37	40	41
45	41	43
44	45	43
25	35	33
37	48	41
33	42	34
28	42	36
38	32	39

2,6-Dimethylnaphthalene

40	46	36
38	40	39
42	43	41
41	46	41
35	48	46
41	40	33
28	42	36
38	32	39

1,5-/1,7-Dimethylphenanthrene

39	35	47
45	40	27
42	42	35
43	42	31
41	44	48
41	32	39
48	38	44
38	41	39

PAHs   AB-48 hr exposure

Naphthalene

43	40	39
37	39	42
38	37	36
35	35	43
36	27	34
40	35	37
39	42	42
41	42	39

Phenanthrene

34	38	42
30	40	33
32	32	34
38	31	27
33	41	30
36	31	36
28	31	32
34	28	32

Pyrene

37	38	29
34	30	33
39	36	38
35	34	33
45	34	36
36	34	36
32	39	32
30	33	35

*Benzo(*a*)pyrene*

38	48	34
32	44	40
31	38	30
25	35	33
31	30	35
36	31	36
39	35	38
40	37	32

2,6-Dimethylnaphthalene

35	34	42
31	41	33
39	35	39
34	38	35
35	37	39
35	38	39
36	44	39
34	36	22

1,5-/1,7-Dimethylphenanthrene

35	42	35
30	48	32
33	34	32
42	27	36
36	39	38
37	36	33
32	35	38
38	39	42

PAHs   CFDA-AM-24 hr exposure

Naphthalene

47	43	59
24	33	25
24	35	54
25	63	48
23	17	21
36	34	32
37	39	29
9	20	30

Phenanthrene

18	22	15
27	10	20
40	37	40
48	49	43
31	38	34
25	28	23
9	14	26
22	29	29

Pyrene

16	17	22
23	28	92
37	44	38
38	45	46
37	38	42
9	15	18
18	26	17
31	12	24

Benzo (a)*pyrene*

21	19	21
42	49	43
42	45	40
36	37	40
37	34	40
12	13	18
20	25	26
29	31	23

1,5-/1,7-Dimethylphenanthrene

18	19	29
26	22	19
42	43	35
42	42	40
35	31	48
16	23	16
21	20	39
18	35	26

2,6-Dimethylnaphthalene

12	15	27
58	43	45
42	38	41
41	36	41
35	38	43
14	15	19
21	28	18
19	31	19

PAHs   CFDA-AM-48 hr exposure

Naphthalene

16	14	26
20	52	26
38	37	34
35	35	43
36	27	34
8	13	16
16	43	36
24	22	26

Phenanthrene

47	43	59
30	25	60
31	21	14
30	18	17
31	23	45
38	42	43
15	44	86
13	18	15

Pyrene

56	33	25
36	33	38
5	25	14
22	25	27
35	48	16
26	22	26
18	25	48
22	16	17

Benzo (a)*pyrene*

21	56	29
60	56	59
26	27	42
20	25	34
20	19	25
34	53	92
22	11	84
30	26	32

1,5-/1,7-Dimethylphenanthrene

49	32	58
32	35	38
10	5	13
40	56	58
18	30	32
22	29	16
53	15	27
23	21	40

2,6-Dimethylnaphthalene

81	36	70
23	20	54
27	21	50
10	18	45
17	28	29
37	59	78
33	20	43
24	29	37

PAHs   mBCI-24 hr exposure

Naphthalene

14	14	17
12	15	12
18	15	14
18	16	12
16	16	11
7	17	14
22	20	19
14	12	11

Phenanthrene

19	21	20
17	26	15
11	16	19
19	15	19
23	16	18
23	18	17
16	23	21
21	9	15

Pyrene

20	21	19
16	16	17
18	16	11
20	22	21
13	23	22
17	14	14
15	20	19
21	17	18

*Benzo(*a*)pyrene*

14	24	20
18	16	19
14	16	18
17	21	19
19	16	23
21	21	23
15	16	25
16	13	23

2,6-Dimethylnaphthalene

23	13	15
17	18	19
20	16	19
18	19	14
21	12	15
21	12	22
9	23	24
20	16	16

1,5-/1,7-Dimethylphenanthrene

17	18	19
13	18	19
21	23	16
24	18	22
21	17	20
22	18	20
27	16	23
20	21	26

PAHs   mBCI-48 hr exposure

Naphthalene

18	20	17
15	27	19
18	13	14
22	14	21
18	19	17
15	21	11
20	16	22
13	15	22

Phenanthrene

18	17	17
11	15	15
11	13	9
12	15	12
15	17	9
19	14	14
20	14	18
10	19	17

Pyrene

19	17	13
14	15	13
9	14	16
19	22	14
19	13	6
19	13	12
11	13	10
5	12	17

*Benzo(*a*)pyrene*

13	20	15
18	12	10
10	12	11
16	12	12
14	12	16
10	12	19
16	26	21
13	11	17

2,6-Dimethylnaphthalene

15	13	17
7	12	16
15	18	17
13	14	16
15	18	15
15	16	18
32	18	22
19	19	10

1,5-/1,7-Dimethylphenanthrene

19	17	14
16	15	14
17	14	16
17	5	14
13	13	20
18	13	21
14	22	15
9	16	15

Copppper Control

AB-24 hr exposure

37	38	43	34	32	38	35	45	49
27	40	35	34	39	41	39	39	46
41	37	36	36	40	32	44	49	49
39	38	49	42	34	35	40	40	50
40	35	41	37	38	39	38	49	47
38	40	39	37	36	35	33	46	42
36	43	30	44	35	41		49	46
21	23	24	24	33	27		44	49
							39	44

Copppper Control

AB-48 hr exposure   **Negative Control**

28	29	29	32	35	33	44	31	39
36	36	31	34	31	28	40	39	41
32	32	29	33	30	28	38	38	34
34	34	34	32	26	38	33	31	39
32	37	36	33	43	36	36	29	33
31	40	32	31	42	29	34	31	39
28	36	28	27	42	30		34	48
0	26	29	29	33	28		26	38
							33	38

CFDA-AM-24 hr exposure   **Negative Control**

5	15	23	34	14	12	25	56	65
3	21	35	14	8	19	23	50	53
6	13	36	36	8	17	16	59	57
9	9	19	42	7	5	64	40	53
0	6	11	27	5	11	57	59	56
9	13	19	27	6	8	54	50	43
12	7	10	14	6	7		50	50
5	6	9	4	5	7		49	50
							50	54

Copppper Control

CFDA-AM-48 hr exposure   **Negative Control**

11	13	26	15	21	13	25	56	65
18	21	7	15	11	12	23	50	53
11	12	10	17	11	7	16	59	57
11	21	10	2	9	5	64	40	53
7	4	3	4	2	1	57	59	56
7	7	4	3	2	7	54	50	43
6	2	4	8	5	1		50	50
0	4	8	4	10	4		49	50
							50	54

Coppper Control

mBCI-24 hr exposure

						Negative Control		
16	21	19	14	18	9	16	25	24
27	15	21	16	18	18	19	20	25
21	16	21	16	21	18	17	19	22
16	14	18	16	18	19	21	23	22
14	20	19	23	22	11	24	22	25
19	21	19	16	19	17	16	21	24
12	19	19	11	15	12		19	23
15	10	10	8	13	12		27	21
							19	25

Coppper Control

mBCI-48 hr exposure

						Negative Control		
13	13	10	14	25	12	13	21	25
12	12	16	14	16	10	20	13	19
13	17	17	19	17	13	11	20	19
11	15	10	15	14	11	17	21	20
13	13	17	11	17	16	17	21	23
16	7	16	16	19	13	10	16	22
14	12	8	11	12	14		22	23
10	6	13	6	10	9		21	25
							22	26