



BIOMARKER ASSESSMENT OF THE ECOTOXICOLOGICAL IMPACT
OF ENVIRONMENTAL CONCENTRATIONS OF OILFIELD CORROSION
INHIBITORS AND PRODUCED WATER

By

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ABSTRACT

The oil and gas industries contaminate the environment through the generation of waste products known as produced water (PW). Globally, large volumes of treated PW, originating from offshore oil and gas installations are discharged into the sea, potentially affecting the marine environment. The substances most commonly present in PW are corrosion (CIs) and scale (SIs) inhibitors. These are chemical compounds comprised of quaternary ammonium salts (QUATs), imidazoline, phosphate esters (PEs) and other chemical substances, and are commonly used in the oil and gas industry to prevent corrosion problems arising during both exploration and production operations (E&P).

The present study evaluates the effects of QUATs, imidazoline, PEs and all components of PW on the marine environment, using a range of biomarker indices. The Comet assay was used to investigate DNA damage, expressed as% tail DNA, in individual haemocytes and gill cells derived from marine mussels *Mytilus edulis* that had been exposed to environmental concentrations of QUATs, imidazoline, PEs and PW. DNA damage is often an indirect effect of the oxidative radicals generated by a chemical or its metabolites. The oxidative stress assay employed was superoxide dismutase (SOD), and the associated lipid peroxidation was determined using the thiobarbituric acid reactive substances (TBARS) assay. Lysosomal membrane instability, a measure of cytotoxicity, was assessed using the neutral red retention (NRR) assay found in haemocytes cells. Bioaccumulation of the QUATs, imidazoline, PEs and PW were also determined.

The results of the study show a significant increase in DNA damage in the haemocytes and gills taken from adult mussels exposed to QUATs, imidazoline, PEs and PW within a concentration range of 0.001, 0.01, 0.1, 0.5 and 1 mg/L, when compared to control groups. Oxidative stress was measured, showing that QUATs, imidazoline, PEs and PW increased SOD activity and lipid peroxidation in a concentration-dependent manner (≥ 0.5 , 0.001, 0.1 and 0.001 mg/L and 0.1, 0.1, 0.5, 0.001 mg/L, respectively). Lysosomal membrane stability was affected at concentrations of ≥ 0.1 mg/L for QUATs, imidazoline and PW. Moreover, it was affected by PEs at concentrations ≥ 0.5 mg/L. In addition, QUATs and imidazoline were found to have accumulated in mussel tissues at concentrations of ≥ 0.1 and 0.001 mg/L, respectively, but PEs were not found in tissues.

This work suggests that very low concentrations of QUATs, imidazoline, PEs and PW may be harmful to marine organisms and the biomarkers described could be further developed as tools for monitoring and regulating the disposal of PW at sea.

DEDICATION

*To my parents Fatima and Mohammed for their
love, support and prayers*

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Of course, it is solely my responsibility if any errors or inadequacies remain in this work.

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List of Abbreviations

AAS	Atomic absorption spectrometry
AH	Aromatic hydrocarbon
ALS	Alkali-labile sites
AP	Alkylated phenols
ATAC	Alkyltrimethylammonium chlorides
BAC	Benzalkonium chloride
BHT	Butylated hydroxytoluene
CA	Chromosomal aberration assay
CIs	Corrosion inhibitors
CO ₂	Carbon dioxide
DCM	Dichloromethane
DDAC	Dialkyldimethylammonium chlorides
DDH ₂ O	Double distilled water
DH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
DSB	Double strand break
E&P	Exploration and production
EBS	Emulsion breakers
EDTA	Ethylenediaminetetraacetic acid
EI	Electron ionization
ESI	Electrospray ionisation
FW	Formation water
GPX	Glutathione peroxidase
GPX	Glutathione peroxidases
GSH	Glutathione
H ₂ O [·]	Hydroperoxyl anion
H ₂ O ₂	Hydrogen peroxide
H ₂ S	Hydrogen sulphide
HO [·]	Hydroxyl radical
HOCL	Hypochlorous acid
IE	Ionization energy
IR	Relative intensity

LC ₅₀	Lethal concentration, 50%
LMP	Low melting agarose
LOOHs	Lipid hydroperoxides
m/s	Mass to charge ratio
MB	Modified bases
MDA	Malonaldehyde
MGE	Microgel electrophoresis
MNT	Micronucleus test
MS	Mass spectrometry
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium
NGA	Normal gel agarose
NO [•]	Nitrogen oxide
NR	Red dye
NRR	Neutral red retention
O ₂ ^{•-}	Superoxide anions
OSPAR	Oslo and Paris Convention
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PEs	Phosphate esters
PLL	Poly-L-Lysine
PPM	Parts per million
PSS	physiological saline solution
PUFA	Polyunsaturated fatty acids
PW	Produced water
QUATs	Quaternary ammonium salts
RNS	Reactive nitrogen species
RO [•]	Alkoxy anion
RO ₂ ⁻	Peroxy anion
ROO [•]	Peroxy radical
ROS	Reactive oxygen species
SCE	Sister-chromatid exchange assay
SCGE	Single cell gill electrophoresis

SI _s	Scale inhibitors
SOD	Superoxide dismutase
SPE	Solid phase extraction
SSB	Single strand break
T [•]	Tocopherol radical
T/Y	Tonnes/year
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
Tris	Tris hydrochloride
V/V	Volume to volume
WST	Working solution
XO	Xanthine oxidase

List of publications at conferences

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Introduction

The oil and gas industry contaminates the marine environment by releasing different types of waste into it; one of the most significant of these waste products is produced water (PW) (Figure 1-1). PW contains many substances, including hydrocarbons and other organic and inorganic chemical compounds and manufactured chemicals such as biocides, surfactants, demulsifiers and corrosion (CIs) and scale (SIs) inhibitors. The focus of this study is on CIs and SIs.

CIs and SIs are typically used in oil and gas fields to prevent corrosion problems arising during exploration and production operations. They are formulated using a large number of chemical compounds, such as quaternary ammonium salts (QUATs), imidazoline and phosphate esters (PEs). These chemical compounds are found in concentrations of up to 30 mg/L in PW prior to its discharge into the marine environment (Grigson and Gagliardi, 2003). These chemical compounds are not chemically transformed after use. CIs such as QUATs have become ubiquitous, having been found in environments such as surface water, wastewater, soil and aquatic sediments at concentrations below inhibitory levels. In aquatic sediments the concentrations of QUATs have been found to range between 0.74 and 10.1 ng/kg (Grigson et al., 2000). In addition, a further study consulted reported that QUATs were present at 0 - 0.6 m in depth, at concentrations of 0.63 mg/L dry in surface water and 9.7 mg/kg dry in solids and at 0.6 – 1.2 m in depth, the concentration was 7.4 mg/kg in dry solids (Tezel and Pavlostathis, 2011). Additionally, researchers in Austria measured QUATs in sewage sludge and marine sediments, finding average concentrations to range between 22 and 103 mg/kg dry in solids and 0.06 and 6.8 mg/kg dry in solids, respectively (Martínez-Carballo et al., 2007a). However, many studies have demonstrated that discharging PW into a receiving environment is an important source of organophosphate esters in marine ecosystems. Indeed, SIs such as organophosphate esters have been found in various environmental media, including surface water, wastewater and aquatic sediments in Austria; where concentrations ranged between 2.6 and 7.9 ng/L and 4.1 and 13 ng/L and 0.48 and 11 µg/kg, respectively (Martínez-Carballo et al., 2007b). Previous to this research, the potential effects of these compounds on marine mussels *M. edulis* has been unknown.

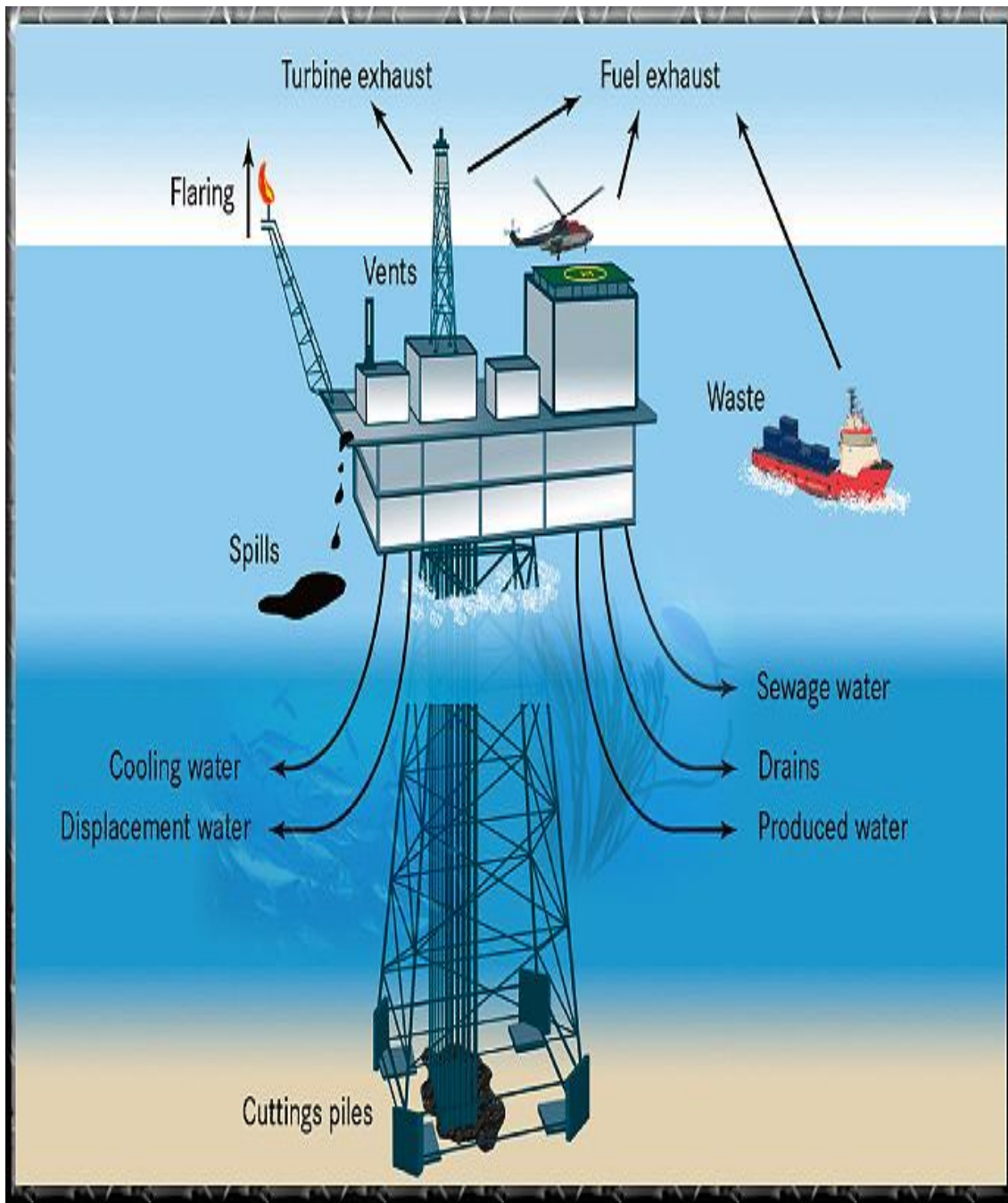


Figure 1-1 Offshore waste discharges into the marine environment (OSPAR, 2010a)

Oil and gas exploration and production

When offshore exploration and production (E&P) installations in the North Sea were first set up in the 1970s, the environmental impact of offshore production was unknown. However, shortly after offshore production began, concerns arose with regards to the potential effects on the marine environment during oil and gas E&P operations. As a consequence, the Oslo and Paris (OSPAR) Convention developed the first set of regulations to control discharges to the environment from oil and gas E&P operations. Regulations for chemicals used offshore

were introduced in 2002 by the UK Parliament (Carol, 2002), to make provision for the decision made by OSPAR to implement a harmonised, mandatory, control system for the use and discharge of chemicals from the offshore oil and gas fields. Subsequently, these regulations were amended in 2011 by the UK Parliament (Carol, 2011).

The nature of offshore discharges

Oil and gas E&P operations result in the generation of two broad categories of waste: the first is directly linked to the production process and the second to support activities. The major wastes are from drilling and production, and include drilling wastes (drill cuttings, drilling mud and fluid) and PW (Orszulik, 2008). PW is an important waste product generated by oil field installations and it is discharged directly into the receiving offshore environment. Part of the PW consists of formation water, which occurs naturally and contains many types of chemical compounds. Over millions of years, water from a variety of sources can become trapped with the oil and gas and this is referred to as formation water (FW). In addition, once extraction has begun, and as the well ages, water is frequently injected into it to maintain reservoir pressure and to improve the oil yield (Orszulik, 2008; Patin, 1999; Utvik, 1999). Together with the FW, this water is discharged as a constituent of so-called PW (Neff et al., 1987; Patin, 1999). To recycle the PW, many oil and gas fields re-inject the PW into other wells. Moreover, because PW can be hot, highly saline and contain oil residues, many national regulatory agencies have set standards for permissible concentrations of total oil, grease and other remaining chemicals in PW before disposal to the receiving environment. Thus, before the PW is discharged to the receiving environment, it is treated to meet local regulatory limits for oil and grease (Fakhru'l-Razi et al., 2009). In the North Sea, the permitted limits for treated PW discharges are 40 mg/L of total oil and grease (Neff, 2002). Before the refining of the crude oil and gas can proceed, the PW must be removed from the production stream. The separation of PW from oil and gas may take place on the production platform or the PW may be transferred through pipelines to onshore treatment facilities (Figure 1-2).

There are no regulations established, as yet, prescribing the discharge of treated QUATs, imidazoline and PEs into the receiving environment. However, Fakhru'l-Razi et al. (2009) suggests bringing the concentrations down to <0.1 mg/L before releasing the PW into the marine environment, so as to protect the marine environment in the North Sea.

Table 1-1 Concentrations of production chemical compounds in produced water and amount discharged into the North Sea

Treatment Chemical	Concentration	Phase association of chemical	Amount discharged to North Sea
	(ppm, v/v)		(t/y)
Corrosion inhibitor	25–100	Oil	216
Scale inhibitor	3–10	Water	1,143
Biocide	10–200	Water	81
Emulsion breaker	10–200	Oil	9
H₂O/O₂ scavenger	5–15	Water	22
Coagulants and flocculants	<3	Water	197
Gas treatment chemicals	Variable	Water	2,846

Source: (Neff et al., 2011a; Neff, 2002)

Composition of Produced Water

PW contains many chemical compounds which either occur naturally or are introduced during the production process. The composition of these chemicals depends on the geological formation of the reservoir, the age of the reservoir and the type of petroleum produced, and can therefore vary substantially between production platforms. In general, the composition of PW includes hydrocarbons, such as aromatic hydrocarbons (AH), polycyclic aromatic hydrocarbons (PAHs), volatile aromatic compounds (xylenes benzene, ethylbenzene, and toluene), trace metals, phenols and additive production chemicals (Binet et al., 2011). These chemical additives are used at oilfields to prevent problems that can occur during production operations, such as foaming, corrosion, bacterial growth, scale formation and hydrogen sulphide formation. CIs, SIs and others are added at different stages throughout the production and treatment process, not only to avoid the above problems but also to improve the effectiveness, efficiency and completeness of the oil/water separation procedure (Table 1-2).

Table 1-2 Chemical classes used to prevent the most frequently occurring problems during oil and gas E&P operations.

Problem	Treatment chemical
Water Vapour	Dehydrator
Mineral Deposits	Scale inhibitor
Chemical Corrosion	Corrosion inhibitor
Bacterial Corrosion	Bactericide
Emulsions (Normal or Reverse)	Emulsion breakers, coagulants, flocculants
Foaming	Defoamer
Paraffin	Paraffin inhibitor, solvent
Hydrate Formation	Hydrate inhibitor

In PW, at the point of discharge into the marine environment the concentration of CIs can range between 25 and 100 mg/L, and for SIs between 3 and 10 mg/L. The concentrations of other additive chemicals are shown in Table 1-2. The large quantities of additive chemicals in PW are usually from CIs and SIs, and more than 50% of these CIs and SIs are ultimately discharged into the marine environment (Neff, 2002). Consequently, CIs and are now found in many environments such as surface water, wastewater, soil and aquatic sediments. According to Tezel and Pavlostathis (2011) the concentration of CIs as QUATs in the marine environment was 0.63 mg/L) and in marine sediment they ranged between 0.74-10.1 ng/kg (Grigson et al., 2000). Furthermore, the concentration of SIs as organophosphate ester in surface water, wastewater and aquatic sediment ranged between 2.6 and 7.9 ng/L and 4.1 and 13 ng/L and 0.4 and 11 µg/kg respectively (Martínez-Carballo et al., 2007b).

Types of production chemical compounds

Many chemical compounds are introduced to the production system as treatment chemicals; the aim of which is to protect the system from corrosion, to assist in recovery, to aid the separation of oil, gas and water and to prevent ice formation as a consequence of the presence of methane hydrates in gas production systems. Treatment chemicals contain biocides, demulsifiers, de-oilers, antifoamers, surfactants, coagulants and antiflocculants, CIs and SIs. These chemicals then remain as pollutants in the PW (Hudgins, 1992; Patin, 1999) (Table 1-2).

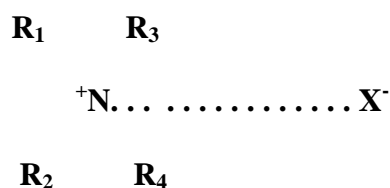
Corrosion inhibitors (CIs)

Corrosion inhibitors (CIs) are chemical compounds which are typically used by the oil and gas industries to reduce the serious problems that would arise if corrosion were to occur. The production system can be subject to corrosive attack from hydrogen sulphide (H₂S), carbon dioxide (CO₂) and other aggressive constituents found in the reservoir fluids. When added in small concentrations, a CI prevents and slows down corrosion in the pipelines. Using a concentration of 80 mg/L of QUATs or imidazoline, a level that is typically used to provide good protection, can achieve a 95% reduction in corrosion; whereas 40 mg/L of QUATs or imidazoline have been reported to provide 90% protection (Jones, 1988). Various inhibitory mechanisms can form a passivation layer, which is a thin film on the surface of the material which prevents corrosive substances from gaining access to the metal, either by inhibiting the oxidation and reduction element of the redox corrosion system (as in the case of cathodic and anodic inhibitors) or by scavenging the dissolved oxygen (Jones, 1988). Inhibitors can be water soluble/oil dispersible or oil soluble/water dispersible. To avoid corrosion it is important to select a type of CI that is suitable for use with the metals present, in particular considering also whether the metal or alloy to be protected is oil-wet or water-wet; although, in general, during oil production an oil-soluble inhibitor is used because it is more effective. Offshore, organic compounds are used as CIs to protect the surface layer of the target metal. The majority of these organic compounds contain nitrogen as part of the key functional group (Henderson, 1999). CIs include amines/amine salts; QUATs and amides/imidazolines.

Quaternary ammonium salts (QUATs)

QUATs are chemical compounds that have the general formula $R_4N^+X^-$, where “R” represents organic groups which are aryl substituents, alkyl or aralkyl groups, and “X” is any anion. R groups may be identical or different. These organic groups have ionic compounds created from primary, secondary and tertiary amines and opposite sulphate, alkyl halide or similar compounds. QUATs are highly soluble when in a mixture comprised of polar organic solvents. Finally, these amines are quaternised with dimethyl sulphate, methyl chloride or benzyl chloride. QUATs’ molecules have at least one hydrophobic hydrocarbon chain linked to a positively charged nitrogen atom; the other alkyl or aryl groups are mostly short-chain substituents, such as benzyl or methyl groups. Due to their amphiphilic characteristics, they are frequently utilised as surfactants, whereby the alkyl chain length determines the physical-chemical properties (adsorption/partition coefficient on the sediments, the octanol/water partition coefficient, water solubility, soils and sludges) (Martínez-Carballo et al., 2007c).

General formula:



QUATs have been used for more than 30 years in large quantities (Callely and Forster, 1977; García et al., 2000). They are not only used in oil and gas field applications, but also in cosmetic and household products, to prevent or retard microbial growth during storage and use. These are positively charged cationic surfactants with an extremely strong affinity for negatively charged surfaces, which is why they are used as disinfectants and fabric softeners (García et al., 2001; Martínez-Carballo et al., 2007c). The oil and gas industries use QUATs as CIs to prevent metal based corrosion in pipelines (Patrauchan and Oriel, 2003). There are many types of QUATs: benzalkonium chloride (BAC), dialkyldimethylammonium chlorides (DDAC) and alkyltrimethylammonium chlorides (ATAC). BAC is the chemical most commonly used in oil and gas fields. DDACs are a mixture of mainly C₁₀ or C₁₈ alkyl chains and are found in commercial application, such as household products. ATAC is generally used in shampoo and conditioning agents (Martínez-Carballo et al., 2007c). The molecular structure of QUATs is shown in Figure 1-3.

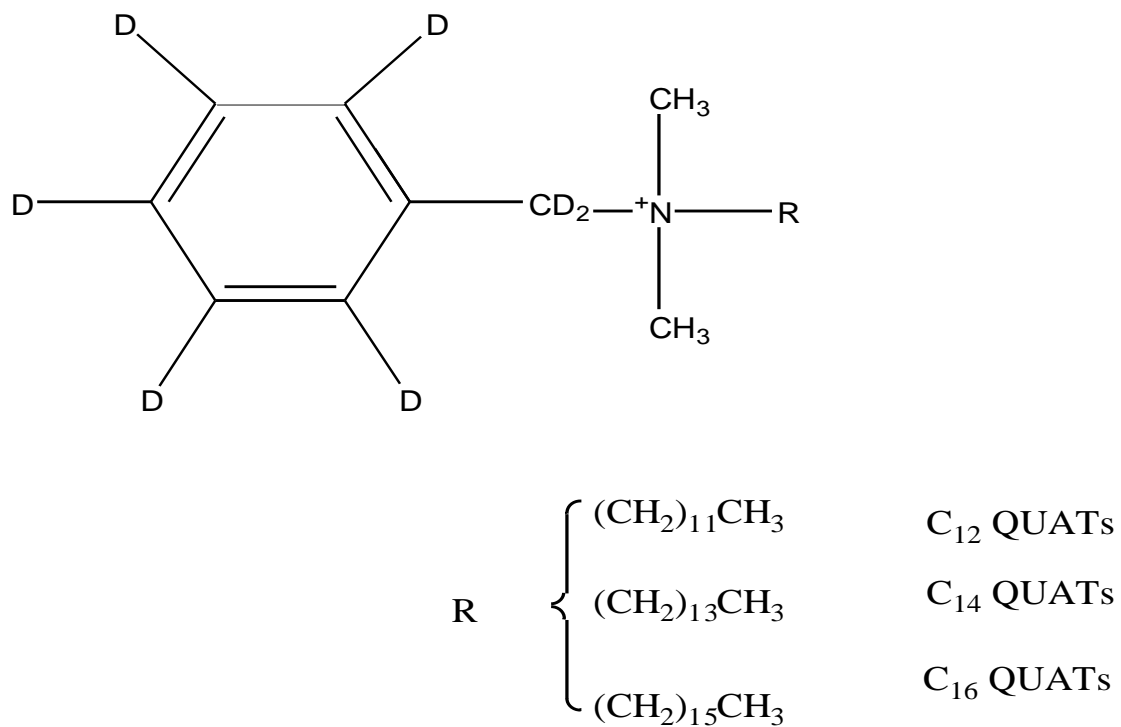


Figure 1-3 Molecular structure of quaternary ammonium salts, (D): deuterated (Gagliardi and Grigson, 2003).

Concentrations of QUATs in marine environments

QUATs are released into the aquatic environment where they accumulate, potentially having toxic effects on aquatic organisms. The quantities of QUATs or other surfactants used is difficult to estimate (McWilliams and Payne, 2002). However, the volume of CIs discharged into the North Sea has been reported to be 216 tonnes per annum (Neff et al., 2011a). The total concentrations of QUATs found in marine sediments around two North Sea oil platforms that were tested ranged between 0.74 to 10.84 ng/kg (Grigson et al., 2000). The concentrations of QUATs in a PW sample collected from a discharge point at Brae Alpha, where North Sea platforms are located, ranged from 10 to 30 mg/L (Gagliardi and Grigson, 2003). Concentrations of 5-20 µg/L QUATs have been found in the environment since 1979 in the Main River in Germany, and also in 1994 in sewage and surface water at concentrations of 350-480 µg/L and 6-12 µg/L respectively (Table 1-3) (Tezel and Pavlostathis, 2011). The highest concentration of QUATs was noted for benzethonium chloride (C₁₂-C₁₆), which is commonly used by the oil and gas industry; it was recorded at 1.9 µg/L, spanning three decades from 1980 up to 2010 (Tezel and Pavlostathis, 2011).

Table 1-3 Concentrations of QUATs in the environment

Place	Type of sample	Concentrations (µg/L)
North Sea	Sediments	0.74 - 10.84 ng/kg
Main river in Germany	Water	5 - 20
Germany	Surface water	6 - 12
Germany	Sewage	350 - 480

Source: (Grigson et al., 2000, Tezel and Pavlostathis, 2011)

In the present study, the most important issues in relation to the use of QUATs or offshore discharge are whether QUATs pose a risk as a result of direct toxicity in the aquatic system, or whether bioaccumulation and biodegradation (and potential metabolites) of QUATs poses a greater risk to aquatic organisms. Several studies into the bioaccumulation and biodegradation of QUATs have been carried out (Basse, 2010; Basse et al., 2009; Grigson et al., 2000; Patrauchan and Oriel, 2003). These studies were concerned with the concentrations of quaternary ammonium surfactant benzyldimethyl hexadecylammonium chloride (BDHAC) in marine sediments, but they did not consider organismal interaction. In the present study, the aims involved determining the impacts of environmental concentrations

of QUATs using biomarkers for exposure at several levels of biological organisation, DNA damage, oxidative stress, cytotoxicity and bioaccumulation in marine mussels (*M. edulis*)

Imidazoline

Imidazoline surfactants belong to the category of cationic surfactants. In the 1930s, cationic surfactants were first used as dye levelling agents in the textile industry, and their softening properties were only discovered as an additional benefit. The general molecular formula for imidazoline is $C_3H_6N_2$. Cationic surfactants are composed of quaternary nitrogen salts and are commonly used in both aqueous and non-aqueous systems. Cationic surfactants can be used in other applications, such as disinfectants, and are classified into fatty diamine salts, fatty amine salts, imidazoline salts and QUATs. Imidazoline possesses desirable storage stability, dispersibility, fabric conditioning properties and viscosity, and is valuable for laundry purposes and in other industrial applications, including at oil and gas fields (Divya and Tyagi, 2006). There are two types of imidazolines: cationic and amphoteric. Cationic imidazolines carry a positive charge on the imidazoline nucleus and the electrical charge on this molecule is unaffected by pH changes. The positive charge remains in an alkaline, neutral or acidic medium. These compounds are used by the oil and gas industries as anti-corrosives, water repellents, emulsifiers and dispersing agents. The advantage of using imidazolines as CIs and for emulsification is that they make water soluble in oil. Imidazolines belong to the family of heterocyclic compounds, including those members with a ring containing two nitrogen atoms. Cationic imidazolines are QUATs of $R_4N^+X^-$ which, as already described above, have an asymmetrical structure (consistent with the tetrahedral carbon scheme) determined by the cation. The structure of an imidazoline, in Figure 1-4, shows that the substitution takes place in three positions: the first position of the ring is with a fatty acid aminoethyl chain, the second position is with a long chain alkyl substituent and the last position is with a methyl group. The tallow alkyl derivative is a widely used representative of this group, and is used mainly as a liquid in fabric softeners (Divya and Tyagi, 2006; Rashmi et al., 2007).

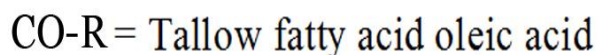
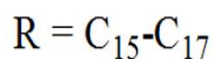
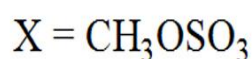
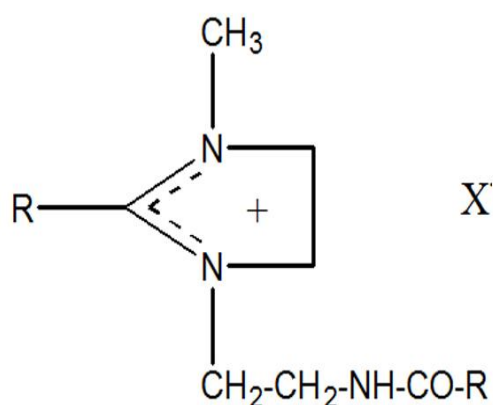


Figure 1-1 structure of an imidazoline (Divya and Tyagi, 2006)

Concentrations of imidazoline in marine environments

Wastes from offshore installations consisting of toxic substances, such as PW, are potentially harmful to the ecological system. As mentioned in the previous chapters, during offshore oil extraction operations, CIs are widely used to protect pipelines and these are usually comprised of complex mixtures (Neff et al., 2011a). The majority of the inhibitors used in oil production systems are nitrogenous compounds, which can be classified into broad groups such as QUATs and amides/imidazolines (Fink, 2012). Imidazolines, in particular, are widely used as CIs (Sastri, 2011). However, their properties and behaviour in complex environments are far from understood (Gagliardi and Grigson, 2003). For instance, although they are very effective, the precise mechanism by which they prevent corrosion is not completely clear (Ash and Ash, 2011; Chen et al., 2000; Sastri, 2011). Generally, an imidazoline consists of an imidazoline head group, a pendant group and a hydrocarbon tail as described in detail in Chapter One (Rashmi Tyagi, 2007). Recent molecular modelling studies (Bajpai and Tyagi, 2006; Chen et al., 2000; Tyagi et al., 2007) suggest that the head and pendant group promote

the bonding of molecules to the surface, while the hydrocarbon tail forms a protective monolayer. This hydrocarbon barrier therefore defends the pipe's surface from corrosive ions and water, thereby making imidazoline more effective as a CI. However, not all imidazolines are effective as CIs: effectiveness depends on their hydrocarbon tail, which must be long enough; i.e. at least C₈ (Jovancicevic et al., 1999).

Very few studies have been undertaken to investigate the properties and behaviour of imidazolines as CIs (Bajpai and Tyagi, 2006; Tyagi et al., 2007; Ash and Ash, 2011; Fink, 2012). Despite the fact that the precise quantities of imidazolines required are difficult to estimate, they are commonly used in the oil and gas industry (McWilliams and Payne, 2002). However, the volume of CIs discharged into the North Sea in 2007 was reportedly 216 tonnes (Neff et al., 2011a). The concentrations of imidazolines ranged from 0.3 up to 12 mg/L, in PW samples collected from a discharge point at Brae Alpha, where North Sea platforms are located, while the concentrations of imidazoline in marine environment were not reported (Gagliardi and Grigson, 2003). The LC₅₀ toxicity of imidazoline after 24hrs has been reported at concentrations < 10 mg/L (Sastri, 2011).

The aims of the present study were to determine the impacts of imidazoline on DNA integrity, biomarkers of oxidative stress, cytotoxicity and bioaccumulation in marine mussels (*M. edulis*).

Scale inhibitors (SIs)

Scale inhibitors (SIs) are chemical compounds commonly used in the oil and gas fields as to prevent mineral deposit problems; these can arise during production processes. Three types of chemical compounds are widely used as scale inhibitors: PEs, phosphonates and acid polymers. These chemicals are all highly soluble in water, thus they cannot be easily removed from the PW and so are normally discharged with it into the marine environment.

Phosphate esters (PEs)

PEs are also known as organophosphates, which is a general name for the esters of phosphoric acids. Acids can also reduce esters. PEs or organophosphates are commonly applied as solvents and SIs. PEs are used as anti-wear, solubilisation, wetting, emulsification, dispersion and anti-corrosion agents, and are commonly used in the oil and gas industry to prevent mineral deposition in and on production equipment. The molecular structure of PEs can be finely tuned to offer specific benefits (Figure 1-5).

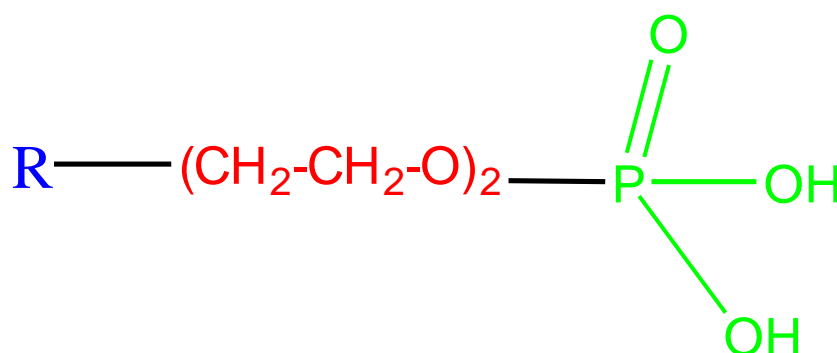


Figure 1-2 Phosphate ester molecular structures (Rhodia, 2006).

Concentrations of PEs in marine environments

Since the revolution in the oil and gas industry, the aquatic environment in particular has been increasingly threatened by the growing number of types and amounts of corrosion inhibitors (CIs) and scale inhibitors (SIs) discharged into the marine environment (Manduzio et al., 2003; Neff, 2002; Orszulik, 2008; Patin, 1999). The exposure of living organisms to these pollutants results in interactions between chemicals and biological systems that can give rise to biochemical disturbances and/or adaptive responses (Manduzio et al., 2003)

PEs are organic compounds, also known as phosphoric esters of organic hydroxyl compounds, which are amphiphilic molecules with a polar phosphate head and 1 to 3 non-polar alkyl tails (Belosinschi et al., 2012). PEs are commonly used as SIs and CIs, due to their excellent stability and surfactant nature, which also makes them appropriate for use as antistatic agents and for cleansing, emulsifying and rust inhibition, as well as for preventing metal deposits (Belosinschi et al., 2012). They are also used as flame retardants in hazardous environments where the use of hydrocarbon oils poses an unacceptable risk (Placek and Shankwalkar, 1994). PEs can also be used as an additive chemical in fluids and lubricant based oils that can be subjected to high temperatures under actual service conditions (Douglass et al., 2012). Unlike the QUATs and imidazolines described in Chapters 3 and 4, PEs are highly water-soluble and will remain with the PW discharged into a marine environment. The concentrations of PEs in PW have been found to range between 3 to 5 mg/L (Gordon, 2008). Indeed, SIs such as organophosphate esters have been found in various environmental media, including surface water, wastewater and aquatic sediments in Austria, where concentrations ranged between 2.6 and 7.9 ng/L and 4.1 and 13 ng/L and 0.48 and 11 µg/L, respectively (Martínez-Carballo et al., 2007b).

In the present study, the aim was to determine the impacts of environmental concentrations of PEs on DNA damage, oxidative stress, cytotoxicity and bioaccumulation biomarkers in marine mussels (*M. edulis*).

Discharges of PW into the Sea

At offshore oil platforms, large volumes of PW, commonly several times greater than the volume of oil, are discharged into the marine environment; this PW is contaminated with the residuals of production chemical compounds (Utvik, 1999). Many production chemical compounds are oil-soluble and remain in the oil phase but others are soluble in PW and are discharged with it. As these chemical compounds are used at oil and gas fields to solve certain problems, the amount of gas treatment chemicals used (both CIs and SIs), is highest in production operations facing corrosion and scaling problems.

Many offshore installations discharge their waste to the sea, including those in the North and East Atlantic. In the North Sea, following treatment, about 19% of the offshore production chemicals used on platforms are discharged with the PW into the marine environment, including 50% or more of the emulsifiers, SIs, oil removing agents and surfactants, and 20% of the CIs, emulsion breakers and oxygen scavengers, gas treatment agents and defoamers (Neff et al., 2011a; Neff, 2002). In the OSPAR region, maritime operations discharging to the sea increased by approximately 50% between 2000 and 2007 (Table 1-4).

Table 1-4 Number of offshore installations discharging PW (in tonnes) into the North-East Atlantic Sea and re-injecting PW before discharge in the period 2000 to 2007

Year	2000	2005	2006	2007
Discharges to the sea	298	407	416	444
Reinjection produced water	9	16	20	23

Data from (OSPAR, 2010b)

The quantity of PW discharged into the sea increased, but not significantly, in the period 2005 up to 2007, and has since stabilised at approximately 1.2 million m³ per day (Table 1-5). Both the United Kingdom and Norway discharge PW, oil and gas wastes into the North Sea. In 2007, the discharge rate was approximately 553,139 and 558,647 m³ of PW per day, respectively. Offshore installations are also increasingly re-injecting PW for reuse during their production operations (Taylor et al., 1998).

Table 1-5 Quantities of chemicals discharged in PW to the North-East Atlantic Sea in the period 2005-2007

Quantity	2005	2006	2007
PW (m ³)	1,275,143	1,217,453	1, 221,082
Produced water injected per year (m ³)	76,709,672	80,185,640	87,721,185
Dispersed oil discharges in PW (tonnes)	8238	7098	5020
Dissolved oil discharged in PW (tonnes)	4992	4880	4575
Total oil discharged in PW (tonnes)	13348	12110	9690

Data from (OSPAR, 2010b)

800 million m³ (667 million metric tonnes) of PW were discharged from offshore platforms into the marine environment throughout the world in 2003 (Neff et al., 2011a). Total volumes of PW included 358-419 million tonnes of PW in Europe, which is released annually from offshore installations into the marine environment of the North Sea (Neff et al., 2011a). This is something that is causing concern in the region in relation to the health of fish stocks (Hylland et al., 2008). Approximately 30% of the PW discharged into the North Sea is from oil and gas production that takes place in the Norwegian sector (Neff et al., 2006). According to Sundt et al.(2012) the total volume of PW discharged into the marine environment in the North Sea from the Norwegian sector was 134 million m³ in 2009, including approximately 1,500 metric tonnes of dispersed oil. However, according to figures given by Farnen (2010) the total volume of PW discharged from Norwegian sector was expected to reach more than 250 million m³ in 2010. The total volume of PW discharged into North Sea by BP from UK platforms was 54.4 million m³ in 2008, containing 205 metric tonnes of oil (BP, 2008). It is to be noted that the volume of PW increases during an oilfield's life time. Effluents are treated and directly discharged into the receiving environments (Wake, 2005; Zrafi-Nouira et al., 2008) containing inorganic and organic compounds including polycyclic aromatic hydrocarbons (PAHs), alkylphenols (APs) and production chemicals. Production chemicals include biocides, demulsifiers, de-oilers, antifoams, surfactants, coagulants and antiflocculants, SIs and CIs in addition to many other potentially toxic chemicals (Sundt et al., 2012; Utvik, 1999).

During the treatment process in the oil and gas industry, biodegradation methods are employed to degrade chemical compounds and to reduce their concentrations. There are many tests in use to evaluate biodegradability, such as the "CO₂ Headspace" test (ISO 14593). This technique allows for the assessment of the ultimate aerobic biodegradability of an organic compound in an aqueous medium at a known concentration of microorganisms via

analysis of inorganic carbon (Gathergood et al., 2006). However, QUATs inhibit the growth of microorganisms and so are able to degrade them. Therefore, many studies employ another technique, called the “screening method” to measure the biodegradability of QUATs. They have suggested that the non-biodegradation of QUATs with a long alkyl chain may be caused by their relatively high concentrations (Gathergood et al., 2006; Larson, 1983). Because imidazoline compounds contain similar charged ammonium ion-species, the potential toxicity of the biodegradable ionic liquids may also have a negative effect on their biodegradation (Gathergood et al., 2006).

There are two different strains of bacteria, that have been isolated from marine sediments and presented to degrade QUATs in a minimal salts medium (Bassey, 2010). The biodegradation, sedimentation, evaporation and adsorption contribute to lower concentrations of PW components in the seawater near to the oil platform discharge (Utvik, 1999).

Both treated and untreated PW are discharged into the marine environment. Because the methods of treatment are very expensive, some oil and gas industries discharge PW without treatment (Zhang et al., 2010). Discharging PW without treatment may result in an increase in the concentrations of chemicals in the marine environment, therefore increasing the risk of pollution. Once discharged, the fate of these chemicals is complex, leading to potential accumulation in sediments and biota, and also possibly subjecting marine organisms to acute or chronic exposure, leading to a substantial toxic response.

Fate and toxicity of produced water discharges

On a daily basis, a substantial volume of PW is discharged into the marine environment by the oil and gas industry; this is potentially toxic to marine organisms. Evidence of exposure can manifest itself in a number of biochemical aberrations on various biological levels. Examples include DNA, protein and lipid damage revealing oxidative stress caused by free radicals and reactive oxygen species (ROS). Aquatic organisms under the oil and gas platforms are regularly exposed to PW during continuous discharge. Some organisms are able to move on to find a better environment but others cannot. The impact of PW on aquatic organisms depends on their particular trophic positions and their dietary and ecological niches. Recent environmental toxicity studies have focused on the impact of PW on mussels because of their sessile nature and filter-feeding activity, which leads to a tendency to accumulate contaminants from the water column.

Gorbi et al. (2008) studied the impact of offshore activities and investigated the effect of heavy metals As, Ba, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb and Zn on mussels caged in the area surrounding a platform in the Adriatic Sea, confirming the effect on antioxidants and lysosomal stability. Hannam et al. (2009) also studied the impact of North Sea PW on mussels. This study exposed the mussels to concentrations of PW from 0.125–0.5% for 21 days. After 7 days a biphasic response was observed at concentrations of 0.25% and 0.5% PW, at which point the cell viability and phagocytosis were inhibited. These results indicated that high concentrations of PW cause adverse effects to exposed biota, such as restricted growth and reproduction and reduced survival rates.

In addition, Harman et al. (2009) assessed the environmental impact of the oilfield chemicals discharged from the offshore oil installations. Polar organic chemical integrative samplers and semi-permeable membrane devices were deployed around a North Sea platform. The exposure to PAHs and alkylated phenols (AP) was determined by passive samplers. They predicted that the exposure was comparatively similar within 1-2km from the discharge point. They reported that these concentrations caused both acute and sub-lethal effects.

Hylland et al. (2008) reported on studies in 2001, 2003 and 2004, which also confirmed that the components of PW, such as PAHs, could be detected in mussels within 500m, 2 km and 10 km of platforms; some biological responses were also observed.

In another study, by Caliani et al. (2009), mosquito fish (*Gambusia affinis*) were exposed to concentrations of 50% PW discharged from an Italian on-shore oil plant for 5 days, and 10% PW for 30 days. After 8 days of exposure, micro-nucleated cells were found in the females and after 30 days, DNA damage, expressed as single strand breaks (SSB), was observed in fish of both genders.

Turja et al. (2012) determined the biological effects of chemical pollutants in a low salinity environment in blue mussels caged at four sites in the vicinity of an oil platform in the Gulf of Finland. The results confirmed that soft tissue growth was reduced and that there was increased oxidative stress and lysosomal membrane damage at highly polluted sites.

Famadas (2009) confirmed that two PW samples collected from different oil platforms in the North Sea showed acute and sub-lethal effects on the mussels *M. edulis*.

In a study of the concentrations in marine sediments in chemical compounds added to the topside during oil and gas production processes, total benzalkonium quaternary ammonium

salts concentrations were found to range from (0.74 to 10.84 ng/kg) around two North Sea oil platforms (Grigson et al., 2000; Neff et al., 2006). In PW, the concentrations of CIs ranged from 25 up to 100 mg/L, and those of scale inhibitors ranged from 3 up to 10 mg/L before discharge into the marine environment. The concentrations of QUATS, imidazoline and PEs in PW have been found to range from 10 to 30, 0.3 to 12 and 0.1 to 5 mg/L respectively (Table 1-6).

Table 1-6 Concentrations of QUATs, Imidazoline and PEs in PW

Chemical Class	Concentration (mg/L)
Quaternary Ammonium Salts (QUATs)	$\leq 10.0 - > 30.0$
Imidazoline	$\leq 0.3 - > 12.0$
Phosphate Ester (PEs)	$\leq 0.1 - > 5.0$

(Gagliardi and Grigson, 2003; Grigson et al., 2000; Neff, 2002)

QUATs, imidazoline and PEs are widely used in oil and gas fields despite the fact that these effluent chemicals may cause changes to the ecological system. In general concentrations can exceed toxic levels for those compounds, therefore affecting those marine organisms that come into contact with them (Gagliardi and Grigson, 2003; Grigson et al., 2000). However, during discharge, some PW components, such as Fe and Mn, rapidly precipitate to the bottom sediment as aggregates, consequently being rapidly removed from the seawater column before contact with filter-feeding bivalves (Gorbi et al., 2008).

Concentrations of other chemicals in PW as total PAHs and other higher molecular weight compounds typically range from 0.04 up to 3 mg/L (Neff et al., 2011a). The metals most regularly found in PW at elevated concentrations, relative to those in seawater are cadmium, zinc, barium, manganese, iron and mercury. Aquatic organisms around the PW discharge point may be exposed to these effluents and possibly accumulate PAH, APs, trace metals and production chemicals through sediments, their food, or ambient water (Neff et al., 2011a).

As many chemical compounds from oil and gas production are known to cause toxic effects, multiple studies have been carried out determining the effect of oilfield waste on aquatic systems; for example on macro-benthic communities (D'Unger et al., 1996; Patin, 2004;

Woodward and Riley, 1983), on sediment quality (Grigson et al., 2000; Hartl, 2010; Scholz-Böttcher et al., 2008; Yu et al., 2003), on aquatic birds (Ramirez, 2005; Rattner et al., 1995), in fish (Brooks et al., 2011; Zhu et al., 2008) and mussels (Brooks et al., 2011; Fernley et al., 2000; Lee et al., 1972; Neff et al., 2006; Sundt et al., 2011).

The present study focuses on PW, in particular it seeks to evaluate the effect of CIs and SIs on marine mussels. The objective is to add to the existing research (e.g. that of: Henderson, 1999; Johnsen et al., 2004; Neff et al., 1987; Neff, 2002; Patin, 1999; Reineke et al., 2006; Yu et al., 2003) into the effects on the marine ecosystem of this important form of waste containing several harmful chemical compounds.

Biomarkers

As shown in the previous section, PW from oil and gas fields contains several chemical compounds that have the potential to present a variety of toxic risks to marine organisms; these can be detected by the observation of biomarkers. Biological markers, known as biomarkers, have been used to detect the presence of diseases in organisms since 1874. There are many definitions given for biomarkers; one of the most widely accepted definitions is “a characteristic that can be objectively measured and evaluated as an indicator of common biological process as well as pathological process or pharmacological response to a therapeutic intervention” (DeCaprio, 2006; Jain and SpringerLink, 2010).

A biomarker can also be defined as any alteration in a biological response, which can be linked to a toxic effect, such as exposure to a chemical or chemicals. For example, serious biological consequences can result due to changes to the structure of the DNA that occurs as a consequence of chemical exposure. Chemicals that have the capacity to interact with DNA and damage the integrity of its molecular structure are referred to as genotoxicants (Shugart, 2000).

Recently many biomarker techniques have been developed to detect DNA damage, superoxide dismutase enzyme and lipid peroxidation. Several techniques are used, including SCGE or Comet assay, SOD, TBARS and NRR assays. Many chemical compounds used in oil and gas production are known to cause toxic effects, and so there have been numerous studies that have examined the effect of oilfield waste on aquatic systems using biomarker techniques. Studies include the measuring of effects on: macrobenthic communities (D’Unger

et al., 1996; Patin, 2004; Woodward and Riley, 1983); on sediment quality (Grigson et al., 2000; Hartl, 2010; Scholz-Böttcher et al., 2008; Yu et al., 2003); on aquatic birds (Ramirez, 2005; Rattner et al., 1995) and on marine mussels (Donkin et al., 1991; Gorbi et al., 2008; Kirchin et al., 1992; Middleditch, 1984; Neff et al., 2006; Reineke et al., 2006; Sundt et al., 2011). Measurements of oxidative stress and DNA damage offer sensitive biomarker techniques with which to determine the genotoxic effects of exposure to oilfield chemicals on marine organisms. Therefore, this study will investigate the potential impact of oilfield chemicals on marine mussels using these biomarker techniques.

Oxidative Stress and DNA damage

Free radical forms of oxygen (O) and nitrogen (N) are continually created as part of the normal physiological function of living cells (Valavanidis et al., 2006). In aerobic organisms, basic cellular metabolism involves the production of free radicals of O_2 and non-radical reactive oxygen species (ROS). Many studies and much laboratory evidence serve to demonstrate that living organisms use free radicals and ROS, such as superoxide anions ($O_2^{\cdot-}$), nitrogen oxide (NO^{\cdot}), peroxy radical (ROO^{\cdot}) and hydrogen peroxide (H_2O_2), to control their physiological processes (Dröge, 2002) and maintain “redox homeostasis” (Ames et al., 1993). However, during these metabolic processes, a small proportion (2-3%) of these free radicals can escape the protective shield of the antioxidant mechanisms, resulting in oxidative damage to the cellular components.

Oxidative stress is defined as an imbalance between antioxidant defence and the production of reactive oxygen/nitrogen species (ROS) / (RNS) (free radicals) and occurs through a significant reduction in the capability of antioxidant defences (Schafer and Buettner, 2001). Oxidative stress can cause damage to the nucleic acid bases in the DNA, the thiol groups in proteins and the unsaturated fatty acyl chains in the cell membranes (Ceconi et al., 2003; Halliwell, 1994).

Free radicals can be defined as any atom or molecule which contains one or more unpaired electron, thereby raising the chemical reactivity of the atoms or molecules (Halliwell and Gutteridge, 1998). Known free radicals include hydroxyl radical (HO^{\cdot}), superoxide anion ($O_2^{\cdot-}$), peroxy (RO_2^{\cdot}), alkoxyl (RO^{\cdot}) and hydroperoxyl (H_2O^{\cdot}) anions. ROS are important in the body and are produced during normal metabolism (Gaté et al., 1999). They are implicated in a variety of functions contributing to biological homeostasis, for example, vasoregulation

(Hensley and Floyd, 2002). However, excessive ROS can result in damage to the macromolecules, which are important for maintaining cell structure and function. Cellular ROS production can be a result of both non-enzymatic and enzymatic reactions and includes the above mentioned species, as well as H_2O_2 , an important non-free radical. A defence against the overproduction of free radicals is supplied by antioxidants, which usually cross the mitochondrial membranes causing neutralisation of the dangerous effects on the cells and also interacting with molecules, such as sugar and fat, resulting in the failure of their absorption and metabolism. Antioxidant defences can cross the mitochondrial double membrane and assault free radicals or ROS, decreasing their production of O_2^- . This will then activate an enzyme called superoxide dismutase (SOD), which accelerates the scavenging of O_2^- . The production of free radicals and peroxide is increased when a disturbance occurs in the normal redox state, causing damage to all the cell components, including the DNA, lipids and protein (Figure 1-6).

Replication in DNA can be also adversely affected by environmental influences such as radiation or exposure to chemicals. Such damage can result in an alteration of the genetic information. The cells' repair mechanisms recognise and remove incorrect or damaged nucleotides. The undamaged strand serves as a template for replacement with an appropriate intact nucleotide (Figure 1-7).

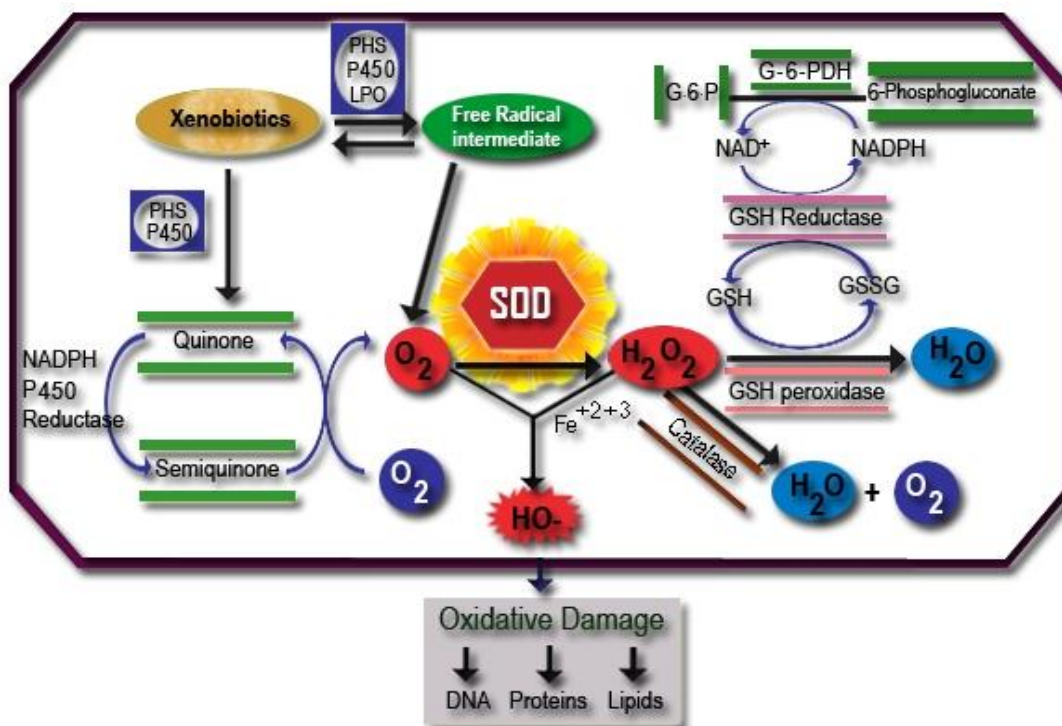


Figure 1-6 The reaction of superoxide dismutase in cells (Sigma, 2012)

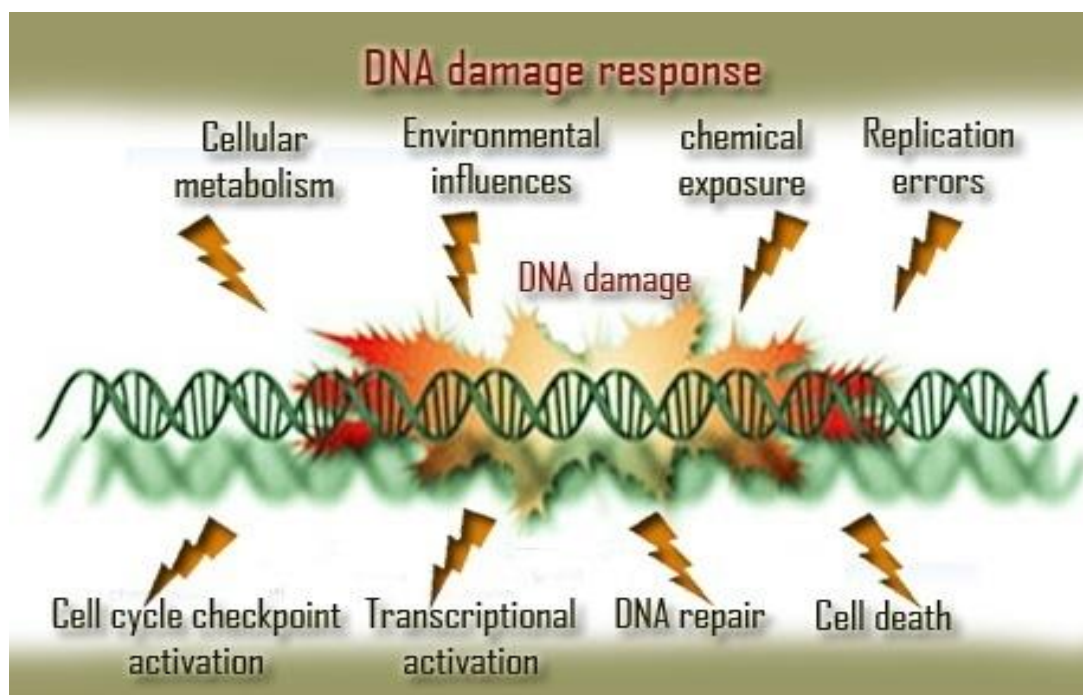
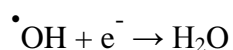
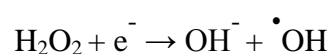
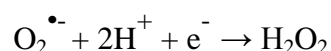
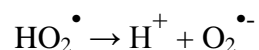
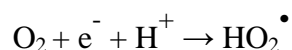


Figure 1-7 Example of DNA damage response (Ahel, 2012)

ROS can cause cumulative damage that may result in several human diseases (Aruoma and Halliwell, 1998), including heart and eye disease (Li et al., 2008), atherosclerosis (Fearon and Faux, 2009) and diabetes (Ceriello and Motz, 2004), while vegetable diets and fruit rich in antioxidants, such as carotenoids, polyphenols and vitamin C can decrease the risk of such diseases (Christen et al., 2008; Dauchet et al., 2006; Dherani et al., 2008).

Free radicals are produced by the numerous different biochemical processes that take place within the body. These include molecular oxygen reduction through aerobic respiration, yielding superoxide and HO^\bullet ; the by-products of chemistry, caused by oxidation of catecholamine and activation of the arachidonic acid cascade product electrons. They are also capable of reducing molecular oxygen to superoxide; production of superoxide and hypochlorous acid (HOCL), a powerful oxidant, arises due to activated phagocytes and nitric oxide production by vascular endothelium and other cell types. Furthermore; free radicals can be produced in response to, or as a result of, external electromagnetic radiation, such as gamma rays, that are capable of splitting water to generate HO^\bullet , as shown in the equations below (Betteridge, 2000).

- Molecular oxygen reduction



The presence of unpaired electrons is due to ROS such as peroxides and free radicals, which are inherently unstable molecules. Consequently, these free radicals can be highly reactive (although there are variations from radical to radical), reacting locally to gain or lose electrons to other molecules to achieve a more stable state. Because many of the molecules are non-radicals, such as H_2O_2 , they will also become involved in the reaction. The reaction of a radical with non-radicals (all biological macromolecules, carbohydrates, nucleic acids, proteins and lipids can be targets) generates a free radical chain reaction with the formation of new radicals, that can react further with macromolecules (Betteridge, 2000). Examples of these reactions include protein damage and lipid peroxidation, e.g. by the addition of cross-linking, fragmentation or carbonyl groups. Carbonylation of amino acid residues can expose proteins to proteolysis (Stadtman and Oliver, 1991). DNA base oxidation also affects the transformation of thymine into thymine glycol and guanine into 8-hydroxyguanine (Figure 1-8), protein/DNA cross-links and SSB and DSB (Hall et al., 1996; Halliwell and Aruoma, 1991; Spear and Aust, 1995).

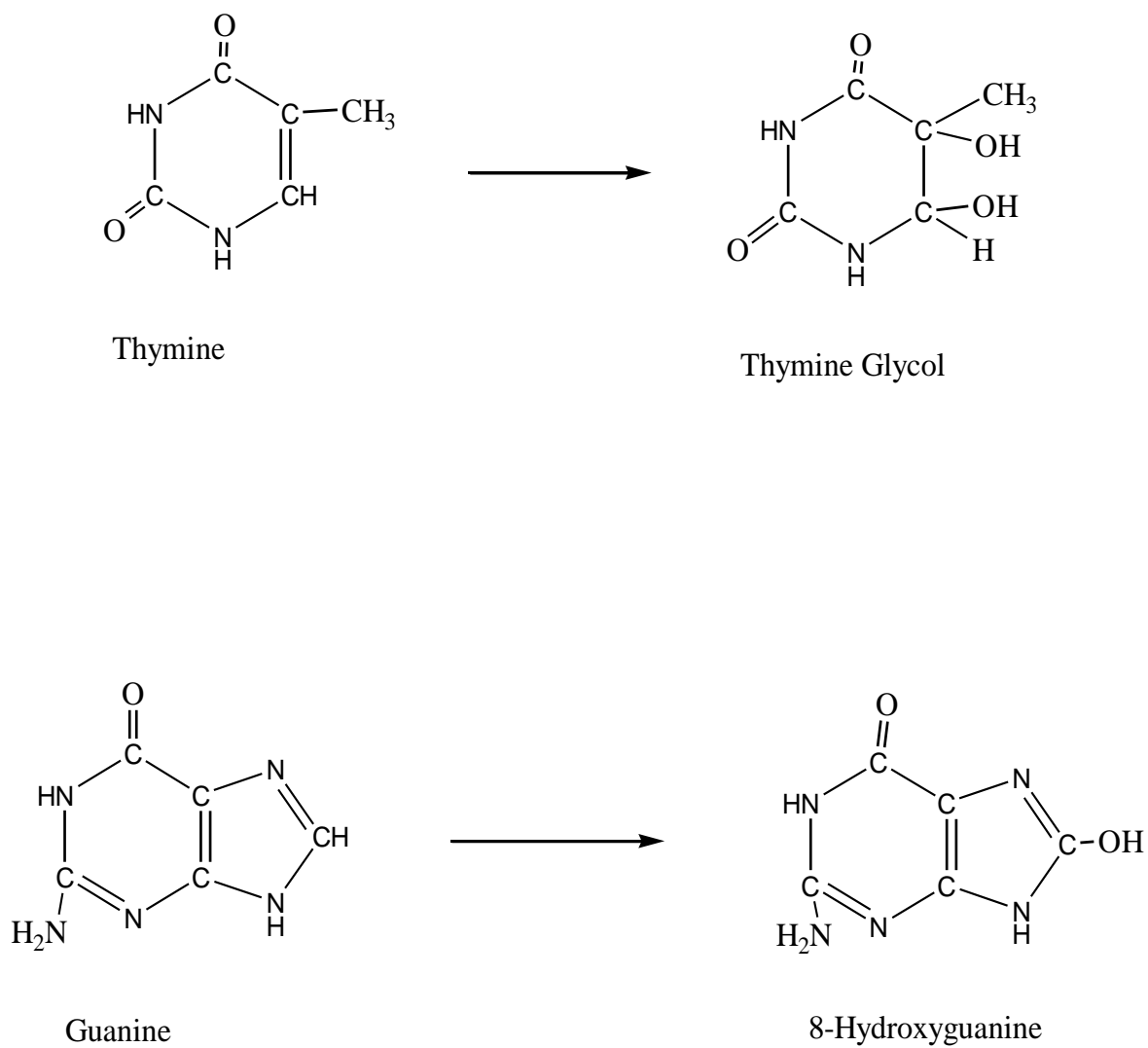


Figure 1-8 Example of DNA base oxidations resulting from hydroxyl radical ($\cdot\text{OH}$) attachment (Di Giulio et al., 1995)

Lipid peroxidation is described in Figure 1-9. Free radical reaction, e.g. the removal of an H from a methylene group (-CH₂-) of fatty acids by •OH, results in an unpaired electron on the carbon (-CH•). Unsaturated, particularly polyunsaturated, fatty acids are prone to free radical attack because of the existence of a deteriorated double bond on the next carbon atom. The remaining carbon radical is capable of undergoing molecular reorganisation, resulting in a conjugated double bond. Conjugate double bonds (conjugated dienes) can combine with O₂, to form a peroxy radical. This can attract more H and initiate a chain reaction, which can continue until either the substrate has been consumed or the reaction has been terminated by a chain-breaking antioxidant, such as vitamin E. The resulting lipid peroxides are reasonably stable compounds. However, the presence of transition metals and complex metals can catalyse their decomposition and leading to the production of peroxy and alkoxy radicals, that can stimulate lipid peroxidation. Lipid peroxidation is more susceptible when tissues are disrupted and this can have profound effects on cellular function. Extensive peroxidation can cause changes in the fluidity of cell membranes and influence the permeability, reduce membrane potential and finally bring about membrane rupture. Lipid peroxidation is also responsible for diabetic vascular damage following a free radical attack (Figure 1-9).

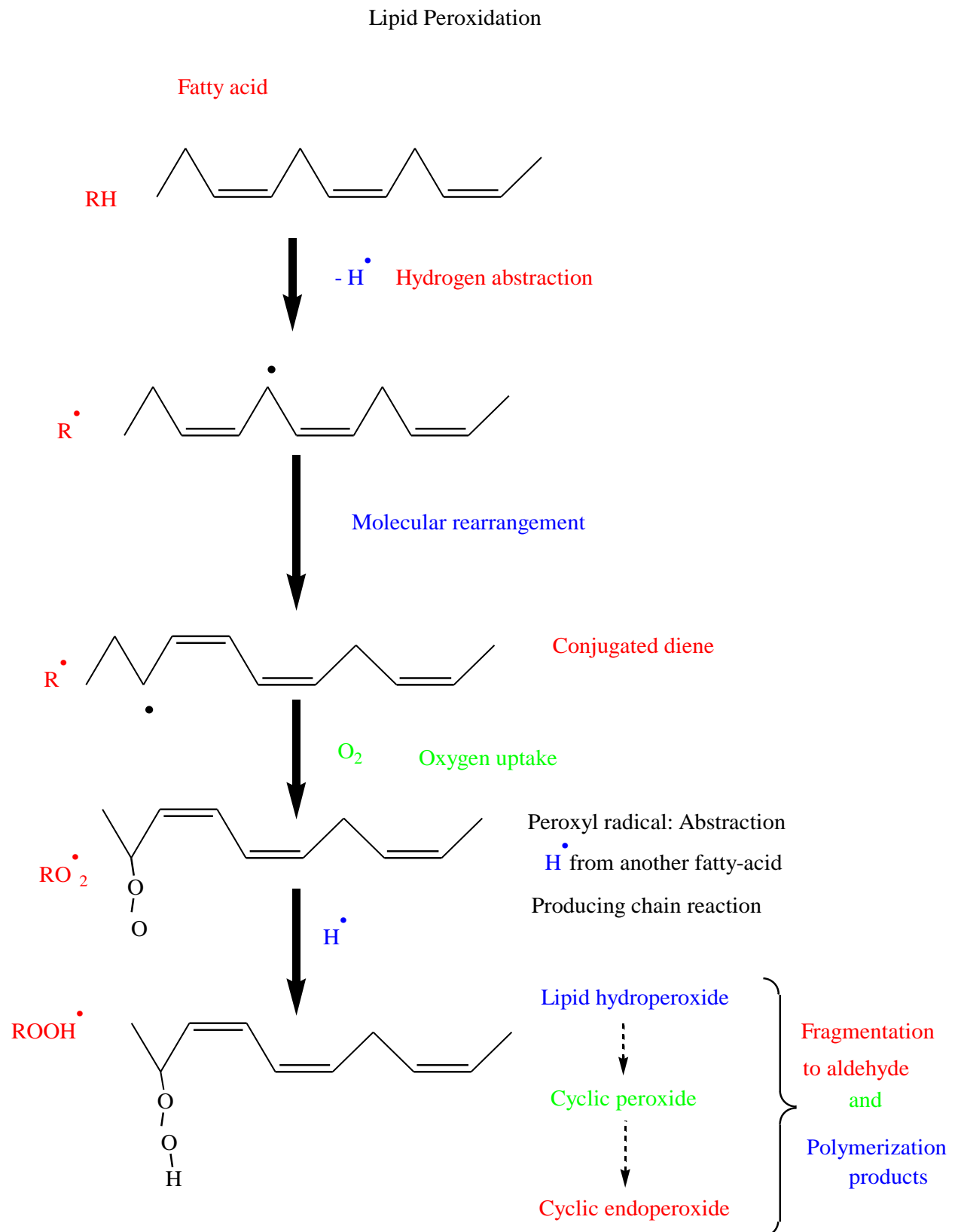
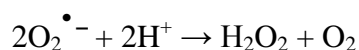
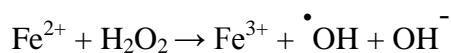


Figure 1-9 Lipid peroxidation as an example of free radical-mediated tissue damage (Betteridge, 2000).

Given the potential for tissue damage, the body has major antioxidant defence mechanisms to protect itself from free radical attack. These antioxidant defence mechanisms can be classified as either extracellular, membrane and cellular. According to Gutteridge (1995), an antioxidant can be defined as any substance which, when found at low concentrations relative to those of the oxidisable substrate, inhibits substrate oxidation. The dismutase, peroxidase and catalase enzymes are known as cellular antioxidant defences. Additionally, the possibility of intracellular free radical production is considerably weakened by the ability of mitochondrial cytochrome oxidase to function catalytically in an electron transport chain, (Chance et al., 1979). Superoxide dismutases (SODs) contain copper, zinc and/or manganese. Cu/Zn-SODs located in cytosol and Mn-SOD in mitochondria catalyses the ($\text{O}_2^{\bullet -}$) into (H_2O_2) and (O_2).

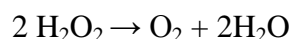


The product of this reaction, H_2O_2 , is relatively stable and it is a weak oxidant. Nevertheless, unlike $2\text{O}_2^{\bullet -}$, H_2O_2 can rapidly diffuse across a cell membrane, and in the presence of transition metal ions it could be translated via Fenton chemistry to HO^{\bullet} .



There are two enzyme systems with the capability of breaking down H_2O_2 : glutathione peroxidase (GPX) and catalase. Glutathione peroxidases (GPX) located in cytosol and mitochondria have an important role in abstracting H_2O_2 by SOD with the oxidation of glutathione (GSH).

When H_2O_2 is present in high concentration, catalase, which is present in peroxisomes within many tissues will break it down as follows:



Vitamin E, coenzyme Q and β -carotin are antioxidant defences located within the cell membranes. When incorporated into the lipid core of the cell membrane, the lipophilic vitamin E (α -tocopherol) is a highly effective antioxidant. It has the capability to scavenge

intermediate ROO^\bullet , thereby disrupting the chain reaction of lipid peroxidation (Burton et al., 1982).

The tocopherol radical (T^\bullet) is converted back to α -tocopherol by vitamin C and is much less reactive. In addition, numerous other lines of antioxidant defences are present in the cells; such as membranes with convenient cholesterol/phospholipid proportions.

The most important extracellular antioxidant defences include metal binding proteins. The free metals Fe and Cu can promote free radical damage, accelerating lipid peroxidation as well as catalysing HO^\bullet formation. Through binding proteins, the body is defended against these potentially adverse effects, ensuring that Cu and Fe are rendered into a nonreactive state. Albumin and haemopexin bind haemoglobin and haem, which can also accelerate lipid peroxidation. In addition to these important metal binding proteins, a variety of low-molecular-weight molecules, for example: urate, vitamin C and bilirubin, have antioxidant properties (Frei et al., 1988; Halliwell and Gutteridge, 1990). In addition, SOD and extracellular forms are also important and as such have been extensively reviewed (Bag and Bag, 2008; Betteridge, 2000; Maddipati and Marnett, 1987; Marklund et al., 1982). The measurement of free radical production and antioxidant balance is frequently used as an indicator of oxidative reactions for *in vitro* experiments. It is particularly important to understand how conflicting experimental conditions, such as the nature of the oxidative conditions applied and the type of substrate employed, can result in the generation of different products.

Generally, oxidative stress can be measured by evaluating products that react to forms of oxidative damage, such as protein oxidation, DNA oxidation and lipid peroxidation. The measurement of the depletion of antioxidants, such as the thiol group, vitamin C and α -tocopherol, is a complementary approach.

Contaminants of the aquatic environment and oxidative stress in aquatic organisms

Oxidative stress is one of the most important subjects of concern in the field of aquatic toxicology (Valavanidis et al., 2006). The substantial amounts of environmental pollutants discharged daily into the aquatic environment have the potential to cause oxidative stress by triggering free radicals and ROS mechanisms in aquatic organisms. The aquatic organisms may uptake the environmental pollutants in their food sources, from suspended particulate matter with toxic properties and from sediments containing toxins. Aquatic organisms were chosen for this study according to their sensitivity to oxidative damage following chronic exposure or sub-lethal concentrations, their filtration capacity and whether they are easy to cage. Furthermore, such organisms can provide model systems to investigate the potential damage to cellular components caused by ROS, how cells respond, how this damage can be ameliorated by repair mechanisms and how oxidative stress causes diseases (Valavanidis et al., 2006).

Many articles have reviewed biomarker techniques, and have provided evidence, for the indirect measurement of formation of free radicals, such as SOD to evaluate superoxide dismutase activity (Fridovich, 1989; Huang et al., 2000; McCord and Fridovich, 1988; Sun et al., 1988; Zelko et al., 2002). A review of oxidative stress in aquatic organisms by Di Giulio et al. (1989), described the general processes responsible for ROS generation in aquatic organisms and highlighted the role of some metal ions, such as chromium, copper, arsenic, mercury and pesticides (fungicides, herbicides and insecticides), along with the oil products, in the induction of oxidative stress. The impact of oil and PAH on marine mussels following the *Don Pedro* oil spill accident was also investigated, by Sureda et al. (2011).

Thiobarbituric acid reactive substances (TBARS) can be used for determining lipid peroxidation; this is achieved by measuring xenobiotic-induced oxidative stress (Alves de Almeida et al., 2007; Crockett, 2008; Labrot et al., 1996; Torres et al., 2002; Wilhelm Filho et al., 2001) and the impact of PAHs on marine mussels and other species has been determined using oxidative stress indicators such as TBARS and others (Nahrgang et al., 2012). Also, single cell gel electrophoresis (SCGE) or Comet assay have been used to assess the integrity of DNA (Collins, 2004; Dhawan and Anderson, 2009; Pereira et al., 2010; Singh, 2000; Singh et al., 1988; Singh and Stephens, 1997; Steinert et al., 1998; Tice et al., 2000).

All these assays are important methods with which to investigate the genotoxicity created by QUATs, imidazolines, PEs and PW. In this study, therefore, genotoxicity, cytotoxicity, oxidative stress and bioaccumulation and bio-concentration will be investigated using different biomarker techniques, as described below. Although single cell gel electrophoresis (SCGE) has been used to determine the impact of oil exposure, it has not yet been used to evaluate exposure to oil field chemicals such as CIs and SIs.

Single cell gel electrophoresis (SCGE) or comet assay

Chemical compounds being discharged by the oil and gas industries into aquatic environments may cause a range of forms of DNA damage in aquatic organisms, including single-strand breaks (SSB) (a single-strand break is created when a DNA lesion causes at least one strand to break), alkali-labile sites (ALS), double-strand breaks (DSB) (a double-strand break is generated when a DNA lesion occurs on both DNA strands), DNA crosslinks and modified bases (MB) (Kurelec, 1993).

Many techniques have been used to investigate DNA damage, such as the chromosomal aberration assay (CA), the sister-chromatid exchange assay (SCE) and the micronucleus test (MNT) (Kim and Hyun, 2006). In particular, metaphase methods, for example CA and SCE, have not been found to be useful for detecting damage *in vivo*, because only a few cells simultaneously occur in metaphase and there are also often karyotype limitations, particularly in triploid fish species (Hooftman and de Raat, 1982). However, MNT has been applied *in vivo* to identify DNA damage in fish and bivalves and thus to monitor water quality (Al-Sabti and Metcalfe, 1995; Bolognesi et al., 1999; Grisolia and Starling, 2001). The alkaline single cell gel electrophoresis (SCGE) or Comet assay, is widely utilised in many field studies investigating genotoxicity and is a useful technique for measuring the electrophoretic migration of DNA strand breaks and ALS, from immobilised nuclear DNA (Dhawan et al., 2009; Singh et al., 1988).

The name Comet assay is used because the DNA migration means the image obtained of the cell resembles a comet, with a head and a tail (Klaude et al., 1996; Singh and Stephens, 1997). SCGE, like Comet assay, is very useful because it can be used to identify DNA lesions in individual cells (Tice et al., 2000). For this reason it is widely used to examine the genotoxicity of pharmaceuticals, agrochemicals, biocides and industrial chemicals (Hartmann et al., 2003). This technique, sometimes also referred to as microgel electrophoresis (MGE), was pioneered by Ostling and Johanson (1984) as a microelectrophoretic technique to be for

detecting DNA damage in individual cells. There are many advantages of using Comet assay, such as its applicability to a wide variety of cells, including bacteria. It is inexpensive, sensitive, rapid and simple and it takes only a short time to achieve a result, making it a helpful technique for environmental monitoring (Dhawan and Anderson, 2009). In addition, during the last 15 years, the method has been further developed into a new tool for evaluating genetic damage, *in vivo* and *in vitro*, in a diversity of cells taken from a variety of fish (Hartl et al., 2007; Kilemade et al., 2004; Monteiro et al., 2011; Pereira et al., 2010) and invertebrate species (Lewis and Galloway, 2008; Mitchelmore and Hyatt, 2004; Monserrat et al., 2009) including bivalves (Coughlan et al., 2002; Hartl et al., 2004; Hartl. et al., 2010; Pisanelli et al., 2009; Rank and Jensen, 2003; Richardson et al., 2008). Singh (1988) also developed a MGE technique for detecting DNA damage in individual cells under highly alkaline (pH > 13) conditions, in particular SSB and ALS.

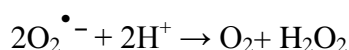
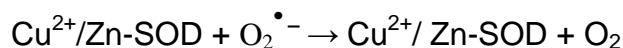
The versatility of the Comet assay has been widely reviewed elsewhere, including by Fairbairn et al. (1995), Anderson et al. (1998), Rojas et al. (1999), Speit and Hartmann (1999), Tice et al. (2000), Hartmann et al. (2003), Kim and Hyun (2006), Dhawan and Anderson (2009), Hartl (2010) and Monteiro et al. (2011).

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is an antioxidant defence enzyme, which was discovered by McCord and Fridovich (1969), and is important for reducing the biological impact on cells of exposure of oxygen.

SODs are metalloenzymes that catalyse the dismutation of ($\text{O}_2^{\bullet -}$) into (O_2) and (H_2O_2) and therefore supply important antioxidant defence mechanisms to counter superoxide radicals (Malstrom et al., 1975). This reaction protects cells from superoxide toxicity and is recognised as an antioxidant system. There are many types of superoxide dismutases, that depend on the central metal ion type. In mammals, there are three important isoforms of SOD, which are identified in different distributions depending on tissue (Zelko et al., 2002). Cu/Zn-SOD (SOD1) is found in lysosomes, cytoplasm and the nuclear compartment of haemocytes (Crapo et al., 1992; Keller et al., 1991; Liou et al., 1993). SOD1 is a protein composed of two identical polypeptide chains called homodimers, which contain one copper ion and one zinc ion in each 16-kDa subunit of 153 amino acids. The Cu^{2+} ions are detained by interaction with imidazolate ligands of the histidine residues in SOD1 at the active enzyme

site. The Zn^{2+} helps to stabilise the enzyme. The reaction below shows the mode of action of SOD1:



SOD1 activities are almost independent of pH in the range of 5.0 to 9.5 and at physiological pH the reaction rates are approximately diffusion-limited ($\sim 10^9 \text{ M}^{-1} \text{ s}^{-1}$) (Liochev and Fridovich, 2007; Miyamoto et al., 2010; Valentine et al., 2005).

Another type of SOD, Mn-SOD (SOD2), is a homotetramer, which has one ion of Mn at its active centre with an individual 22-kDa subunit (Miyamoto et al., 2010). SOD2 is usually found in mitochondria and catalysed as SOD1. The activity of SOD2 is highest in specific forms of tissue: for example the activity of SOD2 is higher in the renal cortex than in other tissue.

There is an additional type of SOD (SOD3), which contains Cu^{2+} and Zn^{2+} . In many species, SOD3 is located in extracellular fluids, such as lymph and plasma, and is present as a tetramer of an identical 30- kDa subunit (Nozik-Grayck et al., 2005). SOD3 acts as a superoxide scavenger working to maintain protection against the overproduction of ROS in tissues. In an extracellular matrix, SOD3 contributes to localisation because it contains a high affinity domain for heparin (Fattman et al., 2003).

To detect the activity of the SOD enzyme in an organism, both direct and indirect methods have been developed. However, many studies have used indirect methods by applying nitro blue tetrazolium (NBT), a substance which is widely used due to its convenience and ease of use. There is a convenient and common indirect method that utilises NBT for detection of superoxide radicals produced by hypoxanthine and xanthine by conversion into formazan dye.

All types of SOD (Cu/Zn, Mn and Fe) can be measured by the SOD assay. The assay represents a simple, rapid and reproducible tool with which to measure activity in tissue homogenates, erythrocyte lysates, serum, cell lysates and plasma. As shown in Figure 1-6, the rate of reduction of the O_2 is linearly correlated with xanthine oxidase (XO) activity and indirectly correlated with SOD inhibition, so a colorimetric method can be used to determine the IC_{50} (50% inhibition activity of SOD or SOD- like materials).

Thiobarbituric acid reactive substances (TBARS)

The thiobarbituric acid reactive substances (TBARS) assay has been widely applied since the 1950s to measure the extent of lipid peroxidation in biological membranes (DeLong and Steffen, 1997; Du and Bramlage, 1992; Heath and Packer, 1968; Sinnhuber et al., 1958). More recently it has been used for the assessment of free radical-mediated oxidation of polyunsaturated fatty acids (PUFA) (Camejo et al., 1998).

Lipid peroxidation occurs when there is a series of reactions in which first dienes are formed, followed by lipid hydroperoxides (LOOHs), and then fragmentation of PUFAs to carbonyl compounds occurs, referred to as TBARS (Camejo et al., 1998). In the present study, the TBARS assay was adapted for *Mytilus* from a variety of sources (Bouskill et al., 2006; Camejo et al., 1998; Smith et al., 2007). It is useful due to its straightforwardness and because its effects correlate highly. In this assay, a thiobarbituric acid (TBA) test is used to evaluate the extent of any reaction. TBARS reacts with two molecules of malonaldehyde (MAD), a by-product of lipid oxidation, developing a pink colour, with an absorption maximum of 530nm.

Integrity of the lysosomal membrane

The lysosome has been identified at the sub-cellular level as a particular target of the toxic effect of contaminants (Moore, 1990); furthermore, pathological alterations in lysosomes have been particularly useful for identifying adverse environmental impacts on marine organisms (Giamberini and Pihan, 1997; Moore et al., 1996). One such characteristic pathological alteration is the reduced integrity of the lysosomal membrane following the exposure of mussels to xenobiotics.

Lysosomal membrane integrity decreases with increases in nonspecific stress (both biotic and abiotic). This alteration in membrane stability is caused by mechanism(s) that are not well

understood, but it is possible that it may be due to direct chemical effects on the membrane or a rise in the frequency of secondary lysosomes in toxicant-stressed cells.

Neutral red retention assay (NRR)

There were two traditional techniques used to assess the stability of the lysosomal membranes, prior to the development of the neutral red retention assay (NRR). These previous techniques were either cytochemical (Moore, 1976) or biological (Baccino and Zuretti, 1975) and were established to measure the activity or amount of the lysosyme leaking through the lysosome membrane in response to pH or osmotic shock, respectively. However, when observing leakage from lysosomes, it was found that neither of these techniques works under the regular physiological states. Furthermore, NRR is much easier to apply and has therefore achieved wide-spread application (Valérie, 2001). It is a sensitive, cost-effective ecotoxicological tool that can be used to consider the impact of multiple stressors on aquatic organisms, particularly marine bivalves (Brown et al., 2004; Buratti et al., 2012; Cheung et al., 1998; Coughlan et al., 2009; Galloway et al., 2004; Guidi et al., 2010; Livingstone et al., 2000; Martins et al., 2005; Moore et al., 1996; Nicholson, 1999; Nigro et al., 2006; Ringwood et al., 1998; Shaw et al., 2011; Wedderburn et al., 2000); it has also been reviewed by various researchers (e.g. Chong Zhao et al., 2011; Luzio et al., 2007; Moore et al., 2008; Svendsen et al., 2004; Valérie, 2001). In the present study, lysosomal stability was determined using the NRR assay, applying the protocol defined by Coughlan et al. (2009).

Originally, NRR was developed as a cytotoxicity assay for use *in vitro* on mammalian cells; it was also adapted for ecotoxicity fields, using fish cells (Babich and Borenfreund, 1990). The NRR assay is based on the uptake and binding of neutral red dye (NR) as a cationic dye in viable cells in a lysosomal matrix, following incubation with toxic agents. This method was widely used in cytotoxicity studies for determining the relative acute cytotoxicity of chemicals, the relationships of structure-toxicity for related chemicals, interactions, and metabolism mediated cytotoxicity; it was also used in other fields, such as for pharmaceutical studies. Lowe et al. (1992) developed the NRR assay following *in vivo* exposure, isolating the cells from organisms, and utilising this technique for studying damage to lysosomal membranes in isolated hepatic cells of fish collected from contaminated water and water lying in clean fields. NRR techniques have been developed that can quantify the leakage of the dye in lysosomes under *in vitro* conditions (Lowe and Pipe, 1994). Lowe et al. (1995a)

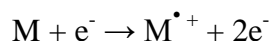
used the NRR assay to assess contaminant-induced lysosome membrane damage in marine mussels. Svendsen and Weeks (1995) implemented further modifications on the basis of this technique and Weeks and Svendsen (1996) established the use of this technique with terrestrial and freshwater invertebrates. Peek and Gabbott (1989) described an NRR assay involving extracting the haemolymph into an equal volume of physiological saline solution (PSS); neutral red dye is then added and the rate of leakage of the dye from the lysosomes recorded.

Mass spectrometry

The characteristics of mass spectrometry, including detection limits, unequalled sensitivity, speed and diversity of functions have led to it becoming an outstanding option amongst analytical methods. Mass spectrometry is an analytical tool commonly used in biochemical fields to detect biochemical problems; therefore it commonly used in drug discovery. In addition, it is routinely used in food control, pollution control, for evaluating natural products and in forensic science or process monitoring.

Mass spectrometry has been defined as a study of systems generating the formation of gaseous ions with or without fragmentation, which can be measured by their mass to charge ratio (m/z) and relative abundances. Mass spectrometry is the study of the effect of energy and ionisation on molecules. Therefore, mass spectrometry is unlike most other forms of spectrometry or spectroscopy, which are concerned with non-destructive interactions between electromagnetic radiation and molecules. Sample molecules are consumed during the formation of ionic and neutral species dependent upon the chemical reactions in the gaseous phase. Although the sample can be destroyed by mass spectrometry, this technique is extremely sensitive, as well as only using trace amounts of material for analysis. The function of a mass spectrometer is to convert sample molecules into ions in the gas phase, thereby separating or resolving the ions formed at the ionisation source according to their m/z , and sequentially recording the individual ion current intensities for each mass across the mass spectrum (Belton, 2000). The values of m/z versus percentage relative intensity (%IR) are represented as a line diagram. When the ion current intensities are drawn in a histogram, the current intensity for the most abundant ion is taken as 100% (Belton, 2000).

In the mass spectrometric analysis of compounds the first step is the production of the compound's gas phase ions, for instance by electron ionisation.



Normally, a molecular ion undergoes fragmentations. If it is a radical cation with an odd number of electrons, it may fragment, giving both a radical and an ion with an even number of electrons, or a molecule and a new radical cation.

Production of molecular ions

When the quantity of energy is equivalent to the ionisation energy (IE) of the molecule as supplied under conditions of electron ionisation (EI), a molecular ion is formed, as denoted by a radical ion, such as $M^{\bullet+}$. The fundamental components of the mass spectrometry system are divided into three groups, namely the ionisation source, the analyser, and the detector (Figure 1-10). In this study, mass spectrometry was applied to analysis of polar molecules ranging from less than 100 Da to more than 1,000,000 Da in molecular mass; therefore electrospray ionisation (ESI) has been used. This will be described in the next section.

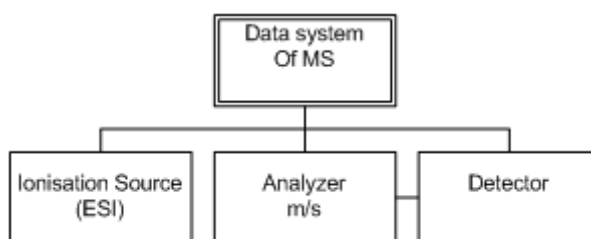


Figure 1-10 Fundamental parts of the mass spectrometry system

Electrospray ionisation (ESI)

From the HPLC pump, the liquid flow enters a stainless steel capillary with a small diameter, and is maintained at a voltage of 3 – 4 kV forming a plume of charged liquid droplets. Via an evaporation mechanism, ions are produced and ionised particles transported into the vacuum of the mass spectrometer via a pumped nozzle-skimmer arrangement (Figure 1-11). To prevent cluster ion formation, a curtain of nitrogen gas is then applied to aid evaporation of the charged droplets. Through an orifice, ions are sampled into the mass analyser. ESI has revolutionised mass spectrometry by allowing mass spectra to be routinely obtained on non-volatile and polar molecules. ESI allows a very high level of sensitivity to be reached and can be used to analyse even those substances with high molecular weights. Multiple charged ions can be obtained from proteins, thereby allowing their molecular weights to be determined within the mass range of a conventional mass spectrometer. These signals are then

mathematically transformed to yield the molecular weight of the sample (Belton, 2000). Many studies have reviewed the principles and biological applications of ESI (Cech and Enke, 2001; Gu et al., 2012; Guha et al., 2012; Mora et al., 2000; Peterson and Cummings, 2006; Rohner et al., 2004).

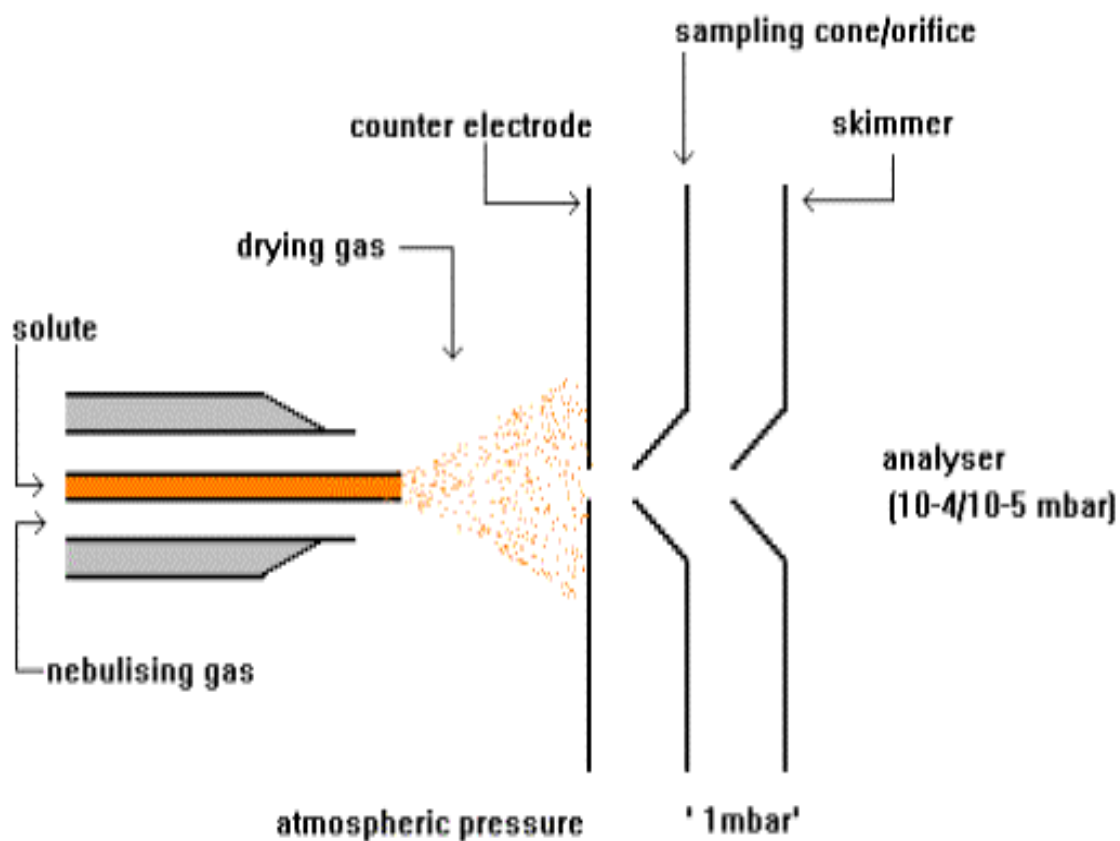


Figure 1-11 Typical electro spray ionisation sources (Ashcroft, 2008)

Marine mussels (*Mytilus edulis*)

Marine mussels are in the phylum Mollusca and belong to the class Bivalvia, and the subclass Pteridomorpha. These animals belong to the family *Mytilidae*, which is very ancient and contains many important byssally-attached genera; for instance, *Aulocomya*, *Perna*, *Choromyilus*, *Modiolus* and *Mytilus*.

Marine mussels belonging to the genus *Mytilus* are widely distributed in the cooler waters of both the northern and southern hemispheres and have been proven as ideal organisms on which to conduct a variety of biochemical, physiological and genetic investigations. There are three classifications within the family of *Mytilus*; these are *Mytilus trossulus*, *Mytilus gallorovincialis* and *Mytilus edulis*, all of which are categorised based on genetic and morphological differences (Innes and Bates, 1999). These morphological differences are fairly distinct assisting identification; however where their distributions overlap, such as in hybridisation and introgression which is a regular occurrence, morphological distinctions are ineffective as diagnostic tools (Beaumont et al., 2008) as the systematic status of these types is unknown. In spite of their systematic status, *M. gallorovincialis* and *M. edulis* do exhibit a zone of overlap in Western Europe where they hybridise extensively (Gardner, 1995).

Ova and spermatozoa from *M. edulis* are derived from germinal epithelium in the genital follicles which are located principally in the mantle and connected through ducts to the exterior. According to environmental circumstances, the timing of the reproductive cycles differs, so as a result gonads grow between October and November and during the winter months gametogenesis occurs. In spring and early summer, successive periods of partial spawning occur (Jabbar and Davies, 1987).

The marine mussel *Mytilus* is typically found in littoral and shallow sub-littoral waters. However, it is also found in deep water, with instances of this having been occasionally recorded. *Mytilus* mussels occur in both open water and brackish estuaries, mostly where there is significant water movement. These animals can live on a diversity of substrata, such as stones, rock, dead shells, shingle and even compacted sand or mud (Seed and Richardson, 1990). They attach themselves by their byssal threads (figure 1-12) to any substrate that provides secure anchorage. These byssal threads are tough protein threads which *Mytilus* secretes through glands positioned along a groove in its foot. Attachment to a surface is achieved by flexing the foot outwards to the substrate and pushing the byssus secretion out along the groove and retracting the foot. The sticky thread then hardens rapidly, leaving the

mussel safely anchored to the substrate. These animals remain prey to many enemies, even after becoming anchored to a suitable substrate. Mussels face attack from multiple enemies, both in and out of the water, for example, predators such as gulls and oystercatchers attack the mussels from above the waterline, whereas eider ducks, crabs and sea stars attack from beneath the water.

The identification of the *Mytilus* species at the species level, is not possible in many cases based solely on morphology (Bayne, 1976). However, other approaches have been explored; for instance, Abalde et al. (2003) described an immunological method for the identification of *Mytilus* larvae although their method did not allow for discrimination at the species level (Dias et al., 2009).

M. edulis is widely distributed in Scotland and is native to the United Kingdom. However, recently, some studies have reported the sympatric occurrence of *M. edulis*, *Mytilus trossulus*, *M. galloprovincialis* and their hybrids in cultivation in Loch Etive; *Mytilus trossulus* is widely present in Loch Etive (Dias et al., 2009). However, *M. edulis* is abundant at Cramond Island, Edinburgh. The sexes in *M. edulis* are separate, with no external signs of dimorphism, although hermaphroditism does occur. These animals mature in their first year, although their size at maturity depends on their growth rate. The reproductive system of mussels contains a number of ducts which ramify over the greater part of the body, each terminating in a genital follicle (Bayne, 1976), see figure 1-12.

M. edulis is widely used as a bio-monitor to investigate genotoxicity. In the present study, the haemocytes and gill cells from the marine mussel, *M. edulis* _Mollusca: Bivalvia has been used. Marine mussel, *M. edulis* is known as a member of an ecologically important group of marine invertebrates, that have been widely monitored to measure the ecotoxicological, genotoxicological and bioaccumulation effects of marine pollutants (Akcha et al., 2004; Almeida et al., 2005; Dailianis et al., 2003; Faria et al., 2009; Nahrgang et al., 2012; Pisanelli et al., 2009; Shaw et al., 2011; Turja et al., 2012). It was chosen for the present study, because of its rapid uptake of mineral oil (Lee et al., 1972). Furthermore, the organisms are sedentary, long-lived, easily identified and sampled, reasonably abundant and available throughout the year, as well as being tolerant of natural environmental fluctuations and pollution (Stankovic et al., 2012). These biological and ecological characteristics allow the

development of a time-integrated picture of local pollution that is not possible with more mobile species.

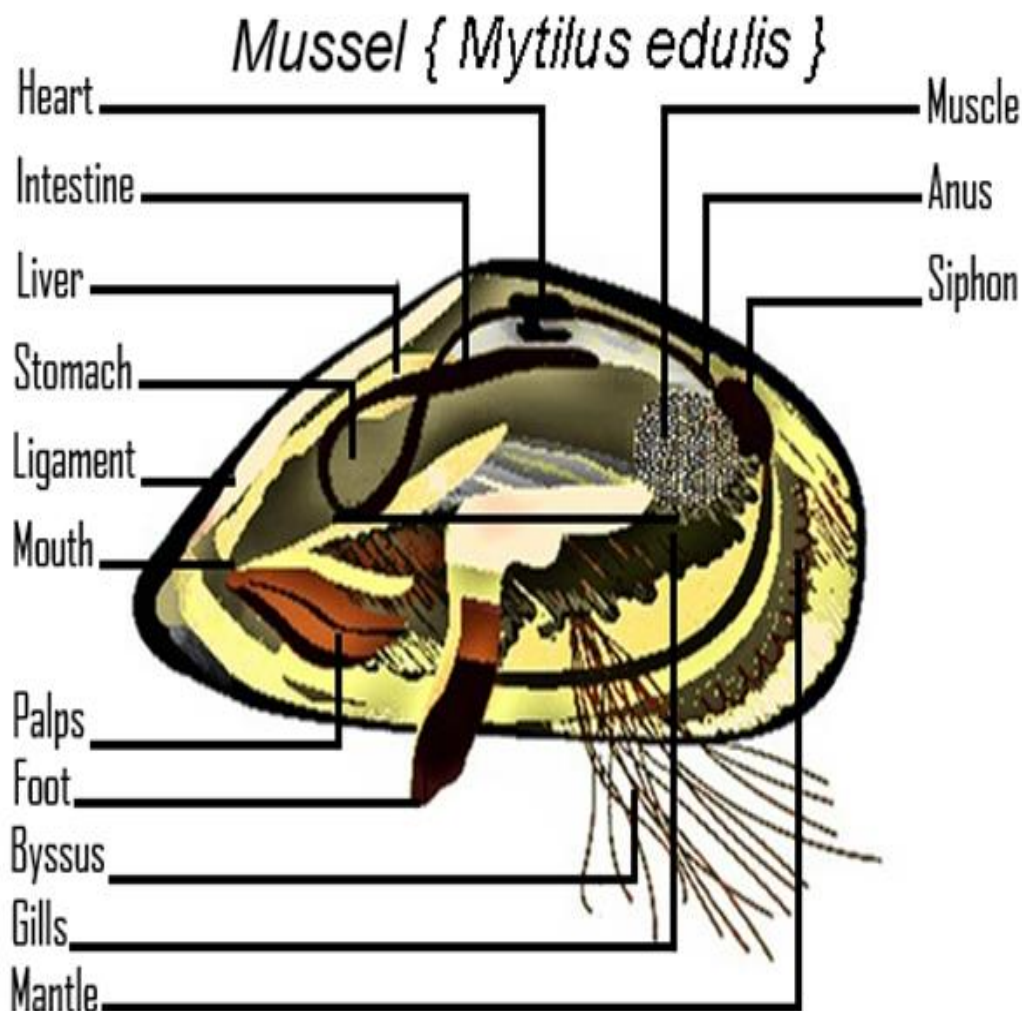


Figure 1-12 Morphology of marine mussel *M. edulis*

The aim of this study was to investigate the potential effect of these compounds on marine mussels *M. edulis* by applying various biomarker techniques; such as single cell gel electrophoresis (SCGE) or Comet, superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS), neutral red retention (NRR) assays, and associated analytical chemistry using atomic absorption spectrometry (AAS) and the solid phase extraction method (SPE) followed by the electrospray ionisation tandem and ESI MS/MS mass spectrometry. All these techniques are in common use to investigate toxicity, cytotoxicity, oxidative stress, and bioaccumulation in marine organisms. All the above chemical compounds were obtained

from oil and gas company where located in the North Sea Brae Alpha platform without disclosing specific details about their chemistry and PW was received from the North Sea Brae Alpha platform discharge point, tapped in 2 m³ steel tank, and kept at 4°C until use.

Aims and objectives

One of the key objectives stated was to determine the potential effects of environmental concentrations of PW and corrosion and scale inhibitors. Therefore, this research study focused on investigating and evaluating the bioavailability and potentially genotoxic effects of QUATs and imidazoline (both of which are used as corrosion inhibitors), of PEs (which are used as scale inhibitors), and also of PW as a whole, on marine mussels *M. edulis* at environmental concentrations, by applying biomarker techniques.

All the following objectives were focused on whilst conducting this research:

Determine the genotoxic effects of environmental concentrations of QUATs, imidazolines, PEs and PW and their impact on DNA damage and oxidative stress biomarkers in haemocytes and the gill cells of marine mussels (*M. edulis*), by applying Comet assay or SCGE, superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS) and neutral red release (NRR) assays.

Identify and evaluate the bioaccumulation and bio-concentration of environmental concentrations of QUATs, imidazolines, PEs and PW in the soft tissues of marine mussels (*M. edulis*), using solid phase extraction methods (SPE) and mass spectrometry.

Some specific objectives

- Investigate the presence of QUATs, imidazoline and PEs all the mussels' soft tissues using mass spectrometry.
- Confirm the uptake of QUATs, imidazoline, PEs and PW by the mussels using mass spectrometry.
- Identify the types of heavy metals in the PW sample and all the mussels' soft tissues using atomic absorption spectrometry (AAS).
- Identify the concentrations of QUATs, imidazoline and PEs in PW samples using mass spectrometry.
- Determine the impact of QUATs, imidazoline, PEs and PW; their impact on lysosomal membrane stability of mussel haemocytes and gill cells using NRR.

- Determine the genotoxicity of QUATs, imidazoline, PEs and PW and their impact on DNA damage to mussel haemocytes and gill cells using Comet assay.
- Provide evidence based on indirect measurements of oxidative stress in the mussels' gill cells using SOD and TBARS assay.

Materials and methods

This chapter explains the process of the collection of marine mussels *M. edulis*, the conditions of exposure, the preparation of cells and the procedures used for the Comet assay, SOD, TBARS, NRR, SPE assays and mass spectrometry.

Sample collection

M. edulis were collected from Cramond Island, Edinburgh, Scotland (Figure 2-1), in a 2.5L plastic bucket. The animals, which were between 4.5-6.5 cm in length, were then immediately relocated to the aquarium at Riccarton where they were kept under controlled temperature conditions ($12^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) in plastic tanks (45cmx 23cmx 15cm), containing aerated seawater (32-35‰) and left to acclimatise for 4 days without feeding, allowing gut evacuation in preparation for exposure experiments.

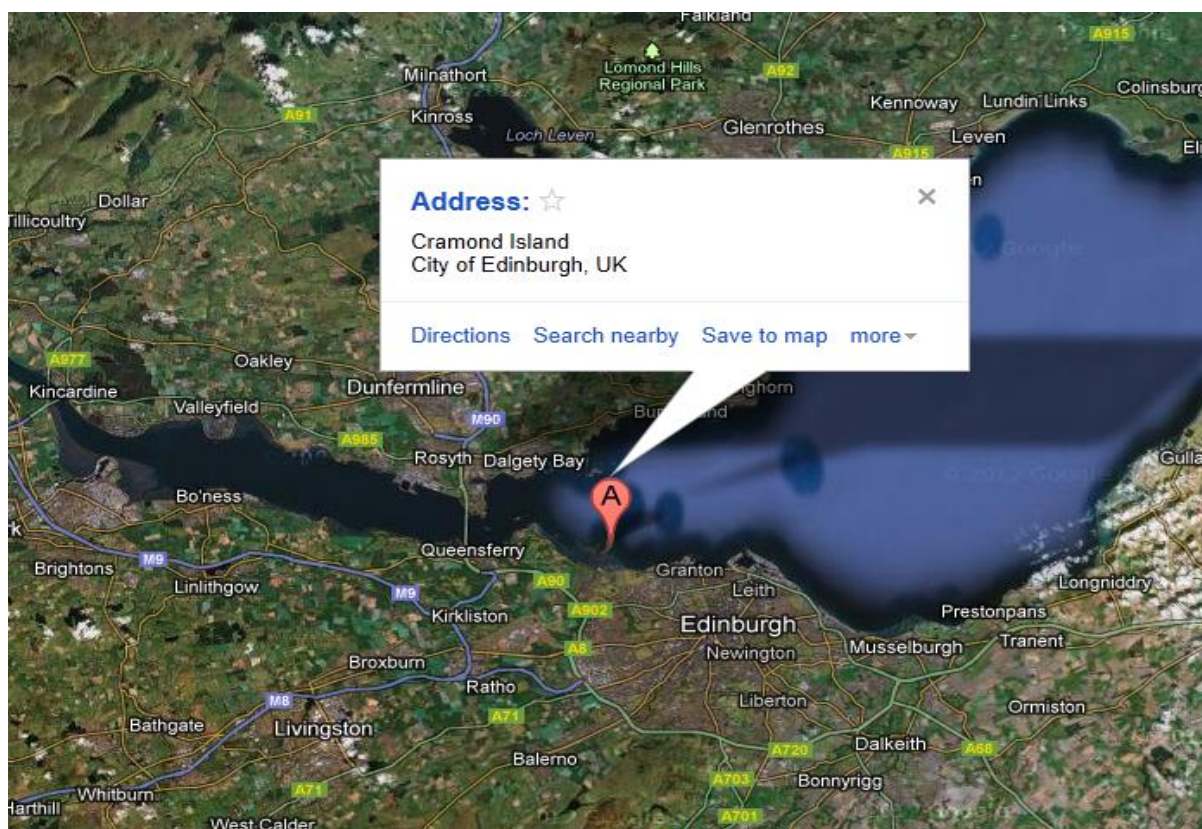


Figure 2-1 Illustration to show that marine mussels were collected from Cramond Island, Edinburgh, Scotland

Conditions of exposure

M. edulis were exposed to environmental concentrations of oilfield chemicals. Five mussels were placed in (1L) glass tanks filled with filtered and aerated seawater (Figure 2-2). The mussels were then exposed to the following chemical compounds for 24 hours:

- 1- Quaternary ammonium salts (QUATs) were prepared by diluting 10 mg of QUATs in 10 ml methanol to obtain 1000 mg/L of QUATs that were then diluted as follows: 1 ml QUATs in 999 ml of seawater, 0.5 ml of QUATs in 999.5 of seawater, 0.1 ml of QUATs in 999.9 of seawater, 10 µl of QUATs in 999.99 of seawater and 1 µl of QUATs in 999.999 of seawater to obtain final concentrations of 1 mg/L, 0.5 mg/L, 0.1 mg/L, 0.01 mg/L and 0.001 mg/L of QUATs respectively.
- 2- Imidazoline was prepared by diluting 10 mg of imidazoline in 10 ml distilled water to obtain 1000 mg/L of QUATs that were then diluted as follows: 1 ml imidazoline in 999 ml of seawater, 0.5 ml of imidazoline in 999.5 of seawater, 0.1 ml of QUATs in 999.9 of seawater, 10 µl of imidazoline in 999.99 of seawater and 1 µl of imidazoline in 999.999 of seawater to obtain final concentrations of 1 mg/L, 0.5 mg/L, 0.1 mg/L, 0.01 mg/L and 0.001 mg/L of imidazoline respectively.
- 3- PEs was prepared by diluting 10 mg of PEs in 10 ml methanol to obtain 1000 mg/L of PEs that was then diluted as follows: 1 ml PEs in 999 ml of seawater, 0.5 ml of PEs in 999.5 of seawater, 0.1 ml of PEs in 999.9 of seawater, 10 µl of PEs in 999.99 of seawater and 1 µl of PEs in 999.999 of seawater to obtain final concentrations of 1 mg/L, 0.5 mg/L, 0.1 mg/L, 0.01 mg/L and 0.001 mg/L of PEs respectively.
- 4- PW was diluted (1:10⁶: 10 µL of PW in 10 ml methanol) to obtain 1000 mg/L of PW that was then diluted as follows: 1 ml PW in 999 ml of seawater, 0.5 ml of PW in 999.5 of seawater, 0.1 ml of PW in 999.9 of seawater, 10 µl of QUATs in 999.99 of seawater and 1 µl of PW in 999.999 of seawater to obtain final concentrations of 1 mg/L, 0.5 mg/L, 0.1 mg/L, 0.01 mg/L and 0.001 mg/L respectively.

A carrier control of 1 mg/L methanol and a negative control for all chemical compounds were run in parallel. All the above chemical compounds were obtained from oil and gas company where located in the North Sea Brae Alpha platform without disclosing specific details about their chemistry and PW was received from the North Sea Brae Alpha platform, tapped in 2 m³ steel tank, and kept at 4°C until use.



Figure 2-2 Conditions of exposure of marine mussels to oilfield chemicals (corrosion inhibitors)

Preparation of cells

Two types of cells, haemolymph and gill cells were investigated to determine the potential effects of environmental concentrations of CIs, SIs and PW. These cells were prepared as described below.

Preparation of haemolymph

Hanks buffer saline solution (HBSS Ca^{2+} and Mg^{2+} free) was prepared and adjusted by adding 11.1 g/L of NaCl to correct for mussel haemolymph osmolarity of 990 mOsm/L. The mussels were removed after 24 hours of exposure, and placed on their beaks (umbo) for dissection. The valves were forced apart with scissors far enough to insert a hypodermic needle and drain any enclosed seawater. Then 0.1 ml haemolymph was collected from the posterior adductor muscle of each mussel (Figure 2-3), following the protocol established by Hartl et al. (2010), using a 1 ml syringe with a 21 gauge needle containing 0.1 ml of osmotically corrected Ca^{2+} and Mg^{2+} and free HBSS to keep the cells alive to avoid clumping (Lowe et al., 1995b). The needle was removed, and 0.2 ml of the contents, which

were a blend of 0.1 HBSS and 0.1 ml haemolymph, were gently transferred to an Eppendorf tube (1.5 ml) and kept on ice.



Figure 2-3 Collection of haemolymph from mussel *M. edulis*

Preparation of gill cells

Gill single-cell suspensions were prepared according to guidelines established by Hartl et al. (2010) and Coughlan et al. (2002). Meanwhile, in the same mussels from which the haemocytes were collected, the adductor muscle was cut and the valves opened completely. Using a scalpel blade and scissors the gills were extracted from both sides (see Figure 2-4). The gills excised from each mussel were then briefly immersed in 2.5 ml of HBSS in Petri dishes and chopped 20 times with a scissor-like motion using two fresh scalpel blades. The resulting pieces, including the HBSS, were transferred to a tube (PP-Test 15ml) containing 2.5 ml 0.1% buffered trypsin solution, to give a final enzyme concentration of 0.05%. The tubes were then laid on a gyro-rocker and gently rocked for 10 mins at room temperature, after which a further 5 ml of HBSS were added. The contents of the tubes were then passed through a 40 μ m cell strainer and the filtrate centrifuged using an MSE Mistral 1000 with a speed of 3000 rpm for 10 mins, after which the supernatant was removed and the pellet re-suspended in 100 μ L of fresh HBSS, and then transferred to an Eppendorf tube and stored on ice.



Figure 2-4 Dissection of mussels to collect gill cells

Single cell gel electrophoresis or Comet assay

To detect DNA damage in individual cells, the SCGE or Comet assay technique pioneered by Ostling and Johanson (1984) as a microelectrophoretic technique was used. There are many advantages derived from using SCGE or Comet assay, in particular its applicability to many types of cells. The SCGE is inexpensive, sensitive, rapid and simple and takes only a short time to achieve a result; it is thus a helpful technique for use in environmental monitoring (Dhawan and Anderson, 2009).

Preparation of solutions

Solutions were prepared following the protocol recorded by Hartl et al. (2007). All solutions obtained are described below.

- **Phosphate buffered saline (PBS)**

Phosphate buffered saline (PBS) was obtained in the form of tablets: 1 tablet was dissolved in 200 ml of distilled water and stored at room temperature.

- **Normal gel agarose (NGA)**

1% (w/v PBS) NGA smear (normal gelling agarose type V): 1g was dissolved in 100 ml of PBS in a conical flask through exposure to heat and stored at 4°C.

- **Low melting agarose (LMP)**

1% (w/v PBS) low melting agarose (LMP type I-B): 1g was dissolved in 100 ml of PBS in a conical flask by exposure to heat and stored at 4°C.

- **Sodium hydroxide (NaOH)**

10 N NaOH was prepared and stored at room temperature.

- **Ethylenediaminetetraacetic acid (EDTA)**

0.2 M of EDTA was prepared and the pH adjusted to 7.

- **Tris hydrochloride (Tris)**

0.4 M of tris was prepared at pH = 7.4

- **Lysing solution stock**

To prepare a lysing solution, several chemical compounds were used. 2.5M NaCl and 100 mM EDTA were dissolved in distilled water, followed by 10 mM Tris. NaOH was then added carefully slowly to adjust the pH level of the solution to 10. The solution was stored at 4°C.

- **Lysis working solution**

In a small light-proof tank (Figure 2-6), the lysis solution was prepared by adding 1.5 ml Triton X-100 to 135 ml pre-prepared lysing solution, followed by 15 ml dimethyl sulfoxide (DMSO) and then stored at 4°C.

- **Electrophoresis solution**

60 ml of 10 N NaOH followed by 10 ml 200 mM EDTA were added to 2L of chilled distilled water to obtain a final concentration of 300mMol of NaOH and 1mMol EDTA and the resulting solution was then stored at 4°C.

SCGE or comet assay procedure

To obtain reliable results, alkaline SCGE was used consistently for all methodological steps. The SGCE or comet assay was used following a protocol established by Woods (1999) and adapted for bivalves by Coughlan et al. (2002). The process involved embedding nucleated single cells into an agarose sandwich on a microscope slide, lysing the cell membranes and

subjecting the exposed DNA to electrophoresis, after which the level of DNA damage can then be assessed using image analysis software.

To offer increased gel bonding and, consequently, stability, microscope slides were “frosted” by applying 1% (w/v PBS) NGA smear (normal gelling agarose type V) and the slide was left to air dry. To the frosted microscope slides, 100 μ L NGA was applied and covered with a cover slip (22mm x 22mm), and the slide then left to set in the fridge for 15 min to produce the first layer of the minigel sandwich. The cover slip was then removed and the second layer, containing 30 μ l of the cells mixed with 70 μ l of 1% (w/v PBS) low melting agarose (LMP) was quickly added onto the first layer. The coverslip was then replaced and the slide left to set at 4°C for at least 20 min. After this, the cover slip was removed again and the third layer was produced by applying 100 μ L of LMP, as described for the first layer. The cover slip was then replaced and the slide left to set at 4°C for at least 30 min. This provides a protective layer, by increasing the distance between the cells and the gel surface as well as filling in any remaining gaps in the second layer.

Once the agarose had solidified, the slides were immersed in 135 ml of lysis solution (see above) and stored in a light-proof tank at 4°C for at least one hour to remove residual salts and detergents (Figure 2-5).



Figure 2-5 Immersing the slides in a black box

The slides were then placed in a horizontal electrophoresis tank containing alkaline buffer and left for 30 mins for the DNA to unwind. Without changing the electrolysis solution, a 25 V, 300 mA current was applied for 25 min, after which the gels were neutralised three times with a tris buffer (0.4 M Tris-HCl, pH 7.4) at 5 min intervals and stained with 25 μ l of the fluorescent dye, ethidium bromide solution (20 μ g/ml in distilled water), for 10 mins (see Figure 2-6), after which the slides were carefully rinsed with cold distilled water (Singh et al., 1988).

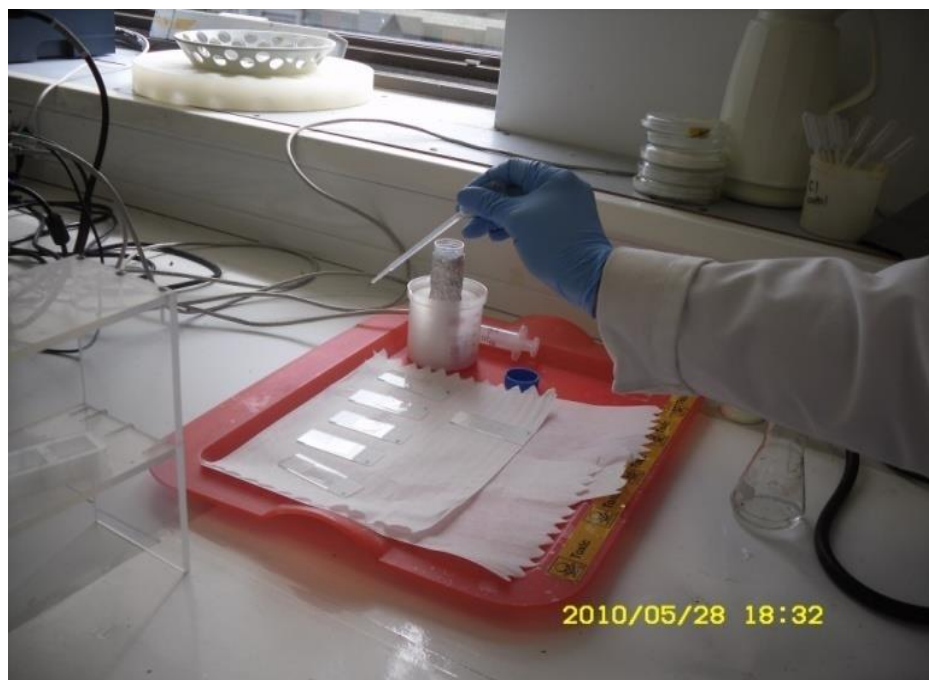


Figure 2-6 Adding the fluorescent dye to stain the slides

The cover slips were then replaced and the slides kept overnight at 4°C in a humidified chamber. After this the slides were viewed under an epifluorescence microscope and 50 cells per slide were then scored using the image analysis software package Comet Assay IV (Perceptive Instruments) and the DNA damage was expressed as % DNA in the tail.

Superoxide dismutase assay

To detect oxidative stress, the activity of SOD enzyme in the mussels can be assessed using tetrazolium salts that produce a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 is linearly related to xanthine oxidase (XO) activity, and is inhibited by SOD. Thus, the IC_{50} (50% inhibition activity of SOD) can be indirectly determined by applying a colorimetric method.

Preparing the solutions

The SOD assay kit was purchased from Sigma-Aldrich (Reference: 19160-1KT-F). This included 5 ml of working solution (WST), 100 μ L of enzyme working solution, 50 ml of dilution buffer and 100 ml of buffer solution (Figure 2-7). All the solutions used during the experiment are detailed below.



Figure 2-7 Contents of the SOD assay kit purchased from Sigma-Aldrich (Reference: 19160-1KT-F)

- **Working solution (WST) (SOD assay kit, Re 53456, 5 ml)**

1 ml of WST was added to 19 ml of buffer solution.

- **Enzyme working solution**

The enzyme solution was centrifuged for 5 seconds, mixed by pipetting, and then diluted with 15 μ L of enzyme solution in 2.5 ml of dilution buffer.

- **Dilution buffer (SOD assay kit, Re 07437, 50 ml)**
- **Buffer solution (SOD assay kit, Re 43525, 100 ml)**
- **Double distilled water (DDH₂O)**

SOD assay procedure

Preparation of the sample was performed according to Almeida et al. (2005). After the mussels were exposed to 0.001, 0.01, 0.1, 0.5 and 1 mg/L of QUATs, imidazoline, PEs and PW for 24 hours, the gills were extracted and shock frozen in liquid nitrogen, then kept at -80°C for a day.

The samples were collected from the freezer, immersed in 750 μL of buffer (tissue buffer = 1:5) (Tris- HCl 50 mM, 0.15M KCl. pH 7.4) and carefully homogenised on ice with a 10 ml glass homogenizing tube and Teflon pestle. The entire homogenate was transferred to a 1 ml Eppendorf tube and centrifuged in a Helena Biosciences Eppendorf Minispin at 10,000 x g for 20 minutes at 4°C. The supernatant was then divided into aliquots for SOD, TBARS and protein analysis. The aliquots for the TBARS and protein were stored at -80°C to await further processing. The SOD aliquots were transferred to a 5 ml Beckmann ultracentrifuge tube and centrifuged again at 40,000 x g for 60 minutes at 4°C. 20 μL of the supernatant containing the cytosolic fraction of each sample was added to a 96-well plate and reagents added, as described in Table 2-1.

Table 2-1 The amount of sample solution, double distilled water, working solution, enzyme working solution and dilution buffer for Sample, Blank 1, Blank 2 and Blank 3.

Solutions	Sample	Blank 1	Blank 2	Blank 3
Sample solution	20 μL	-	20 μL	
ddH ₂ O	-	20	-	20 μL
WST	200 μL	200 μL	200 μL	200 μL
Enzyme working solution	20 μL	20 μL	-	-
Dilution buffer	-	-	20 μL	20 μL

The 96-well microplate was then incubated at 37°C for 20 minutes and read at 450 nm with a spectrophotometer (spectra Max M5). Subsequently, the SOD activity (expressed as % inhibition) was calculated using the following equation:

$$\text{SOD activity (\% inhibition)} = \frac{[(\text{Abs blank1} - \text{Abs blank2}) - (\text{Abs Sample1} - \text{Abs Sample 2})]}{(\text{Abs blank1} - \text{Abs blank2})} \cdot 100 \quad \text{Eq 2-1}$$

Thiobarbituric acid reactive substances (TBARS)

The thiobarbituric acid (TBA) assay was selected due to its straightforwardness and simplicity when measuring thiobarbituric acid reaction substances (TBARS). TBA reacts with two molecules of malonaldehyde (MDA) (which is a by-product of lipid oxidation), developing a pink colour with an absorption maximum at 530nm.

Preparation of Solutions

1. PBS solution

100 μ L of EDTA (pH 7.4) diluted in 19.9 ml PBS.

2. BHT solution (1 mol/l BHT)

0.22 g/ml of butylated hydroxytoluene (2, 6-Di-O-tert-4-methyl phenol) (BHT) dissolved in ethanol.

3. TCA solution (50% (w/v) TCA in dH₂O)

0.5 g/ml of trichloroacetic acid (TCA) dissolved in distilled water.

4. Thiobarbituric acid (TBA) solution (1.3% (w/v) in 0.3% (w/v) NaOH)

13.5 g/ml of TBA dissolved in NaOH.

TBARS assay

The frozen samples of gill cells were thawed and 40 μ L added in triplicate to each well of a 96-well microtiter plate. Then reagents were added in the following order: 10 μ L of BHT, to inhibit the oxidation step, 140 μ L of PBS, 50 μ L of TCA and 75 μ L of TBA. Following a 60 minute incubation period at 60°C, the plate was read using a spectrophotometer (spectra Max M5), first at 530 nm and then at 630 nm, for normalisation of the samples for any turbidity, before reading against 1, 10, 3, 30-tetraethoxypropane (0.5–25 nmol/L). All data points from the assays were then normalised per mg of total protein in the homogenates.

The total protein concentration of the sample was determined on a separate microtiter plate, according to the method detailed by Bradford (1976). Briefly, 10 μ L of sample was pipetted into 5 replicate wells and 290 μ L of Bradford reagent added; then the plate was incubated in the dark for 5 minutes and read using a spectrophotometer (spectra Max M5) at 595 nm (Bradford, 1976). Subsequently, the TBARS concentration was calculated using the following equations:

$$Abs = A_{530\text{ nm}} - A_{630\text{ nm}} \quad \text{Eq 2-2}$$

$$TBARS = \frac{Abs}{Protein} \text{ nmol mg protein}^{-1} \quad \text{Eq 2-3}$$

Neutral red retention assay (NRR)

The NRR assay was used to determine the cytotoxicity in the haemocyte cells by assessing the integrity of lysosomal membranes. Peek and Gabbott (1989) described a NRR assay which involved extracting the haemolymph into an equal volume of physiological saline solution (PSS) and then measuring the rate of dye leakage in lysosomes by adding neutral red.

In the present study, the lysosomal stability was determined by applying the NRR assay according to the protocol specified by Coughlan et al. (2009).

Preparation of solutions

- 1- Poly-L-lysine solution (PLL 1:10 in distilled water) was prepared. 10 μ L of PLL solution were pipetted onto and smeared across each slide before being left to dry overnight. The smearing helps the cells attach to the slide during the assay.
- 2- The neutral red dye
0.02 g of Neutral Red was dissolved in 1 ml dimethylsulphoxide (DMSO) in a smoked amber vial.
- 3- The contents of the physiological saline solution are specified in Table 2-2.

Table 2-2 Contents of the physiological saline solution

MgSO ₄	0.072M	= 8.67g (/2=4.33)
NaCl	0.4M	= 23038g (/2=11.69)
CaCl ₂	0.01M	= 1.11g (/2=0.555)
KCl	0.01M	= 0.75g (/2=0.375)
HEPES	0.02	= 4.77g (/2=2.385)
Salinity = 38.5‰		

The chemicals in Table 2-3 were added to 700 ml of distilled water and mixed using a magnetic stirrer. The pH was adjusted to 7.4 pH, using NaOH and the solution made up to 1 L in a glass flask with distilled water.

Neutral Red retention (NRR) procedure

Mussels were collected from each tank following 24 hours of exposure and haemolymph extracted from the posterior adductor muscle into an equal volume of PS solution, using a 1 ml syringe with a 21 gauge needle. The needle was removed and the haemolymph transferred gently into a 1.7 ml Eppendorf tube and kept on ice. 40 μ L of each single cell suspension were tipped onto separate Poly-L-Lysine (PLL) (0.01% solution, Re P4707, 50 ml)-treated slides and incubated in a light-proof humidity chamber for 15 minutes to allow the cells to adhere.

In the meantime, a NR working solution was prepared as follows: in a smoked amber vial, 10 μ L of NR stock solution was added to 5ml of PS solution and mixed. Following the 15 minute incubation period, the slides were removed from the chamber and the excess fluid drained.

40 μ L of the working NR solution were added to each slide and a cover slip applied. The slides were once again kept in the light-proof humidity chamber for 15 minutes. After this time had elapsed, the slides were examined systematically every 15 minutes for the first hour, and then every 30 minutes thereafter, until over 50% of the cells had shown leakage of NR into lysosomes and the vacuole formation had been recorded using a light microscope. The cells were observed to identify both structural abnormalities and NR probe retention time. The conditions were verified at each time interval. The NR retention time was investigated by examining the lysosomes and estimating the proportion of cells showing leakage of the lysosomes into the cytosol and/or displaying abnormalities in cell size, shape, colour and also lysosome size.

Chemical analysis

The second part of this study was focused on chemical analysis. In this section, the bioaccumulation and bio-concentration of corrosion inhibitors used in the offshore oil industry in the soft tissues of the mussels was investigated using mass spectrometry (MS) and atomic absorption spectrometry (AA) through a solid phase extraction method (SPE).

Preparation of chemical samples

Whole soft tissues were collected from the mussels, homogenised using a homogeniser (Ystral GmbH, Re D-7801, Dottingen, Type X 1020, 220V, 05HZ, Nr 3132, 1.25 A, 260 W, 25000 upm) and incubated for 48 hours at 50°C to dry (Figure 2-8). Tables 2-3, 2-4, 2-5 and 2-6 show the dry weight of the whole soft tissues of mussels. The tissues were then refluxed for 4 hours with 100ml methanol in a round bottomed flask (Figure 2-9) using an electromantle (ME) then filtered using GF/C filter paper in a 250ml flask (Figure 2-10).

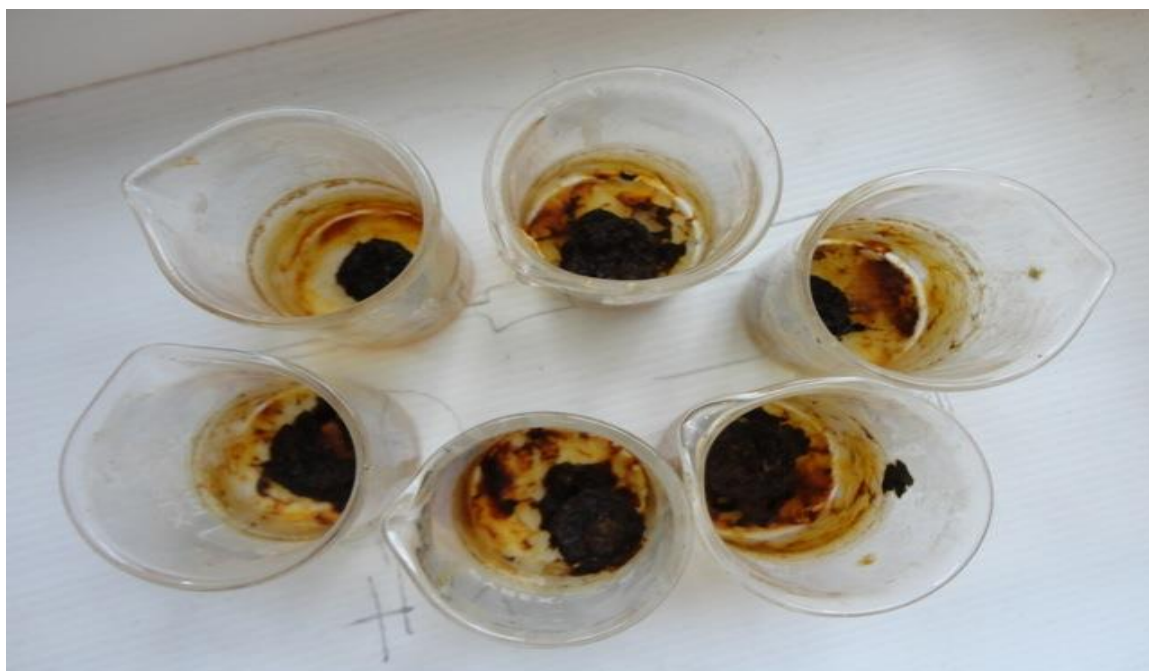


Figure 2-8 Dried complete soft tissues from mussels

Table 2-3 Amount of whole dry soft tissues of mussels exposed to QUATs

Exposure concentrations mg/L	Control	0.001	0.01	0.1	0.5	1
Number of mussels	5	5	5	5	5	5
Beaker including dry tissues (g)	34.8	34.8	34.9	35.9	34.5	34.5
Empty beaker (g)	32.5	32.4	32.8	33.6	32.4	32.4
Dry tissues (g)	2.3	2.4	2.1	2.3	2.1	2.1

Table 2-4 Amount of whole dry soft tissues of mussels exposed to imidazoline

Exposure concentrations mg/L	Control	0.001	0.01	0.1	0.5	1
Number of mussels	5	5	5	5	5	5
Beaker including dry tissues (g)	34.9	37.1	35.5	34.9	34.7	35.5
Empty beaker (g)	32.5	34.7	33.0	32.8	32.7	33.4
Dry tissues (g)	2.4	2.4	2.5	2.1	2	2.1

Table 2-5 Amount of whole dry soft tissues of mussels exposed to PEs

Exposure concentrations mg/L	Control	0.001	0.01	0.1	0.5	1
Number of mussels	5	5	5	5	5	5
Beaker including dry tissues (g)	34.9	35.9	35.5	34.8	34.5	34.5
Empty beaker (g)	32.8	33.7	33.3	32.6	32.0	32.4
Dry tissues (g)	2.1	2.2	2.2	2.2	2.0	2.1

Table 2-6 Amount of whole dry soft tissues of mussels exposed to PW

Exposure concentrations mg/L	Control	0.001	0.01	0.1	0.5	1
Number of mussels	5	5	5	5	5	5
Beaker including dry tissues (g)	34.9	34.5	35.5	34.8	34.5	34.5
Empty beaker (g)	32.9	32.3	33.2	32.5	32.4	32.2
Dry tissues (g)	2.0	2.2	2.3	2.3	2.1	2.3



Figure 2-9 Round bottomed flasks after refluxing the tissues for 4 hours and preparing for filtration.



Figure 2-10 Filtering tissues using GF/C Filter paper

Solid phase extraction (SPE) is a method used to remove salts and any other chemicals that are not of interest from the samples (Figure 2-11). In this method, the Strata X 33u Universal Sorbent 30 mg/ml tube and a tubular glass vial placed under the 30 mg/ml tube were used. 1 ml methanol was then added, to activate the column, followed by 1 ml distilled water to prepare and equilibrate the column. 1 ml of sample was loaded into the column, followed by 1 ml of distilled water to wash the salt out; after this the tubular glass vial was changed. Once the new tubular glass vial was under the column, 1 ml of acidified methanol was added to elute the QUATs or imidazoline or PEs (Figure 2-12) and the samples were then analysed using ESI MS/MS.

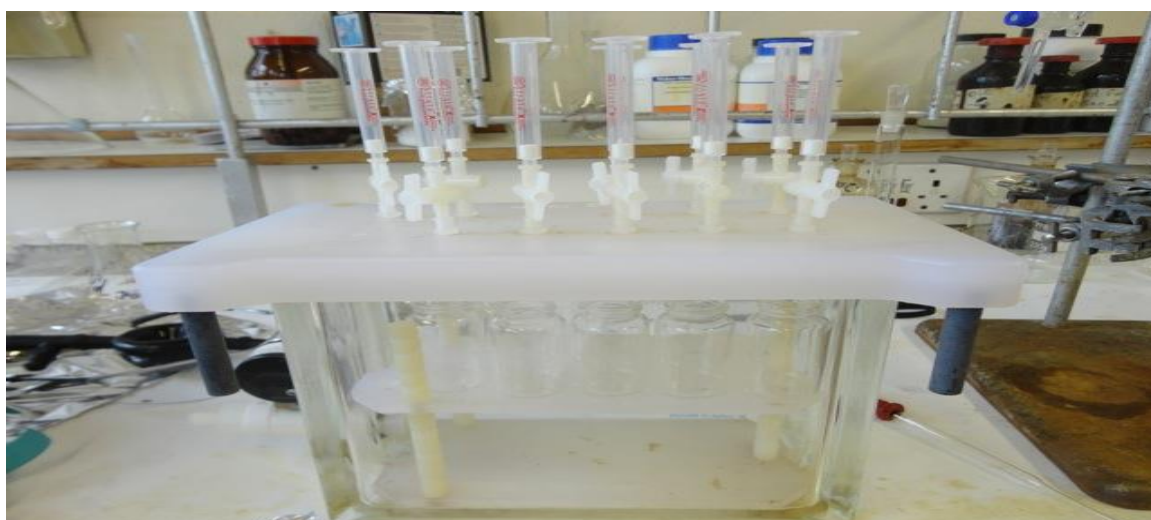


Figure 2-11 Preparing the tubes using the solid phase extraction method (SPE)

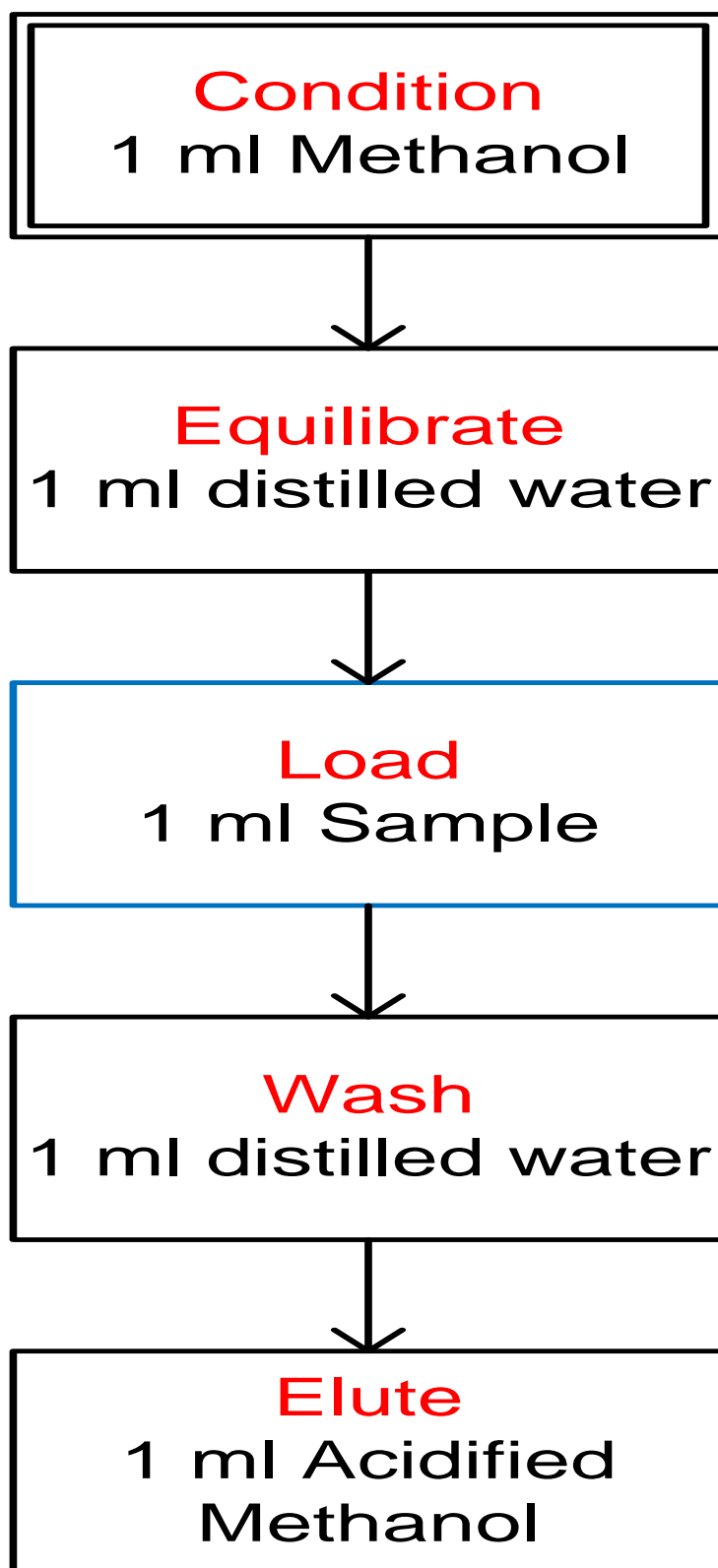


Figure 2-12 Instructions for SPE

PW sample preparation

To fractionate the PW samples, two techniques were used to analyse PW containing QUATs, imidazoline and PEs: these were SPE and dichloromethane (DCM) extraction.

SPE was used to remove non-polar compounds and other chemical compounds that were not of interest from the PW sample (Figure 2-13). In the SPE stage, Strata C18-E (55 μ m, 70 Å) 1000 was prepared in a 30 mg/ml tube from Phenomenex and a tubular glass vial was placed under the 30 mg/ml tube. 6 ml of methanol was added to activate the column, after which 6 ml of distilled water was added in order to prepare and equilibrate the column. 6 ml was taken from the PW sample and added to load the column, followed by 6 ml of distilled water as a wash, after which the tubular glass vial was changed. Once the new tubular glass vial was positioned under the column, 6 ml acidified methanol was added to elute the QUATs, imidazoline and PEs (Figure 2-14). DCM extraction was used to obtain the polar organic fraction: in 100 ml of PW 10ml x2 of DCM was added and shocked strongly. The extract was blown until dry under N₂, then the residue was re-dissolved in MeOH: H₂O (1:1, v/v). The solvent was then ready to run. All these techniques were followed by tandem ESI MS/MS.



Figure 2-13 Fractionating PW sample using Strata C18-E to remove non-polar compounds

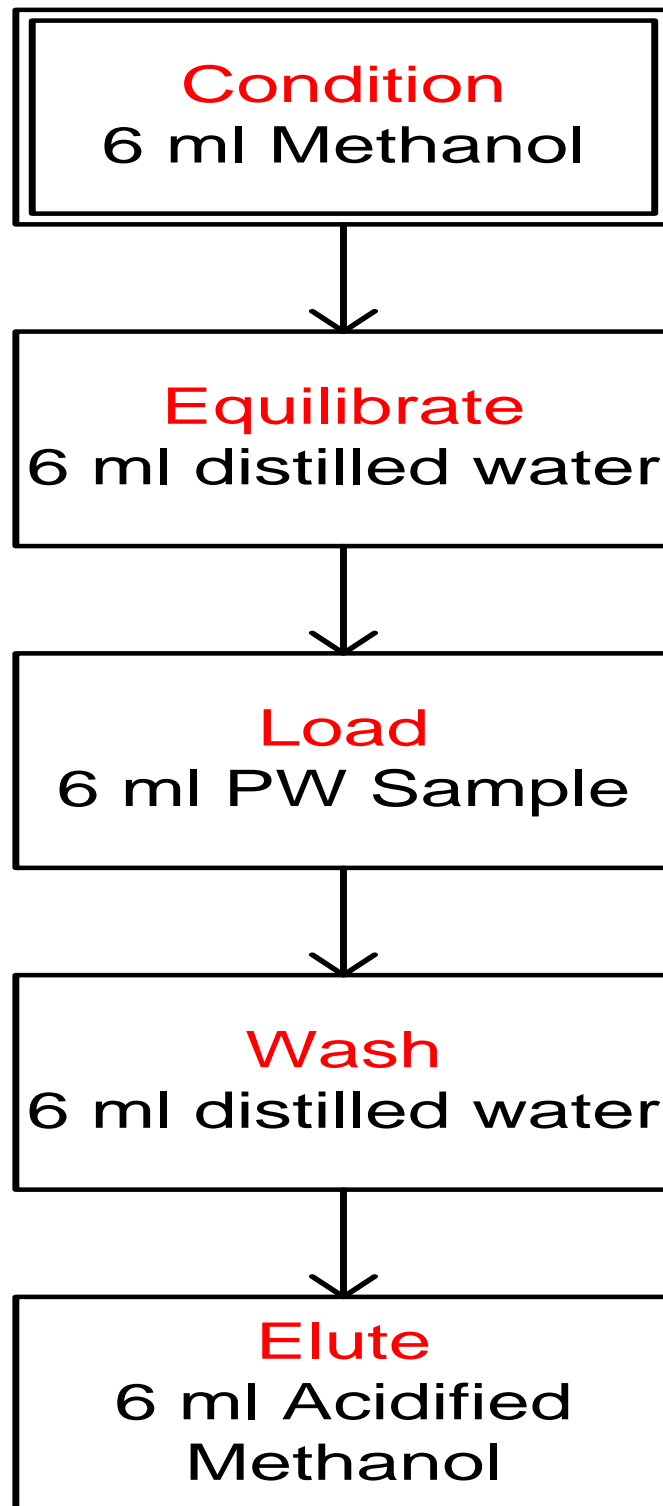


Figure 2-14 PW fractionation instructions

Mass spectrometry analysis

Mass spectrometry (MS) is an analytical technique used to determine the molecular mass of a sample. 10 μ L of sample was injected into the MS (micromass Quatro LC) and Electrospray Ionization (ESI) was used to analyse the sample in the positive ion mode (Tables 2-7 and 2-8). A Waters 600 S controller and Waters 616 pump were used in the mobile phase 90:10 for all samples.

Table 2-7 Mass spectrometer settings for measuring the molecular weight of chemical compounds

Chemical Compounds	Molecular weight	Analyser	Mass	Span (AMU)	Dwell time (Sec)	Cone voltage (V)
QUATs C ₁₆	360.4	SIR	360.4	0.06	0.02	28
Imidazoline	612	SIR	70.08	0.06	0.02	28
Phosphate esters	116	SIR	116	0.06	0.02	28

SIR= Selected Ion Recording, AMU= Atomic Mass Units

Table 2-8 Mass spectrometer settings for obtaining parent and daughter mass of QUATs

Chemical Compound	Molecular weight (MW)	Analysis type	Mass	Energy (eV)	Cone voltage (V)
C ₁₆ QUATs	360.4	MRM	360.4→91	30	28

MRM= Mass Reactive Monitoring.

In this study 2 amu to 402 amu was scanned in 3 seconds, with an interscan delay of 0.1 sec.

Spectrophotometer analysis

After SPE, a spectrophotometer (spectra Max M5) was also used to investigate the amount of imidazoline and PEs in the mussels' entire soft tissues and in the PW sample. 300 μ L of each tissue sample and PW sample were added in triplicate to each well of a 96-well microtiter plate, measured at wavelengths 200-229 nm and 320-380 nm for imidazoline and PEs, respectively. Ultraviolet spectroscopy was also used to estimate the concentration of imidazoline and PEs in tissues and in the PW samples. 1 ml of sample was added into a glass cuvette, and then wavelengths of 229 nm and 345 nm read for imidazoline and PEs respectively.

Atomic absorption spectrometry (AA) standards preparation

Cadmium standard solution (1000ppm), Reference: 20/21/22-36/38, Zinc Standard Solution (1000ppm), Reference: 36/38, Copper standard solution (1000ppm), Reference: J/8025/15, Mercury standard solution (1000ppm), Reference: 36/37 and Barium standard solution (1000ppm), Reference:141342D from Spectrosol, were prepared as standards from concentrations of 0.625, 1.25, 2.5, 5 and 10 mg/L, then run through an atomic absorption spectrometer (AA) (AAnalyst 200; Reference (2005526), from Perkin Elmer).

Preparation of samples for atomic absorption (AA) spectrometry

After 24 hrs of exposure to PW at different concentrations (see Exposure Conditions, above), the mussels were collected from the tank. Complete soft tissues were collected from the mussels, homogenised using a homogeniser (Figure 2-15) then dried for 48 hrs at 50°C (Figure 2-16). The beaker was then weighed, both with and without the dry tissues to obtain the final weight of dry tissues (Table 2-9). To extract the heavy metals, 5 ml of nitric acid (S.G.1.42 (70%); Batch (0755171) from Fisher Scientific) was added to each sample and kept overnight at room temperature. After this, 45 ml of distilled water was added to each sample to make it up to 50 ml (Figure 2-17); the samples were then left in an aquatic water bath (SUB36, Reference; 2008254, from Grant) for 4 hrs at 60°C to extract the heavy metals from the tissues. Then the samples were filtered into a 250ml flask using GF/C filter paper (Figure 2-18) and analysed using AA.

Table 2-9 Weight of empty beaker and with dry whole soft tissues

Exposure concentrations mg/L	Control	0.001	0.01	0.1	0.5	1
Number of mussels	5	5	5	5	5	5
Beaker including dry tissues (g)	34.9	37.1	35.5	34.9	34.7	35.5
Empty beaker (g)	32.7	34.6	32.8	32.6	32.4	32.5
Dry tissues (g)	2.2	2.5	2.7	2.3	2.3	3



Figure 2-15 Homogenising the tissues, using a homogeniser (Ystral GmbH, Re D-7801, Dottingen, Type X 1020, 220V, 05HZ, Nr 3132, 1.25 A, 260 W, 25000 upm)



Figure 2-16 Drying the tissues in an oven at 50°C



Figure 2-17 Extracting heavy metals from the tissues by adding nitric acid



Figure 2-18 Filtering tissues into a 250 ml flask using GF/C filter paper

Statistical analysis

In the eventual analysis of the data and to make the study more reliable an analysis using the variance ANOVA test was applied (Dytham, 2011). A common problem that occurs with statistical tests is that when the level of significance $P = 0.05$ (5%) is consistently accepted, an erroneous conclusion can be drawn on average with every twentieth test completed. There is a good chance that at least 1 false conclusion will be drawn when the means of our hypothetical 7 samples in 21 z-tests, are compared. Thus, the risk of committing a Type 1 error, that is, rejecting null hypothesis (H_0) when it should be accepted, is reduced by setting the acceptable significance level to a more stringent (1%) $P = 0.01$. However, this will increase the risk of creating a Type 2 error, which is failing to reject H_0 when it should be rejected. It is to overcome these difficulties the ANOVA technique should be applied.

An ANOVA test allows for comparisons to be made between any numbers of sample means, all within a single test. This test is applied in this way to compare the means of many samples while only one factor is referred to as a one-way ANOVA (Fowler et al., 1998). One-way ANOVA is an extension of the independent samples t-test and is commonly applied when comparing more than two groups or treatments (Dytham, 2011).

In the present study, to avoid the risk of creating statistical errors and to assist in drawing the right conclusions, the SimaStat programme was used for comparisons between more than two groups. This programme allows the selection of an Assumption to check the tab from the options dialog box to view equal Variance and options the normality. The normality and the equal variance assumption tests check for a normally distributed population and variability about the group means respectively. Therefore, a one-way ANOVA followed by Tukey multiple comparison tests were carried out to determine the statistical differences between the treatment groups and the control when checking the normality and the equal variance assumption tests. Statistical significance was accepted at $P < 0.05$.

Oilfield corrosion inhibitor (Quaternary ammonium salts (QUATs)): biomarkers of exposure in marine mussels *Mytilus edulis*

Results

The results of this study showed significant increases in DNA damage, lysosomal stability and oxidative stress, in the haemocytes and gill cells taken from adult marine mussels (*M. edulis*) exposed to QUATs at concentrations ranging from 0.001 to 1 mg/L (Figures 3-1 to 3-5).

A significantly increased level of DNA damage ($P>0.05$) in haemocytes, compared to control and MeOH groups, were observed at all concentrations of QUATs, even those as low as 0.001 mg/L (Figures 3-1). However, there was no significant difference between 0.01 and 0.1mg/L of QUATs. However, in gill cells, there was a significant increase in DNA damage from the lowest concentration of 0.001 mg/L and the damage in the comet tail varied significantly with 0.001, 0.01 and 1 mg/L from that of the control group at $P<0.05$ (Figure 3-2). However, gill cells contain large numbers of haemocytes.

Oxidative stress was also assessed. The inhibition of SOD activity was significantly increased at concentrations ≥ 0.5 mg/L. However, at concentrations ≤ 0.1 mg/L, the level of inhibition was not significantly higher between each group and the control group ($P>0.05$; Figure 3-3). Despite this, the levels of lipid peroxidation in gill cells was significantly elevated compared to the control group at concentrations ≥ 0.1 mg/L QUATs ($P<0.05$; Figure. 3-4). In addition, there was a significant increase at concentrations ≥ 0.1 mg/L compared to 0.001 mg/L and between 0.1 and 1 mg/L; no significant difference was observed between 0.1 and 0.5 or between 0.5 and 1mg/L.

Lysosomal membrane integrity was assessed and this showed a significant increase ($P<0.05$) in lysosomal membrane damage occurred in haemocytes at all concentrations, compared to the control group, at concentrations ≥ 0.1 mg/L; however there were no significant differences between the treatment groups ($P>0.05$). There was also no significant increase between 0.01 and 0.001 mg/L and the control group ($P>0.05$; Figure 3-5).

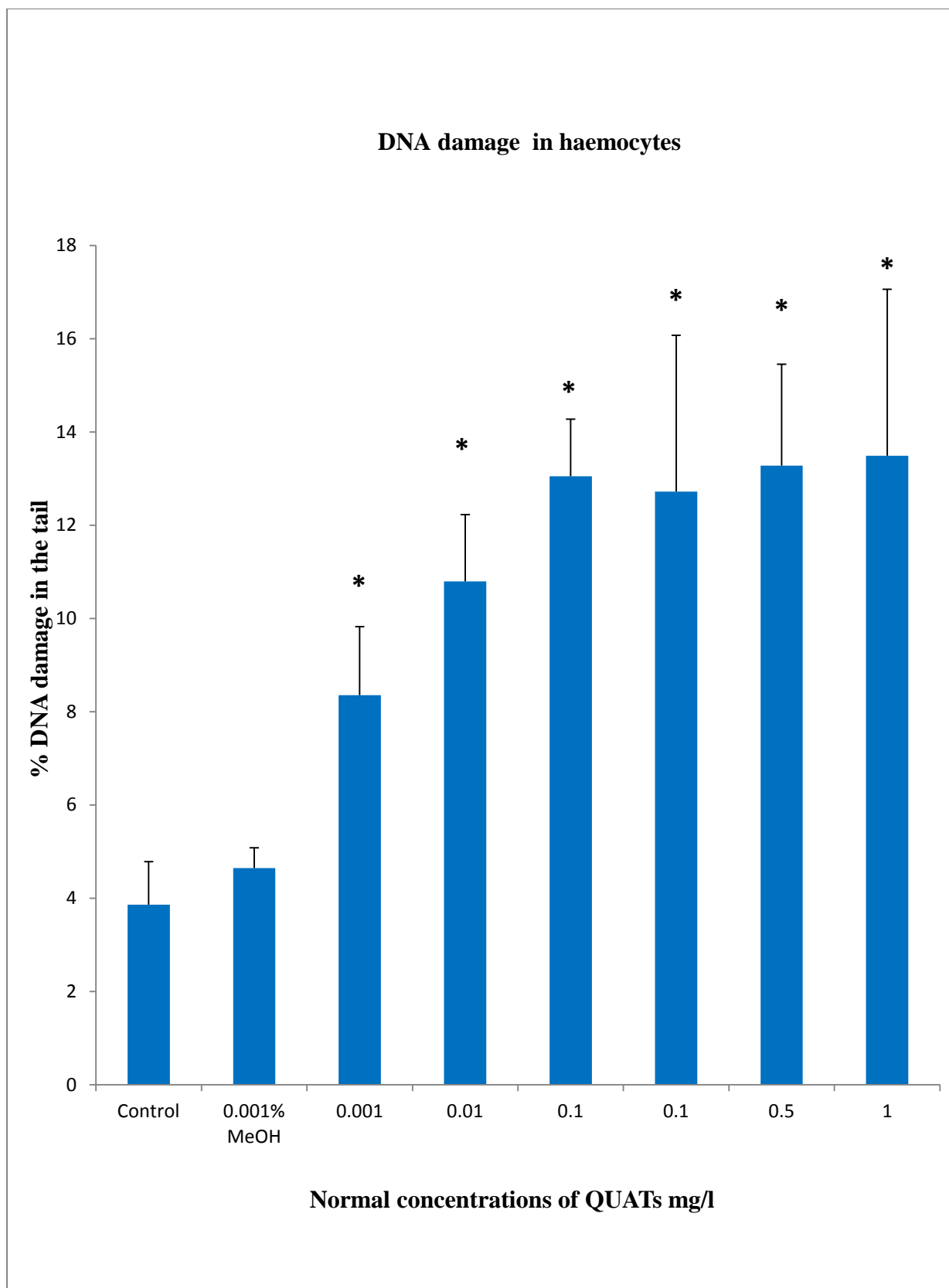


Figure 3-1 Average (\pm SD; n=5) percent DNA in the comet tail in the haemocytes of mussels exposed to QUATs. (*): There is a significant difference compared to the control group and methanol group at (one-way ANOVA; $P < 0.05$).

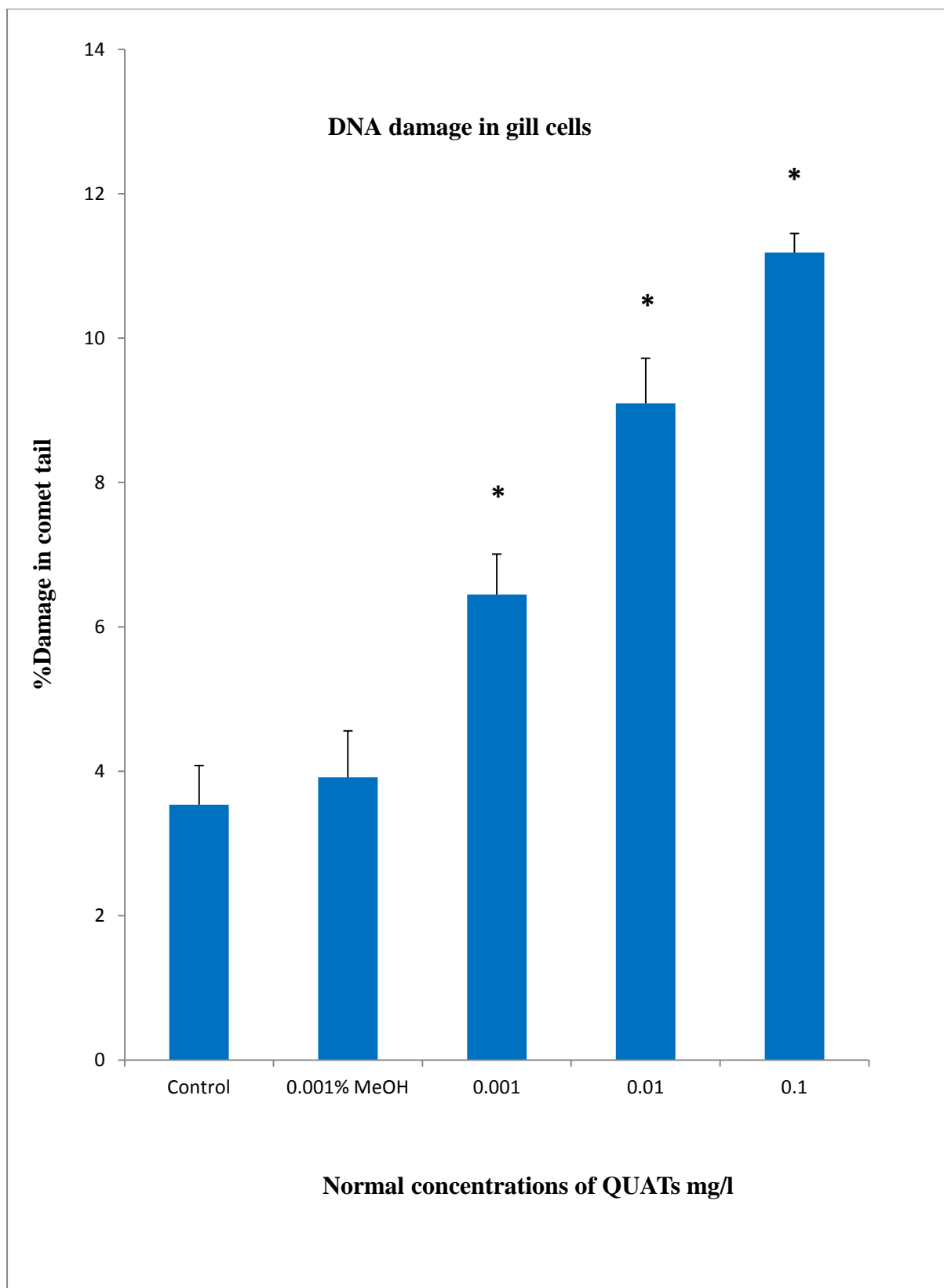


Figure 3-2 Average (\pm SD n=5) percent DNA in the tail in gill cells of mussels exposed to QUATs. (*): There is a significant difference compared to the control group.

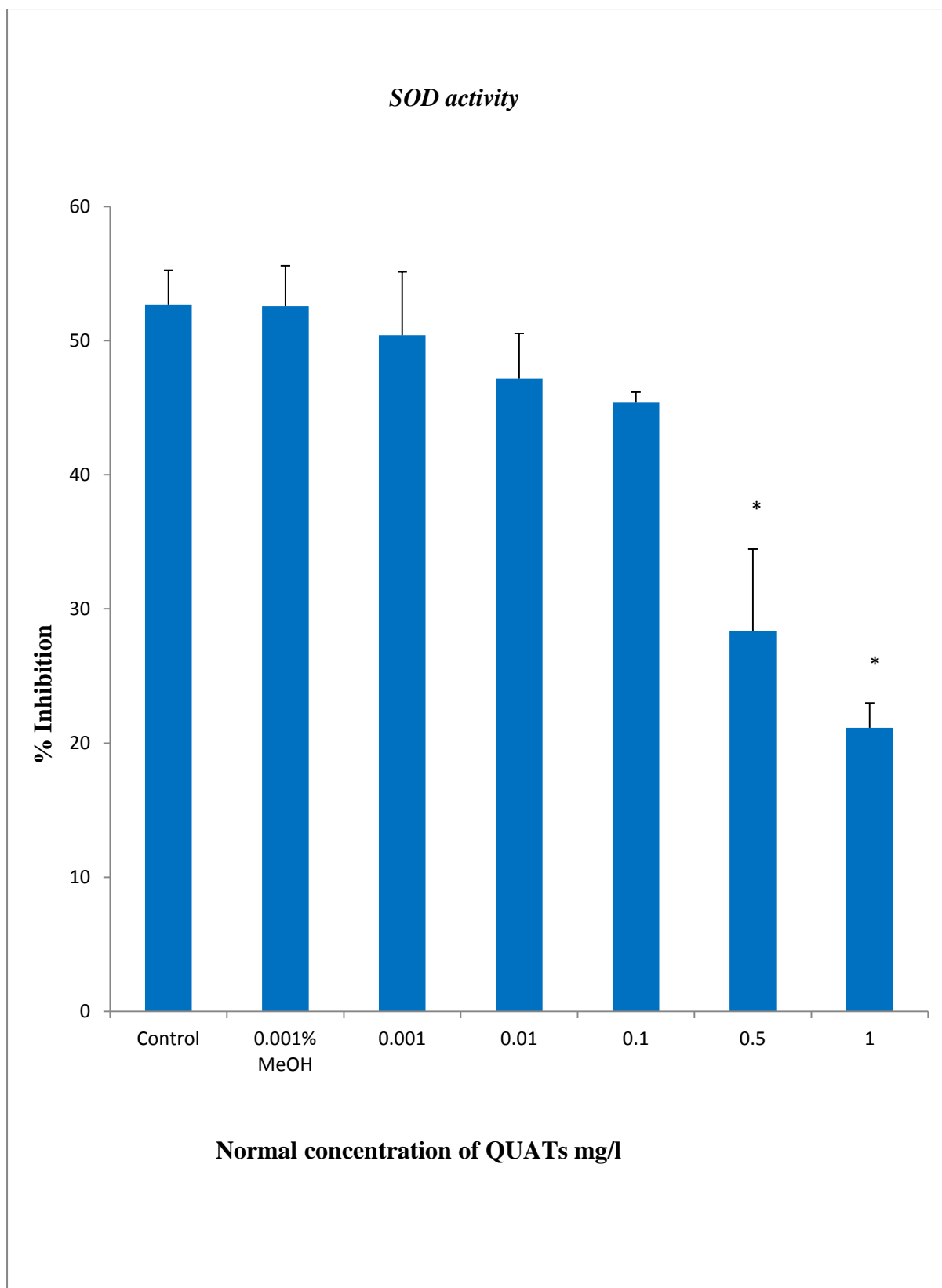


Figure 3-3 Average (\pm SD; n=5) SOD inhibition in gill tissue homogenates of mussels exposed to QUATs. (*) There is a significant reduction in activity between the control group and 0.001 mg/L, but no significant difference between these groups at (one-way ANOVA; $P < 0.05$).

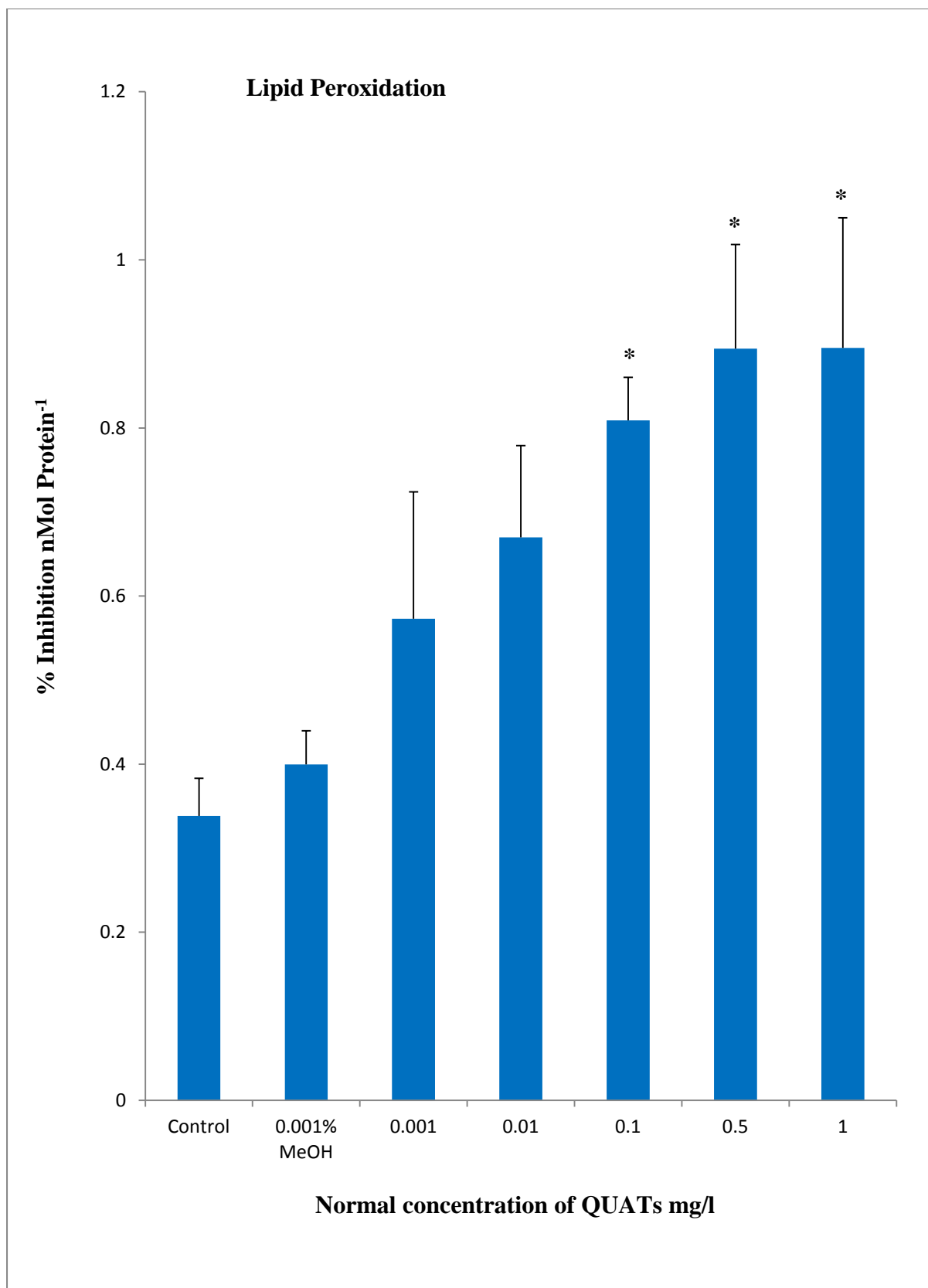


Figure 3-4 Average (\pm SD; n=5) % TBARS lipid peroxidation in gill mussels exposed to QUATs. (*): a significant increase between these groups compared to methanol and the control groups.

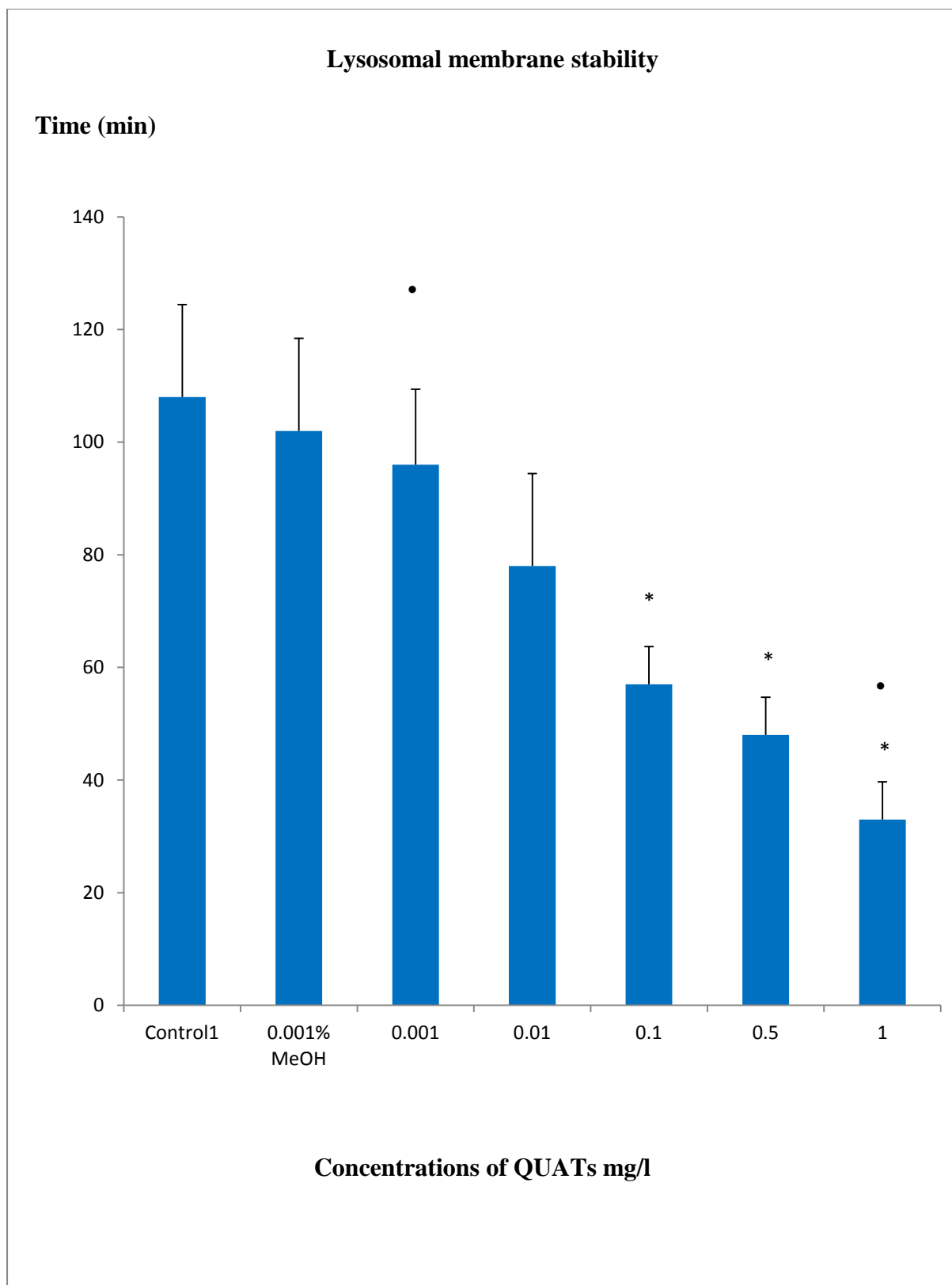


Figure 3-5 Average (\pm SD; n=5) lysosome membrane stability in haemocytes of mussels exposed to QUATs. (*): significant difference compared to control group but not between each other. (•): significant difference between these groups at ($P < 0.05$) and no significant difference between 0.01, 0.1 and 0.5 mg/L, (one-way ANOVA; $P > 0.05$).

Bioaccumulation and bio-concentrations of QUATs were measured in all the soft tissues using SPE, followed by ESI MS/MS quantitative analysis to investigate the amount of C₁₆ QUATs in all the soft tissues of the mussels. To check the extent of a possible linear relationship between the peak and the level of concentration, tests were applied on standard solutions containing known concentrations of QUATs, prior to analysis of the QUATs treatment groups. The spectrum of the QUATs was confirmed after SPE (Figure 3-6). The spectra for the daughters of QUATs was also recorded (Figure 3-7). Calibration curves obtained with methanol and acidified methanol ranging from 0.015, 0.03125, 0.0625, 0.125, 0.25, 0.5 and 1 mg/L are shown in Figures 3-8 & 3-9. All the samples for the control and treatment groups were prepared and 10 µL of sample was directly injected into the MS system. The amount of QUATs present in all the soft tissues and the seawater samples with SPE at a concentration ≥ 0.1 mg/L was measured. However, the signals in the tissue samples were clearer and better than in the seawater samples (Figure 3-10). The amounts were calculated by direct comparison with the amounts in the standard solutions, using equation 3-1. QUATs were found in tissues, when they were exposed to normal concentrations 0.1, 0.5 and 1 mg/L, and the concentrations were presented at 0.075, 0.127 and 0.4 mg/kg respectively.

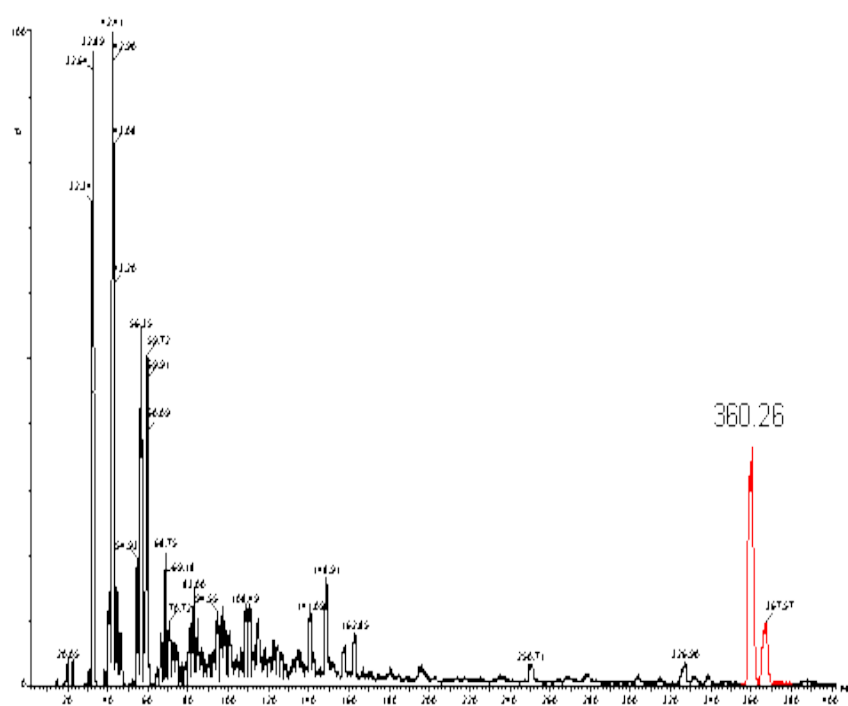


Figure 3-6 Spectrum of C₁₆ QUATs after SPE, showing a strong signal

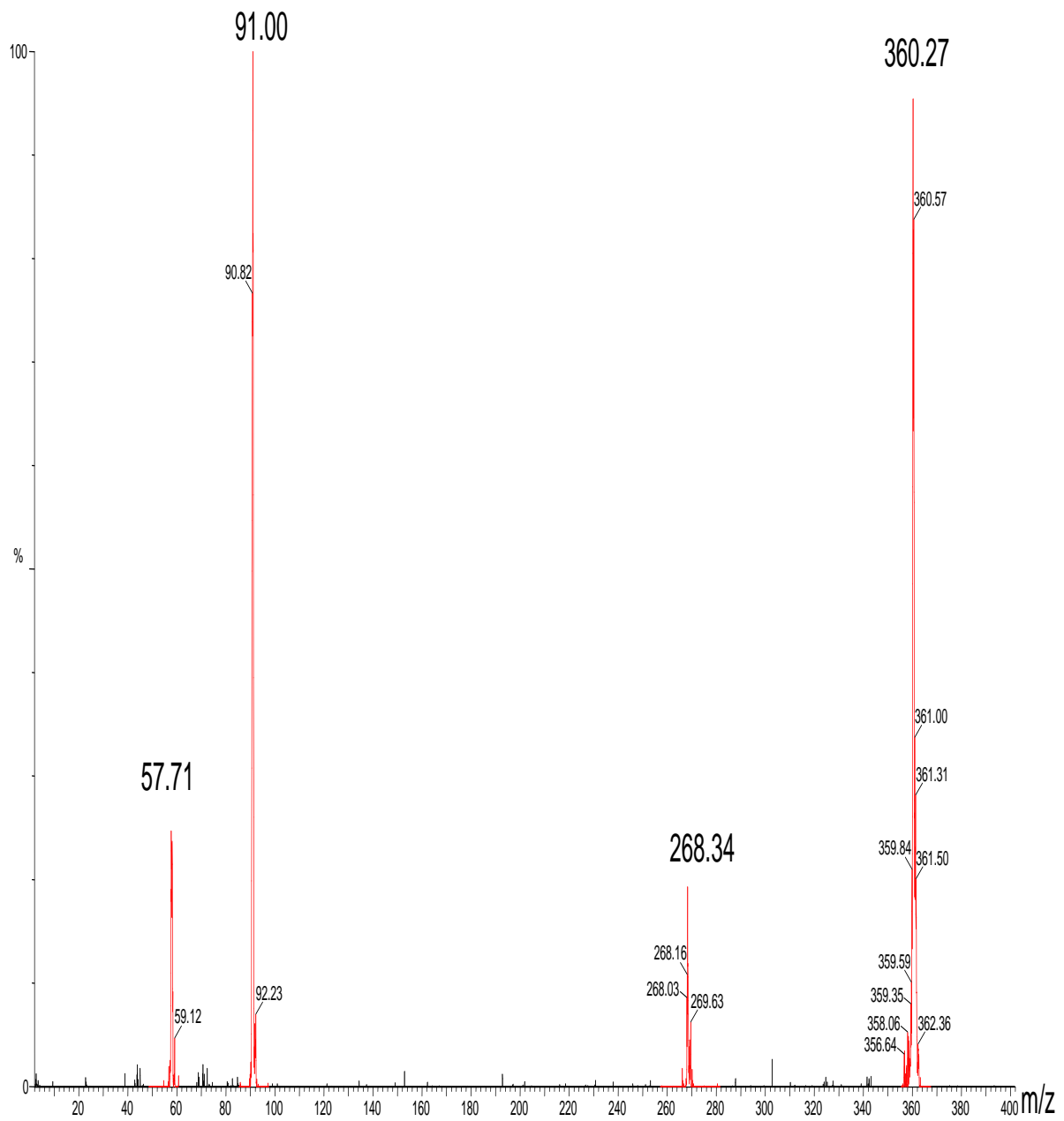


Figure 3-7 Spectrum of daughters of C₁₆ QUATs

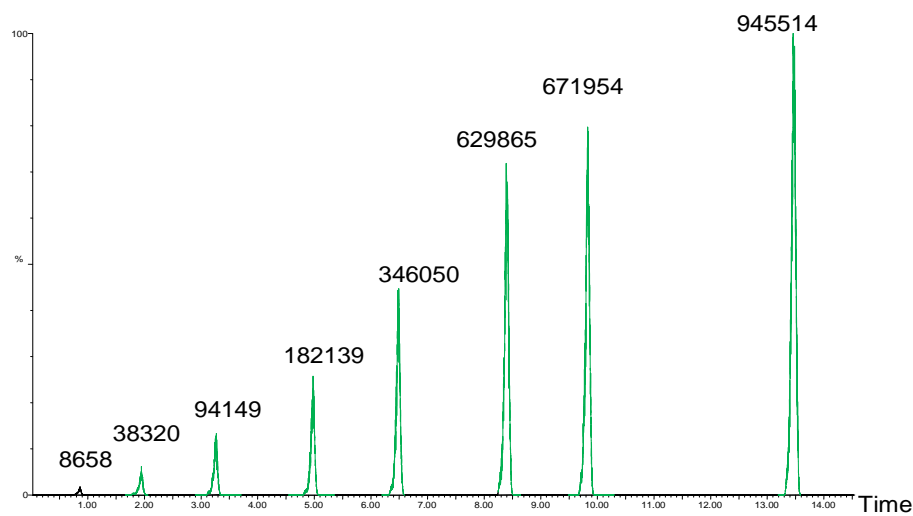


Figure 3-8 Calibration of C₁₆ QUATs using MRM (parent-daughter transition) starting from blank, 0.015, 0.03125, 0.0625, 0.125, 0.25, 0.5 to 1 mg/L

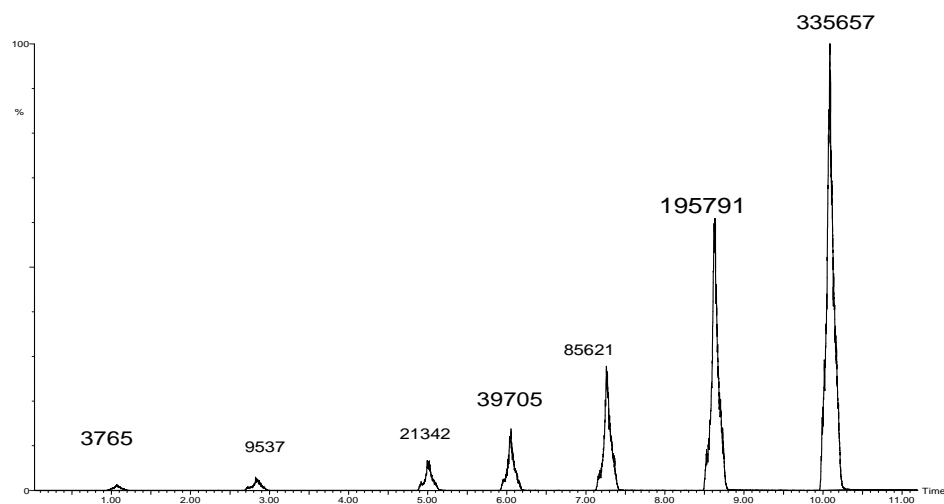


Figure 3-9 Calibration of C₁₆ QUATs using acidified methanol, starting from blank, 0.015, 0.03125, 0.0625, 0.125, 0.25, 0.5 to 1 mg/L)

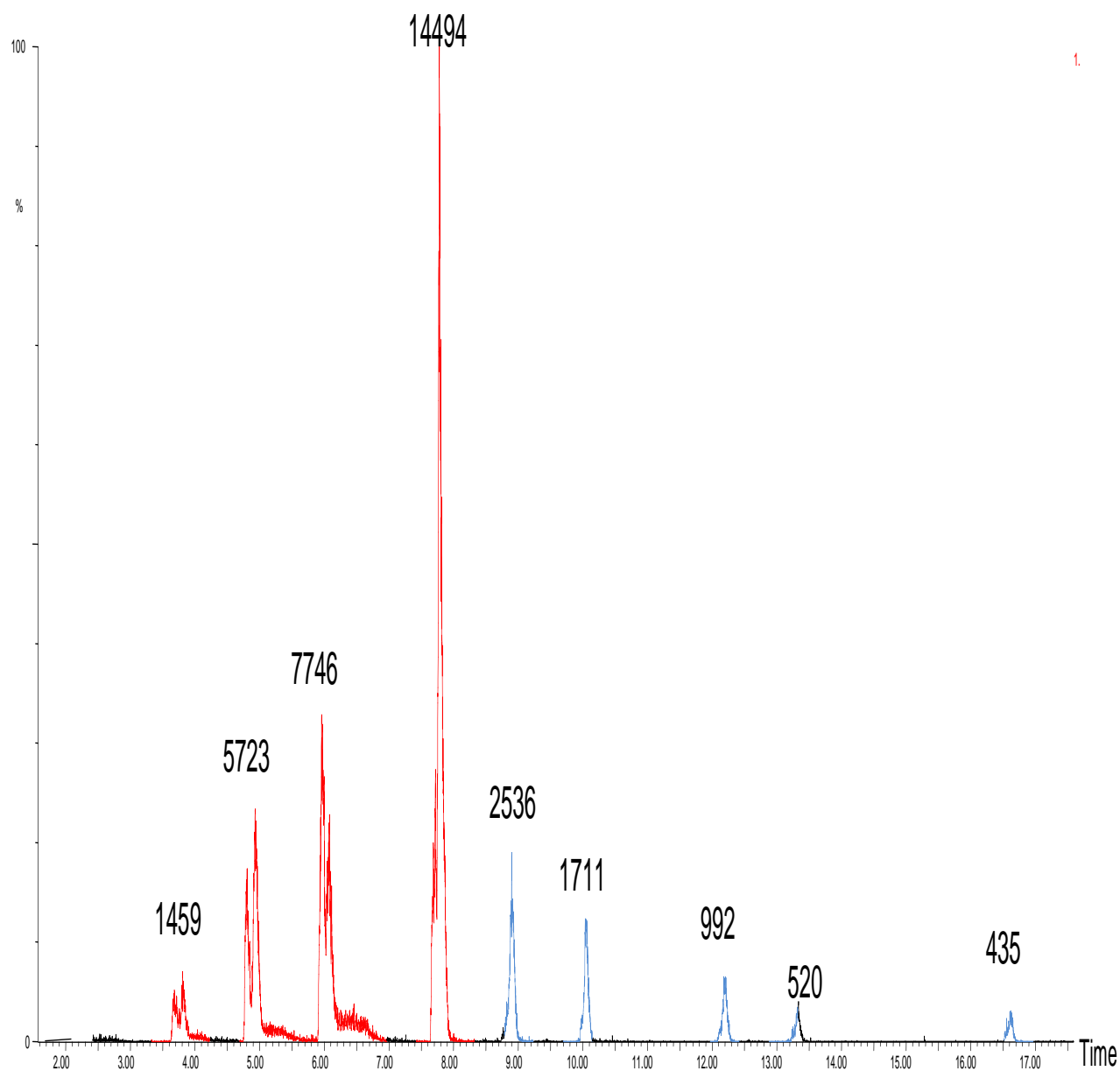


Figure 3-10 Spectrum of C₁₆ QUATs presented in all the tissues of the mussels (n=5). The red colour shows the concentration of C₁₆ QUATs in mussel tissues, starting from control, 0.1, 0.5 to 1 mg/L of QUATs and the blue colour shows concentrations in seawater.

Discussion

Every day, the oil and gas industries discharge a substantial volume of PW contaminated with petroleum hydrocarbons, including PAHs and several other additive chemical compounds, such as QUATs, that can cause potential deleterious effects in exposed organisms (Neff, 2002), and which can accumulate in the aquatic environment (Grigson et al., (2000)). In the present study, an effect on mussel tissues caused by QUATs was confirmed, even at the lowest concentrations comparable to environmental levels, following dilution during discharge (Harman et al., 2009; Hylland et al., 2008). This suggests that QUATs can cause significant DNA damage to the haemocytes and gill cells of exposed mussels. The sensitivity of mussels towards QUATs may increase the percentage in the comet tail. In the present study, the comet tails were clearly increased for both haemocytes and gill cells. The comet tails from the control up to 0.1 mg/L of QUATs in haemocytes were between (3.86±14.2%) and in the gill cells were between (4.1±13.1%), respectively. The percentage of DNA in the haemocytes' comet tails was higher than in the gill cells. However, some previous studies have agreed that the percentage of DNA in a comet tail is typically higher in gill cells than in haemocytes (Jha et al., 2005; Rank and Jensen, 2003; Rank et al., 2005; Rank et al., 2007; Taban et al., 2004; Tran et al., 2007). A reason for this has been suggested, based on the fact that QUATs are organic chemical compounds, which heightens the level of toxicity so that it becomes acute (García et al., 2001).

In addition the cationic compound cetrimonium bromide, which is based on quaternary ammonium cations, was found to be highly toxic to algae at 0.1 mg/L and fish and crustaceans at concentrations of 0.1 up to 1 mg/L (Aherns, 2008). Understanding the impact of below environmental concentrations of QUATs on DNA damage is important, especially as Grigson (2000) found concentrations of QUATs between 0.74 to 10.84 ng/kg in marine sediment close to two off-shore North Sea platforms. The low concentrations (0.001 mg/L) of QUATs used in the present study caused greater DNA damage to the haemocytes than the gill cells, suggesting that QUATs maybe more toxic to mussel haemocytes than to their gill cells.

In general, QUATs were reported to cause adverse effects to marine organisms. LC₅₀ toxicity of QUATs to species of invertebrates was observed at concentration 1 mg/L at 24 hours (Cooper, 1988). A study by Caliani et al. (2009) reported that DNA damage, expressed as SSB and micro-nucleated cells, was observed in fish of both genders after exposure to PW. In addition, DNA damage was also observed in digestive gland cells of *M. edulis* exposed to fractions of light crude oil (Hamoutene et al., 2002).

In the present study, the gill cells of the mussels showed increased SOD activity after 24 hours of exposure. The percentage of inhibition of SOD enzyme after 24 hours of exposure in control and the highest concentration (1 mg/L) were between 49.6 ± 54.2 and $22.3 \pm 19\%$ inhibitions respectively.

The reduction of SOD enzyme activity inhibition was confirmed at concentrations ≥ 0.5 mg/L. This fits well with the observation of SOD activity in gill homogenates, which was affected only by high levels of QUATs exposure. This suggests that the concentration ≥ 0.5 mg/L QUATs may generate overproduction of ROS, possibly leading to an increase in oxidation and the activity of the SOD enzyme. However, there was no increase in SOD activity at concentrations < 0.5 mg/L. This may be explained by the fact that QUATs are designed to be sticky and so may have adhered to the tank rather than partitioning in to the seawater phase, making the majority of the QUATs unavailable to the mussels at low concentrations. A study by Almeida et al. (2005) observed the increase of SOD activity in gill cells of mussel *p. perna* exposed to air for 4 hrs.

Levels of lipid peroxidation have been evaluated in the gill cells of mussels and used as indicators of environmental stress. Lipid peroxidation in gill cells was observed only at high concentrations of QUATs. *M. edulis* exposed to QUATs showed higher levels of TBARS at concentrations of 1 mg/L (0.33% inhibition nmol mg protein⁻¹) compared to animal control (0.89% inhibition nmol mg protein⁻¹). A previous study showed similar levels of lipid peroxidation in mussels' digestive glands and gill cells after exposure to 45 µg/L HgCl₂ (Verlecar et al., 2007), and Aloísio Torres et al. (2002) reported DNA damage and lipid peroxidation in the digestive glands of mussels collected at polluted sites. The increase of SOD activity, lipid and DNA damage is probably due to formation of ROS. Other studies have been presented evidence that formation of ROS can increase by exposed animal to chemical compounds and cause damage to Protein, lipid and DNA (Halliwell, 1994; Ceconi et al., 2003; Halliwell, 2012).

Many studies have used NNR assay to determine lysosomal membrane damage as early indicators of adverse effects, including effects caused by exposure to pollutants (Lowe et al., 1992; Moore, 2002). Therefore, the function of lysosomal damage can be used across a range of animals such as fish, annelids, crustaceans and molluscs, to identify biological responses to environmental stress (Galloway et al., 2004; Lowe et al., 1992; Lowe et al., 1995b; Svendsen and Weeks, 1995).

In the present study, lysosome membrane damage was confirmed at concentrations of 0.1 mg/L and above. The retention time for the treatment groups for mussels *M. edulis* exposed to concentrations from the lowest 0.001 up to the highest (1 mg/L) of QUATs were between 96 ± 33 minutes. The bioaccumulation and bio-concentrations of QUATs in mussels exposed to 0.1, 0.5 and 1 mg/L has been shown in both tissues and seawater at 0.1 mg/L in Figure 3-11. The 0.075 mg/kg of QUATs in tissue samples were confirmed at concentration ≥ 0.1 mg/L. Lee et al. (1972) confirmed the ability of the filter-feeding blue mussel *M. edulis* to rapidly take up mineral oil from seawater during 24 hrs of exposure.

In the present study, the seawater samples were also investigated but the signals were poor, probably because QUATs are sticky and may have adhered to the tanks but possibly also due to salinity of seawater. Therefore, the seawater samples presented low concentrations or showed poor signals due to the presence of large quantity of salts in the samples.

As shown in Figure 3-11, the concentration of QUATs in the initial sea water control was higher than in the treatment group and, in addition, the lowest concentration detected was higher than 1 mg/L. It was hypothesised that this may have been because the samples contained large amounts of salts which, upon ionisation and evaporation of the sample, were deposited on the sampling orifice and this is likely to have prevented the sample from reaching the analyser part of the MS. To overcome this problem, the researcher attempted to use SPE to remove the salts but without success.

The findings of the present study suggest that environmentally relevant concentrations of QUATs caused oxidative stress at concentrations ≥ 0.1 mg/L and inflicted significant DNA damage at concentrations ≥ 0.001 mg/L in both haemocytes and gill cells, and lysosomal membrane damage at concentrations ≥ 0.1 mg/L on marine mussels, under laboratory conditions. QUATs were accumulated in the soft tissues of the mussels when exposed to concentrations of 0.1, 0.5 and 1 mg/L, and the concentration was present in tissues at 0.075, 0.127 and 0.4 mg/kg respectively. QUATs were confirmed in mussel tissues after 24 hrs of exposure at concentrations ≥ 0.1 mg/L, which means that mussels may be capable of up taking the QUATs, which could therefore have consequences for the disposal of produced water at sea.

Oilfield corrosion inhibitor (Imidazoline): biomarkers of exposure in the marine mussel (*Mytilus edulis*)

Results

The results of this study showed significant increases in DNA damage, lysosomal stability and oxidative stress in haemocytes and gill cells taken from adult marine mussels (*M. edulis*), which were exposed to imidazoline at concentrations ranging between 0.001 and 1 mg/L (Figure 4-1 - 4-5).

DNA integrity in haemocytes and the gill cells of mussels exposed to imidazoline was detected by Comet assay. The level of DNA single strand breakages in both haemocytes and gill cells was significantly increased ($P < 0.001$) at concentrations as low as 0.001 mg/L, compared to the control group ($P < 0.001$; Figure 4-1 – 4-2).

SOD activity was found to be significantly increased, even at the lowest concentration ≥ 0.001 mg/L, compared to the control group ($P > 0.05$; Figure 4-3). In addition, the levels of lipid peroxidation in gill cells were significantly increased as compared to the control group at concentrations ≥ 0.1 mg/L ($P < 0.05$; Figure 4.4).

The damage to the lysosomal membranes was also determined as was damage to the membrane at concentrations ≥ 0.1 mg/L; the damage observed was not significantly different from the effects seen at concentrations as high as 1 mg/L ($P < 0.001$; Figure 4-5). However, there was a significant difference between concentrations ≤ 0.01 mg/L and ≥ 0.5 mg/L (Figure 4-5).

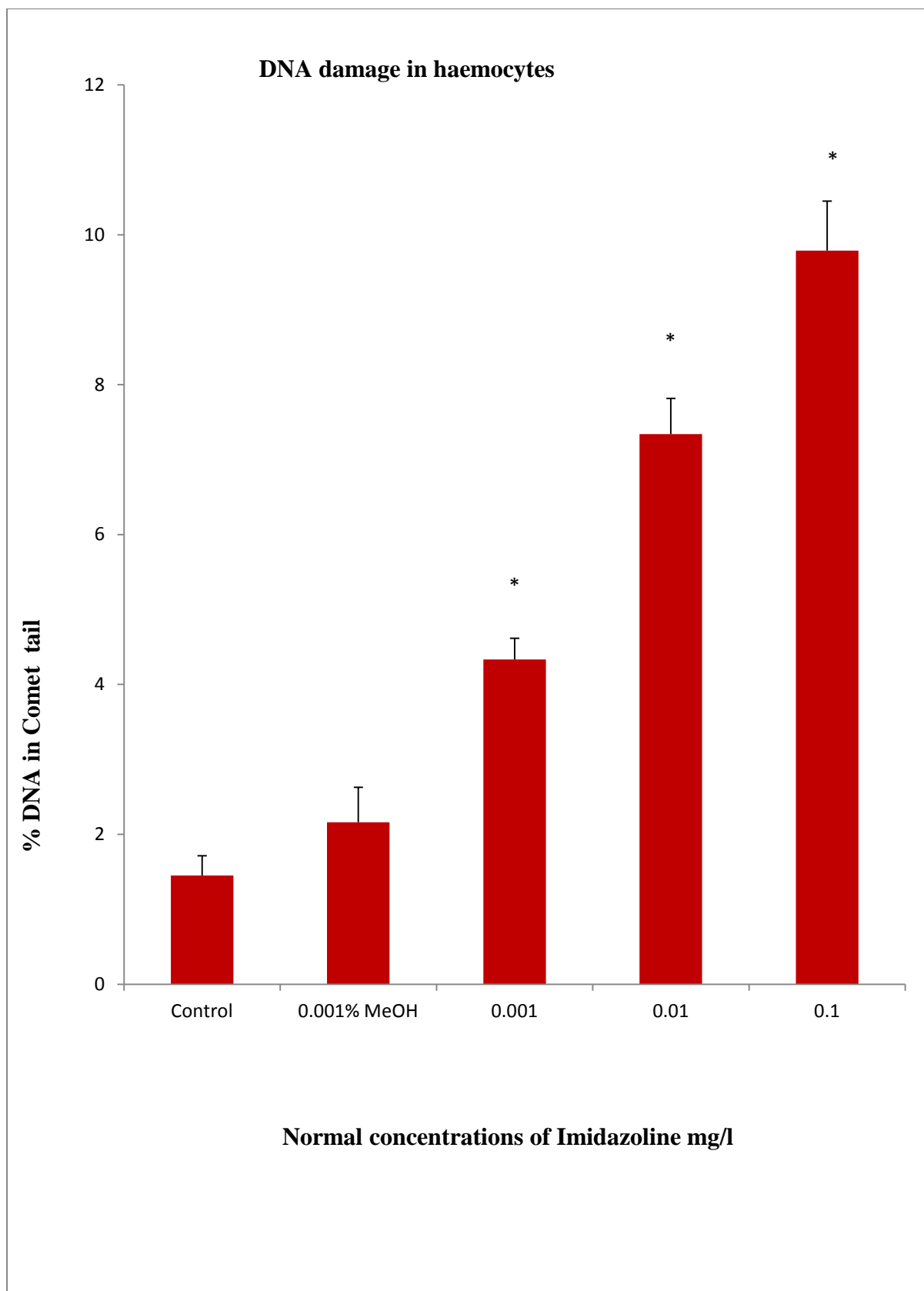


Figure 4-1 Average (\pm SD; n=5) percentage DNA damage in the tail of mussel haemocytes exposed to imidazoline. (*): significant differences from the control group and each other, (one-way ANOVA; $p \leq 0.001$).

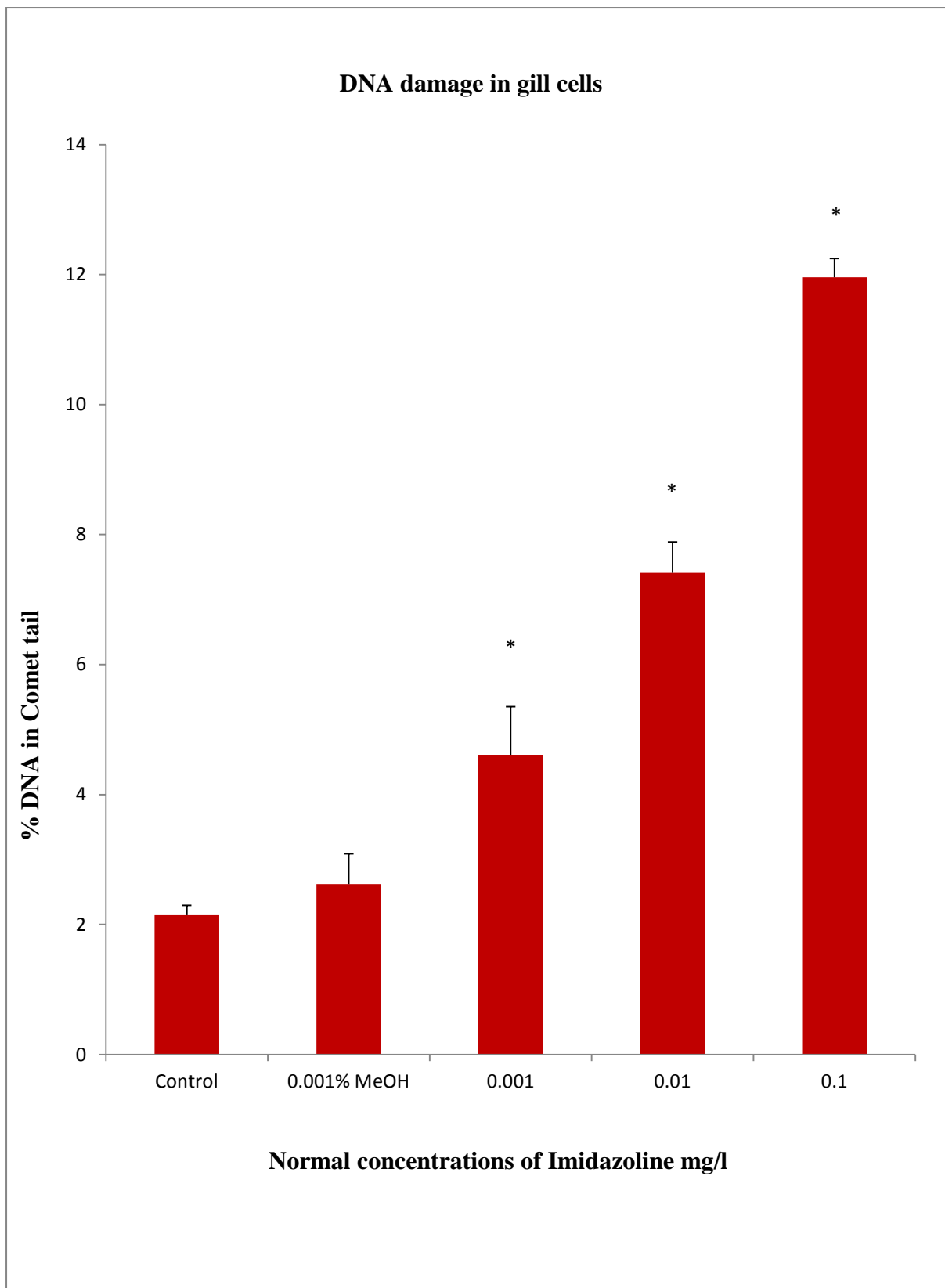


Figure 4-2 Average (\pm SD; n=5) percent DNA in the tail of gill cells taken from mussels exposed to imidazoline. (*): significant difference between the groups and from the control group (one-way ANOVA; $p \leq 0.001$).

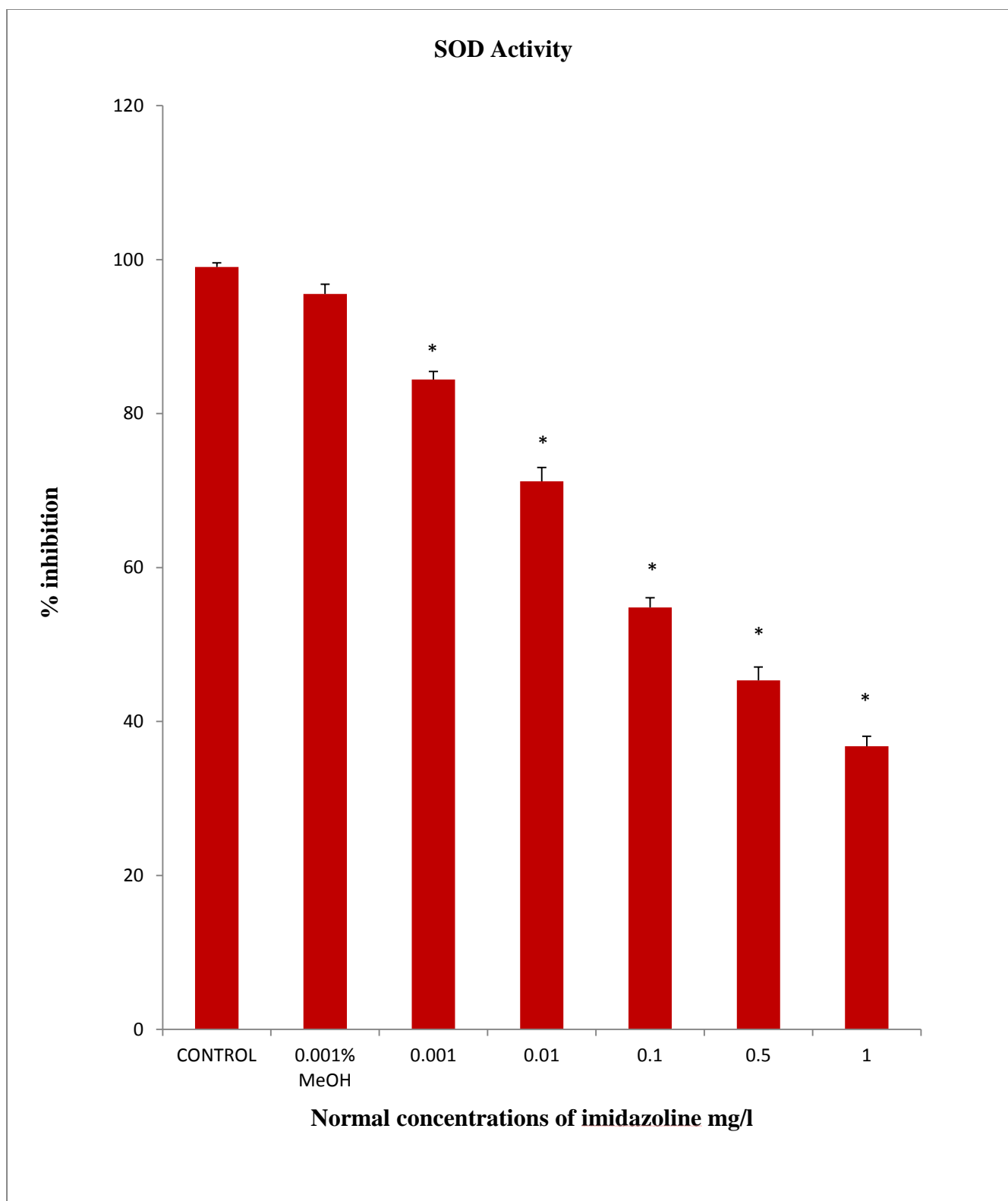


Figure 4-3 Average (\pm SD; n=5) percent inhibition of SOD activity in gill tissue homogenates of mussels exposed to imidazoline. (*): a significant difference between the groups and from the control group (one-way ANOVA; $p>0.05$).

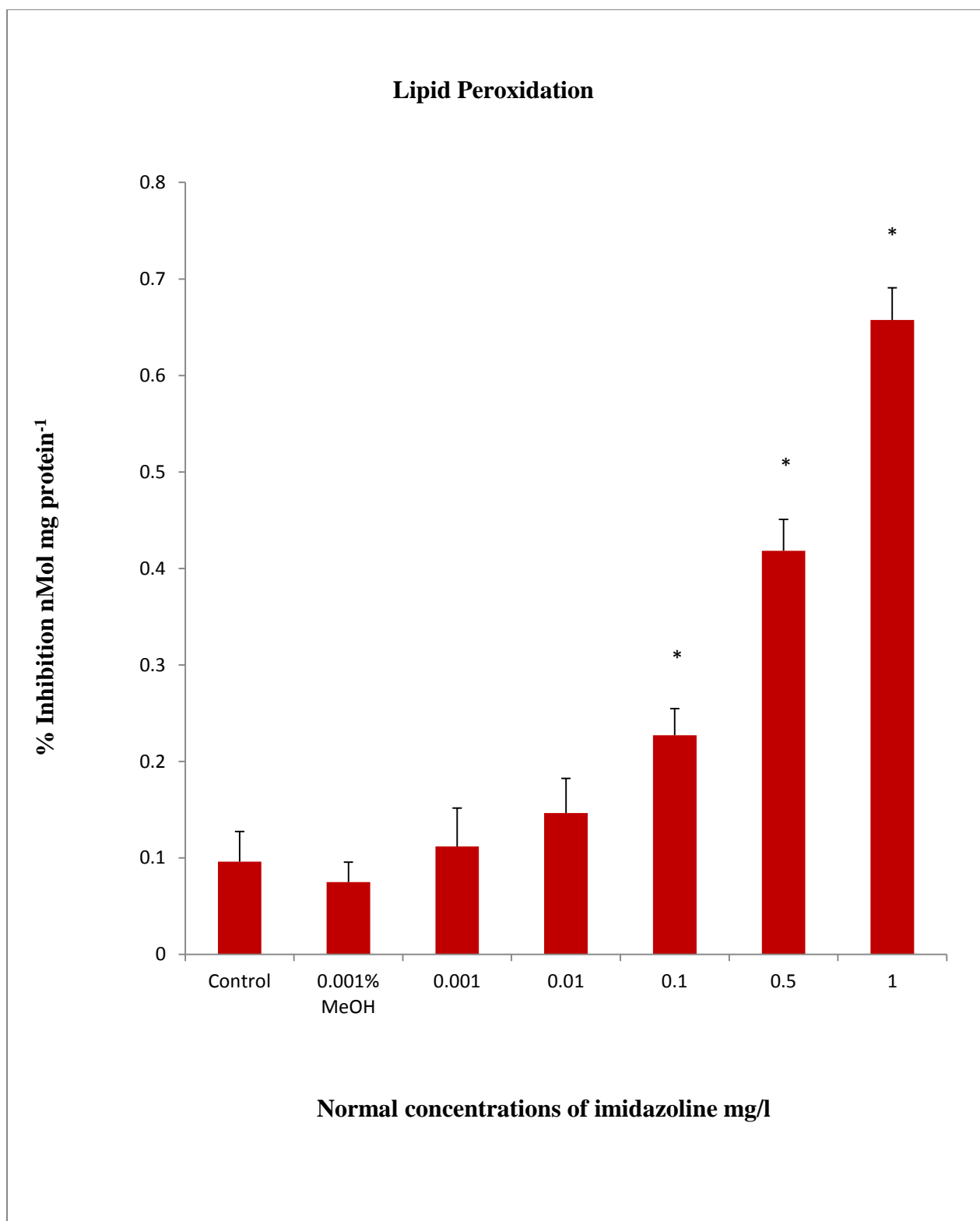


Figure 4-4 Average (\pm SD; n=5) TBARS in gill cells of mussels exposed to imidazoline: (*) significant difference between these groups and from the control group (one-way ANOVA; $p \leq 0.001$).

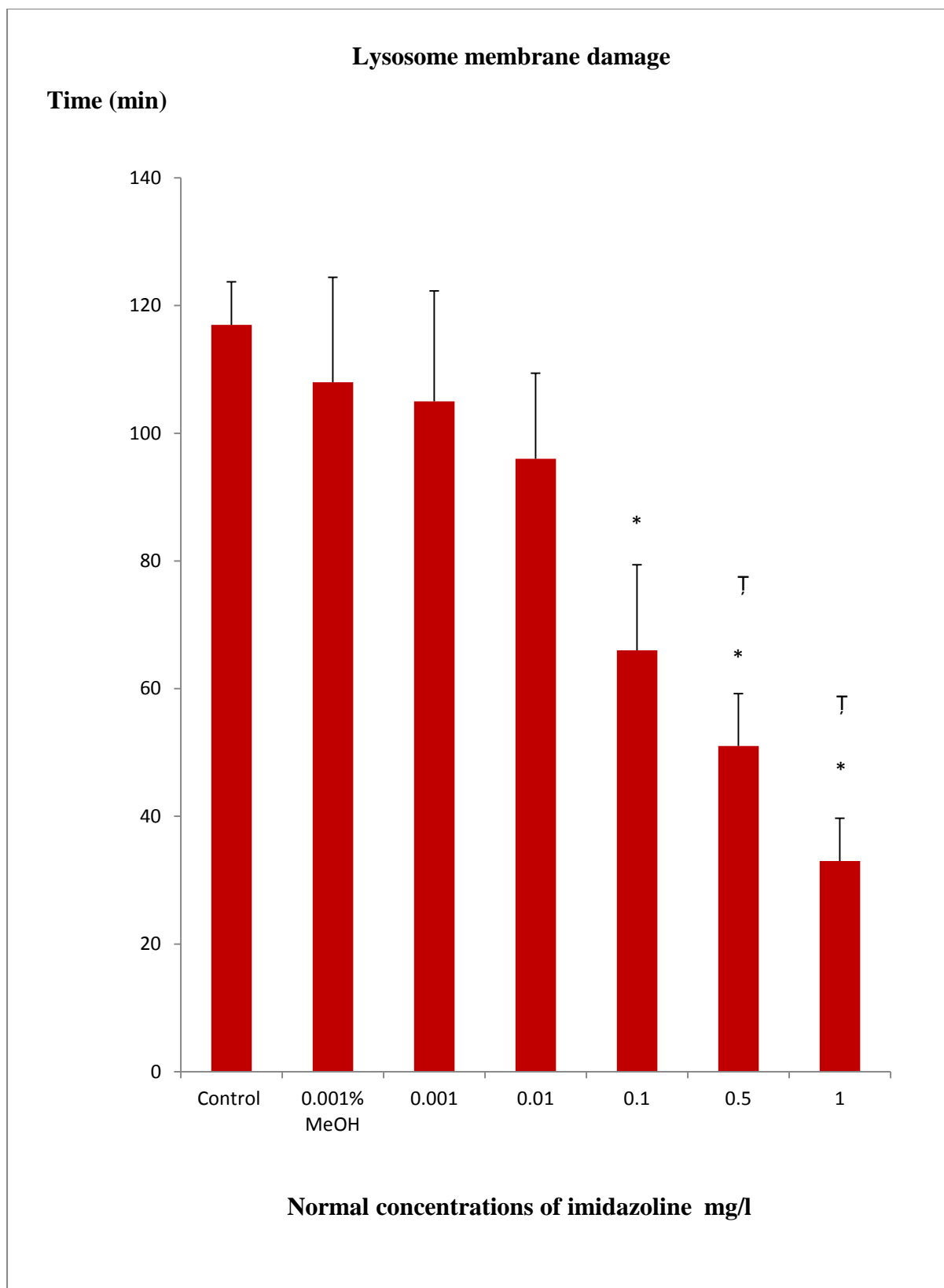


Figure 4-5 Average (\pm SD; n=5) lysosome membrane stability in haemocytes of mussels exposed to imidazoline. (*): significant difference compared to the control group and between each group (one-way ANOVA; $P < 0.001$) as well as (†): significant difference from concentrations ≤ 0.01 mg/L (one-way ANOVA; $P < 0.05$).

Bioaccumulation and bio-concentration of imidazoline were measured in all soft tissues using SPE, followed by spectrophotometry (spectra Max M5) and ultraviolet (UV). ESI MS/MS was also used to investigate the amount of imidazoline in all the mussel's soft tissues, but the signals were very poor. ESI MS/MS could not identify the standard solutions containing a known concentration of imidazoline, and this gave a very poor signal at concentrations of 5 mg/L and a poor signal at concentrations of 10 mg/L (Figure 4-8). Imidazoline is sticky and may adhere to the tank; the animals may not uptake all the imidazoline or some amount of the imidazoline may remain in the seawater. Therefore, the spectrophotometer (spectra Max M5) was used to determine the bioaccumulation of imidazoline in tissues at the wavelength 229 nm. The results show the concentrations of imidazoline in tissues are higher than the exposure concentrations. The bio-concentration factor (BCF) was applied to investigate bio-concentration in 1g of tissues by using the equation (4-1), where BCF is the ratio of the imidazoline concentration in mussels' tissues (C_B) to the concentration in water (C_W) Equation 4-1.

$$BCF = C_B/C_W \quad \text{Eq. 4.1}$$

The results show the BCF in the entire soft tissues of the mussel is high at exposure at low concentrations. The results are shown in Table 4-1.

Table 4-1 Concentration (mg/kg) of imidazoline in whole soft tissues of mussel exposed to environmental concentrations of imidazoline

UV wavelength	229 nm					
Normal concentrations of imidazoline (mg/L)	Control	0.001	0.01	0.1	0.5	1
Concentrations of imidazoline in tissues	0	0.29	0.42	0.65	0.73	0.87
BCF		291.6	42.1	6.5	1.4	0.87

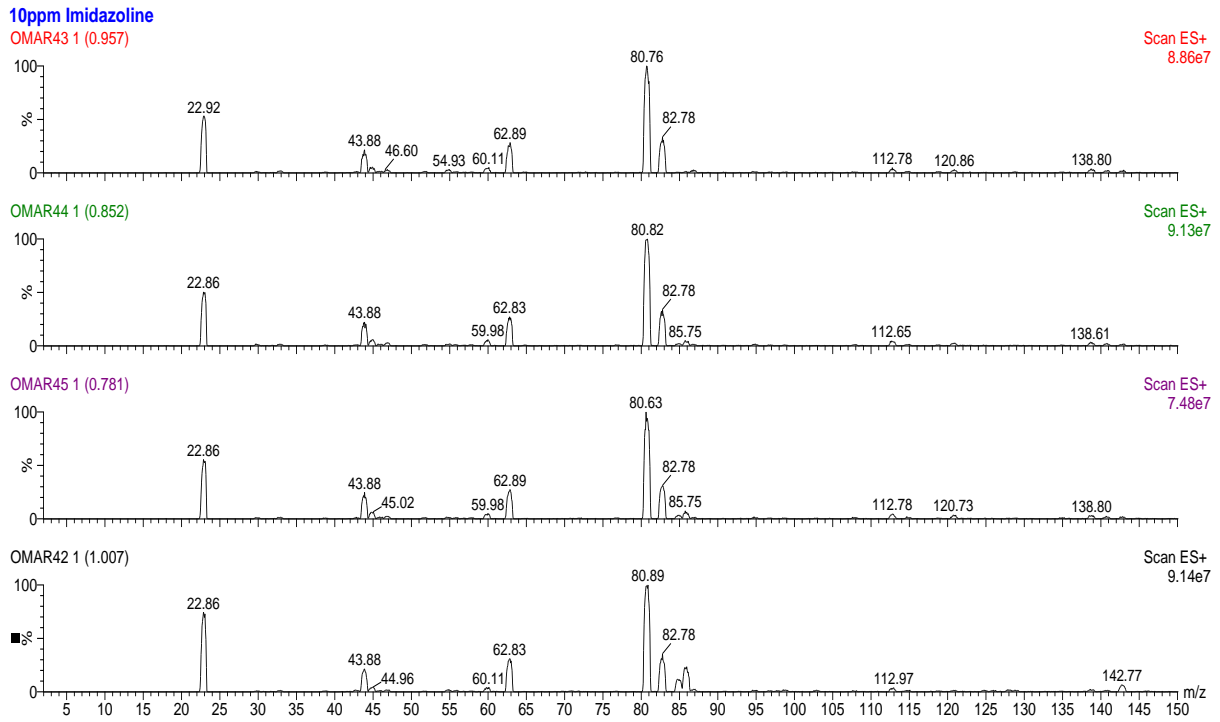


Figure 4-6 Calibration of imidazoline using ESI MS/MS starting from 1.25, 2.5, 5 to 10 mg/L, showing poor signals for all concentrations

Discussion

The oil and gas industry has largely stopped using inorganic compounds as CIs, due to their known toxicity to aquatic environments. They have therefore transferred to organic compounds such as benzimidazole and imidazoline, which are added to the boilers during treatment process to prevent adherent deposits of calcium (Ca^{2+}) and magnesium (Mg^{2+}) (Arora et al., 2012). However, many organic chemical compounds now used as CIs are harmful to the aquatic environment, such as benzotriazole, which was found to cause toxicity to marine organisms at concentrations over 10 $\mu\text{L/L}$ (Burkhardt, 2011).

In the present study, imidazoline was confirmed to cause significant damage to DNA even at its lowest concentrations (0.001 mg/L), in both the haemocytes and gill cells of marine mussels. However, the level of DNA damage in gill cells is higher than that in haemocytes. Despite this gill tissues contain large numbers of haemocytes (Burge et al., 2007). The comet tails from control up to 0.1 mg/L of imidazoline in haemocytes were between (1.5±9.8%), and in gill cells were between (2.2±11.9%) respectively. This may be because the concentrations of oxygen were higher in gill cells than in haemocytes (Jha et al., 2005; Singh and Hartl, 2012). Furthermore, the DNA-damaging chemicals that have dissolved or bound to particles in the water can attack and affect the gill cells directly during the filtration process (Rank and Jensen, 2003). In the present study, the percentage of the DNA comet tail in haemocytes and gill cells is comparable to that found in other studies (Rank and Jensen, 2003; Rank et al., 2007; Singh and Hartl, 2012).

The results in the present study have illustrated that the imidazoline compound caused a genotoxic effect in both haemocytes and gill cells of mussel *M. edulis*. These results are comparable to those for QUATs compounds reported in Chapter Three. Furthermore, imidazoline caused significant damage to the lysosome membrane at concentrations ≥ 0.1 mg/L. The retention time for the treatment groups for mussels *M. edulis* exposed to concentrations from the lowest 0.001 up to the highest (1 mg/L) of imidazoline were between 117 ± 33 minutes. These results are comparable to Rank et al. (2007) and may occur because during enzymatic detoxification of imidazolines, ROS are generated, which can cause significant increases in SSB and DSB (Ceconi et al., 2003; Halliwell, 1994).

In the present study, the mussels' gill cells showed increased SOD activity after 24 hours of exposure to imidazoline. The percentage of the inhibition of SOD enzyme after 24 hours of exposure in control and highest concentrations (1 mg/L) were between 99.2±99.4 and

37.3±35.3% inhibition respectively. However, even the lowest concentrations caused oxidative stress by increasing SOD activity at concentrations of ≥ 0.1 mg/L.

Lipid peroxidation also showed an increase in the gill cells of the mussels and the level of TBARS after 24 hours of exposure to imidazoline were at concentrations ≥ 0.1 mg/L. The higher TBARS level after 24 hours of exposure were at concentrations (1 mg/L) between 0.62±0.7% inhibition nmol mg protein⁻¹ compared to control samples (0.05±0.13).

The reduction in the SOD enzyme, could be due to the high concentration of oxygen in the gill cells (Santovito et al., 2005). Basically, certain organic chemicals and pollutants in PW are well-known as possible sources of oxidative stress and causes of variation in the activities of antioxidant enzymes (Santovito et al., 2005). Actually, good correlations have been reported between impairment of antioxidant capability, NNR retention time, and changes in DNA integrity in different organisms (Binelli et al., 2009). Furthermore, previous authors have suggested that reactive free radicals such as ROS and xenobiotic derivative can contribute to reduce lysosomal membrane stability and show that the intralysosomal environment is previously a site of free radicals production (Kirchin et al., 1992, Winston et al., 1996) (Binelli et al., 2009). Moreover, the increase of the genotoxicity at concentrations ≥ 0.5 mg/L (Figure 4-1 up to 4-5) suggest that imidazoline toxicity may result from the observed inhibition of antioxidant activities as a consequence of raised oxidative stress as also reported by many studies in biological models exposed to different chemical compounds (Binelli et al., 2009). Some studies investigated the properties and behaviour of imidazoline (Bajpai and Tyagi, 2006; Tyagi et al., 2007; Ash and Ash, 2011; Fink, 2012). A study by Sastri (2011) reported that imidazoline causes adverse effect to organisms and LC₅₀ toxicity of imidazoline after 24hrs has been reported at concentrations < 10 mg/L.

The overproduction of ROS can elevate lipid peroxidation and inhibit the SOD enzyme, causing oxidative stress and leading to chronic diseases such as heart disease, aging and cancer (Halliwell, 2012).

Tandem ESI MS/MS was used to determine the concentration of imidazoline in all the mussel *M. edulis*' soft tissues. The results did not produce any signals for tissue samples after SPE and, in addition, the signals for the standard solutions containing a known concentration of imidazoline also produced very poor peak resolution. However, the tissues samples from the QUATs were analysed and resulted in a clear peak, without any problems. (See Chapter Three). The poor results in this part of the study may be attributed to some fragments of the

imidazoline compound and the fact that the available MS cannot detect mass between 600-620 m. Therefore, chromatographic analysis of the tissues samples was undertaken at 229 nm.

The results from chromatography confirmed that imidazoline had accumulated and concentrated in the mussel's tissues. This suggests that the mussel may uptake and concentrate imidazoline through all possible routes, including dietary absorption or transport across the respiratory surface, thereby resulting in levels of concentration which exceed those in seawater (Gobas and Morrison, 2000). As Mackay and Fraser (2000) observed, organisms can achieve high concentrations of certain organic contaminants relative to concentrations of these substances in the environment they inhabit. Additionally, some authors have observed the BCF of aquatic organisms and how these chemicals are accumulated and concentrated; such as in fish exposed to chlorophenols (Kishino and Kobayashi, 1995, 1996), or in mussels exposed to Triclosan (Riva et al., 2012). In the present study, the biomarker tests under controlled conditions confirmed that imidazoline caused a biomarker response in the exposed mussels.

The findings of the present study validate the hypothesis that environmentally relevant concentrations of imidazoline could cause significant DNA damage and inhibit SOD activity, even at the lowest concentration (0.001 mg/L), and furthermore, increase lipid peroxidation and damage to lysosomal membranes at concentrations of 0.1 mg/L in mussels *M. edulis*, under laboratory conditions. Imidazolines were found, even at the lowest concentration (0.001 mg/L), and were concentrated in mussel tissues after 24 hours of exposure, which could therefore confirm that imidazolines increase the formation of ROS. These compounds can, therefore, be expected to accumulate and concentrate and cause genotoxicity, oxidative stress and cytotoxicity in both haemocytes and gill cells, resulting in many biological responses in both mussels and other aquatic organisms when these chemicals are discharged into the marine environment.

Scale inhibitor phosphate esters (PEs): biomarkers of exposure in the marine mussel (*Mytilus edulis*)

Results

Adult marine mussels were exposed to PEs and the levels of DNA damage present in the haemocytes and gill cells was detected using the Comet assay. The results showed significant increases in damage at concentrations ≥ 0.001 mg/L in both the haemocytes and the gill cells ($P < 0.001$ Fig 5-1, 5-2). However, gill tissues contain large numbers of haemocytes.

The SOD activity in the gill cells was also determined for all concentrations of PEs and showed a significant increase in activity at concentrations ≥ 0.1 mg/L, compared to the control groups and concentrations ≤ 0.01 mg/L. However there was no significant change in activity between 0.1 and 0.5 mg/L at ($P > 0.05$; Figure 5-3).

Figure 5-4 shows that the levels of lipid peroxidation in mussel gills were significantly increased at concentrations ≥ 0.5 mg/L, as compared to the control group ($P < 0.005$).

The damage to the lysosomal membranes was also determined and these were shown to be damaged at concentrations ≥ 0.5 mg/L. However, there was a significant difference between 0.5 and 1mg/L, when compared to the control group ($P < 0.001$; Figure 5-5).

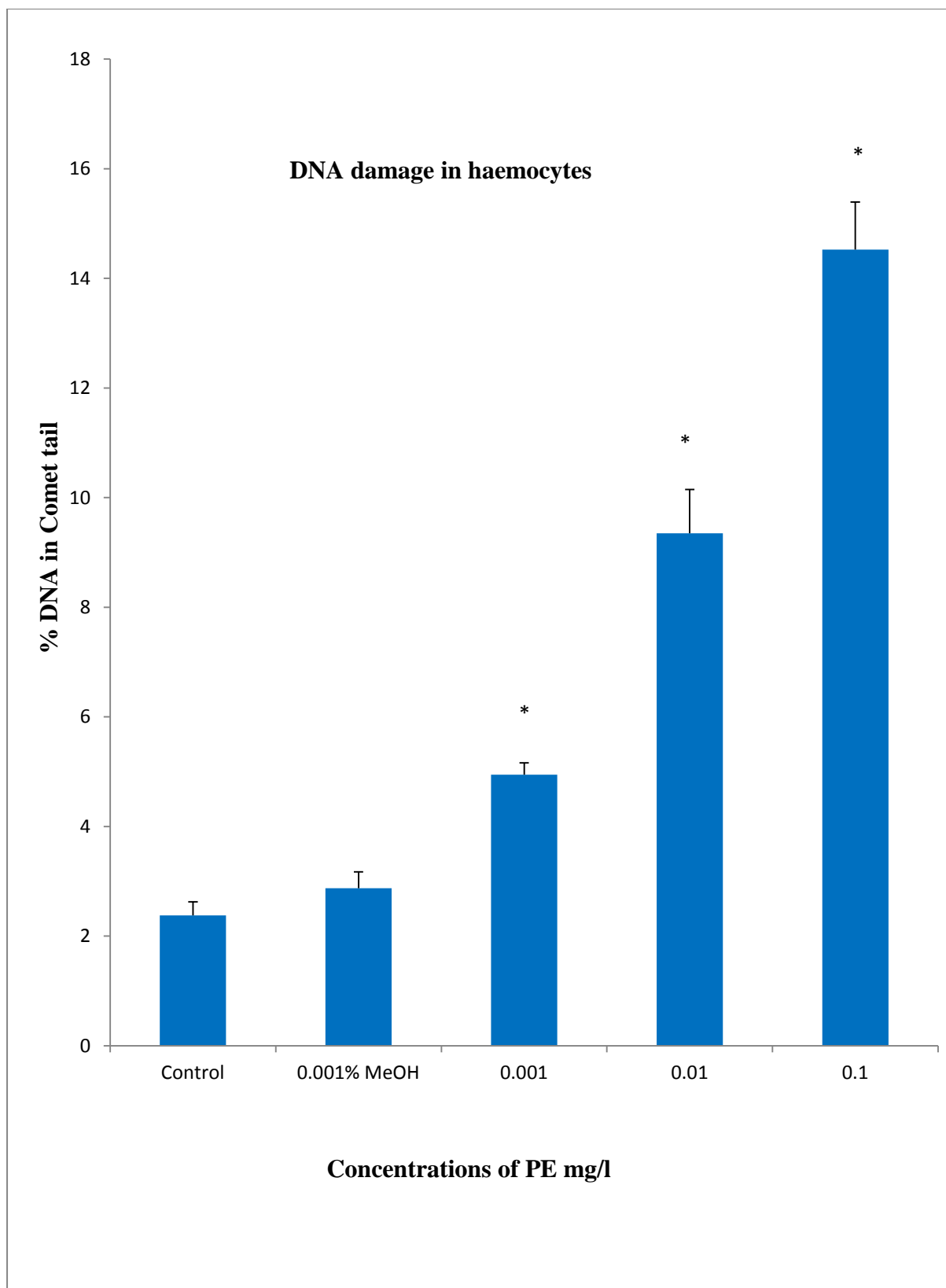


Figure 5-1 Average (\pm SD; n=5) percentage DNA in the comet tail of mussel haemocytes exposed to PEs. (*): a significant difference between groups and from the control group (one-way ANOVA; $p \leq 0.001$).

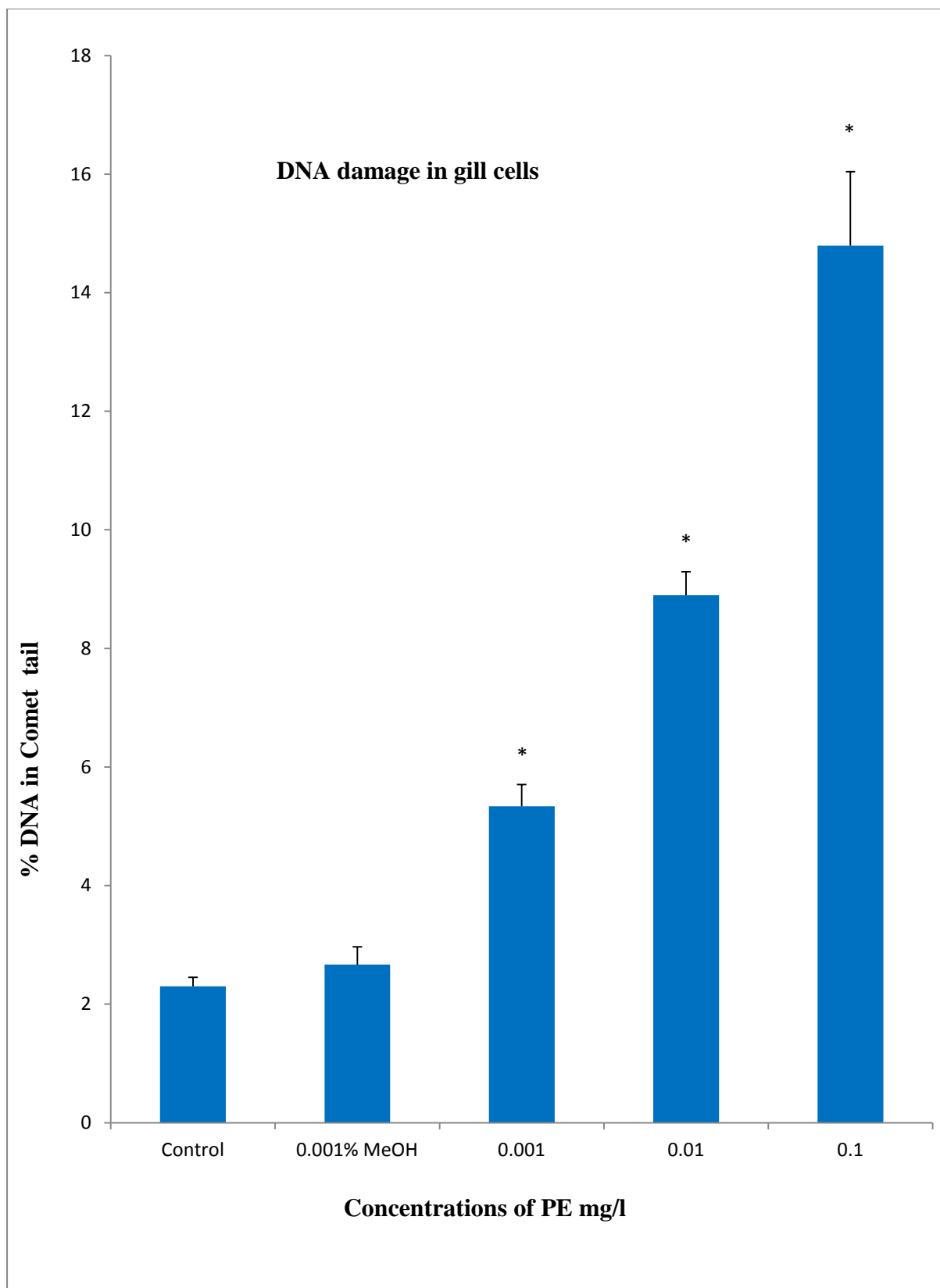


Figure 5-2 Average (\pm SD; n=5) percentage DNA in the comet tail of gill cells of mussels exposed to PEs (*): a significant difference between each other and the control group (one-way ANOVA; $p \leq 0.001$).

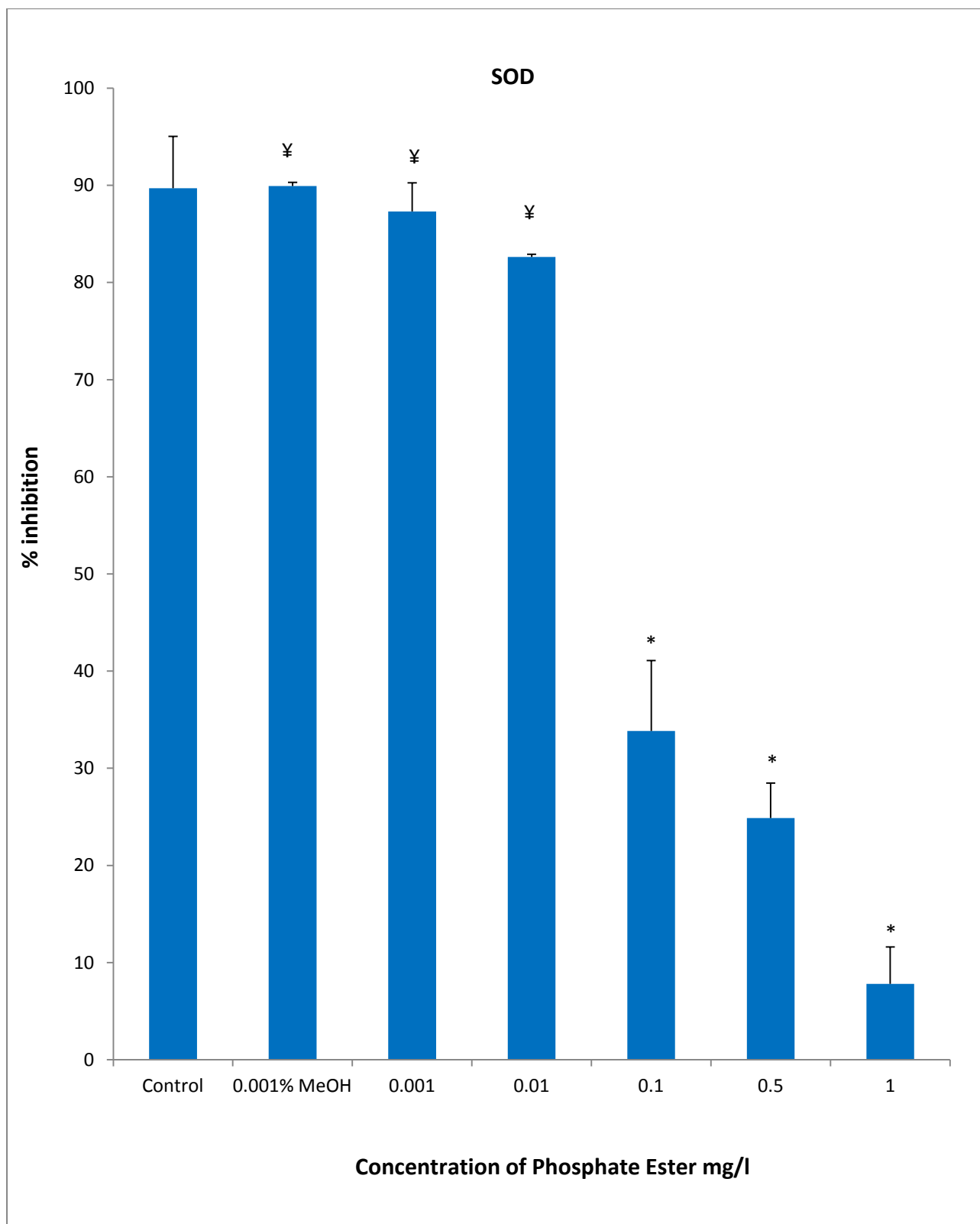


Figure 5-3 Average (\pm SD; n=5) percentage inhibition of SOD activity in the gill tissue homogenates of mussels exposed to PEs. (*): a significant difference between these groups compared to the control group (¥): no significant differences between the groups and from the control (one-way ANOVA; $P>0.05$).

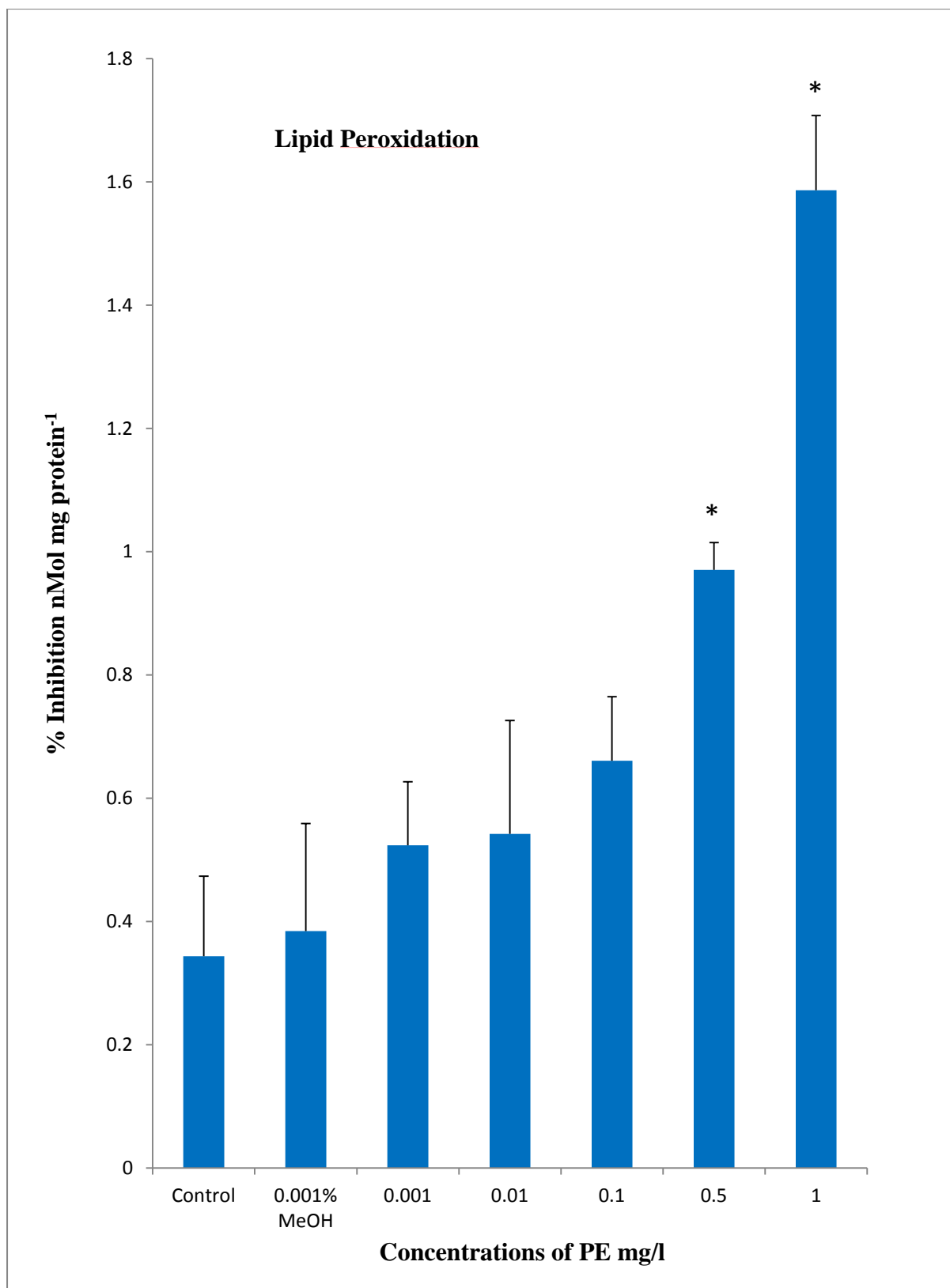


Figure 5-4 Average (\pm SD; n=5) TBARS in gill cells of mussels exposed to PEs. (*): a significant difference between these groups and from the control group. (¥): no significant difference between these groups and the control (one-way ANOVA; $P \leq 0.05$).

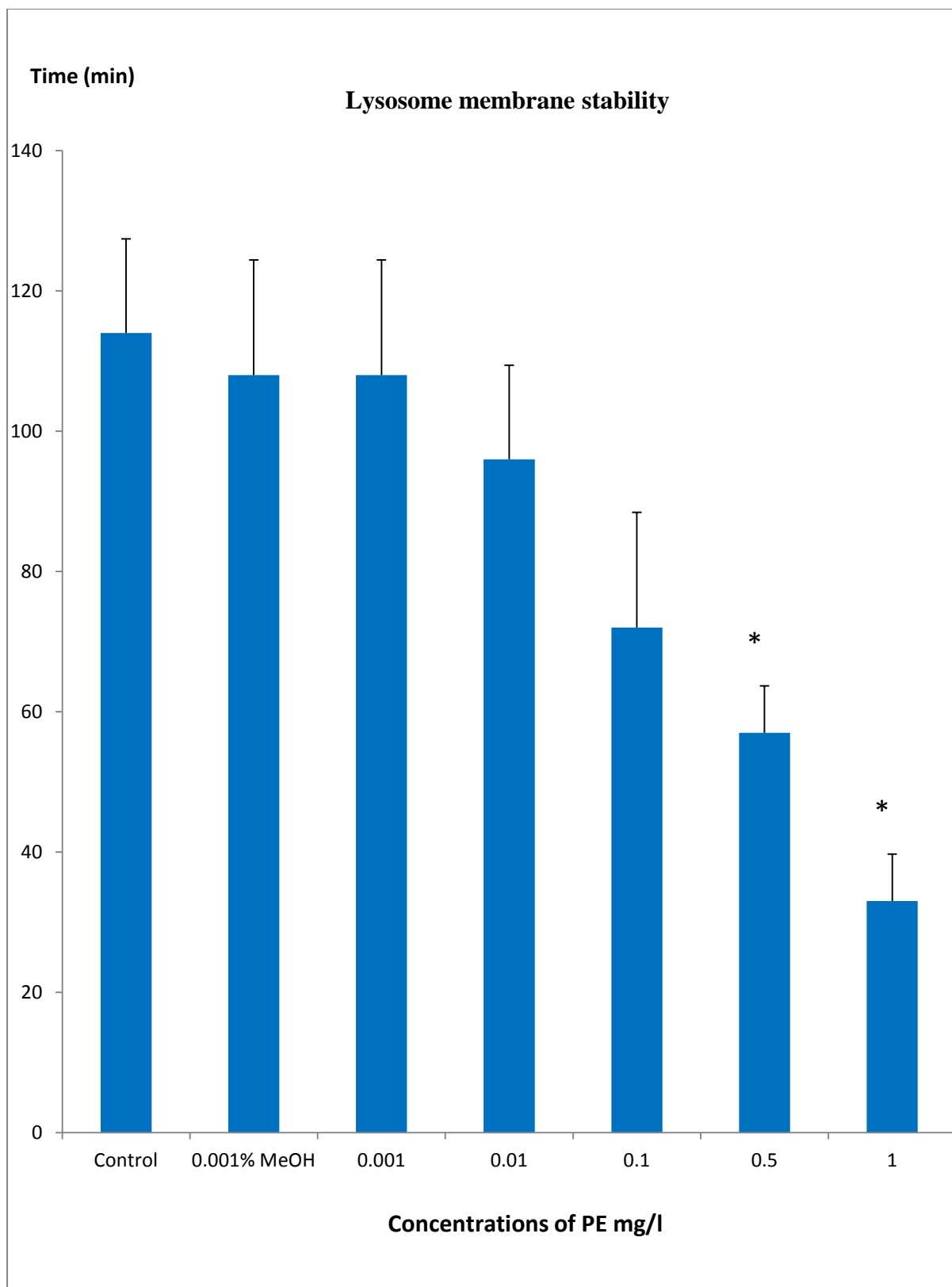


Figure 5-5 Average (\pm SD; n=5) lysosome membrane stability in the haemocytes of mussels exposed to PEs. (*): a significant difference in these groups compared to the control group and each group (one-way ANOVA; $P < 0.001$).

Bioaccumulation and bio-concentrations of PEs were measured in all soft tissues using SPE followed by spectrophotometry (spectra Max M5). ESI MS/MS was also used to investigate the amount of PEs in all the mussels' soft tissues but no signals were detected. ESI MS/MS could not identify the standard solutions containing known concentrations of PEs. This did not give a signal at concentration up to 10 mg/L. Therefore, the spectrophotometer (spectra Max M5) was used to determine the bioaccumulation of PEs in tissues at wavelength 345 nm. PEs were not found in soft tissues at concentrations from 0.001 up to 1 mg/L.

Discussion

PEs is highly water-soluble and will remain in the PW discharged into the marine environment. In the present study it was confirmed that PEs caused DNA damage in haemocytes and gill cells even at low concentrations. The percentage of DNA damage in the comet tail in both haemocytes and gill cells were between ($2.1\pm 14.5\%$ and $2.3\pm 16.0\%$) respectively. Consequently, the% in the comet tail in gill cells was higher than that in the haemocytes. However, gill tissues contain large numbers of haemocytes (Burge et al., 2007).

These results are comparable to those for imidazoline Chapter Four. There are many studies, which are comparable to this one, that have detected biological changes in both haemocytes and gill cells from mussels exposed to oilfield chemicals such as crude oil and PAH. A previous study reported that a response was seen in comet tails at concentrations of between 1 and 33 mg/L methansulfonate after 2 days of exposure in both haemocytes and gill cells.

In the present study, a response was seen in lysosome membrane at concentrations ≥ 0.5 mg/L. The NNR retention time of treatment groups for mussels *M. edulis* exposed to concentrations from the lowest 0.001 up to the highest (1 mg/L) of PEs was between 114 ± 33 minutes. These results are comparable to those of Famadas (2009) and Rank et al. (2007).

In addition, in the present study, a significant increase in SOD activity at concentrations ≥ 0.1 mg/L was shown and the inhibitions of SOD activity from the control up to 1 mg/L of PEs were between ($7.81\pm 89.7\%$, Figure 3-5) affecting the levels of lipid peroxidation. However, a higher TBARS level was registered at concentrations (1 mg/L) between $1.43\pm 1.76\%$ inhibition $\text{nmol mg protein}^{-1}$ when compared to control samples (0.22 ± 0.4).

The inhibitions of TBARS level and SOD activity were at a significantly higher level at concentrations of 1 mg/L of PEs compared to the control group. A previous study by (Torres et al., 2002) reported that mussels are able to increase their antioxidant level in response to contaminants.

Thus it may be predicted that PEs generate extensive amount of O₂ in the body which leads to the distribution of a large number of the free radicals and ROS which are known to be responsible for causing biological responses (Ceconi et al., 2003; Halliwell, 1994). These free radicals and ROS can inflict damage on the DNA, inhibit the SOD enzyme, increase lipid peroxidation and cause damage to the lysosomal membrane, leading to a range of diseases (Halliwell, 2012). Some studies have provided evidence of ROS production in the livers of fish that have exposed to toxic pollutants in field experiments (Valavanidis et al., 2006). Farmen et al. (2010) also investigated oxidative stress and cytotoxicity in fish (rainbow trout), primary hepatocytes exposed to water-soluble and a particulate organic fraction of PW from ten different platforms of North Sea oil production. The results showed that PW fractions caused a concentration-dependent increase in ROS after 1 hrs exposure and, after 96 hrs, there were changes in levels of cytotoxicity. Interestingly, major contributors to oxidative stress and cytotoxicity were due to the water soluble organic compounds in the PW.

The results of this study are the first indication of genotoxic, oxidative stress and cytotoxicity effects of environmentally relevant concentrations of PEs in marine mussels. However, the results for the presence of PEs did not confirm the accumulation and concentrations of PEs in the mussel tissues, which is unsurprising given their high water solubility. Previously, bioaccumulations of QUATs were measured at concentrations ≥ 0.1 mg/L (see Chapter 3) and imidazoline at concentrations over 0.001 mg/L (see Chapter 4). In the previous study, (Sundt et al., 2011) PAH was investigated and it was found that this accumulated in mussel's soft tissue, even at the lowest exposure dose. In this study, although many techniques were tried to neither investigate the presence of PEs in the mussels' tissues, such as ESI MS/MS, UV chromatography and SPE, neither positive nor negative results could be obtained. The first technique used was ESI MS/MS. The results show that ESI MS/MS cannot detect a standard solution of PEs containing known concentrations ranging from 1 up to 10 mg/L of PEs, because of the low sensitivity of the available MS: concentrations up to 10 mg/L were too weak to register; it would only register with much higher concentrations.

Overall this study confirms that a low concentration of PEs causes significant damage to DNA, and damage to lysosomal membranes, after 24 hrs of exposure due to overproduction of ROS and free radicals, which then cause damage to the SSB and DSB (Halliwell, 1994).

The findings also indicated that PEs caused a great damage to the DNA of marine mussels exposed to environmental concentration of PEs for 24 hours, even at the lowest concentrations (0.001 mg/L) in both haemocytes and gill cells. Furthermore, they caused oxidative stress in gill cells through inhibition of the SOD enzyme, at concentrations ≥ 0.1 mg/L, and increased lipid peroxidation at concentrations ≥ 0.5 mg/L. They also caused lysosomal membrane damage at concentrations ≥ 0.5 mg/L in haemocytes. In the present study, after 24 hours of exposure, chemical analyses were performed using ESI MS/MS and spectrophotometry. However, the accumulation and concentration of PEs were not confirmed throughout all the mussels' soft tissues.

These results suggest that environmentally relevant concentrations of PEs can be expected to cause genotoxicity at the lowest concentrations, in the form of oxidative stress and cytotoxicity, in both haemocytes and gill cells; thus, this might have consequences for the disposal of PW at sea.

Oilfield effluent (Produced water (PW)): biomarker of exposure in mussels (*Mytilus edulis*)

Results

Mussels were exposed to PW to investigate the genotoxicity in haemocytes and gill cells. The level of DNA damage was detected using the Comet assay technique, and it was shown that even the lowest concentrations of PW can cause damage to DNA. In haemocytes and gill cells this damage significantly increased at concentrations ≥ 0.001 mg/L ($P < 0.001$; Fig 6-1 and 6-2). However, gill cells contain large numbers of haemocytes.

Oxidative stress was also measured. SOD activity was significantly increased in each group, at all concentrations, as compared to the control group. There was a significant increase even at the lowest concentration ($P > 0.001$; Fig 6-3).

The level of lipid peroxidation also showed a significant increase across all concentrations when compared to the control group. However, there were no significant differences between the groups at different concentrations (Figure 6-4).

Cytotoxicity was also investigated. The lysosome membrane damage in haemocytes showed a significant increase at concentrations ≥ 0.1 mg/L, compared to the control group. There was no significant increase between concentrations of 0.001, 0.01 and 0.1 mg/L respectively, ($P > 0.001$; Figure 6-5).

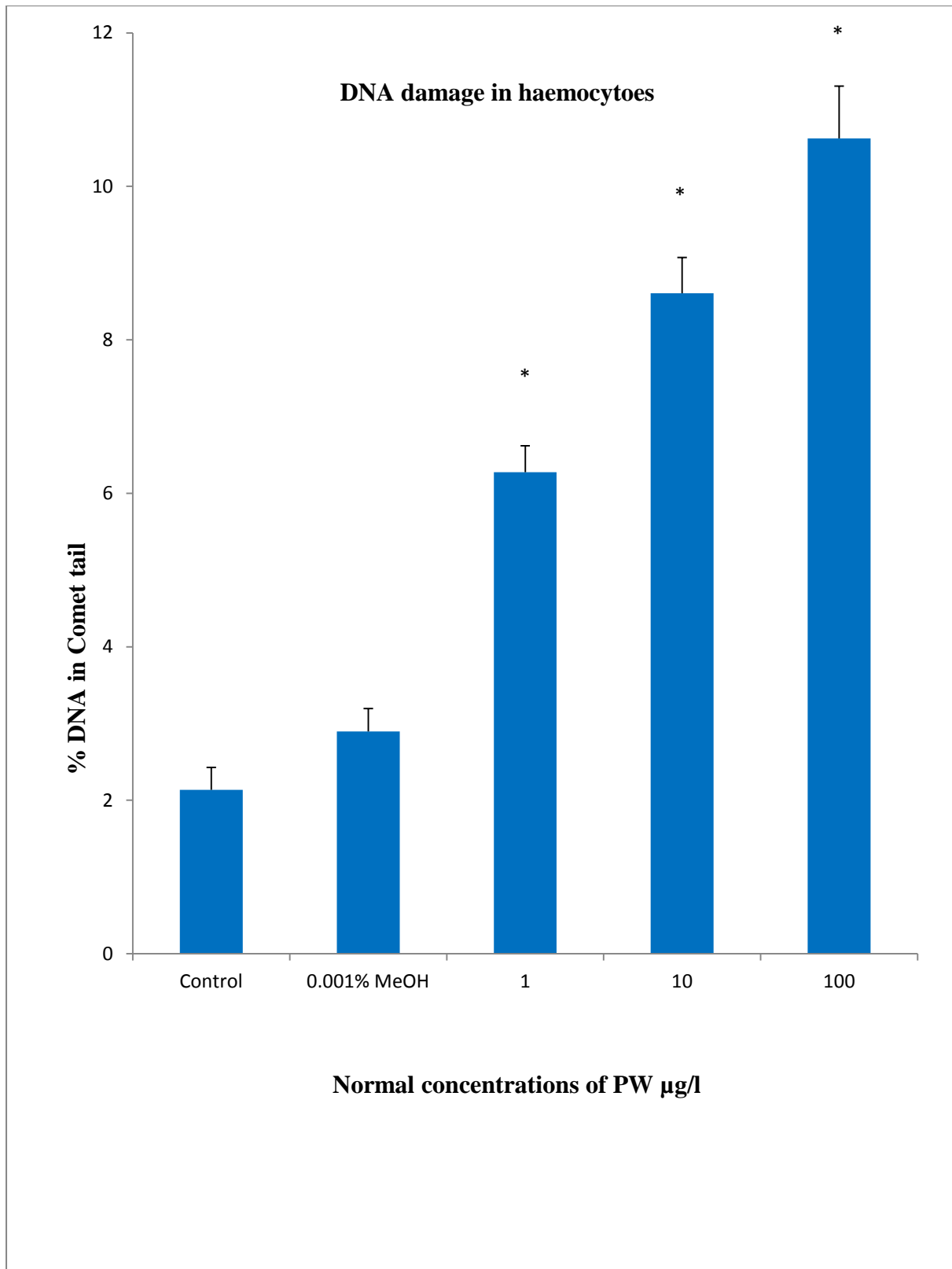


Figure 6-1 Average (\pm SD; n=5) percent of DNA in the tail for mussel haemocytes exposed to PW. (*): a significant difference between groups and from the control (one-way ANOVA; $P < 0.01$).

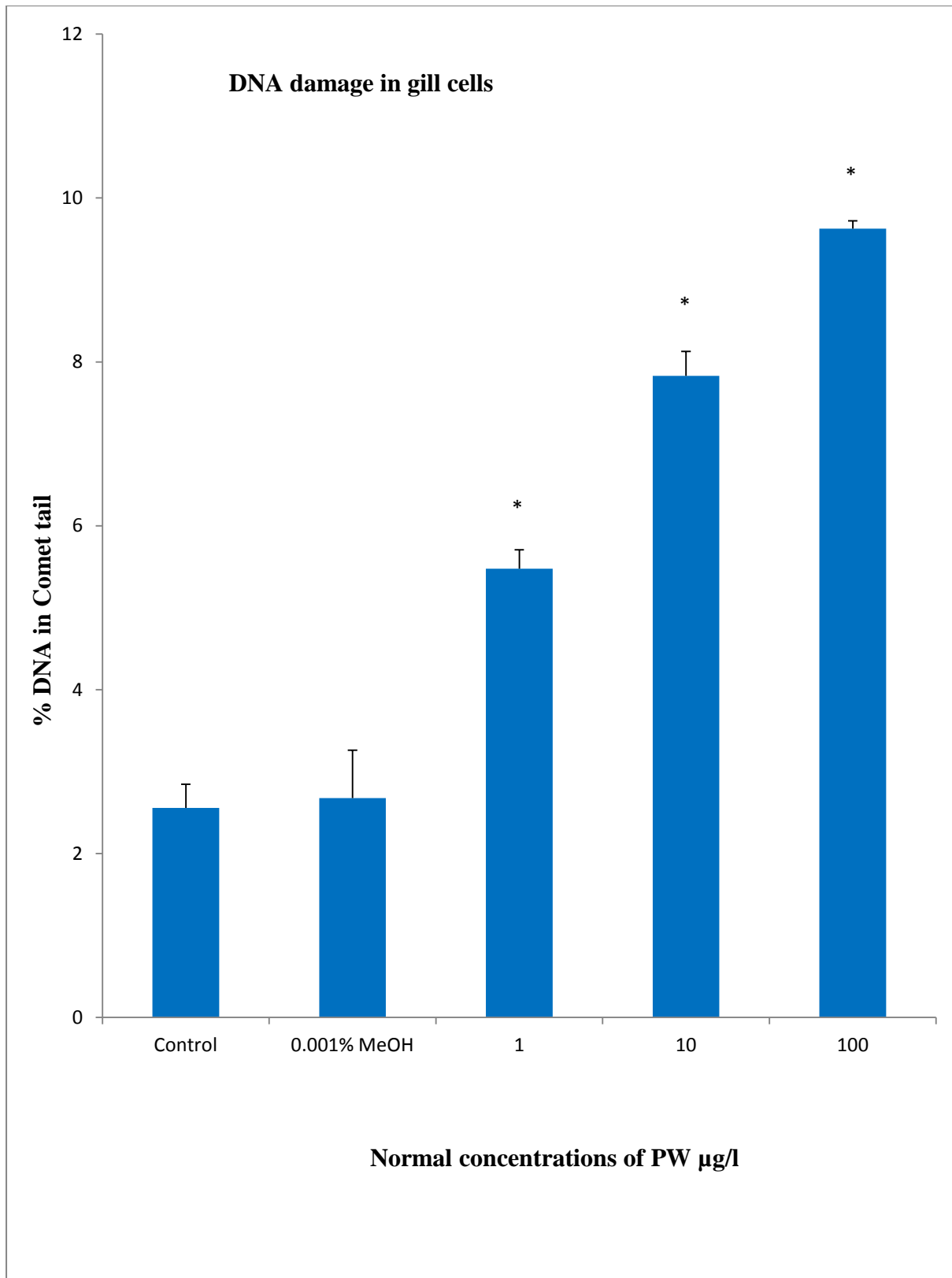


Figure 6-2 Average (\pm SD; n=5) percentage of DNA in the tail in the gill cells of mussels exposed to PW (*): a significant difference between groups and from the control group (one-way ANOVA; $P < 0.001$).

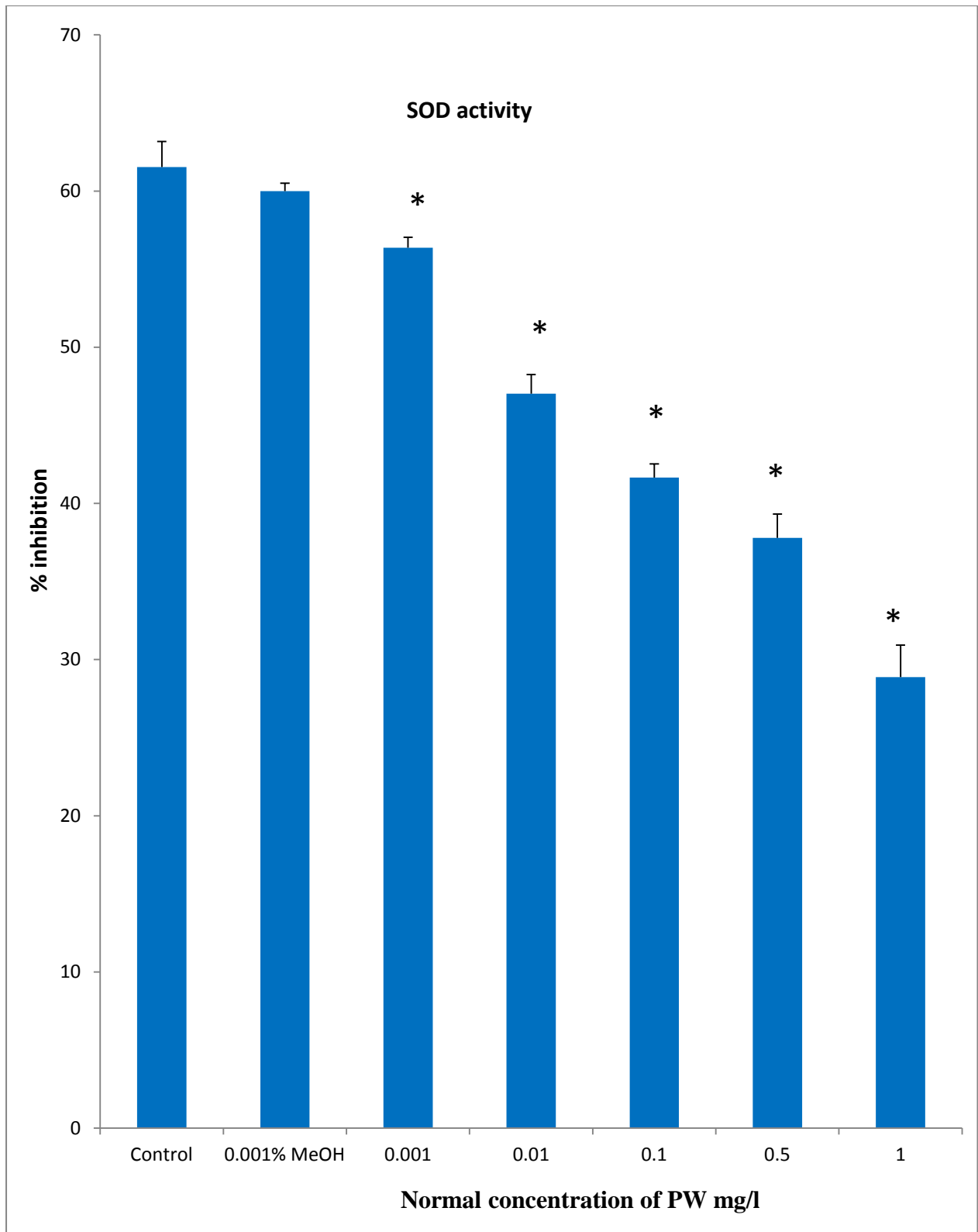


Figure 6-3 Average (\pm SD; n=5) percent inhibition of SOD activity in the gill tissue homogenates of mussels exposed to PW. (*): a significant increase between these groups compared to the control group (one-way ANOVA; $P < 0.001$).

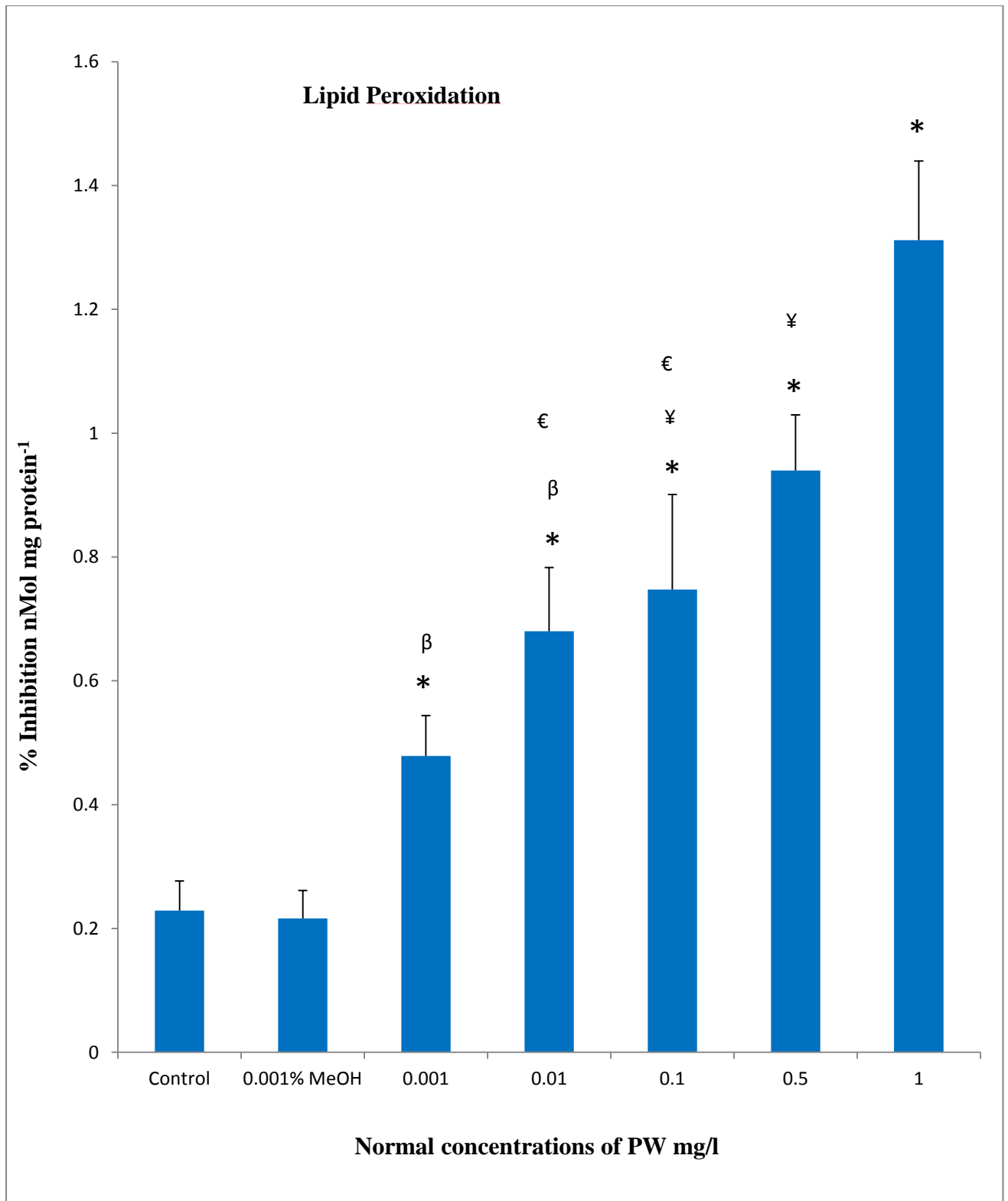


Figure 6-4 Average (\pm SD; n=5) TBARS in the gill cells of mussels exposed to PW. (*): significant increase between all concentrations compared to control group. (¥, € and β): no significant difference between these groups but significant difference from the control group (one-way ANOVA; $p \leq 0.05$).

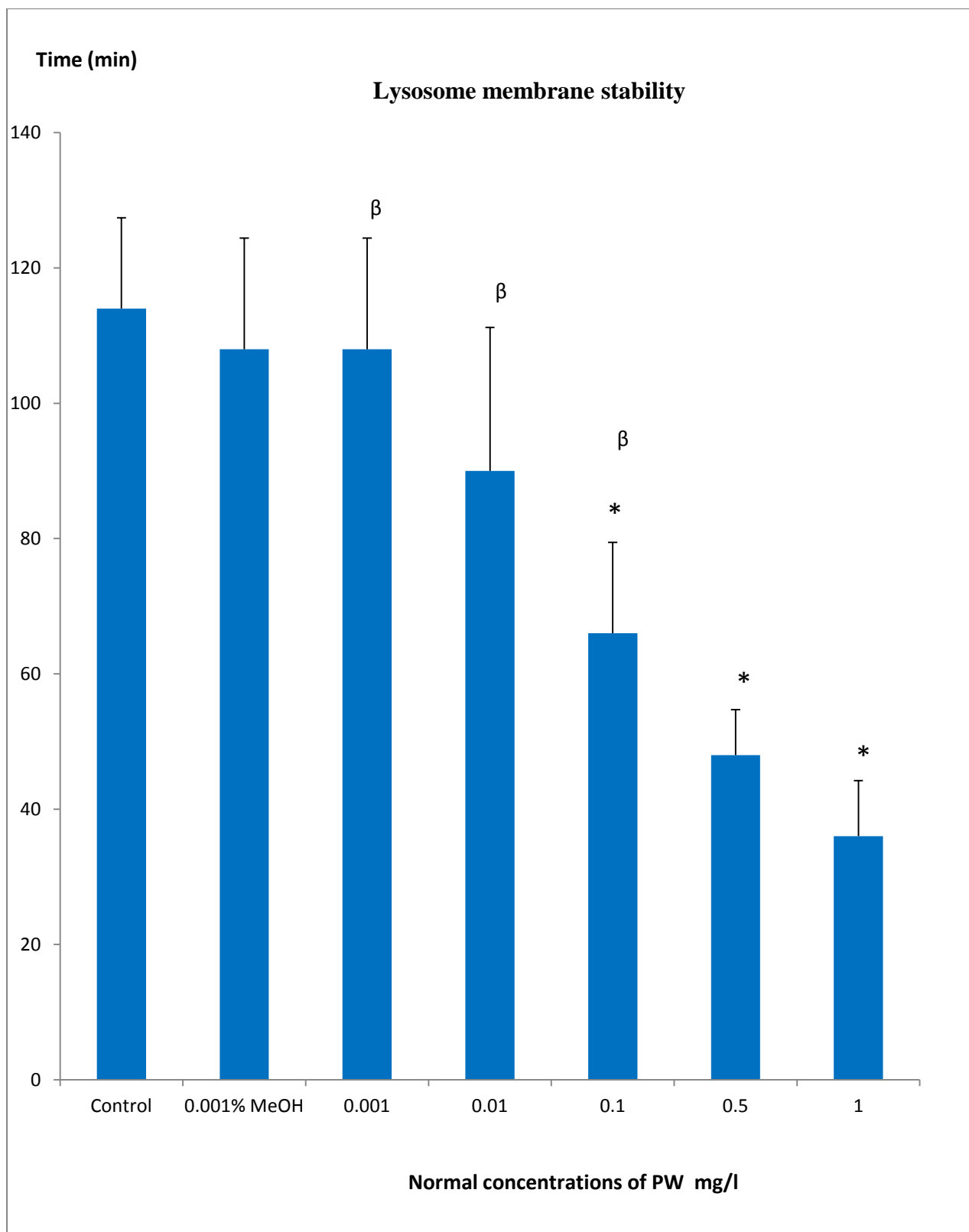


Figure 6-5 Average (\pm SD; $n=5$) lysosome membrane stability in mussel haemocytes exposed to PW. (*): significant decrease in stability compared to the control group but no significant difference between these groups. (β): no significant difference between these groups (one-way ANOVA; $P<0.001$) compared to the control group and each other.

Bioaccumulation and bio-concentrations of constituent contaminants in PW were investigated in mussels' soft tissues using SPE, followed by ESI MS/MS quantitative analysis and UV chromatography. ESI MS/MS and UV chromatography were used to investigate the amount of QUATs, imidazoline and PEs in PW in the tissue samples. The results showed no concentrations either of QUATs, imidazoline and PEs in the samples or in the PW samples (Figure 6-6). The common trace metals in PW, Zn, Hg, Cu, Cd and Ba (Stephenson, 1992), were present in undiluted PW and there was bioaccumulation of these metals in the soft tissues of the mussels. The concentrations of Zn, Hg, Cu, Cd and Ba in 1 g of whole soft tissues is shown in Tables 6-1. The tissue concentrations of Cd, a highly genotoxic metal, following exposure at nominal concentrations of 0.001, 0.01, 0.1, 0.5 and 1 mg/L of PW resulted in tissue concentrations of 0.02, 0.023, 0.04, 0.06 and 0.1 mg/kg, respectively. Among the other metals found in the PW sample, Barium was in the highest concentrations found in PW water samples, at 113.83 mg/L. The concentrations of Barium in tissues after exposure to normal concentrations of 0.001, 0.01, 0.1, 0.5 and 1 mg/L of PW were 4.12, 4.2, 4.7, 5.1 and 5.6 mg/kg, respectively. The basic parameters such as pH and salinity were measured and pH found to be at a neutral level and salinity at 60‰ (Table 6-2).

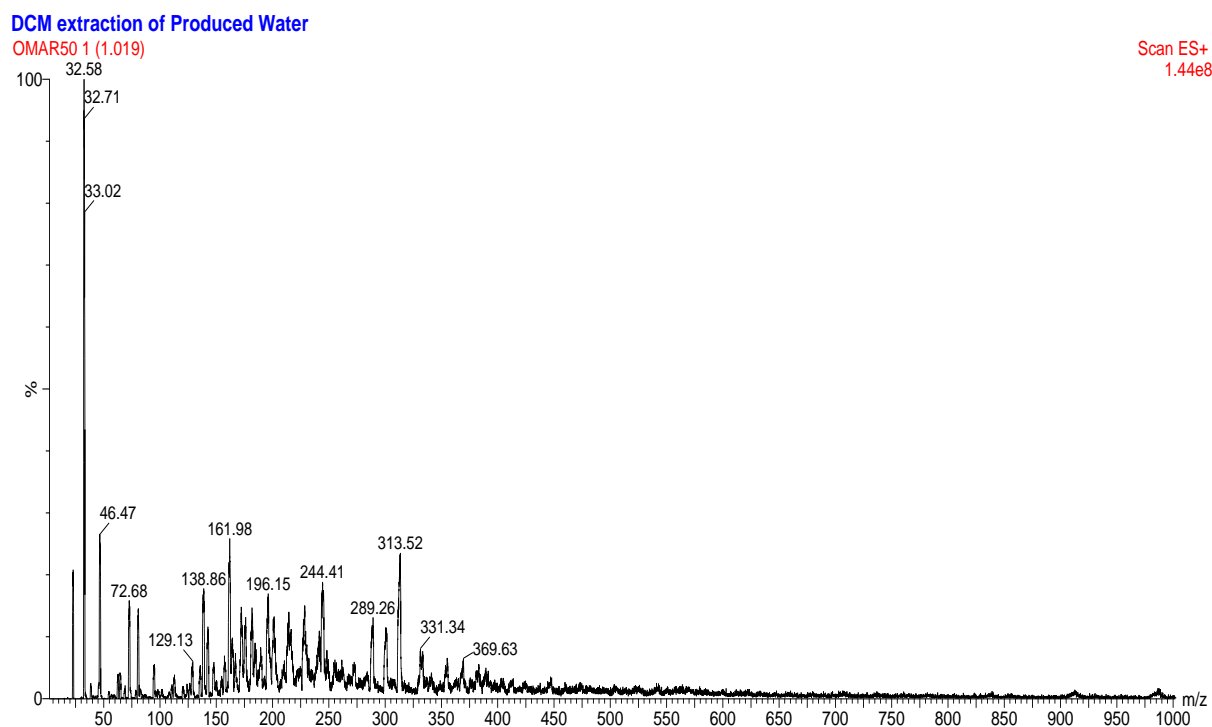


Figure 6-6 Spectrum of QUATs, imidazoline and PEs in a PW sample

Table 6-1 Concentrations (mg/kg) of common trace metals in 1 g of mussel's whole soft tissues after 24 hrs of exposure to PW

Concentrations in 1 g of whole tissues mg/kg	Control	0.001	0.01	0.1	0.5	1
Zn	0.2	0.64	0.6	0.8	0.8	0.8
Hg	0.3	0.64	0.8	0.8	0.9	1.1
Cu	0.05	0.12	0.1	0.2	0.2	0.2
Cd	0.005	0.02	0.023	0.04	0.06	0.1
Ba	2.8	4.12	4.2	4.7	5.1	5.6

Table 6-2 Parameters (pH and salinity) of undiluted PW

Parameters	PW sample
pH	7.45
Salinity (‰)	60
Observations	There are some suspended residues and a thin layer of oil on the top of the bottle

Discussion

PW is a complex mixture containing many potential toxicants; including major ions, SO_4^{2-} , Ca^{2+} , Mg^{2+} , K^+ , Cl^- and Na^+ and compounds such as hydrogen sulphide, ammonia, petroleum hydrocarbons (PH), BTEX (benzene, ethylbenzene, xylenes and toluene), PAHs such as aphanthalenes, phenols as well as Cd, Zn, Hg, Cu, Ba and other trace metals and production chemicals such as CIs as QUATs and imidazoline, SIs as PEs and other additive chemical compounds (Fisher et al., 2010; Neff et al., 2011a).

Aquatic organisms near the discharge point are exposed daily to these effluents, and so the possibility of accumulation of the effluents in their tissues and corresponding biological responses is high (Neff et al., 2011a). Utvik (1999) suggested that aromatic compounds are the most important contributors to toxicity. In addition, PW contains many chemical compounds that contribute to toxicity, such as oil-soluble and water-soluble production

chemicals. However, according to Farmen et al. (2010) water-soluble organic compounds of PW were major contributors to oxidative stress and cytotoxicity, and such effects are not correlated to the components of total oil in PW. In the present study, mussels exposed to PW from the Brae Alpha platform displayed genotoxic and other biological responses, consistent with oxidative stress in haemocytes and gill cells, even at the lowest dilution screened, 0.001 mg/L.

In addition, a previous study by Stromgren (1994) exposed four organisms, representing different trophic levels and biota, such as (*Abra alba*, *Skeletonema*, *Crassostrea gigas* embryos and *M. edulis*), to PW from three different oilfields in the North Sea and reported that components of PW caused toxic effects on all species. The EC₅₀ values were applied and *M. edulis* was confirmed to be more sensitive than the other test species to the toxicants present in the PW, such as AHPs, QUATs, imidazoline, PEs and other organic compounds, for all fields, and it is suggested that this is probably related to the high filtering capacity of *M. edulis*. In addition, a study by Stagg (1996) examined three marine species and measured the acute toxicity of PW from four oil production platforms, using these species, and confirmed PW to be acutely toxic to all species with LC₅₀ between 30 and 0.6%, depending on the animal used in the test. The genotoxicity of pollutants from the petroleum industry, through the generation of free radicals, has previously been investigated in mussels (Baršienė et al., 2006; Gorbi et al., 2008; Hylland et al., 2008; Rybakovas et al., 2009; Sundt et al., 2011). Essentially, the production of free radicals in higher organisms and the maintenance of “redox homeostasis” is regulated, to establish the physiological health of organisms (Ames et al., 1993). However, during these metabolic processes of the body, a small proportion (2-3%) of the free radicals can escape the protective shield of the antioxidant mechanisms, resulting in oxidative damage to cellular components. Excessive ROS can result in damage to those macromolecules that are important for cell structure and function (Betteridge, 2000).

As is already known, overproduction of ROS beyond the capacity of a mussel or any marine organism to reduce these reactive species can cause damage to DNA, lipids and proteins (Lesser, 2006). Therefore, these reaction species may be predicted to cause lipid, protein and DNA damage in both haemocytes and gill cells of mussels *M. edulis*, even at the lowest concentrations, through SSB and cross-linking to proteins.

The results of the comet assay in the present study showed significant DNA damage in both haemocytes and gill cells. The comet tails from control up to 0.1 mg/L of PW in haemocytes

were between (2.1±9.6%) and in gill cells were between (2.3±10) respectively. Therefore, the percentages of DNA in the comet tail for haemocytes and gill cells were similar (Figure 6-1 and 6-2); gill cells also contain large numbers of haemocytes (Burge et al., 2007). These results are comparable to those of a previous study: Rank and Jensen (2003), found the comet tails in DNA for haemocytes and gill cells were very similar after exposure to 3.3 mg/L of methyl methansulphonate. These results may be because PW contains several complex chemical compounds and high concentrations of toxicants, leading to more effect on the comet tail in both haemocytes and gill cells (Rank and Jensen, 2003).

In the present study, the SOD activities were increased and the inhibitions of SOD activity in the control group and 1 mg/L of PW were between (25.8±30.02 and 60.6±64.4,% inhibition, Figure 3-5, respectively). In addition, the results show that increased lipid peroxidation and the higher TBARS level occurred at concentrations of 1 mg/L (1.31 nmol mg protein⁻¹) compared to the control group (0,22 nmol mg protein⁻¹, Figure 6-4).

Furthermore, the results show that concentrations of 0.1 mg/L PW cause damage to the lysosomal membrane stability (Figure 6-5). Moreover, the NNR retention time of the treatment groups for mussels *M. edulis* exposed to concentrations from the lowest 0.001 to the highest (1 mg/L) of PW were between 114 ± 36 minutes. These results are comparable with those from previous studies (Gorbi et al., 2008; Hannam et al., 2010a; Hannam et al., 2010b; Hannam et al., 2009). As a result, the effects induced by PW components in aquatic organisms can still be observed several kilometres away from the platform in the North Sea (Hylland et al., 2008). In the area surrounding the PW discharge point it has been confirmed that there is a modulation of cellular immunity (Hannam et al., 2009). Mussels *M. edulis* were also exposed to oil or its components and suffered genotoxic and cytotoxic effects after 1, 2, 4 and 8 days (Baršienė et al., 2010). PW components such as PH and PAHs can trigger overproduction of free radicals which can readily bind to DNA leading to DNA strand damage. Furthermore, these reactive radicals can generate ROS, which can cause damage to the DNA and cell membranes, resulting in oxidative stress in aquatic organisms. Some previous studies also confirm that ROS can induce a number of lesions in DNA, causing many genetic effects such as deletions, mutations and susceptibility to oxidation, resulting in SSB, cross linking to proteins and degradation (Imlay and Linn, 1988; Imlay, 2003).

According to Sundt et al. (2011), bioaccumulations of PAH increased in mussel soft tissue even at the lowest exposure dose. In the present study, however, following exposure to PW, the bioaccumulation of organic chemical compounds such as QUATs, imidazoline, and PEs was not confirmed, although Gagliardi and Grigson (2003) found a high concentration ranging between 10-30 mg/L of these chemicals in a PW sample taken from the same platform, but at a different time. It is possible that the sample of PW used in the present study did not contain these compounds or contained concentrations below the detection limits (lower than 0.1 mg/L) of the available analytical techniques.

It is also possible that, owing to the sticky nature as QUATs and imidazoline when dissolved in water as PEs (Jones, 1988), and some of other production chemical compounds, that they may have adhered to the tanks as the PW had been moved from tank to tank before it reached the laboratory.

In the present study, heavy metals such as Cd, Cu, Zn, Hg and Ba, which are typically present in PW with high concentrations (Neff et al., 2011a), were found to have accumulated in the entire soft tissues of the mussels *M. edulis*. Cd is a very toxic metal and was found in both the tissues and the PW sample in the present study; it is known to cause SSB in DNA (Hartl, 2010). The heavy metals (Hg, Cd and Cr) bind to DNA and can directly cause damage; producing SSB, DNA-DNA and DNA-protein crosslinks, as well as causing cells to produce ROS (Lee and Steinert, 2003). Sea urchin embryos exposed to Cr had DNA-protein crosslinks (Lee and Steinert, 2003). In the present study trace metals were found in high concentrations and measured in both tissues and the PW sample (Table 1-6).

The salinity of PW is usually higher than seawater (Neff, 2002). In the present study the pH and salinity were measured and pH was at a neutral level although salinity was 60%. The salinity result is comparable to that found in a previous study (Neff, 2011a). As known, high salinity can cause damage to DNA and lysosomal membranes (Neff, 2002).

In the present study, the potential effects of PW on mussels *M. edulis* was investigated after exposure for 24 hrs to different concentrations of PW, ranging from 0.001, 0.01, 0.1, 0.5 to 1 mg/L.

The results of the study confirm that PW caused damage to DNA at the lowest concentrations, and inhibited the SOD enzyme and lipid peroxidation causing considerable damage to the lysosomal membrane in both haemocytes and gill cells. The findings from the

study also confirmed the presence of trace metals in PW and the whole soft tissues of marine mussels, after exposure conditions, but did not demonstrate the presence of any concentration of QUATs, imidazoline and PEs in PW.

PW is the major waste discharged from the oil and gas industry, and as this study confirms, the consequences can be genotoxicity, cytotoxicity and oxidative stress. Moreover, the toxic compounds or elements in the PW can accumulate in aquatic organisms affecting their environment.

General discussion

The oil and gas industry widely uses many traditional components in oilfield CIs and SIs which can accumulate, concentrate or cause toxicity in the marine environment, potentially affecting and contaminating marine organisms. Effective oilfield CIs and SIs, such as QUATs, imidazoline and PEs, are generated and discharged by the oil and gas industries into the marine environment in the North Sea. PW contains multiple complex chemical compounds, including very small dispersed oil droplets between 1-10 μm (Neff et al., 2011a), high concentrations (5-30 mg/L) of organic compounds, such as QUATs, imidazoline and PEs (Grigson and Gagliardi, 2003), 20–30 mg/L of ammoniacal nitrogen and many inorganic components (Somerville et al., 1987). Neff et al., (2011a) report that concentrations of other chemicals in PW, such as total PAHs and other higher molecular weight compounds typically range from 0.04 up to 3 mg/L. In a study of the concentrations in marine sediments of chemical compounds added to the topside during oil and gas production processes, QUATs concentrations were found to range from (0.74 to 10.84 ng/kg) around two North Sea oil platforms (Grigson et al., 2000; Neff et al., 2006). In PW, the concentrations of CIs ranged from 25 up to 100 mg/L, and those of SIs ranged from 3 up to 10 mg/L, before discharge into the marine environment. The concentrations of QUATs, PEs and imidazoline in PW and the nearby environment have been found to range from 10 to 30, 0.1 to 5 and 0.3 to 12 mg/L and 0.74 to 10.84 ng/kg and 0.48 to 11 $\mu\text{g/kg}$ respectively.

Regular surveys of benthic communities in the vicinity of platforms have monitored the chronic toxic effect of oil production activity compounds (Gray et al. 1990; Stagg, 1996; Stagg, 2011). Particularly, the components of PW, such as QUATs, imidazoline and PEs have been reported to cause chronic toxic effect on marine organisms (Stagg, 1996; Sastri, 2011). For example, in a study, by Caliani et al. (2009), mosquito fish (*Gambusia affinis*) were exposed to concentrations of 50% PW discharged from an Italian on-shore oil plant for 5 days, and 10% PW for 30 days. After 8 days of exposure, micro-nucleated cells were found in the females and after 30 days, DNA damage, expressed as SSB, was observed in fish of both genders.

The relationship between actual adverse impact and observed effect is that all of them indicate exposure of the organisms to the chemical compounds. However, although observed effects can not give any information about the degree of the adverse effect, observation of the biological responses

indicates the presence of a xenobiotic substance or its metabolites which can be measured within a part of an organism in the absence of adverse effects.

Over the last two decades, many previous studies have tested and validated a number of biomarker techniques for their suitability to investigate and detect biological responses as indicators of chemical stress at different levels in biological organisation (Akcha et al., 2004; Bolognesi et al., 2004; Bolognesi et al., 1999; Coughlan et al., 2002; de Lafontaine et al., 2000; Gutteridge, 1995; Hartl, 2010; Hartl et al., 2010; Lüchmann et al., 2011; Pérez-Cadahía et al., 2004; Sundt et al., 2012; Sureda et al., 2011; Taban et al., 2004; Torres et al., 2002; Wedderburn et al., 1998; Zorita et al., 2006). Marine mussels' haemocytes and gill cells have been extensively used for genotoxicological studies (Jha et al., 2005; Rank et al., 2007). In the biomarker assays, these cells have proved to be sensitive and reliable under laboratory conditions, following exposure to indirectly acting genotoxins.

Marine mussels have been widely used as indicator organisms, due to their ability to rapidly take up chemical compounds, and also because they are sedentary organisms, long-lived, easily identified and sampled, reasonably abundant and available throughout the year, as well as being tolerant of natural environmental fluctuations and pollution (Lee et al., 1972; Stankovic et al., 2012). A study by Stagg, (1996) examined three species, *S. costata*, *T. battagliai* and *A. tonsa*, in laboratory toxicity tests and the most sensitive species used in the study was found to be *A. tonsa* (48 h LC₅₀ 2 – 0.6 %). Additionally, a study by Stromgren (1994) used four organisms, representing different trophic levels and biota (*Abra alba*, *Skeletonema*, *Crassostrea gigas* embryos and *M. edulis*) in laboratory toxicity tests and recorded that the EC₅₀ values confirmed that *M. edulis* is more sensitive than the other test species to the toxicants found in the PW, such as AHPs, QUATs, imidazoline, PEs and other organic compounds for all fields. Consequently, many studies have used mussels as the indicator organism to identify genotoxicity and oxidative stress (Akcha et al., 2004; Almeida et al., 2005; Baussant et al., 2011; Binelli et al., 2009; Faria et al., 2009; Hannam et al., 2009; Lemiere et al., 2005a; Nahrgang et al., 2012; Nicholson and Lam, 2005; Pisanelli et al., 2009; Shaw et al., 2011; Sundt et al., 2011; Turja et al., 2012)

In the present study, the mussel *M. edulis* was used as an indicator organism and biomarker techniques were employed to detect the biological effects of oilfield chemicals, in particular CIs. The mussels *M. edulis* used in this study were of comparable size (6.5 cm ± 0.5) and collected from a well

characterised site (clean area) in the Firth of Forth which flows into the North Sea; it is located between Fife to the north and West Lothian, the city of Edinburgh and East Lothian to the south minimising inter-individual variability and assuring the necessary resolution required to identify the subtle differences between treatment groups (Singh and Hartl, 2012). In this study, *M. edulis* were exposed to QUATs, imidazoline, PEs and PW at concentrations ranging between 0.001 and 1 mg/L and multi-biomarkers were applied to investigate genotoxicity, cytotoxicity and oxidative stress in haemocytes and gill cells after 24 hours of exposure. QUATs, imidazoline, PEs and the components of PW were all found to cause damage to DNA and affected the genetic information in both haemocytes and gill cells of *M. edulis*. These results were not dissimilar to the previous study by Stagg, (1996) that confirmed the components of PW were acutely toxic to marine organisms and Sastri's (2011) finding that LC₅₀ toxicity of imidazoline was at concentration 10 mg/L at 24 hours. In addition, LC₅₀ toxicity of QUATs to species of invertebrates was at concentration 1 mg/L at 24 hours (Cooper, 1988). The results also show that *M. edulis* take up the QUATs, imidazoline and heavy metals present in the PW sample. This may confirm that *M. edulis* is more sensitive to exposure to chemical compounds and is able to take up these compounds, which is probably related to the high filtering capacity of this species.

In the present study, oxidative stress was also confirmed by observed inhibition of SOD enzyme and lipid peroxidation. These effects are comparable to previous studies by Hylland et al., (2008) who reported that the components of PW caused biological responses on studies in 2001, 2003 and 2004 and Turja et al., (2012) who found that soft tissue growth was reduced and that there was increased oxidative stress in *M. edulis* caged at four sites in the vicinity of an oil platform.

Lysosome membrane damage was also observed when *M. edulis* was exposed to QUATs, imidazoline, PEs and PW for 24 hrs. These results show similar findings to previous studies by Famadas (2009), which found that two PW samples collected from different oil platforms at Brae Alpha in the North Sea showed acute and sub-lethal effects and lysosomal membrane damage on the mussels *M. edulis*, and by Turja et al. (2012) reporting that lysosomal membrane instability was observed in *M. edulis* at highly polluted sites. In addition, significant effects on antioxidant enzymes and lysosomal stability were reported from *M. edulis* caged in the area surrounding a platform in the

Adriatic Sea (Gorbi et al. 2008). Therefore, it seems likely that aquatic organisms around the PW discharge point may be exposed to these effluents and possibly accumulate PAH, APs, trace metals, QUATs, imidazoline and PEs through sediments, their food, or ambient water. Thus, in the present study, accumulation of QUATs, imidazoline and heavy metals present in PW was found in mussels *M. edulis* after 24 hours of exposure and a study by Neff et al. (2011b) has also reported that PAHs were accumulated in mussels *M. edulis*.

There are many reasons why the components of PW cause toxic effects on marine organisms, such as digestion of particulate material and absorption of water-soluble components throughout their surface epithelia (e.g. gills and body surface) and oral ingestion. In addition, the reasons for these biological responses may be due to oilfield chemical compounds generating excessive ROS which can result in damage to the macromolecules (Betteridge, 2000).

These biological responses may occur because QUATs, imidazoline and PEs from PW are found in both water and other parts of the marine environment, such as sediment, and thus available to the total ecosystem (Grigson et al., 2000; Grigson and Gagliardi, 2003; Martínez-Carballo et al., 2007b; Tezel and Pavlostathis, 2011).

DNA damage

In many marine organisms, DNA damage has been associated with abnormal development, reduced growth and the reduced survival of embryos, larvae and adults (Lee and Steinert, 2003; Steinert, 1999). In the present study, QUATs, imidazoline, PEs and PW generated a high percentage of DNA in the comet tail in both mussel haemocytes and gill cells; gill tissues are contain large numbers of haemocytes (Burge et al., 2007). QUATs can cause significant DNA damage in haemocytes and gill cells of exposed mussels. The sensitivity of mussels towards QUATs may cause alteration in the genetic information which will increase the percentage in comet tail. In the present study, QUATs, imidazoline, PEs and PW registered in both haemocytes and gill cells after 24 hours of exposure. The comet tails from control up to 0.1 mg/L of QUATs in haemocytes were between (3.86±14.2%) and in gill cells were between (4.1±13.1%). However, the comet tails in DNA after exposure to imidazoline in haemocytes were between (1.5±9.8%) and in gill cells were between (2.2±11.9%). The highest comet tails were registered in QUATs haemocytes and QUATs gill cells, and were higher than then

imidazoline gill cells in the comet tail of DNA. However, the comet tail of DNA on gill cells after exposure to PEs and PW were between (2.3±16.0%) and (2.3±10%) respectively. The comet tails of DNA on PEs gill cells was higher than QUATs and imidazoline and also PW in gill cells and QUATs haemocytes was higher than for imidazoline and PW haemocytes (2.1±9.6%), although it was a similar level when compared with PEs haemocytes (2.1±14.5%). The lowest comet tails in the DNA were registered on PW and imidazoline as (2.1±9.6%) and (1.5±9.8%) respectively; however, all reported that the level of DNA damage significantly increased when compared to the control groups. All these differences in the percentage of the comet tail of the DNA may be due to many factors, such as the types and the toxicity of chemical compounds and the characteristic functions of mussels *M. edulis* such as habitat, lifespan, the body size and the physiology of the animals (Bayne, 1976).

In the present study, a significant increase in damage in haemocytes from concentrations of 0.001 up to 1 mg/L QUATs was found, upon comparison with the control group. However, there was no further significant increase from 0.1 up to 1 mg/L QUATs, suggesting that above 0.1 mg/L QUATs does not affect DNA in a dose-dependent manner. Accordingly, even extremely low concentrations of QUATs caused DNA lesion in both haemocytes and gill cells of mussels. It is therefore plausible to suggest that chronic exposure to QUATs, which is likely in the case of sessile mussels in the vicinity of a production platform, could lead to cell death and higher order pathological effects. Cooper (1988) reported that QUATs caused toxicity to various species of invertebrates at LC₅₀= 1 mg/L at 24 hours.

Imidazoline and PEs also cause significant DNA damage at the lowest concentrations, 0.001 mg/L, in both haemocytes and gill cells. This is because PW contains harmful complex chemical compounds and because of the ability of mussels to uptake substances rapidly, for example mineral oil (Lee et al., 1972). This increased damage to DNA indicates a potential for these chemical compounds, including treated PW, to result in genetic toxicity; as even some low molecular weight compounds, such as mono and diaromatics, rather than PAHs, result in damage to DNA (Hamoutene et al., 2002).

In this study, it was confirmed that imidazoline and PEs caused a higher percentage of DNA in the comet tail in mussel's gill cells than in haemocytes. However, QUATs caused a higher percentage of DNA in the comet tail in the mussel haemocytes than in the gill cells. The results of imidazoline experiments agree with previous findings by other authors that suggest the percentage of DNA in a

comet tail is typically higher in gill cells than in haemocytes (Jha et al., 2005; Rank and Jensen, 2003; Rank et al., 2005; Rank et al., 2007; Taban et al., 2004; Tran et al., 2007). The reason for this is that gills are in direct contact with the medium, including particle- or colloid-bound contaminants, and there are higher concentrations of O₂ in gill cells (Singh and Hartl, 2012). In addition, there are many differences in the DNA repair activities and enzymatic activities of gill cells.

Abiotic factors, such as temperature, pH and salinity can often cause higher cell turnover rates in haemocytes. This is because haemocytes need to be recruited rapidly in response to environmental stress (Jha et al., 2005; Singh and Hartl, 2012). However, some previous studies have shown similar levels of DNA damage in haemocytes and gill cells (Akcha et al., 2004; Coughlan et al., 2002; Hartl et al., 2004). The present study confirmed that QUATs, imidazoline, PEs and PW all caused responses and DNA lesions in both the haemocytes and gill cells of mussels at different levels of the DNA. DNA damage in haemocytes mirrors such damage in gill cells at cancer development risk (Halliwell, 2000).

Hylland et al. (2008) and Baussant et al. (2011) found that PW caused biomarker responses and DNA lesions in tissue cells of mussels caged along a transect from an offshore oil producing platform, and a considerable effect on the immune function was reported in mussels in the laboratory by Hannam et al. (2009). Crude oil contains approximately 3 mg/L of PAHs in PW Lemiere et al. (2005b) and Neff et al. (2011a). Taban et al. (2004) found that 0.06 mg/L oil caused a high percentage of DNA in the comet tail in mussel haemocytes. In addition, a significant increase in DNA damage after exposure to PAHs (Lee et al., 2011). These previous studies are comparable to the present study, which confirmed that QUATs, imidazoline, PEs and PW inflict damage on DNA, even at the lowest concentrations of 0.001mg/L.

Lysosomal membrane damage

The principle of the NRR assay is based on the fact that only lysosomes in healthy cells take up and retain the vital of neutral red dye. The damage to lysosomal membranes caused by the impact of the test substances, of natural stressors and of the cytotoxic nature of NR decreases the NRR times, by inducing the leaking of lysosomal components (Dailianis et al., 2003).

The lysosomal membrane stability at the sub-cellular level is widely used as particular target for identification of the toxic effects of environmental contaminants (Moore, 1990); further, pathological alterations in lysosomes have been particularly useful in the identification of adverse environmental impacts on aquatic organisms (Moore et al., 1996). NRR is widely applied in haemocytes of mussels *M. edulis* and in the digestive cells of oyster (Dailianis et al., 2003; Lowe and Pipe, 1994; Lowe et al., 1995b; Martinez-Gomez et al., 2008).

According to Hauton, et al., (1998) natural factors influence the NRR assay, with higher NRR times being measured at intermediate temperature and high salinity. In clams and mussels, *M. edulis*, NNR time has been found to decrease following exposure to metals (Matozzo et al., 2001) and to organic chemicals (Lowe et al., 1995a) or PW (Brooks et al., 2011; Hannam et al., 2009; Hylland et al., 2008).

A normal range of retention time in unpolluted areas is considered as being between 90-180 minutes for *M. edulis* (Hylland et al., 2008). The retention time for *M. edulis* collected from the vicinity of industrial and urban areas was 20 and 100 minutes, respectively, depending on the degree of anthropogenic contamination at sample sites (Castro et al., 2004). However, the retention time for mussels *M. edulis* caged from the discharge point of PW near an oil platform were between 20 and 60 minutes (Hylland et al., 2008).

In the present study, the average retention time of the control and control with methanol groups for mussels *M. edulis* exposed to QUATs, imidazoline, PEs and PW were between 108 and 102 minutes, 117 and 108 minutes, 114 and 108 minutes and 114 and 108 minutes, respectively. These results are within the normal range for unexposed mussels and comparable to previous studies (Brooks et al., 2011; Famadas, 2009; Hannam et al., 2009; Hylland et al., 2008; Sundt et al., 2011).

In the present study the retention times of the treatment groups for mussels *M. edulis* exposed to concentrations from the lowest 0.001 up to the highest 1 mg/L of QUATs, imidazoline, PEs and PW were between 96 ± 33 minutes, 105 ± 33 minutes, 108 ± 33 minutes and 108 ± 36 minutes, respectively. When comparing the effects of QUATs, imidazoline, PEs and PW, the haemocytes exposed to QUATs, imidazoline and PEs leaked faster than PW in the highest concentration of 1 mg/L: for example QUATs, imidazoline and PEs presented 50% leakage of haemocytes in 33 minutes, while the PW sample required a time of 36 minutes. However, lysosome membrane damage

in haemocytes was also observed at concentrations ≥ 0.1 mg/L of QUATs, imidazoline and PW and PEs ≥ 0.5 mg/L. Therefore, reduced lysosomal stability was confirmed in mussel haemocytes, presumably because one of the characteristic pathological alterations is the decreased integrity of the lysosomal membrane (Moore 1988) and continuous overload of lysosomal capacity could also reduce cell viability over the period of exposure (Hannam et al., 2009).

In addition, it has previously been found in the mussel *M. edulis* haemocytes, that lysosomes sequester, accumulate and metabolise a range of organic xenobiotics and metals (Moore, 1985). Reduced lysosomal stability has also been observed in mussel haemocytes when they have been exposed to crude oil, (Baussant et al., 2009; Fernley et al., 2000). In addition, mussels were exposed to two different PWs collected from offshore platforms in North Sea and also suffered damage to the lysosomal membrane (Famadas, 2009; Hannam et al., 2009) compared to clams (Martins et al., 2005) exposed to petroleum related hydrocarbons in sediments, to scallops exposed to oil, (Baussant et al., 2009) and to adult shrimps exposed to oil-water dispersions (Bechmann et al., 2010). Thus, pollutants are capable of causing direct damage to the cell membrane (Hannam et al., 2009). The results in the present study are comparable with those found in previous studies (Baussant et al., 2009; Brooks et al., 2011; Famadas, 2009; Hannam et al., 2009; Sundt et al., 2011; Turja et al., 2012). QUATs, imidazoline and PW all therefore seem to cause severe stress in mussel haemocytes at concentrations of 0.1 mg/L and PEs at concentrations of 0.5 mg/L, at least under present experimental conditions.

In the present study show that indication of NRR assay may constitute a good marker of lysosomal damage, as it is sensitive to pollution gradient, precise, and minimally affected by variation of natural factors. Additionally, the results of previous studies concerning Lysosomal membrane stability evaluation of the digestive gland and the results further enhance the use of lysosomal damage evaluation as a good biomonitoring tool (Dailianis et al., 2003).

Oxidative stress

Measurement of oxidative stress is crucial when identifying biological effects on marine organisms exposed to environmental contaminants. Previous studies serve to demonstrate that living organisms use free radicals and ROS, such as $O_2^{\cdot-}$, NO^{\cdot} , ROO^{\cdot} and H_2O_2 , to control their physiological processes (Dröge, 2002) and maintain “redox homeostasis” (Ames et al., 1993). ROS are important in the body and are produced during normal metabolism (Gaté et al., 1999). However, overproduction of ROS can affect the protective shield of the antioxidant mechanisms, resulting in oxidative damage to the cellular components. Thus, oxidative stress can cause damage to the nucleic acid bases in the DNA, the thiol groups in proteins and the unsaturated fatty acyl chains in the cell membranes (Ceconi et al., 2003; Halliwell, 1994). Free radicals are produced by the numerous oilfield chemical compounds as well as by different biochemical processes that take place within the body. These include molecular oxygen reduction through aerobic respiration, yielding superoxide and HO^{\cdot} ; the chemical by-products caused by oxidation of catecholamine and activation of the arachidonic acid cascade product electrons. These processes are also capable of reducing molecular oxygen to superoxide; production of superoxide and HOCL, a powerful oxidant, arises due to activated phagocytes and nitric oxide production by vascular endothelium and other cell types (Betteridge, 2000). Oxidative stress can be measured by evaluating products that react to forms of oxidative damage, such as protein oxidation, DNA oxidation, lipid peroxidation and inhibition of SOD enzyme (Betteridge, 2000). Many articles have reviewed biomarker techniques, and have provided evidence, for the indirect measurement of formation of free radicals, such as SOD to evaluate superoxide dismutase activity (Fridovich, 1989; Huang et al., 2000; McCord and Fridovich, 1988; Sun et al., 1988; Zelko et al., 2002). A review of oxidative stress in aquatic organisms by Di Giulio et al. (1989), described the general processes responsible for ROS generation in aquatic organisms and highlighted the role of some metal ions, such as chromium, copper, arsenic, mercury and pesticides (fungicides, herbicides and insecticides), along with the oil products, in the induction of oxidative stress. The impact of oil and PAH on marine mussels following the *Don Pedro* oil spill accident was also investigated, by Sureda et al. (2011).

In the present study marine mussels *M. edulis* were exposed to environmental concentration of QUATs, imidazoline, PEs and PW and SOD and TBARS assays were used to determine the oxidative stress. Oxidative stress was confirmed after exposure for 24 hours to QUATs, imidazoline, PEs and

PW. Mussels exposed to QUATs were found to have increased SOD activity at concentrations ≥ 0.5 mg/L, when compared with the control group.

The average higher TBARS level and inhibition of SOD activity were registered at concentrations (1 mg/L) of PEs and were 1.58% inhibition nmol mg protein⁻¹ and 7.81% inhibition, respectively, compared to the high TBARS level and inhibition of SOD activity of QUATs, imidazoline and PW which were between (0.89% inhibition nmol mg protein⁻¹ and 21.1% inhibition, 0.65% inhibition nmol mg protein⁻¹ and 36.7% inhibition, 1.31% inhibition nmol mg protein⁻¹ and 28.88% inhibition respectively. However, the average inhibition of SOD activity by QUATs was 21.1% inhibition; higher than that for imidazoline and PW. However, PW registered as higher than QUATs and imidazoline, using the TBARS level. Imidazoline was found to have a low effect on both TBARS level and inhibition of SOD activity and the averages were (0.65% inhibition nmol mg protein⁻¹ and 36.7% inhibition respectively. However, QUATs, imidazoline, PEs and PW all showed a significant increase in inhibition when compared to the control groups. The activities of SOD for QUATs, imidazoline, PEs and PW were higher in *M. edulis* than activities observed in the bivalve *P. Perna* and *Chamaelea galina* (Almeida et al., 2005). In addition, the SOD activities were much higher than the values reported for the horse mussel (*Modiolus*) (Lesser, 2006). The reasons for causing these differences can be possibly depend on the influence of different environmental conditions such as laboratory and seawater temperature or availability of oxygen, also may due to interspecific differences related to biological condition such as the reproductive cycles and biological rhythms.

Oilfield chemical compounds are accepted as being involved in ROS production, especially additive chemical compounds during the E&P, such as biocides and corrosion and scale inhibitors. Therefore, the inverse correlation of TBARS concentrations (Torres et al., 2002), while it has an effect on oilfield chemicals-induced oxidative stress, also increased the TBARS level and inhibited SOD activity at concentrations (1 mg/L) of PEs; as derived from Fenton's which can generate HO[•], and the mainly toxic ROS (Halliwell, 2012; Halliwell and Gutteridge, 1998; Halliwell and Gutteridge, 2007; Winston, 1991).

It is also suggested that this increase in activity is due to the fact that QUATs generate an overproduction of ROS, which, if unchecked can cause damage to DNA, proteins, lipids and other structurally important molecules. However, the structure of QUATs does not contain any O₂ atoms

that could cause oxidation in the body. It may therefore be the case that compounds are metabolised by a chemical reaction into a metabolic pathway, where they are then transformed into another chemical by a sequence of enzymes acting as catalysts (Friedrich, 1997), or by P450 enzymes which are able to interact with the cells macromolecules to initiate tumour formation (Cavalieri and Rogan, 1992), thereby generating free radicals and ROS, leading to oxidation and an increase in SOD activity (Halliwell and Gutteridge, 1998).

In the present study, it was also found that PEs increased the activity of the SOD enzyme at concentrations ≥ 0.1 mg/L in gill cells. Furthermore, imidazoline and PW had a significant impact on increased SOD activity in gill cells, even at the lowest concentrations ≥ 0.001 mg/L. These biological effects caused damage to DNA, lipids and proteins, and high SOD activity was predicted due to the production and accumulation of ROS (Valavanidis et al., 2006). In the present study, these chemical compounds were generated effects consistent with oxidative stress. SOD provides a degree of cytoprotection against free radicals by converting O_2^- generated in peroxisomes and mitochondria to H_2O_2 . Accordingly, H_2O_2 , is then further converted to H_2O and O_2 through catalysis (CAT) (Otitolaju and Olagoke, 2011).

Both lipid peroxidation and high SOD activity were also confirmed after 24 hrs of exposure to these chemical compounds. QUATs and imidazoline increased the level of lipid peroxidation in the gill cells at concentrations ≥ 0.1 mg/L, as compared to the control group. PEs increased the levels of concentrations ≥ 0.5 mg/L, compared to the control group. Furthermore, PW increased the level of lipid peroxidation even at the lowest concentrations of 0.001 mg/L. It is suggested that these chemical compounds cause overproduction of ROS, which degrades polyunsaturated lipids, resulting in production of malondialdehyde (MDA), leading to toxic stress in cells. The reduction or low level of enzymatic antioxidant defence can be correlated with an increase in oxidative damage, as reflected by the increasing level of damage in the gill cells of the mussels. The inhibition of the SOD enzyme will therefore cause high oxidative stress in gill cells exposed to QUATs, imidazoline, PEs and PW (Otitolaju and Olagoke, 2011). These results are similar to those detailed in previous studies covering exposure to different chemical compounds such as heavy metals (Torres et al., 2002) in offshore gas platform effluents (PW) (Gorbi et al., 2008).

Bioaccumulation and bio-concentration

The environmental fates of many oilfield chemicals are unknown (Bassey et al., 2009), since few techniques exist for their characterisation *in situ*. Investigations into the use of multistage ESI (MS/MS) have shown a potential for the identification and quantification of compounds in specialty oilfield chemicals (CIs and SIs) and PW (Grigson et al., 2000; McCormack et al., 2001). In the present study, marine mussels were exposed to concentrations of oilfield chemicals (QUATs, imidazoline, PEs and PW) from 0.001 up to 1 mg/L to identify bioaccumulation and bio-concentrations in whole soft tissues and in PW samples. In addition, trace metals were identified in both tissues and PW water samples. After 24 hours of exposure to these chemicals, both seawater from the tank and mussels were collected to carry out chemical analysis. Bioaccumulations were confirmed in complete soft tissues when exposed to QUATs at concentrations of 0.1 mg/L and over but could not be confirmed in the seawater sample; this is predictable as the sample it contains large amount of salts and the concentrations were very low, in this case, below the detection limit.

The present study confirmed and quantified QUATs and imidazoline in complete soft tissues of mussels. The concentrations of imidazoline in these tissues were greater than in the surrounding environment. Bioaccumulation and bio-concentration are most commonly relevant to aquatic organisms with high body lipid content, such as fish, mussels and some aquatic invertebrates (Arnot and Gobas, 2006). In the present study it is suggested that QUATs and imidazoline accumulated and concentrated in the mussels soft tissues. However, PEs was not confirmed to have accumulated and concentrated in the soft tissues of the mussels; this is because they are water-soluble. However, Farmen et al. (2010) confirmed that water-soluble and particulate organic fractions of PW from ten different platforms of North Sea oil production compounds can cause oxidative stress and cytotoxicity in fish.

Many chemical compounds found in PW are known to accumulate and concentrate in aquatic organisms, including PAH (Baussant et al., 2001; Bechmann et al., 2010; Neff et al., 2011b), Benzo(a)pyrene (Reynaud et al., 2012), hydrocarbons and trace metals (Neff et al., 2011b). Some metals, such as Cd and Pb, have long been known to accumulate within the aquatic food chain. Neff (2002) found that the concentrations of trace metals in PW after treatment are often higher than those occurring naturally in the marine ecosystem. Trace metals have the ability to bio-concentrate in aquatic organisms directly following the discharge of PW to the marine environment,

bioaccumulating and biomagnifying in the food chain, thus causing higher trophic organisms to become contaminated with high concentrations of trace metal pollutants. Trace metals can interact and be transformed in seawater, sediments and marine biota, due to physical, chemical, microbial or light-mediated mechanisms (Stankovic et al., 2012). Some of the most common trace metals studied by industrial and government organisations and found in PW are Ba, Cd, Hg, Cu and Zn (Stephenson, 1992).

In the present study, trace metals such as Ba, Cd, Hg, Cu and Zn were all identified in entire soft tissues exposed to PW and in PW samples. The accumulation of trace metals in mussel tissues is due to their biological and ecological characteristics, and the fact that mussels are sedentary organisms that take up and concentrate considerable quantities of metals that may cause toxic effects to higher trophic level organisms. As Stankovic et al. (2012) observed, trace metals such as Cd and Pb, readily accumulate in the mussels' soft tissues (in this case *M. galloprovincialis*); they are also known to accumulate within the aquatic food chain, affecting many organisms.

In conclusion, the aim of the present study was to investigate the potential effects of environmental concentrations of oilfield chemicals on marine mussels, *M. edulis*, by applying several biomarker techniques. *M. edulis* was shown to be a suitable biomarker organism for this purpose and the biomarkers applied for assessing genotoxicity, cytotoxicity and oxidative stress applied in the present study are well established in the field (Bolognesi et al., 1999; Brooks et al., 2011; de Lafontaine et al., 2000; Faria et al., 2009; Gorbi et al., 2008; Hannam et al., 2009; Hartl et al., 2010; Pérez-Cadahía et al., 2004; Sundt et al., 2011; Sureda et al., 2011; Turja et al., 2012). In the present study the biological effects and concentration of QUATs, imidazoline, PEs and PW were investigated by Comet, NRR, SOD and TBARS assays as well as SPE followed by ESI MS/MS and UV chromatography.

A genotoxic response was detected and expressed as a percentage of DNA strand breaks using the comet assay for measuring haemocytes and gill cells. Oxidative stress was also measured by investigating the inhibition of SOD activity and the levels of lipid peroxidation in gill cells. Lysosome membrane stability in haemocytes was also investigated. Bioaccumulations were measured in complete soft tissues using SPE followed by ESI MS/MS quantitative analysis. ESI MS/MS was used to investigate the amount of C₁₆ QUATs, imidazoline and PEs in the mussels' soft tissues. In addition, the presence of these compounds in PW sample was investigated. Furthermore, UV chromatography

was also used to investigate the bio-concentration of imidazoline and PEs. The animals were exposed to different concentrations of QUATs, imidazoline, PEs and PW ranged between 0.001, 0.01, 0.1, 0.5 and 1 mg/L for 24 hours of exposure.

The findings of the present study suggest that environmentally relevant concentrations of QUATs, imidazoline, PEs and PW increased SOD activity and lipid peroxidation at concentration ≥ 0.5 , 0.001, 0.1 and 0.001 mg/L and 0.1, 0.1, 0.5, 0.001 mg/L respectively. All these chemical compounds inflicted significant DNA damage on marine mussels under laboratory conditions at concentrations ≥ 0.001 mg/L, and lysosomal membrane damage at concentrations ≥ 0.1 mg/L. This was with the exception of PEs that did not cause damage below concentrations ≥ 0.5 mg/L. QUATs and imidazoline were found in mussel tissues at concentrations ≥ 0.1 and 0.001 mg/L respectively, which means that mussels may be uptake the QUATs and imidazoline, which could therefore have implications for the disposal of PW at sea.

- The presence of QUATs, imidazoline and PEs in the whole soft tissues of mussels was found after 24 hours of exposure at concentration 0.1 and 0.001 mg/L respectively.
- The uptake of QUATs and imidazoline by mussels was confirmed after 24 hours of exposure but that of PEs was not, due to its solubility in seawater.
- Five common heavy metals (Cd, Ba, Cu, Zn and Hg) were found in the treated PW sample the concentrations were 8.8, 113.83, 11.4, 8.5 and 15.3 mg/L respectively.
- Concentrations of heavy metals (Cd, Ba, Cu, Zn and Hg) were found even at the lowest concentration of PW in whole soft tissues of mussels after exposure to PW for 24 hours.
- Concentrations of QUATs, imidazoline and PEs were not found in PW samples because these maybe did not contain the compounds or contained concentrations below detection limits (lower than 0.1 mg/L) using the analytical techniques available.
- The impacts of QUATs, imidazoline, PEs and PW were observed on the lysosomal membrane stability of mussel haemocytes using NRR assay.
- The genotoxicity of QUATs, imidazoline, PEs and PW and their impact on DNA damage to mussel haemocytes and gill cells were observed using Comet assay.
- Oxidative stress caused by QUATs, imidazoline, PEs and PW in the gill cells of mussels were observed using SOD and TBARS assay.

- ESI MS/Ms presented a poor signal for imidazoline at concentrations 10 mg/L; therefore lower concentrations could not be measured as this may be attributed to some fragments of the imidazoline compound and the fact that available MS cannot detect masses between 600-620 m. However, imidazoline was confirmed to have accumulated and concentrated in mussel tissues by using UV chromatography at a wavelength of 229 nm.
- ESI MS/Ms did not present any signals for PEs at concentrations of 10 mg/L. Therefore, PEs had not accumulated in mussel tissues even when using UV chromatography at wavelength 345 nm; this may be due to their high water solubility.

Overall QUAT, imidazoline, PEs and PW caused biological effects and QUATs, imidazoline and some trace metals were accumulated and concentrated in the mussel's tissues. It should be mentioned here that the industry is developing and, in some cases, already applying alternative compounds, especially water soluble and biodegradable ones. However, it is time for government regulation of the discharge of these compounds into the receiving environment.

This study has observed the impact of oilfield chemicals corrosion inhibitors (QUATs, imidazoline and PEs) to the marine mussel *M. edulis* in terms of causing DNA damage, oxidative stress, damage to lysosome membrane and the fact that these can accumulate becoming concentrated. Therefore, it is reasonable to suppose that these chemicals may be reaching the food chain and may affect humans also by causing several diseases. As is known, humans have been eating mussels as a food for many years; therefore, humans are also experiencing bioaccumulations of these chemicals (Geyer et al., 1982). Mussels *M. edulis* are commonly eaten by humans because they are easy to collect; they may also enter the food chain as they are consumed by other marine species and marine predators.

It is strongly recommended that this work should be continued to allow comparisons between these results and those obtained from other marine species, such as other types of mussels (*M. galloprovincialis*, *M. trossellus* and *Perna canaliculus*), fish and clams.

To answer the important question: "can these compounds enter the human food chain?" it is recommended that further trophic transfer studies be conducted.

References

- Ahel, I. (2012). *DNA damage response* [online]. Manchester: Paterson institute for cancer research. Available: <http://www.paterson.man.ac.uk/images> [Accessed: 17thDecember 2012].
- Aherns, M. (2008). *Literature Review of Organic Chemicals of Emerging Environmental Concern in Use in Auckland*. Auckland: Auckland Regional Council, p. 193.
- Akcha, F., Tanguy, A., Leday, G., Pelluhet, L., Budzinski, H. and Chiffolleau, J.F. (2004). Measurement of DNA single-strand breaks in gill and hemolymph cells of mussels, *Mytilus* sp., collected on the French Atlantic Coast. *Marine Environmental Research* 58, 753-756.
- Al-Sabti, K. and Metcalfe, C.D. (1995). Fish micronuclei for assessing genotoxicity in water. *Mutation Research/Genetic Toxicology* 343, 121-135.
- Almeida, E.A., Bairy, A.C.D., Dafre, A.L., Gomes, O.F., Medeiros, M.H.G. and Di Mascio, P. (2005). Oxidative stress in digestive gland and gill of the brown mussel (*Perna perna*) exposed to air and re-submersed. *Journal of Experimental Marine Biology and Ecology* 318, 21-30.
- Almeida, E.A., Bairy, A.C.D., Loureiro, P.M.A., Martinez, R.G., Miyamoto, S., Onuki, J., Barbosa, F.L., Garcia, C.M.C., Prado, M.F., Ronsein, E.G., et al. (2007). Oxidative stress in *Perna perna* and other bivalves as indicators of environmental stress in the Brazilian marine environment: Antioxidants, lipid peroxidation and DNA damage. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* 146, 588-600.
- Ames, B.N., Shigenaga, M.K. and Hagen, T.M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences* 90, 7915-7922.
- Anderson, D., Yu, T.-W. and McGregor, D.B. (1998). Comet assay responses as indicators of carcinogen exposure. *Mutagenesis* 13, 539-555.
- Arnot, J.A. and Gobas, F.A. (2006). A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. *Environmental Reviews* 14, 257-297.
- Arora, A., Pandey, S., Kumar, Gandhi, R. and Institute Of Petroleum Technology, R.B., India (2012). Review on Materials for Corrosion Prevention in Oil Industry. *In SPE International Conference & Workshop on Oilfield Corrosion*. Aberdeen, UK: Society of Petroleum Engineers, p. 10.
- Aruoma, O.I. and Halliwell, B. (1998). *Molecular biology of free radicals in human diseases*. London: OICA International.
- Ash, M. and Ash, I. (2011). *Handbook of corrosion inhibitors*. 2nd edn. Endicott, NY: Synapse Information Resources.
- Ashcroft, A., E. (2008). *An introduction to mass spectrometry*. Astbury centre for structural molecular biology, p. 5.

- Babich, H. and Borenfreund, E. (1990). Applications of the neutral red cytotoxicity assay to in vitro toxicology. *Atla Alternatives to Laboratory Animals* 18, 129-144.
- Baccino, F.M. and Zuretti, M.F. (1975). Structural equivalents of latency for lysosome hydrolases. *The Biochemical Journal*, 146, 97-108.
- Bag, A. and Bag, N. (2008). Target Sequence Polymorphism of Human Manganese Superoxide Dismutase Gene and Its Association with Cancer Risk: A Review. *Cancer Epidemiology Biomarkers & Prevention* 17, 3298-3305.
- Bajpai, D. and Tyagi, V.K. (2006). Fatty Imidazolines: Chemistry, Synthesis, Properties and Their Industrial Applications. *Journal of Oleo Science*, 55, 319-329.
- Baršienė, J. andreikėnaitė, L. and Bjornstad, A. (2010). Induction of micronuclei and other nuclear abnormalities in blue mussels *Mytilus edulis* after 1-, 2-, 4- and 8-day treatment with crude oil from the North Sea. *Ekologija* 56, 124-131.
- Baršienė, J., Schiedek, D., Rybakovas, A., Šyvokienė, J., Kopecka, J. and Förlin, L. (2006). Cytogenetic and cytotoxic effects in gill cells of the blue mussel *Mytilus* spp. from different zones of the Baltic Sea. *Marine Pollution Bulletin*, 53, 469-478.
- Bassey, D. (2010). An investigation into the microbial degradation of Benzyltrimethyl Hexadecylammonium Chloride used in oilfield chemical formulations. PhD thesis. *In Life Sciences*. Edinburgh: Heriot-Watt University, p. 165.
- Bassey, D.E., Grigson, S.J.W. and Mitchell, W.J. (2009). Microbial Degradation of Benzyltrimethyl Hexadecylammonium Chloride (BDHAC) Used in Oilfield Chemical Formulations. *In SPE Americas E&P Environmental and Safety Conference*. San Antonio, Texas: Society of Petroleum Engineers, p. 12.
- Baussant, T., Bechmann, R.K., Taban, I.C., Larsen, B.K., Tandberg, A.H., Bjørnstad, A., Torggrimsen, S., Nævdal, A., Øysæd, K.B., Jonsson, G. and Sanni, S. (2009). Enzymatic and cellular responses in relation to body burden of PAHs in bivalve molluscs: A case study with chronic levels of North Sea and Barents Sea dispersed oil. *Marine Pollution Bulletin* 58, 1796-1807.
- Baussant, T., Ortiz-Zarragoitia, M., Cajaraville, M.P., Bechmann, R.K., Taban, I.C. and Sanni, S. (2011). Effects of chronic exposure to dispersed oil on selected reproductive processes in adult blue mussels (*Mytilus edulis*) and the consequences for the early life stages of their larvae. *Marine Pollution Bulletin* 62, 1437-1445.
- Baussant, T., Sanni, S., Jonsson, G., Skadsheim, A. and Børseth, J.F. (2001). Bioaccumulation of polycyclic aromatic compounds: 1. Bioconcentration in two marine species and in semipermeable membrane devices during chronic exposure to dispersed crude oil. *Environmental Toxicology and Chemistry* 20, 1175-1184.
- Bayne, B. (1976). *Marine mussels: their ecology and physiology*. Cambridge: C.U.P.

- Beaumont, A.R., Hawkins, M.P., Doig, F.L., Davies, I.M. and Snow, M. (2008). Three species of *Mytilus* and their hybrids identified in a Scottish Loch: natives, relicts and invaders? *Journal of Experimental Marine Biology and Ecology* 367, 100-110.
- Bechmann, R.K., Larsen, B.K., Taban, I.C., Hellgren, L.I., Møller, P. and Sanni, S. (2010). Chronic exposure of adults and embryos of *Pandalus borealis* to oil causes PAH accumulation, initiation of biomarker responses and an increase in larval mortality. *Marine Pollution Bulletin* 60, 2087-2098.
- Belosinschi, D., Chabot, B. and Brouillette, F. (2012). Release Paper: Can Phosphate Esters be an Alternative to Silicone? *BioResources*, 7, 902-903.
- Belton, P.S. (2000). *Introduction to principles and practice of mass spectrometry*. In *Mass Spectrometry of Natural Substances in Foods*. F.A. Mellon, R. Self, and J.R. Startin, eds. Cambridge: The Royal Society of Chemistry, pp. 1-32.
- Betteridge, D.J. (2000). What is oxidative stress? *Metabolism*, 49, 3-8.
- Binelli, A., Parolini, M., Cogni, D., Pedriali, A. and Provini, A. (2009). A multi-biomarker assessment of the impact of the antibacterial trimethoprim on the non-target organism Zebra mussel (*Dreissena polymorpha*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 150, 329-336.
- Binet, M.T., Stauber, J.L. and Winton, T. (2011). *The Effect of Storage Conditions on Produced Water Chemistry and Toxicity Produced Water*. K. Lee, and J. Neff, eds. New York: Springer New York, pp. 163-179.
- Bolognesi, C., Frenzilli, G., Lasagna, C., Perrone, E. and Roggieri, P. (2004). Genotoxicity biomarkers in *Mytilus galloprovincialis*: wild versus caged mussels. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 552, 153-162.
- Bolognesi, C., Landini, E., Roggieri, P., Fabbri, R. and Viarengo, A. (1999). Genotoxicity biomarkers in the assessment of heavy metal effects in mussels: Experimental studies. *Environmental and Molecular Mutagenesis* 33, 287-292.
- Bouskill, N.J., Handy, R.D., Ford, T.E. and Galloway, T.S. (2006). Differentiating copper and arsenic toxicity using biochemical biomarkers in *Asellus aquaticus* and *Dreissena polymorpha*. *Ecotoxicology and Environmental Safety* 65, 342-349.
- BP (2008). Environmental matters. In *Environmental statement*. [online] available from <http://www.bp.com/bodycopyarticle.do?categoryId=1&contentId=7052055> [Accessed March 25th 2013].
- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-254.
- Brooks, S.J., Harman, C., Grung, M., Farmen, E., Ruus, A., Vingen, S., Godal, B.F., Baršienė, J. andreikėnaitė, L., Skarphéðinsdóttir, H., et al. (2011). Water Column Monitoring of the

- Biological Effects of Produced Water from the Ekofisk Offshore Oil Installation from 2006 to 2009. *Journal of Toxicology and Environmental Health, Part A* 74, 582-604.
- Brown, R.J., Galloway, T.S., Lowe, D., Browne, M.A., Dissanayake, A., Jones, M.B. and Depledge, M.H. (2004). Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. *Aquatic Toxicology* 66, 267-278.
- Buratti, S., Ramos-Gómez, J., Fabbri, E., DelValls, T. and Martín-Díaz, M. (2012). Application of neutral red retention assay to caged clams (*Ruditapes decussatus*) and crabs (*Carcinus maenas*) in the assessment of dredged material. *Ecotoxicology* 21, 75-86.
- Burge, E.J., Madigan, D.J., Burnett, L.E. and Burnett, K.G. (2007). Lysozyme gene expression by hemocytes of Pacific white shrimp, *Litopenaeus vannamei*, after injection with *Vibrio*. *Fish & Shellfish Immunology* 22, 327-339.
- Burkhardt, P. (2011). *Linking Water Quality to Human Health and Environment: The Fate of Micropollutants*. (Institute of Water Policy), p. 56.
- Burton, G.W., Joyce, A. and Ingold, K.U. (1982). First proof that vitamin E is major lipid-soluble, chain-breaking antioxidant in human blood plasma. *Lancet* 2, 327.
- Caliani, I., Porcelloni, S., Mori, G., Frenzilli, G., Ferraro, M., Marsili, L., Casini, S. and Fossi, M. (2009). Genotoxic effects of produced waters in mosquito fish (*Gambusia affinis*). *Ecotoxicology* 18, 75-80.
- Callely, A.G. and Forster, C.F. (1977). *Treatment of industrial effluents*. London: Hodder and Stoughton.
- Camejo, G., Wallin, B. and Enojärvi, M. (1998). *Analysis of Oxidation and Antioxidants Using Microtiter Plates Free Radical and Antioxidant Protocols*. D. Armstrong, ed. Totawa, NJ: Humana Press, pp. 377-387.
- Carol, T. (2002). *Environmental protection: The Offshore Chemicals Regulations*. (UK), p. 14.
- Carol, T. (2011). *Environmental protection: The Offshore Chemicals (Amendment) Regulations*. (UK), p. 7.
- Castro, M., Santos, M.M., Monteiro, N.M. and Vieira, N. (2004). Measuring lysosomal stability as an effective tool for marine coastal environmental monitoring. *Marine Environmental Research* 58, 741-745.
- Cavalieri, E.L. and Rogan, E.G. (1992). The approach to understanding aromatic hydrocarbon carcinogenesis. The central role of radical cations in metabolic activation. *Pharmacology & Therapeutics* 55, 183-199.
- Cech, N.B. and Enke, C.G. (2001). Practical implications of some recent studies in electrospray ionization fundamentals. *Mass Spectrometry Reviews* 20, 362-387.

- Ceconi, C., Boraso, A., Cargnoni, A. and Ferrari, R. (2003). Oxidative stress in cardiovascular disease: myth or fact? *Archives of Biochemistry and Biophysics* 420, 217-221.
- Ceriello, A. and Motz, E. (2004). Is Oxidative Stress the Pathogenic Mechanism Underlying Insulin Resistance, Diabetes, and Cardiovascular Disease? The Common Soil Hypothesis Revisited. *Arteriosclerosis, Thrombosis, and Vascular Biology* 24, 816-823.
- Chance, B., Sies, H. and Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiological Reviews* 59, 527-605.
- Chen, Y., Hong, T., Gopal, M. and Jepson, W.P. (2000). EIS studies of a corrosion inhibitor behavior under multiphase flow conditions. *Corrosion Science* 42, 979-990.
- Cheung, C.C.C., Siu, W.H.L., Richardson, B.J., Luca-Abbott, S.B., Lam, P.K.S. (2004). Antioxidant responses to benzo[a]pyrene and Aroclor 1254 exposure in the green-lipped mussel, *Perna viridis*. *Environmental pollution* 128, 393-403.
- Cheung, V.V., Wedderburn, R.J. and Depledge, M.H. (1998). Molluscan lysosomal responses as a diagnostic tool for the detection of a pollution gradient in Tolo Harbour, Hong Kong. *Marine Environmental Research* 46, 237-241.
- Chong Zhao, Xiaoxu Li, Shibin Luo, and Yaqing Chang (2011). Assessments of lysosomal membrane responses to stresses with neutral red retention assay and its potential application in the improvement of bivalve aquaculture. *African Journal of Biotechnology* 10, 13968-13973.
- Christen, W.G., Liu, S., Glynn, R.J., Gaziano, J.M. and Buring, J.E. (2008). Dietary Carotenoids, Vitamins C and E, and Risk of Cataract in Women: A Prospective Study. *Arch Ophthalmol* 126, 102-109.
- Collins, A. (2004). The comet assay for DNA damage and repair. *Molecular Biotechnology* 26, 249-261.
- Cooper, J.C. (1988). Review of the environmental toxicity of quaternary ammonium halides. *Ecotoxicol Environ Saf* 16, 65-71.
- Coughlan, B.M., Hartl, M.G., O'Reilly, S.J., Sheehan, D., Morthersill, C., van Pelt, F.N., O'Halloran, J. and O'Brien, N.M. (2002). Detecting genotoxicity using the Comet assay following chronic exposure of Manila clam *Tapes semidecussatus* to polluted estuarine sediments. *Marine Pollution Bulletin* 44, 1359-1365.
- Coughlan, B.M., Moroney, G.A., Pelt, F.N.A.M.v., O'Brien, N.M., Davenport, J. and O'Halloran, J. (2009). The effects of salinity on the Manila clam (*Ruditapes philippinarum*) using the neutral red retention assay with adapted physiological saline solutions. *Marine Pollution Bulletin* 58, 1680-1684.
- Crapo, J.D., Oury, T., Rabouille, C., Slot, J.W. and Chang, L.Y. (1992). Copper, zinc superoxide dismutase is primarily a cytosolic protein in human cells. *Proceedings of the National Academy of Sciences* 89, 10405-10409.

- Crockett, E. (2008). The cold but not hard fats in ectotherms: consequences of lipid restructuring on susceptibility of biological membranes to peroxidation, a review. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* 178, 795-809.
- D'Unger, C., Chapman, D. and Carr, R. (1996). Discharge of oilfield-produced water in nueces bay, texas: A case study. *Environmental Management* 20, 143-150.
- Dailianis, S., Domouhtsidou, G.P., Raftopoulou, E., Kaloyianni, M. and Dimitriadis, V.K. (2003). Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. *Marine Environmental Research* 56, 443-470.
- Dauchet, L., Amouyel, P., Hercberg, S. and Dallongeville, J. (2006). Fruit and Vegetable Consumption and Risk of Coronary Heart Disease: A Meta-Analysis of Cohort Studies. *The Journal of Nutrition* 136, 2588-2593.
- De Lafontaine, Y., Gagné, F., Blaise, C., Costan, G., Gagnon, P. and Chan, H.M. (2000). Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and monitoring of water quality of the St Lawrence River (Canada). *Aquatic Toxicology* 50, 51-71.
- DeCaprio, A.P. (2006). *Toxicologic biomarkers*. New York & London: Taylor & Francis.
- DeLong, J.M. and Steffen, K.L. (1997). Photosynthetic function, lipid peroxidation, and α -tocopherol content in spinach leaves during exposure to UV-B radiation. *Canadian Journal of Plant Science* 77, 453-459.
- Dhawan, A. and Anderson, D. (2009). *The Comet Assay in Toxicology*. United Kingdom: RSC Publishing.
- Dhawan, A., Bajpayee, M. and Parmar, D. (2009). Comet assay: a reliable tool for the assessment of DNA damage in different models. *Cell Biology and Toxicology* 25, 5-32.
- Dherani, M., Murthy, G.V.S., Gupta, S.K., Young, I.S., Maraini, G., Camparini, M., Price, G.M., John, N., Chakravarthy, U. and Fletcher, A.E. (2008). Blood Levels of Vitamin C, Carotenoids and Retinol Are Inversely Associated with Cataract in a North Indian Population. *Investigative Ophthalmology & Visual Science* 49, 3328-3335.
- Di Giulio, R.T., Benson, W.H., Sanders, B.M. and Van Veld, P.A. (1995). Biochemical mechanisms: metabolism, adaptation and toxicity. In *Fundamentals of aquatic toxicology: effects, environmental fate, and risk assessment*, G.M. Rand, ed. (Washington: Taylor & Francis), pp. 523-561.
- Di Giulio, R.T., Washburn, P.C., Wenning, R.J., Winston, G.W. and Jewell, C.S. (1989). Biochemical responses in aquatic animals: A review of determinants of oxidative stress. *Environmental Toxicology and Chemistry* 8, 1103-1123.
- Dias, P.J., Dordor, A., Tulett, D., Piertney, S., Davies, I.M. and Snow, M. (2009). Survey of mussel (*Mytilus*) species at Scottish shellfish farms. *Aquaculture Research* 40, 1715-1722.

- Divya, B. and Tyagi, V.K. (2006). Fatty Imidazolines: Chemistry, Synthesis, Properties and Their Industrial Applications. *Journal of Oleo Science* 55, 319-329.
- Donkin, P., Widdows, J., Evans, S.V. and Brinsley, M.D. (1991). QSARs for the sublethal responses of marine mussels (*Mytilus edulis*). *Science of The Total Environment* 109–110, 461-476.
- Douglass, M.R., Johnson, D. and Ardito, S. (2012). *Method for Maintaining Antiwear Performance of Turbine Oils Containing Polymerized Amine Antioxidants and for Improving the Deposit Formation Resistance Performance of Turbine Oils Containing Monomeric and/or Polymeric Antioxidants*. Annandale, NJ, US: Exxonmobil Research And Engineering Company, p. 13.
- Dröge, W. (2002). Free Radicals in the Physiological Control of Cell Function. *Physiological Reviews* 82, 47-95.
- Du, Z. and Bramlage, W.J. (1992). Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts. *Journal of Agricultural and Food Chemistry* 40, 1566-1570.
- Dytham, C. (2011). *Choosing and Using Statistics: A Biologist's Guide*. India: Wiley.
- Fairbairn, D.W., Olive, P.L. and O'Neill, K.L. (1995). The comet assay: a comprehensive review. *Mutation Research/Reviews in Genetic Toxicology* 339, 37-59.
- Fakhru'l-Razi, A., Pendashteh, A., Abdullah, L.C., Biak, D.R.A., Madaeni, S.S. and Abidin, Z.Z. (2009). Review of technologies for oil and gas produced water treatment. *Journal of Hazardous Materials* 170, 530-551.
- Famadas, M. (2009). An investigation of the toxicity of produced water from a north sea platform. In School of life sciences (Edinburgh, Heriot-Watt University), p. 135.
- Faria, M., Carrasco, L., Diez, S., Riva, M.C., Bayona, J.M. and Barata, C. (2009). Multi-biomarker responses in the freshwater mussel *Dreissena polymorpha* exposed to polychlorobiphenyls and metals. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 149, 281-288.
- Farmen, E., Harman, C., Hylland, K. and Tollefsen, K.E. (2010). Produced water extracts from North Sea oil production platforms result in cellular oxidative stress in a rainbow trout in vitro bioassay. *Marine Pollution Bulletin* 60, 1092-1098.
- Fattman, C.L., Schaefer, L.M. and Oury, T.D. (2003). Extracellular superoxide dismutase in biology and medicine. *Free Radical Biology and Medicine* 35, 236-256.
- Fearon, I.M. and Faux, S.P. (2009). Oxidative stress and cardiovascular disease: Novel tools give (free) radical insight. *Journal of Molecular and Cellular Cardiology* 47, 372-381.
- Fernley, P.W., Moore, M.N., Lowe, D.M., Donkin, P. and Evans, S. (2000). Impact of the Sea Empress oil spill on lysosomal stability in mussel blood cells. *Marine Environmental Research* 50, 451-455.

- Fink, J.K. (2012). *Petroleum Engineer's Guide to Oil Field Chemicals and Fluids*. Boston: Gulf Professional Publishing, pp. 217-252.
- Fisher, J.C., Belden, J.B. and Bidwell, J.R. (2010). Can site-specific heuristic toxicity models predict the toxicity of produced water? *Chemosphere* 80, 542-547.
- Fowler, J., Cohen, L. and Jarvis, P. (1998). *Practical Statistics for Field Biology*. Chichester: John Wiley & Sons.
- Frei, B., Stocker, R. and Ames, B.N. (1988). Antioxidant defenses and lipid peroxidation in human blood plasma. *Proceedings of the National Academy of Sciences* 85, 9748-9752.
- Fridovich, I. (1989). Superoxide Dismutases - An Adaptation to a Paramagnetic Gas. *Journal of Biological Chemistry* 264, 7761-7764.
- Friedrich, C.G. (1997). Physiology and Genetics of Sulfur-oxidizing Bacteria. In *Advances in Microbial Physiology*, R.K. Poole, ed. Germany: Academic Press, pp. 235-289.
- Gagliardi, S. and Grigson, S. (2003). Chrmval project. *In Final Report* Edinburgh, School of life sciences, pp. 1-104.
- Galloway, T.S., Brown, R.J., Browne, M.A., Dissanayake, A., Lowe, D., Jones, M.B. and Depledge, M.H. (2004). A Multibiomarker Approach To Environmental Assessment. *Environmental Science & Technology* 38, 1723-1731.
- García, M.T., Campos, E., Sánchez-Leal, J. and Ribosa, I. (2000). Anaerobic degradation and toxicity of commercial cationic surfactants in anaerobic screening tests. *Chemosphere* 41, 705-710.
- García, M.T., Ribosa, I., Guindulain, T., Sánchez-Leal, J. and Vives-Rego, J. (2001). Fate and effect of monoalkyl quaternary ammonium surfactants in the aquatic environment. *Environmental Pollution* 111, 169-175.
- Gardner, J.P.A. (1995). Developmental stability is not disrupted by extensive hybridization and introgression among populations of the marine bivalve molluscs *Mytilus edulis* (L.) and *M. galloprovincialis* (Lmk.) from south-west England. *Biological Journal of the Linnean Society* 54, 71-86.
- Gaté, L., Paul, J., Ba, G.N., Tew, K.D. and Tapiero, H. (1999). Oxidative stress induced in pathologies: The role of antioxidants. *Biomedicine & Pharmacotherapy* 53, 169-180.
- Gathergood, N., Scammells, P.J. and Garcia, M.T. (2006). Biodegradable Ionic Liquids. Part 3. The First Readily Biodegradable Ionic Liquids. *ChemInform* 37, no-no.
- Geyer, H., Sheehan, P., Kotzias, D., Freitag, D. and Korte, F. (1982). Prediction of ecotoxicological behaviour of chemicals: Relationship between physico-chemical properties and bioaccumulation of organic chemicals in the mussel *Mytilus edulis*. *Chemosphere* 11, 1121-1134.

- Giamberini, L. and Pihan, J. (1997). Lysosomal changes in the hemocytes of the freshwater mussel *Dreissena polymorpha* experimentally exposed to lead and zinc. *Diseases of Aquatic Organisms* 28, 221-227.
- Gobas, F. and Morrison, H. (2000). Bioconcentration and Biomagnification in the Aquatic Environment. In *Handbook of Property Estimation Methods for Chemicals*. United States of America: CRC Press.
- Gorbi, S., Virno Lamberti, C., Notti, A., Benedetti, M., Fattorini, D., Moltedo, G. and Regoli, F. (2008). An ecotoxicological protocol with caged mussels, *Mytilus galloprovincialis*, for monitoring the impact of an offshore platform in the Adriatic sea. *Marine Environmental Research* 65, 34-49.
- Gordon, H. (2008). *Oil & gas industry water chemicals treatment 101*. Toronato: Hayward Gordon Let, p. 6.
- Grigson, S. and Gagliardi, S. (2003). Measurement of the concentration of oilfield chemical residues in produced water discharges: A comparison to CHARM predictions. *Progress in Mining and Oilfield* 5, 345-356.
- Grigson, S.J.W., Wilkinson, A., Johnson, P., Moffat, C.F. and McIntosh, D. (2000). Measurement of oilfield chemical residues in produced water discharge and marine sediments. *Rapid Communications in Mass Spectrometry* 14, 10.
- Grisolia, C.K. and Starling, F.L.R.M. (2001). Micronuclei monitoring of fishes from Lake Paranoá, under influence of sewage treatment plant discharges. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 491, 39-44.
- Gu, H., Xu, N. and Chen, H. (2012). Direct analysis of biological samples using extractive electrospray ionization mass spectrometry (EESI-MS). *Analytical and Bioanalytical Chemistry*, 1-9.
- Guha, S., Li, M., Tarlov, M.J. and Zachariah, M.R. (2012). Electrospray–differential mobility analysis of bionanoparticles. *Trends in Biotechnology* 30, 291-300.
- Guidi, P., Frenzilli, G., Benedetti, M., Bernardeschi, M., Falleni, A., Fattorini, D., Regoli, F., Scarcelli, V. and Nigro, M. (2010). Antioxidant, genotoxic and lysosomal biomarkers in the freshwater bivalve (*Unio pictorum*) transplanted in a metal polluted river basin. *Aquatic Toxicology* 100, 75-83.
- Gutteridge, J.M. (1995). Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clinical Chemistry* 41, 1819-1828.
- Hall, D.B., Holmlin, R.E. and Barton, J.K. (1996). Oxidative DNA damage through long-range electron transfer. *Nature* 382, 731-735.
- Halliwell, B. (1994). Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet* 344, 721-724.

- Halliwell, B. (2000). Why and how should we measure oxidative DNA damage in nutritional studies? How far have we come?. *Am J Clin Nutr* 72, 1082-1087.
- Halliwell, B. (2012). Free radicals and antioxidants: updating a personal view. *Nutrition Reviews* 70, 257-265.
- Halliwell, B. and Aruoma, O.I. (1991). DNA damage by oxygen-derived species Its mechanism and measurement in mammalian systems. *FEBS Letters* 281, 9-19.
- Halliwell, B. and Gutteridge, J. (1998). *Free Radicals in Biology and Medicine*. Oxford: Oxford Science Publications.
- Halliwell, B. and Gutteridge, J.M.C. (1990). The antioxidants of human extracellular fluids. *Archives of Biochemistry and Biophysics* 280, 1-8.
- Halliwell, B. and Gutteridge, J.M.C. (2007). *Free radicals in biology and medicine*. 4th edn. Oxford: Oxford: Oxford University Press.
- Hamoutene, D., Payne, J.F., Rahimtula, A. and Lee, K. (2002). Use of the Comet assay to assess DNA damage in hemocytes and digestive gland cells of mussels and clams exposed to water contaminated with petroleum hydrocarbons. *Marine Environmental Research* 54, 471-474.
- Hannam, M.L., Bamber, S.D., Galloway, T.S., John Moody, A. and Jones, M.B. (2010a). Effects of the model PAH phenanthrene on immune function and oxidative stress in the haemolymph of the temperate scallop *Pecten maximus*. *Chemosphere* 78, 779-784.
- Hannam, M.L., Bamber, S.D., John Moody, A., Galloway, T.S. and Jones, M.B. (2010b). Immunotoxicity and oxidative stress in the Arctic scallop *Chlamys islandica*: Effects of acute oil exposure. *Ecotoxicology and Environmental Safety* 73, 1440-1448.
- Hannam, M.L., Bamber, S.D., Sundt, R.C. and Galloway, T.S. (2009). Immune modulation in the blue mussel *Mytilus edulis* exposed to North Sea produced water. *Environmental Pollution* 157, 1939-1944.
- Harman, C., Thomas, K.V., Tollefsen, K.E., Meier, S., Bøyum, O. and Grung, M. (2009). Monitoring the freely dissolved concentrations of polycyclic aromatic hydrocarbons (PAH) and alkylphenols (AP) around a Norwegian oil platform by holistic passive sampling. *Marine Pollution Bulletin* 58, 1671-1679.
- Hartl, M.G.J. (2010). Biomarkers in Integrated Ecotoxicological Sediment Assessment. In *Sedimentology of Aqueous Systems*. UK: Wiley-Blackwell, pp. 147-170.
- Hartl, M.G.J., Coughlan, B.M., Sheehan, D., Mothersill, C., van Pelt, F.N.A.M., O'Reilly, S.J., Heffron, J.J.A., O'Halloran, J. and O'Brien, N.M. (2004). Implications of seasonal priming and reproductive activity on the interpretation of Comet assay data derived from the clam, *Tapes semidecussatus* Reeves 1864, exposed to contaminated sediments. *Marine Environmental Research* 57, 295-310.

- Hartl, M.G.J., Grigson, S.J.W. and Sinet, E. (2010). Maintenance of bivalve hemocytes for the purpose of delayed DNA strand break assessment using the comet assay. *Environmental and Molecular Mutagenesis* 51, 64-68.
- Hartl, M.G.J., Kilemade, M., Sheehan, D., Mothersill, C., O'Halloran, J., O'Brien, N.M. and Pelt, F.N.A.M.v. (2007). Hepatic biomarkers of sediment-associated pollution in juvenile turbot, *Scophthalmus maximus* L. *Marine Environmental Research* 64, 17.
- Hartl, M.G.J., J.W. Grigson, S. and Sinety, E. (2010). Maintenance of Bivalve Hemocytes for the Purpose of Delayed DNA Strand Break Assessment Using the Comet Assay. *Environmental and Molecular Mutagenesis* 51, 4.
- Hartmann, A., Agurell, E., Beevers, C., Brendler-Schwaab, S., Burlinson, B., Clay, P., Collins, A., Smith, A., Speit, G., Thybaud, V. and Tice, R.R. (2003). Recommendations for conducting the in vivo alkaline Comet assay. *Mutagenesis* 18, 45-51.
- Hauton, C., Hawkins, L. E., & Hutchinson, S. (1998). The use of the neutral red retention assay to examine the effects of temperature and salinity on haemocytes of the European flat oyster *Ostrea edulis* (L.). *Comparative Biochemistry and Physiology B*, 119, 619–623.
- Heath, R.L. and Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* 125, 189-198.
- Henderson, S.B. (1999). *An investigation into the effect on total toxicity predictions of interactions between components of a typical North Sea produced water*. [electronic resource]. Edinburgh: Heriot-Watt University.
- Hensley, K. and Floyd, R.A. (2002). Reactive Oxygen Species and Protein Oxidation in Aging: A Look Back, A Look Ahead. *Archives of Biochemistry and Biophysics* 397, 377-383.
- Hooftman, R.N. and de Raat, W.K. (1982). Induction of nuclear anomalies (micronuclei) in the peripheral blood erythrocytes of the eastern mudminnow *Umbra pygmaea* by ethyl methanesulphonate. *Mutation Research Letters* 104, 147-152.
- Huang, P., Feng, L., Oldham, E.A., Keating, M.J. and Plunkett, W. (2000). Superoxide dismutase as a target for the selective killing of cancer cells. *Nature* 407, 390-395.
- Hudgins, C.M.J. (1992). Chemical treatments and usage in offshore oil and gas production systems. Journal Name: *Journal of Petroleum Technology* 44:5, X, 604-611.
- Hylland, K., Tollefsen, K.-E., Ruus, A., Jonsson, G., Sundt, R.C., Sanni, S., Røe Utvik, T.I., Johnsen, S., Nilssen, I., Pinturier, L., et al. (2008). Water column monitoring near oil installations in the North Sea 2001–2004. *Marine Pollution Bulletin* 56, 414-429.
- Imlay, J. and Linn, S. (1988). DNA damage and oxygen radical toxicity. *Science* 240, 1302-1309.
- Imlay, J.A. (2003). Pathways of Oxidative Damage. *Annual Review of Microbiology* 57, 395-418.

- Innes, D.J. and Bates, J.A. (1999). Morphological variation of *Mytilus edulis* and *Mytilus trossulus* in eastern Newfoundland. *Marine Biology* 133, 691-699.
- Jabbar, A. and Davies, J.I. (1987). A simple and convenient biochemical method for sex identification in the marine mussel, *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology* 107, 39-44.
- Jain, K.K. and SpringerLink (2010). *The handbook of biomarkers*. [electronic resource]. New York: Springer.
- Jha, A.N., Dogra, Y., Turner, A. and Millward, G.E. (2005). Impact of low doses of tritium on the marine mussel, *Mytilus edulis*: Genotoxic effects and tissue-specific bioconcentration. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 586, 47-57.
- Johnsen, S., Statoil., Røe Utvik, T.I., Emmanuel, G., Norsk Hydro., de Vals, T., Bruno. and Campbell., O.J. (2004). 'Environmental fate and effect of contaminants in produced water'. In *SPE International Conference on Health, Safety, and Environment in Oil and Gas Exploration and Production*. Canada: Society of Petroleum Engineers.
- Jones, L.W. (1988). *Corrosion and water technology for petroleum producers*. Tulsa, Okla.: OGCI Publications.
- Jovancicevic, V., Ramachandran, S. and Prince, P. (1999). Inhibition of Carbon Dioxide Corrosion of Mild Steel by Imidazolines and Their Precursors. *Corrosion* 55, 7.
- Keller, G.A., Warner, T.G., Steimer, K.S. and Hallewell, R.A. (1991). Cu,Zn superoxide dismutase is a peroxisomal enzyme in human fibroblasts and hepatoma cells. *Proceedings of the National Academy of Sciences* 88, 7381-7385.
- Kilemade, M., Hartl, M.G.J., D.Sheehan, Mothersill, C., Pelt, F.N.A.M.v., Brien, N.M.O. and Halloran, J.O. (2004). An assessment of the pollutant status of surficial sediment in Cork Harbour in the South East of Ireland with particular reference to polycyclic aromatic hydrocarbons. *Marine Pollution Bulletin* 49, 12.
- Kim, I.-Y. and Hyun, C.-K. (2006). Comparative evaluation of the alkaline comet assay with the micronucleus test for genotoxicity monitoring using aquatic organisms. *Ecotoxicology and Environmental Safety* 64, 288-297.
- Kirchin, M.A., Moore, M.N., Dean, R.T. and Winston, G.W. (1992). The role of oxyradicals in intracellular proteolysis and toxicity in mussels. *Marine Environmental Research* 34, 315-320.
- Kishino, T. and Kobayashi, K. (1995). Relation between toxicity and accumulation of chlorophenols at various pH, and their absorption mechanism in fish. *Water Research* 29, 431-442.
- Kishino, T. and Kobayashi, K. (1996). Acute toxicity and structure-activity relationships of chlorophenols in fish. *Water Research* 30, 387-392.
- Klaude, M., Eriksson, S., Nygren, J. and Ahnström, G. (1996). The comet assay: mechanisms and technical considerations. *Mutation Research/DNA Repair* 363, 89-96.

- Kurelec, B. (1993). The genotoxic disease syndrome. *Marine Environmental Research* 35, 341-348.
- Labrot, F., Ribera, D., Denis, M.S. and Narbonne, J.F. (1996). In vitro and in vivo studies of potential biomarkers of lead and uranium contamination: lipid peroxidation, acetylcholinesterase, catalase and glutathione peroxidase activities in three non-mammalian species. *Biomarkers* 1, 21-28.
- Larson, R.J. (1983). Comparison of biodegradation rates in laboratory screening studies with rates in natural waters. *Residue reviews* 85, 159-171.
- Lee, H.J., Shim, W.J., Lee, J. and Kim, G.B. (2011). Temporal and geographical trends in the genotoxic effects of marine sediments after accidental oil spill on the blood cells of striped beakperch (*Oplegnathus fasciatus*). *Marine Pollution Bulletin* 62, 2264-2268.
- Lee, R.F., Sauerheber, R. and Benson, A.A. (1972). Petroleum Hydrocarbons: Uptake and Discharge by the Marine Mussel *Mytilus edulis*. *Science* 177, 344-346.
- Lee, R.F. and Steinert, S. (2003). Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. *Mutation Research/Reviews in Mutation Research* 544, 43-64.
- Lemiere, S., Cossu-Leguille, C., Bispo, A., Jourdain, M.-J., Lanhers, M.-C., Burnel, D. and Vasseur, P. (2005b). DNA damage measured by the single-cell gel electrophoresis (Comet) assay in mammals fed with mussels contaminated by the 'Erika' oil-spill. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 581, 11-21.
- Lesser, M.P. (2006). Oxidative stress in marine environments: biochemistry and physiological ecology. *Annual review of physiology* 68, 253-278.
- Lewis, C. and Galloway, T. (2008). Genotoxic damage in polychaetes: A study of species and cell-type sensitivities. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 654, 69-75.
- Li, L., Duker, J.S., Yoshida, Y., Niki, E., Rasmussen, H., Russell, R.M. and Yeum, K.J. (2008). Oxidative stress and antioxidant status in older adults with early cataract. *Eye* 23, 1464-1468.
- Liochev, S.I. and Fridovich, I. (2007). The effects of superoxide dismutase on H₂O₂ formation. *Free Radical Biology and Medicine* 42, 1465-1469.
- Liou, W., Chang, L.-Y., Geuze, H.J., Strous, G.J., Crapo, J.D. and Slot, J.W. (1993). Distribution of CuZn superoxide dismutase in rat liver. *Free Radical Biology and Medicine* 14, 201-207.
- Livingstone, D.R., Chipman, J.K., Lowe, D.M., Minier, C. and Pipe, R.K. (2000). Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates: recent molecular, genotoxic, cellular and immunological studies on the common mussel (*Mytilus edulis* L.) and other mytilids. *International Journal of Environment and Pollution* 13, 56-91.
- Lowe, D.M., Fossato, V.U. and Depledge, M.H. (1995a). Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: an in vitro study. *Marine Ecology Progress Series* 129, 189-196.

- Lowe, D.M., Moore, M.N. and Evans, B.M. (1992). Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. *Marine Ecology Progress Series* 91, 135-140.
- Lowe, D.M. and Pipe, R.K. (1994). Contaminant induced lysosomal membrane damage in marine mussel digestive cells: an in vitro study. *Aquatic Toxicology* 30, 357-365.
- Lowe, D.M., Soverchia, C. and Moore, M.N. (1995b). Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. *Aquatic Toxicology* 33, 105-112.
- Lüchmann, K.H., Mattos, J.J., Siebert, M.N., Granucci, N., Dorrington, T.S., Bicego, M.C., Taniguchi, S., Sasaki, S.T., Daura-Jorge, F.G. and Bainy, A.C.D. (2011). Biochemical biomarkers and hydrocarbons concentrations in the mangrove oyster *Crassostrea brasiliana* following exposure to diesel fuel water-accommodated fraction. *Aquatic Toxicology* 105, 652-660.
- Luzio, J.P., Pryor, P.R. and Bright, N.A. (2007). Lysosomes: fusion and function. *Nature Reviews Molecular Cell Biology* 8, 622-632.
- Mackay, D. and Fraser, A. (2000). Bioaccumulation of persistent organic chemicals: mechanisms and models. *Environmental Pollution* 110, 375-391.
- Maddipati, K.R. and Marnett, L.J. (1987). Characterization of the major hydroperoxide-reducing activity of human plasma. Purification and properties of a selenium-dependent glutathione peroxidase. *Journal of Biological Chemistry* 262, 17398-17403.
- Malstrom, B., Andreasson, L. and Reinhammer, B. (1975). *The Enzymes*, Vol 12. New York: XIIB, Academic Press.
- Manduzio, H., Monsinjon, T., Rocher, B., Leboulenger, F. and Galap, C. (2003). Characterization of an inducible isoform of the Cu/Zn superoxide dismutase in the blue mussel *Mytilus edulis*. *Aquatic Toxicology* 64, 73-83.
- Marklund, S.L., Holme, E. and Hellner, L. (1982). Superoxide dismutase in extracellular fluids. *Clinica Chimica Acta* 126, 41-51.
- Martínez-Carballo, E., González-Barreiro, C., Sitka, A., Kreuzinger, N., Scharf, S. and Gans, O. (2007a). Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part II. Application to sediment and sludge samples in Austria. *Environmental Pollution* 146, 543-547.
- Martínez-Carballo, E., González-Barreiro, C., Sitka, A., Scharf, S. and Gans, O. (2007b). Determination of selected organophosphate esters in the aquatic environment of Austria. *Science of The Total Environment* 388, 290-299.
- Martínez-Carballo, E., Sitka, A., González-Barreiro, C., Kreuzinger, N., Furrhacker, M., Scharf, S. and Gans, O. (2007). Determination of selected quaternary ammonium compounds by liquid

- chromatography with mass spectrometry. Part I. Application to surface, waste and indirect discharge water samples in Austria. *Environmental Pollution* 145, 8.
- Martínez-Carballo, E., Sitka, A., González-Barreiro, C., Kreuzinger, N., Fürhacker, M., Scharf, S. and Gans, O. (2007c). Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part I. Application to surface, waste and indirect discharge water samples in Austria. *Environmental Pollution* 145, 489-496.
- Martinez-Gomez, C., Benedicto, J., Campillo, J.A. and Moore, M. (2008). Application and evaluation of the neutral red retention (NRR) assay for lysosomal stability in mussel populations along the Iberian Mediterranean coast. *Journal of Environmental Monitoring* 10, 490-499.
- Martins, L.K.P., Nascimento, I.A., Fillmann, G., King, R., Evangelista, A.J.A., Readman, J.W. and Depledge, M.H. (2005). Lysosomal responses as a diagnostic tool for the detection of chronic petroleum pollution at Todos os Santos Bay, Brazil. *Environmental Research* 99, 387-396.
- Matozzo, V., Ballarin, L., Pampanin, D.M. and Marin, M.G. (2001). Effects of Copper and Cadmium Exposure on Functional Responses of Hemocytes in the Clam, *Tapes philippinarum*. *Archives of Environmental Contamination and Toxicology* 41, 163-170.
- McCord, J.M. and Fridovich, I. (1988). Superoxide dismutase: The first twenty years (1968-1988). *Free Radical Biology and Medicine* 5, 363-369.
- McCormack, P., Jones, P., Hetheridge, M.J. and Rowland, S.J. (2001). Analysis of oilfield produced waters and production chemicals by electrospray ionisation multi-stage mass spectrometry (ESI-MSn). *Water Research* 35, 3567-3578.
- McWilliams, P. and Payne, G. (2002). Bioaccumulation potential of surfactants: A review. In *Chemistry in the Oil Industry VII: Performance in a Challenging Environment*, T. Balson, H.A. Craddock, J. Dunlop, H. Frampton, G. Payne, and P. Reid, eds. *The Royal Society of Chemistry*, pp. 44-55.
- Meyer, R.G. and Keith, A. (2012). *Corrosion inhibitors for oil and gas applications*. United States: Patent, p. 11.
- Middleditch, B.S. (1984). Ecological effects of produced water effluents from offshore oil and gas production platforms. *Ocean Management* 9, 191-316.
- Mitchelmore, C.L. and Hyatt, S. (2004). Assessing DNA damage in cnidarians using the Comet assay. *Marine Environmental Research* 58, 707-711.
- Miyamoto, S., Arai, H. and Terao, J. (2010). *Enzymatic Antioxidant Defenses*. In *Biomarkers for Antioxidant Defense and Oxidative Damage: Principles and Practical Applications*. U.S: Wiley-Blackwell, pp. 21-33.
- Monserrat, J.M., Ferreira-Cravo, M., Ventura-Lima, J., Sandrini, J.Z., Amado, L.L., Geracitano, L.A., Rebelo, M. and Bianchini, A. (2009). Antioxidant responses in different body regions of the

- polychaeta *Laeonereis acuta* (Nereididae) exposed to copper. *Ecotoxicology and Environmental Safety* 72, 388-393.
- Monteiro, V., Cavalcante, D.G.S.M., Viléla, M.B.F.A., Sofia, S.H. and Martinez, C.B.R. (2011). In vivo and in vitro exposures for the evaluation of the genotoxic effects of lead on the Neotropical freshwater fish *Prochilodus lineatus*. *Aquatic Toxicology* 104, 291-298.
- Moore, M. (1990). Lysosomal cytochemistry in marine environmental monitoring. *The Histochemical Journal* 22, 187-191.
- Moore, M.N. (1976). Cytochemical demonstration of latency of lysosomal hydrolases in digestive cells of the common mussel, *Mytilus edulis*, and changes induced by thermal stress. *Cell and Tissue Research* 175, 279-287.
- Moore, M.N. (1985). Cellular responses to pollutants. *Marine Pollution Bulletin* 16, 134-139.
- Moore, M.N. (2002). Biocomplexity: the post-genome challenge in ecotoxicology. *Aquatic Toxicology* 59, 1-15.
- Moore, M.N., Koehler, A., Lowe, D. and Viarengo, A. (2008). Chapter Thirty-Three Lysosomes and Autophagy in Aquatic Animals. In *Methods in Enzymology*, J.K. Daniel, ed. Italy Moore, M.N., Koehler, A., Lowe, D. and Viarengo, A. (2008). Chapter Thirty-Three Lysosomes and Autophagy in Aquatic Animals. In *Methods in Enzymology*, J.K. Daniel, ed.: Academic Press, pp. 581-620.
- Moore, M.N., Wedderburn, R.J., Lowe, D.M. and Depledge, M.H. (1996). Lysosomal reaction to xenobiotics in mussel hemocytes using BODIPY-FL-verapamil. *Marine Environmental Research* 42, 99-105.
- Mora, J.F.d.l., Van Berkel, G.J., Enke, C.G., Cole, R.B., Martinez-Sanchez, M. and Fenn, J.B. (2000). Electrochemical processes in electrospray ionization mass spectrometry. *Journal of Mass Spectrometry* 35, 939-952.
- Nahrgang, J., Brooks, S.J., Evenset, A., Camus, L., Jonsson, M., Smith, T.J., Lukina, J., Frantzen, M., Giarratano, E. and Renaud, P.E. (2012). Seasonal variation in biomarkers in blue mussel (*Mytilus edulis*), Icelandic scallop (*Chlamys islandica*) and Atlantic cod (*Gadus morhua*)—Implications for environmental monitoring in the Barents Sea. *Aquatic Toxicology*.
- Neff, J., Durell, G., Utvik, T.R., Johnson, S. and Frost, T. (2006). Oil well produced water discharges to the North Sea Part I: Comparison of deployed mussels (*Mytilus edulis*), semi-permeable membrane devices, and the DREAM model predictions to estimate the dispersion of polycyclic aromatic hydrocarbons. *Marine Environmental Research* 62, 29.
- Neff, J., Lee, K. and DeBlois, E.M. (2011a). Produced Water: Overview of Composition, Fates, and Effects of Produced Water. New York: Springer New York, pp. 3-54.
- Neff, J., M., Rabalais, N.N. and Boesh, D., F. (1987). *Offshore oil and gas development activities potentially causing Long-term environment effects*. New York: Elsevier Applied Science Publishers.

- Neff, J., Sauer, T.C. and Hart, A.D. (2011b). *Bioaccumulation of Hydrocarbons from Produced Water Discharged to Offshore Waters off the US Gulf of Mexico*. K. Lee, and J. Neff, eds. New York: Springer New York, pp. 441-477.
- Neff, J.M. (2002). *Bioaccumulation in marine organisms: effect of contaminants from oil well*. Oxford, UK: Elsevier Ltd.
- Nicholson, S. (1999). Cytological and Physiological Biomarker Responses from Green Mussels, *Perna viridis* (L.) Transplanted to Contaminated Sites in Hong Kong Coastal Waters. *Marine Pollution Bulletin* 39, 261-268.
- Nicholson, S. and Lam, P.K.S. (2005). Pollution monitoring in Southeast Asia using biomarkers in the mytilid mussel *Perna viridis* (Mytilidae: Bivalvia). *Environment International* 31, 121-132.
- Nigro, M., Falleni, A., Barga, I.D., Scarcelli, V., Lucchesi, P., Regoli, F. and Frenzilli, G. (2006). Cellular biomarkers for monitoring estuarine environments: Transplanted versus native mussels. *Aquatic Toxicology* 77, 339-347.
- Nozik-Grayck, E., Suliman, H.B. and Piantadosi, C.A. (2005). Extracellular superoxide dismutase. *The International Journal of Biochemistry & Cell Biology* 37, 2466-2471.
- Orszulik, S.T. (2008). *Environmental technology in the oil industry*. UK: Springer Science.
- OSPAR (2010a). *Offshore Oil and Gas Industry - What are the problems?* In Quality status report, p. 65.UK: OSPAR
- OSPAR (2010b). *Overview assessment of the implementation of OSPAR Recommendation 2001/1 for the management of produced water from offshore installations*.UK: OSPAR
- Ostling, O. and Johanson, K. (1984). Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Commun* 123, 8.
- Otitoloju, A. and Olagoke, O. (2011). Lipid peroxidation and antioxidant defense enzymes in *Clarias gariepinus* as useful biomarkers for monitoring exposure to polycyclic aromatic hydrocarbons. *Environmental Monitoring and Assessment* 182, 205-213.
- Patin, S. (1999). Environmental impact of the offshore oil and gas industry. New York, USA: EcoMonitor Publishing, East Northport.
- Patin, S.A. (2004). Assessment of Anthropogenic Impact on Marine Ecosystems and Biological Resources in the Process of Oil and Gas Field Development in the Shelf Area. *Water Resources* 31, 413-422.
- Patrauchan, M.A. and Oriel, P.J. (2003). Degradation of benzyldimethylalkylammonium chloride by *Aeromonas hydrophila* sp. K. *Journal of Applied Microbiology* 94, 266-272.
- Peek, K. and Gabbott, P.A. (1989). Adipogranular cells from the mantle tissue of *Mytilus edulis* L. I. Isolation, purification and biochemical characteristics of dispersed cells. *Journal of Experimental Marine Biology and Ecology* 126, 203-216.

- Pereira, C., Guilherme, S., Barroso, C., Verschaeve, L., Pacheco, M. and Mendo, S. (2010). Evaluation of DNA Damage Induced by Environmental Exposure to Mercury in *Liza aurata* Using the Comet Assay. *Archives of Environmental Contamination and Toxicology* 58, 112-122.
- Pérez-Cadahía, B., Laffon, B., Pásaro, E. and Méndez, J. (2004). Evaluation of PAH bioaccumulation and DNA damage in mussels (*Mytilus galloprovincialis*) exposed to spilled Prestige crude oil. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 138, 453-460.
- Peterson, B.L. and Cummings, B.S. (2006). A review of chromatographic methods for the assessment of phospholipids in biological samples. *Biomedical Chromatography* 20, 227-243.
- Pisanelli, B., Benedetti, M., Fattorini, D. and Regoli, F. (2009). Seasonal and inter-annual variability of DNA integrity in mussels *Mytilus galloprovincialis*: A possible role for natural fluctuations of trace metal concentrations and oxidative biomarkers. *Chemosphere* 77, 1551-1557.
- Placek, D.G. and Shankwalkar, S.G. (1994). Phosphate ester surface treatment for reduced wear and corrosion protection. *Wear* 173, 207-217.
- Ramirez, P. (2005). Oil field-produced water discharges into wetlands: Benefits and risks to wildlife. *Environmental Geosciences* 12, 65-72.
- Rank, J. and Jensen, K. (2003). Comet assay on gill cells and hemocytes from the blue mussel *Mytilus edulis*. *Ecotoxicology and Environmental Safety* 54, 323-329.
- Rank, J., Jensen, K. and Jespersen, P.H. (2005). Monitoring DNA damage in indigenous blue mussels (*Mytilus edulis*) sampled from coastal sites in Denmark. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 585, 33-42.
- Rank, J., Lehtonen, K.K., Strand, J. and Laursen, M. (2007). DNA damage, acetylcholinesterase activity and lysosomal stability in native and transplanted mussels (*Mytilus edulis*) in areas close to coastal chemical dumping sites in Denmark. *Aquatic Toxicology* 84, 50-61.
- Rashmi, T., Tyagi, V.K. and Pandey, S.K. (2007). Imidazoline and Its Derivatives: An Overview. *Journal of Oleo Science* 56, 211-222.
- Rattner, B.A., Capizzi, J.L., King, K.A., LeCaptain, L.J. and Melancon, M.J. (1995). Exposure and effects of oilfield brine discharges on western sandpipers (*Calidris mauri*) in Nueces Bay, Texas. *Bulletin of Environmental Contamination and Toxicology* 54, 683-689.
- Reineke, V., Rullkötter, J., Smith, E.L. and Rowland, S.J. (2006). Toxicity and compositional analysis of aromatic hydrocarbon fractions of two pairs of undegraded and biodegraded crude oils from the Santa Maria (California) and Vienna basins. *Organic Geochemistry* 37, 1885-1899.
- Reynaud, S., Worms, I.A.M., Veyrenc, S., Portier, J., Maitre, A., Miaud, C. and Raveton, M. (2012). Toxicokinetic of benzo[a]pyrene and fipronil in female green frogs (*Pelophylax kl. esculentus*). *Environmental Pollution* 161, 206-214.
- Rhodia (2006). Tailor-made phosphate ester for industrial application. (Needham, USA), p. 2.

- Richardson, B.J., Siu, S.Y.M., Lam, P.K.S., Martin, M. and Caldwell, C.W. (2008). The use of selected genotoxicity assays in green-lipped mussels (*Perna viridis*): A validation study in Hong Kong coastal waters. *Marine Pollution Bulletin* 57, 479-492.
- Ringwood, A.H., Connors, D.E. and Hoguet, J. (1998). Effects of natural and anthropogenic stressors on lysosomal destabilization in oysters *Crassostrea virginica*. *Marine Ecology Progress Series* 166, 163-171.
- Riva, C., Cristoni, S. and Binelli, A. (2012). Effects of triclosan in the freshwater mussel *Dreissena polymorpha*: A proteomic investigation. *Aquatic Toxicology* 118–119, 62-71.
- Rohner, T.C., Lion, N. and Girault, H.H. (2004). Electrochemical and theoretical aspects of electrospray ionisation. *Physical Chemistry Chemical Physics* 6, 3056-3068.
- Rojas, E., Lopez, M.C. and Valverde, M. (1999). Single cell gel electrophoresis assay: methodology and applications. *Journal of Chromatography B: Biomedical Sciences and Applications* 722, 225-254.
- Rybakovas, A., Baršienė, J. and Lang, T. (2009). Environmental genotoxicity and cytotoxicity in the offshore zones of the Baltic and the North Seas. *Marine Environmental Research* 68, 246-256.
- Santovito, G., Piccinni, E., Cassini, A., Irato, P. and Albergoni, V. (2005). Antioxidant responses of the Mediterranean mussel, *Mytilus galloprovincialis*, to environmental variability of dissolved oxygen. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 140, 321-329.
- Sastri, V.S. (2011). *Green corrosion inhibitors: theory and practice*. [electronic resource] Hoboken, N.J.: Wiley.
- Schafer, F.Q. and Buettner, G.R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Biology and Medicine* 30, 1191-1212.
- Scholz-Böttcher, B.M., Ahlf, S., Vazquez-Gutierrez, F. and Rullkötter, J. (2008). Sources of hydrocarbon pollution in surface sediments of the Campeche Sound, Gulf of Mexico, revealed by biomarker analysis. *Organic Geochemistry* 39, 1104-1108.
- Seed, R and Richardson, C. A. (1990). *Mytilus* growth and its environmental responsiveness. In: Stefano, G. B *Neurobiology of Mytilus edulis*. Manchester: Manchester University Press. pp. 1-30.
- Shaw, J.P., Dondero, F., Moore, M.N., Negri, A., Dagnino, A., Readman, J.W., Lowe, D.R., Frickers, P.E., Beesley, A., Thain, J.E. and Viarengo, A. (2011). Integration of biochemical, histochemical and toxicogenomic indices for the assessment of health status of mussels from the Tamar Estuary. *U.K. Marine Environmental Research* 72, 13-24.
- Shugart, L.R. (2000). A Simple technique for quantitation of low level of DNA damage in individual cells. *Ecotoxicology* 9, 11.

Sigma. 2012. *Superoxide dismutase* [Online]. Sigma-alorich. Available: <http://www.sigmaaldrich.com/content/dam/sigma-aldrich> [Accessed: 17thDecember 2012].

- Singh, N.P. (2000). Microgels for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 455, 111-127.
- Singh, N.P., McCoy, M.T., Tice, R.R. and Schneider, E.L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research* 175, 184-191.
- Singh, N.P. and Stephens, R.E. (1997). Microgel electrophoresis: Sensitivity, mechanisms, and DNA electrostretching. *Mutation Research/DNA Repair* 383, 167-175.
- Singh, R. and Hartl, M. (2012). Fluctuating estuarine conditions are not confounding factors for the Comet assay assessment of DNA damage in the mussel *Mytilus edulis*. *Ecotoxicology*, 1-6.
- Sinnhuber, R.O., Yu, T.C. and Yu, T.C. (1958). Characterization of the Red Pigment Formed in the 2-Thiobarbituric Acid Determination of Oxidative Rancidity a, b. *Journal of Food Science* 23, 626-634.
- Smith, C.J., Shaw, B.J. and Handy, R.D. (2007). Toxicity of single walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects. *Aquatic Toxicology* 82, 94-109.
- Somerville, H.J., Bennett, D., Davenport, J.N., Holt, M.S., Lynes, A., Mahieu, A., McCourt, B., Parker, J.G., Stephenson, R.R., Watkinson, R.J. and Wilkinson, T.G. (1987). Environmental effect of produced water from North Sea oil operations. *Marine Pollution Bulletin* 18, 549-558.
- Spear, N. and Aust, S.D. (1995). Hydroxylation of Deoxyguanosine in DNA by Copper and Thiols. *Archives of Biochemistry and Biophysics* 317, 142-148.
- Speit, G. and Hartmann, A. (1999). *The Comet Assay (Single-Cell Gel Test) DNA Repair Protocols*. United States of America: Humana Press, pp. 203-212.
- Stadtman, E.R. and Oliver, C.N. (1991). Metal-catalyzed oxidation of proteins. Physiological consequences. *The Journal of Biological Chemistry* 266, 2005-2008.
- Stagg, R., Gore, D.J., Whale, G.F., Kirby, M.F., Blackburn, M., Bifield, S., McIntosh, A.D., Vance I., Flynn, S.A. and Foster, A. (1996). Field evaluation of toxic effects and dispersion of produce water discharge from North Sea oil platforms: Implications for monitoring acute impacts in the environment. M. Reed and S. Jonsen eds. Plenum Press: *Environmental Issues and Mitigation Technoloies*, New York, pp. 81-99.

- Stankovic, S., Jovic, M., Stankovic, A.R. and Katsikas, L. (2012). *Heavy Metals in Seafood Mussels. Risks for Human Health Environmental Chemistry for a Sustainable World*. E. Lichtfouse, J. Schwarzbauer, and D. Robert, eds. Springer Netherlands: *Nanotechnology and Health Risk*, pp. 311-373.
- Steinert, S.A. (1999). DNA damage as a bivalve biomarker. *Biomarkers* 4, 492-496.
- Steinert, S.A., Streib-Montee, R., Leather, J.M. and Chadwick, D.B. (1998). DNA damage in mussels at sites in San Diego Bay. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 399, 65-85.
- Stephenson, M.T. (1992). Components of produced water; A compilation of industry studies. *Journal of Petroleum Technology*, 44:5, 548-550.
- Stromgren, T., Sorstrom, S.E., Schou, L., Kaarstad, I., Aunaas, T., Brakstad, O.G. and Jonasen, O. (1995). Acute toxic effects of produced water in relation to chemical composition and dispersion. *Marine Environmental Research*, 40:2, 147-169.
- Sun, Y., Oberley, L.W. and Li, Y. (1988). A simple method for clinical assay of superoxide dismutase. *Clinical Chemistry* 34, 497-500.
- Sundt, R.C., Pampanin, D.M., Grung, M., Baršienė, J. and Ruus, A. (2011). PAH body burden and biomarker responses in mussels (*Mytilus edulis*) exposed to produced water from a North Sea oil field: Laboratory and field assessments. *Marine Pollution Bulletin* 62, 1498-1505.
- Sundt, R.C., Ruus, A., Jonsson, H., Skarphéðinsdóttir, H., Meier, S., Grung, M., Beyer, J. and Pampanin, D.M. (2012). Biomarker responses in Atlantic cod (*Gadus morhua*) exposed to produced water from a North Sea oil field: Laboratory and field assessments. *Marine Pollution Bulletin* 64, 144-152.
- Sureda, A., Box, A., Tejada, S., Blanco, A., Caixach, J. and Deudero, S. (2011). Biochemical responses of *Mytilus galloprovincialis* as biomarkers of acute environmental pollution caused by the Don Pedro oil spill (Eivissa Island, Spain). *Aquatic Toxicology* 101, 540-549.
- Svendsen, C., Spurgeon, D.J., Hankard, P.K. and Weeks, J.M. (2004). A review of lysosomal membrane stability measured by neutral red retention: is it a workable earthworm biomarker? *Ecotoxicology and Environmental Safety* 57, 20-29.
- Svendsen, C. and Weeks, J.M. (1995). The use of a lysosome assay for the rapid assessment of cellular stress from copper to the freshwater snail *Viviparus contectus* (Millet). *Marine Pollution Bulletin* 31, 139-142.
- Taban, I.C., Bechmann, R.K., Torgrimsen, S., Baussant, T. and Sanni, S. (2004). Detection of DNA damage in mussels and sea urchins exposed to crude oil using comet assay. *Marine Environmental Research* 58, 701-705.
- Taylor, K.C., Burke, R.A., Nasr-El-Din, H.A. and Schramm, L.L. (1998). Development of a flow injection analysis method for the determination of acrylamide copolymers in brines. *Journal of Petroleum Science and Engineering* 21, 129-139.

- Tezel, U. and Pavlostathis, S.G. (2011). *Antimicrobial Resistance in the Environment*, Canada: John Wiley & Sons, Inc., pp. 349-387.
- Tice, R.R., Agurell, E. anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C. and Sasaki, Y.F. (2000). Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis* 35, 206-221.
- Torres, A.s., Moacir, Pires Testa, C., Gáspari, C., Beatriz Masutti, M., Maria Neves Panitz, C., Curi-Pedrosa, R., Alves de Almeida, E., Di Mascio, P. and Wilhelm Filho, D. (2002). Oxidative stress in the mussel *Mytella guyanensis* from polluted mangroves on Santa Catarina Island, Brazil. *Marine Pollution Bulletin* 44, 923-932.
- Tran, D., Moody, A.J., Fisher, A.S., Foulkes, M.E. and Jha, A.N. (2007). Protective effects of selenium on mercury-induced DNA damage in mussel haemocytes. *Aquatic Toxicology* 84, 11-18.
- Turja, R., Soirinsuo, A., Budzinski, H., Devier, M.H. and Lehtonen, K.K. (2012). Biomarker responses and accumulation of hazardous substances in mussels (*Mytilus trossulus*) transplanted along a pollution gradient close to an oil terminal in the Gulf of Finland (Baltic Sea). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 157, 80-92.
- Tyagi, R., Tyagi, V.K. and Pandey, S.K. (2007). Imidazoline and Its Derivatives: An Overview. *Journal of Oleo Science* 56, 211-222.
- Utvik, T.I., Røe (1999). Chemical characterisation of produced water from four offshore oil production platforms in the North Sea. *Chemosphere* 39, 2593-2606.
- Valavanidis, A., Vlahogianni, T., Dassenakis, M. and Scoullou, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety* 64, 178-189.
- Valentine, J.S., Doucette, P.A. and Zittin Potter, S. (2005). Copper-Zinc Superoxide Dismutase and Amyotrophic Lateral Sclerosis. *Annual Review of Biochemistry* 74, 563-593.
- Valérie, Z. (2001). The neutral red release assay: A review. *Fund for the Replacement of Animals in Medical Experiments* 29, 24.
- Wake, H. (2005). Oil refineries: a review of their ecological impacts on the aquatic environment. *Estuarine, Coastal and Shelf Science* 62, 131-140.
- Wedderburn, J., Cheung, V., Bamber, S., Bloxham, M. and Depledge, M.H. (1998). Biomarkers of biochemical and cellular stress in *Carcinus maenas*: an in situ field study. *Marine Environmental Research* 46, 321-324.
- Wedderburn, J., McFadzen, I., Sanger, R.C., Beesley, A., Heath, C., Hornsby, M. and Lowe, D. (2000). The Field Application of Cellular and Physiological Biomarkers, in the Mussel *Mytilus edulis*, in Conjunction with Early Life Stage Bioassays and Adult Histopathology. *Marine Pollution Bulletin* 40, 257-267.

- Weeks, J.M. and Svendsen, C. (1996). Neutral red retention by lysosomes from earthworm (*Lumbricus rubellus*) coelomocytes: A simple biomarker of exposure to soil copper. *Environmental Toxicology and Chemistry* 15, 1801-1805.
- Wilhelm Filho, D., Tribess, T., Gáspari, C., Claudio, F.D., Torres, M.A. and Magalhães, A.R.M. (2001). Seasonal changes in antioxidant defenses of the digestive gland of the brown mussel (*Perna perna*). *Aquaculture* 203, 149-158.
- Winston, G.W. (1991). Oxidants and antioxidants in aquatic animals. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 100, 173-176.
- Woods, J.A., O'Leary, K.A., McCarthy, R.P. and O'Brien, N.M. (1999). Preservation of comet assay slides: comparison with fresh slides. *Elsevier Science B.V.* 429, 6.
- Woodward, D.F. and Riley, R.G. (1983). Petroleum hydrocarbon concentrations in a salmonid stream contaminated by oil field discharge water and effects on macrobenthos. *Archives of Environmental Contamination and Toxicology* 12, 327-334.
- Yu, S.-p., Lu, J.-f. and Sun, G.-y. (2003). Impact of oil field exploitation on eco-environment of the Daqing lakes. *Chinese Geographical Science* 13, 175-181.
- Zelko, I.N., Mariani, T.J. and Folz, R.J. (2002). Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radical Biology and Medicine* 33, 337-349.
- Zhang, Y., Gao, B., Lu, L., Yue, Q., Wang, Q. and Jia, Y. (2010). Treatment of produced water from polymer flooding in oil production by the combined method of hydrolysis acidification-dynamic membrane bioreactor-coagulation process. *Journal of Petroleum Science and Engineering* 74, 14-19.
- Zhu, S., King, S.C. and Haasch, M.L. (2008). Biomarker induction in tropical fish species on the Northwest Shelf of Australia by produced formation water. *Marine Environmental Research* 65, 315-324.
- Zorita, I., Ortiz-Zarragoitia, M., Soto, M. and Cajaraville, M.P. (2006). Biomarkers in mussels from a copper site gradient (Visnes, Norway): An integrated biochemical, histochemical and histological study. *Aquatic Toxicology* 78, Supplement, S109-S116.
- Zrafi-Nouira, I., Khedir-Ghenim, Z., Zrafi, F., Bahri, R., Cheraeif, I., Rouabhia, M. and Saidane-Mosbahi, D. (2008). Hydrocarbon Pollution in the Sediment from the Jarzouna-Bizerte Coastal Area of Tunisia (Mediterranean Sea). *Bulletin of Environmental Contamination and Toxicology* 80, 566-572.