

Oxidative damage and antioxidant metabolism of
Ulva pertusa and the associated grazer
Micrelenchnus tenebrosus in response to
fluoranthene exposure

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Abstract

Coastal marine systems are both ecologically and economically important worldwide, yet over the last few decades these environments have become increasingly impacted by contaminant inputs, particularly from urban runoff, industrial discharge and marine traffic. Some of the most prevalent contaminants of the coast are Polycyclic Aromatic Hydrocarbons (PAHs), originating from diesel fuels, sewage and as products of industrial incineration. Low level concentrations of PAHs continuously enter populated coastal areas, fluoranthene (FLA) being one of the most common. *Ulva* spp are cosmopolitan marine macroalgae and are often the trophic base of ecologically diverse coastal systems, providing food, shelter and breeding grounds for many coastal marine organisms. *Ulva* spp are typically very hardy, existing in nutrient deficient and rich waters throughout low to high gradients of pollution. The aforementioned attributes, along with its easy accessibility and structural uniformity (single cell type), make *Ulva* spp ideal candidates for PAH monitoring. Little research exists on the biochemical changes occurring in seaweed exposed to PAH pollutants and their potential use as a monitoring tool. This thesis explores that possibility by analysing biochemical changes, in the form of oxidative stress, macromolecule damage and antioxidant responses in *Ulva pertusa* exposed to four environmentally relevant concentrations of FLA, ranging from constant low level input concentrations (0.01nmol/l) to extreme solitary-event levels (10nmol/l). All FLA exposure induced some production of reactive oxygen species (ROS) and subsequent up-regulation of many key antioxidants and enzymes including: catalase (CAT) ascorbate (AsA), ascorbate peroxidase (APX), mono-dehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR) and glutathione S-transferase (GST). Additionally, *U. pertusa* accumulated FLA in its tissue and was unable to recover from damage incurred at high FLA exposure (10nmol/l), while all *U. pertusa* exposed to lower concentrations recovered after a few days.

The ecological importance of *Ulva* spp to other trophic level species prompted an investigation into the effect of three varying levels of FLA contamination (0.01, 0.1 and 1nmol/l) on an associated gastropod grazer (*Micrelenchus tenebrosus*). This was conducted under two independent contaminant regimes: the first being seawater contamination with *U. pertusa* as a food-source, the second; grazing of FLA contaminated *U. pertusa* in uncontaminated seawater. Both contaminant regimes produced an increased level of cellular FLA in the grazer, where levels increased according to concentration and duration of

exposure. Interestingly, *M. tenebrosus* grazing on contaminated *U. pertusa* accumulated 2-3 times more FLA than specimens exposed to contaminated seawater alone. Both contaminant regimes caused oxidation of lipids, proteins and DNA in *M. tenebrosus* and the up-regulation of antioxidants, including: CAT, glutathione (GSH), glutathione peroxidase (GPX), glutathione reductase (GR) and GST. Levels of antioxidant and enzyme activity were again higher overall in snails under the food-source contamination regime, suggesting dietary uptake of FLA by grazing invertebrates enhances its toxicity to the consumer.

In conclusion the antioxidant activity of both seaweeds and grazing invertebrates may be useful bioindicators for future monitoring of coastal marine PAH contamination globally.

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Abbreviations

- APX** – ascorbate peroxidase
- BaP** – benzo[a]pyrene
- AsA** – ascorbate
- CAT** – catalase
- CO₂** – carbon dioxide
- DHA** – dehydroascorbate
- DHAR** – dehydroascorbate reductase
- DNA** – deoxyribose nucleic acid
- EPA** – environmental protection agency
- ERA** – environmental risk assessment
- FESW** – filtered enriched seawater
- FLA** – fluoranthene
- GC-MS** – gas chromatography-mass spectrometry
- GPX** – glutathione peroxidase
- GR** – glutathione reductase
- GSH** – glutathione
- GSSG** – oxidised glutathione
- GST** – glutathione S-transferase
- H₂O₂** – hydrogen peroxide
- HPLC** – high performance liquid chromatography
- MDAR** – mono-dehydroascorbate reductase
- MDA** – mono-dehydroascorbate
- PAH** – polycyclic aromatic hydrocarbon
- PCB** – Polychlorinated biphenyl
- POP** – persistent organic pollutant
- PUFA** – polyunsaturated fatty acid
- ROS** – reactive oxygen species
- SOD** – superoxide dismutase
- UV** – ultra-violet

Chapter 1 – General Introduction

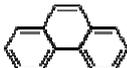
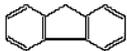
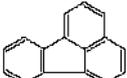
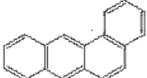
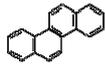
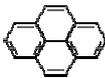
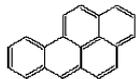
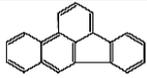
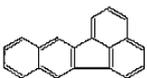
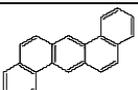
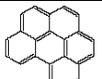
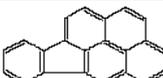
Polycyclic Aromatic Hydrocarbons (PAH's)

Polycyclic aromatic hydrocarbons (PAH's) belong to a group of compounds known as persistent organic pollutants (POP's) (Harvey, 1997). There are 16 PAHs described as priority pollutants by the US environmental protection agency (EPA), each of which consists of between two and six fused benzene rings (EPA, 1987, Bojes and Pope, 2007). These priority pollutants have been identified as such due to their potential toxicity towards terrestrial and aquatic organisms, including humans (Samanta et al., 2002, Bojes and Pope, 2007) and are: naphthalene (NAP), acenaphthene (ACN), acenaphthylene (ACL), anthracene (ANT), phenanthrene (PHE), fluorine (FLU), fluoranthene (FLA), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CRY), pyrene (PYR), benzo[a]pyrene (BaP), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), dibenzo[a,h]anthracene (DBA), benzo[g,h,i]perylene (BPR) and indeno[1,2,3-cd]pyrene (INP) (Latimer and Zheng, 2003, Bojes and Pope, 2007). Though each PAH has unique physical and chemical properties, in accordance with structure and number of rings (Table. 1.1), they are typically solid at ambient temperatures and are characterised by high melting and boiling points, low vapour pressures and low solubility in water (particularly those with higher molecular weights) (Douben, 2003).

PAHs in the Environment

PAHs principally originate from petroleum derived products; they exist in fossil fuels, coal, tar and even cigarettes, and primarily enter the atmosphere as a by-product of processing or incomplete combustion of such products, as well as the incineration of waste products (Samanta et al., 2002, Douben, 2003). Atmospheric PAHs are typically associated with particulate matter, but also exist as gases (Chang et al., 2006), and vary in abundance with geographic location (Latimer and Zheng, 2003). Total atmospheric PAH concentrations throughout Asia ranges from 1.97 to 500ng/m³ (Chang et al., 2006, Latimer and Zheng, 2003), while those in North America are between 3.7 and 450ng/m³ (Latimer and Zheng, 2003) and those observed across Europe range from 0.2ng/m³ in Germany (Schnelle-Kreis et al., 2001) to 137ng/m³ in London (Wild and Jones, 1995). Urban and industrial areas typically have much higher concentrations of gaseous and particle phase PAHs (Bae et al., 2002, Fang et al., 2004a, Fang et al., 2004b).

Table 1.1: The 16 priority PAH pollutants defined by the EPA, along with diagrams of their molecular structure and some defining properties associated with each, including: number of rings, molecular weight (g/mole) and solubility in water (mg/L). Mean (ng/L \pm SD) PAH concentrations from marine waters of Hong Kong (Latimer and Zheng, 2003, Douben, 2003) and Narragansett Bay, North America (Quinn et al., 1988, Haritash and Kaushik, 2009) are also presented.

POLYCYCLIC AROMATIC HYDROCARBONS (PAH's)	STRUCTURE	NO. OF RINGS	MOLECULAR WEIGHT (g/mole)	SOLUBILITY (mg/L)	MEAN MARINE WATER CONCENTRATION (ng/L \pm SD)	
					N. Bay	Hong Kong
Naphthalene		2	128.17	31	0.50 \pm 0.81	199.3 \pm 164.8
Acenaphthene		3	154.21	3.8	0.22 \pm 0.40	128.6 \pm 103.9
Acenaphthylene		3	152.20	16.1	0.06 \pm 0.30	23.3 \pm 26.6
Anthracene		3	178.23	0.045	0.46 \pm 1.15	28.0 \pm 24.0
Phenanthrene		3	178.23	1.1	3.30 \pm 3.76	66.5 \pm 54.6
Fluorene		3	166.22	1.9	0.29 \pm 0.63	48.9 \pm 33.6
Fluoranthene		4	202.26	0.26	6.14 \pm 7.92	30.2 \pm 28.4
Benzo(a)anthracene		4	228.29	0.011	2.04 \pm 3.61	na
Chrysene		4	228.29	0.0015	5.37 \pm 8.64	24.9 \pm 30.4
Pyrene		4	202.26	0.132	5.95 \pm 7.23	54.1 \pm 65.5
Benzo(a)pyrene		5	252.32	0.0038	2.70 \pm 4.30	70.4 \pm 69.5
Benzo(b)fluoranthene		5	252.32	0.0015	6.99 \pm 9.71	18.9 \pm 17.1
Benzo(k)fluoranthene		5	252.32	0.0008	na	17.0 \pm 15.9
Dibenz(a,h)anthracene		6	278.35	0.0005	0.48 \pm 1.17	29.8 \pm 36.1
Benzo(g,h,i)perylene		6	278.35	0.00026	2.60 \pm 5.37	15.3 \pm 16.2
Indeno[1,2,3-cd]pyrene		6	278.35	0.062	1.96 \pm 3.12	17.2 \pm 16.8

PAHs readily enter the coastal marine environment both directly and indirectly (Latimer and Zheng, 2003). Atmospheric PAHs are a major contributing source of direct contamination from urban runoff, wastewater effluents, industrial outfalls, oil leaks and catastrophic oil spill events (Latimer and Zheng, 2003), as are the indirect inputs of atmospheric deposition and particle precipitation (Wania et al., 1998). Concentrations of PAHs in marine environments vary dramatically and can range from undetectable to 11 µg/L (Douben, 2003) (Table 1.1 for examples). Coastal marine environments exhibit higher levels of PAH contamination than oceanic water bodies, as they are commonly associated with high urbanisation and industrialisation (as are atmospheric PAH concentrations) (Kim et al., 1999, Zheng and Richardson, 1999). Coastal marine PAHs are partitioned between the water column and sediment (Latimer and Zheng, 2003). In accordance with their low solubility and hydrophobic nature, marine PAH deposits have a tendency to reside in suspended particles and accumulate in sediments, rather than the water itself (Latimer and Zheng, 2003).

Toxicity

Many PAHs are considered among the most harmful contaminants with recognised toxicity towards both terrestrial and aquatic organisms (Eisler, 1987, Burgess et al., 2003). Direct contact, inhalation and absorption of soluble PAH pollutants, can cause both acute toxicity and carcinogenesis in organisms (Eisler, 1987). Acute toxicity is associated with fewer-ringed PAHs, while those with high molecular weights and low solubility are typically considered carcinogenic (Bojes and Pope, 2007). The most recognised carcinogenic PAH is benzo[a]pyrene (BaP), which can induce mutations by binding directly to DNA and forming adducts (Leung et al., 2007, Singh et al., 2007). In addition to inducing carcinogenesis other PAHs can interact with the physiological mechanisms of an organism, causing oxidative stress (Torres et al., 2008).

Bioavailability, Uptake and Accumulation

Common background PAH concentrations in coastal marine systems range from tens to hundreds of parts per billion and are dominated by low molecular weight PAHs (3- rings) (Burgess et al., 2003, Latimer and Zheng, 2003). The higher molecular weight PAHs tend to have a more recalcitrant nature which ensures their deposit and accumulation in marine sediments (Burgess et al., 2003, Kanaly and Harayama, 2000). Aquatic organisms typically achieve uptake of PAHs by passive diffusion, whereby the difference in pressure between organism tissues and the external environment control the rate of diffusion and tissue concentration (Meador, 2003). Though it is reasonable to assume hydrophobicity of

individual PAHs would affect their uptake rate, little variability has been shown between contaminants of varied hydrophobic properties and their uptake rate (Bender et al., 1988).

PAH availability and specific organism physiology are the two key variables that influence uptake in any marine environment (Meador, 2003). In aquatic systems algae can assimilate PAH contaminants rapidly, removing them from sediments and the water column (Greenberg, 2003). Other marine organisms can passively take up PAHs through integument and gill diffusion, or accumulate them via ingestion of contaminated detritus and prey items (Meador, 2003, Burgess et al., 2003). The biomagnification of PAHs through trophic transfer is not well understood, though some low trophic level primary consumers and detritivores may acquire higher levels of PAH contamination (den Besten et al., 2003, Meador, 2003).

Oxidative stress

Oxidative stress is a process whereby activated forms of oxygen (O_2), known as reactive oxygen species (ROS), form as free radicals in the tissues of an organism and subsequently cause 'oxidative' damage to that organism (Dalton, 1995, Lesser, 2006). ROS are produced as by-products of routine metabolic processes, such as photosynthesis and respiration, and are usually not harmful to the organism (Gould, 2003, Mittler, 2002, Moller et al., 2007, Davies, 2000). However during periods of increased biotic and abiotic stress, including exposure to toxic PAH pollutants, ROS can accumulate and subsequently cause damage to lipids, proteins and DNA, inhibiting physiological processes (Mittler, 2002, Moller et al., 2007). There are many types of ROS including singlet oxygen (1O_2), superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO\cdot$) (Dalton, 1995, Lesser, 2006). The less well characterised reactive nitrogen species (RNS) also exist, often in conjunction with ROS, in particular peroxynitrite ($ONOO^-$) (Gould, 2003).

Some ROS are free radicals ($O_2^{\cdot-}$, $OH\cdot$), described as being capable of independent existence and containing at least one unpaired electron, while others are non radicals (H_2O_2 , 1O_2) (Halliwell and Gutteridge, 2007). Non-radicals may become radicals by either losing or gaining a single electron (Halliwell and Gutteridge, 2007). O_2 itself is a free radical, though it is sometimes referred to as a bi-radical as it has two unpaired electrons, and thus O_2 has a limited ability to react with organic molecules unless 'activated' (Lesser, 2006). Oxygen is activated via two main methods, the first being oxidation whereby energy is transferred by the gain of O_2 or the loss of an electron, in contrast the reduction method involves the transfer of electrons and is accomplished by the loss of O_2 or the successive addition of electrons or hydrogen (H^+) (Ahmad, 1995, Halliwell and Gutteridge, 2007). Refer to Table 1.2 for more details on specific ROS formation.

Table 1.2: The dominant reactive oxygen and nitrogen species, including a description of how they are formed and some defining properties. Adapted from (Lesser, 2006) and (Moller et al., 2007).

ROS and RNS	Formation	Half Life	Reacts With
$^1\text{O}_2$ (Singlet oxygen)	Produced primarily through photochemical pathways. Energy increases in molecules absorbing specific light wavelengths are passed on to O_2 forming $^1\text{O}_2$.	1 μs	- Lipids (PUFA) - DNA (guanine) - Proteins (trp, his, tyr, met, cys)
$\text{O}_2^{\cdot-}$ (Superoxide)	An electron reduced state of O_2 , produced during auto-oxidation reactions in biological systems. Acts as both an oxidant and a reductant.	1 μs	- Lipids (slightly) - Proteins (FE-S centres)
H_2O_2 (Hydrogen peroxide)	Dismutation of $\text{O}_2^{\cdot-}$ catalysed by superoxide dismutase (SOD). $\text{O}_2^{\cdot-}$ can also dismutase spontaneously.	1ms	- Lipids (slightly) - Proteins (Cysteins)
$\text{HO}\cdot$ (Hydroxyl radical)	Produced by the reduction of H_2O_2 , by utilising electrons from the conversion of iron (Fe) from ferrous to ferric forms.	1ns	- Lipids (rapidly) - DNA (rapidly) - Proteins (rapidly)
ONOO^- (Peroxynitrite)	Nitric oxide ($\text{NO}\cdot$) produced in cells readily diffuses across biological membranes to react with $\text{O}_2^{\cdot-}$ producing ONOO^- .	<0.1s	- Lipids (highly) - DNA (guanine) - Proteins (tyr, trp, phe, met)

Protein Damage

Direct attack of proteins by ROS, particularly $\text{OH}\cdot$ and $^1\text{O}_2$, can cause degradation of cellular proteins and amino acids known as protein oxidation (Halliwell and Gutteridge, 2007). In addition to direct damage the assortment of end products created by lipid peroxidation can cause secondary damage to other proteins, resulting in the irreparable fragmentation of peptide chains (Lesser, 2006, Davies, 1987). Protein oxidation often impairs the function of cellular receptors, transport proteins and signalling enzymes, including but not limited to DNA repair enzymes (Halliwell and Gutteridge, 2007). In turn the inhibition of such enzymes results in increased oxidative damage to DNA, amplifying the frequency of mutations (Halliwell and Gutteridge, 2007). Due to the prevalence of protein oxidation, it is often considered a reliable indicator of oxidative stress, particularly in mammalian tissues (Moller et al., 2007). Carbonylation or, the formation of carbonyls, resulting from the oxidation of amino acids, is a particularly useful measure of oxidative stress and is used extensively as such (Moller et al., 2007, Halliwell and Gutteridge, 1999). Oxidised proteins may be completely degraded to amino acids by proteases and these amino acid products can be reutilised as carbon sources for ATP and protein synthesis, enabling replacement protein molecules to be synthesised (Davies, 2000).

Lipid Damage

As one of the most prevalent mechanisms of cellular injury caused by ROS, lipid peroxidation of polyunsaturated fatty acids (PUFAs) can result in a decrease in cell membrane fluidity, increased 'leakiness' of the cell and secondary damage to membrane proteins (as mentioned above) (Lesser, 2006, Moller et al., 2007). PUFAs exist as side-chains in cellular membranes and contain at least two carbon-carbon double bonds (Halliwell and Gutteridge, 2007). Lipid peroxidation is initiated by either the addition of a ROS onto the chain or the removal of H^+ from a methylene ($-CH_2-$) group by a ROS (Halliwell and Gutteridge, 2007). The latter creates carbon-centered lipid radicals that react rapidly with O_2 to produce peroxy radicals ($ROO\cdot$) which in turn attack adjacent PUFA side-chains, proliferating a chain reaction of lipid peroxidation and continued damage, $ROO\cdot$ combines with H^+ to produce lipid peroxides ($ROOH$) (Fig. 1.1). In addition degradation of $ROOH$ produces aldehyde by-products, such as malondialdehyde, that form conjugates with DNA and proteins (Moller et al., 2007). Prevention of ROS production is the best method of avoiding lipid peroxide damage, but in the event of lipid peroxidation cells can maintain homeostasis by repairing damaged lipid components (Davies, 2000). In particular, fatty acid hydroperoxides are substrates for GPX which reduces lipid peroxides to hydroxy fatty acids, removing the threat of reactive aldehyde products being produced (Ursini et al., 1985).

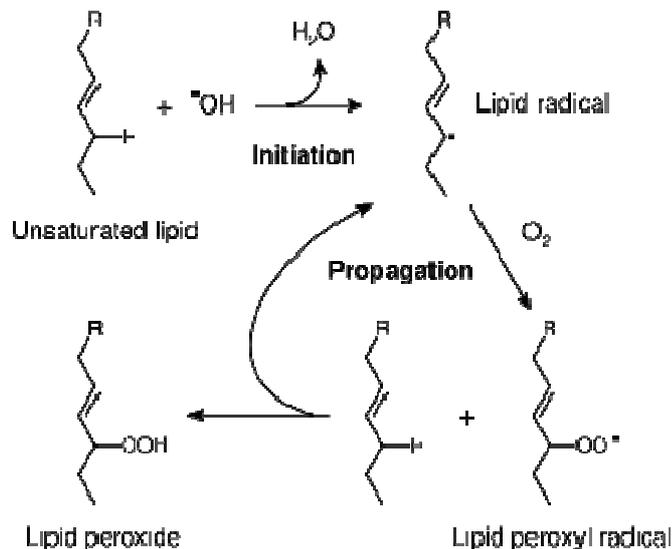


Figure 1.1: Radical chain reaction mechanism of lipid peroxidation. Adapted from (Young and McEneny, 2001) by (Vickers, 2007).

DNA Damage

DNA typically undergoes spontaneous decomposition but this can be amplified in the presence of oxidative stress, in particular $\text{OH}\cdot$ reacts with the base sugars of both DNA and RNA, specifically guanine (Halliwell and Gutteridge, 2007). DNA can undergo a multitude of modifications as a result of $\text{OH}\cdot$ exposure, including the conjugation of PUFA breakdown products mentioned previously, and the direct formation of OH-DNA adducts (Fig. 1.2). Such modifications result in deletions, strand breakage, base degradation, mutations and cross-linking of proteins causing lethal genetic effects (Lesser, 2006). Continued oxidative damage can lead to programmed cell death, known as apoptosis, or the involuntary death of cells through swelling and rupture (necrosis), and the eventual death of the organism if defences against ROS are 'overwhelmed' (Halliwell and Gutteridge, 2007). The mechanism of DNA repair is complex and dependant on the type of damage that has occurred (Halliwell and Gutteridge, 2007), yet antioxidant enzymes GST and GPX are believed to play an important role in the removal and excretion of oxidised DNA (Davies, 2000).

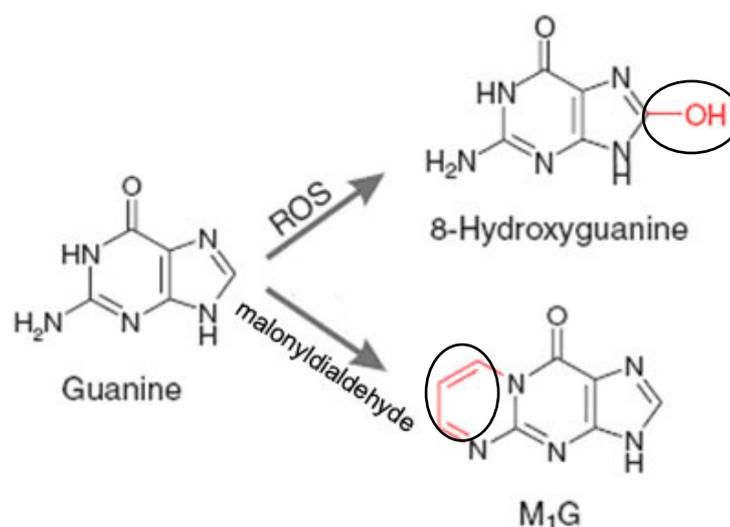


Figure 1.2: Oxidative modification product of DNA from both direct (ROS) and indirect (malondialdehyde) conjugation. Guanine is drawn as free although it would normally be oxidized while attached to the DNA. Modifications are circled. Modified from (Moller et al., 2007).

Antioxidant defences

Due to the prevalence and nature of O_2 , oxidative stress is almost unavoidable, and most organisms experience it to some extent, but the balance between cell damage and repair determines the fate of that organism (Lesser, 2006). Fortunately organisms have a variety of defences, known as antioxidants, to combat harmful ROS and stave off oxidation and subsequent damage (Dalton, 1995). Antioxidants prevent free radical production, scavenge existing ROS and unpaired electrons and purge chain reactions, and as such they are

considered primary defences against oxidative stress (Gould, 2003). The term antioxidant applies to ‘any substance that significantly delays or inhibits oxidation’ (Halliwell and Gutteridge, 2007), this incorporates both enzymes (Table 1.3) and other soluble compounds (e.g. vitamins) (Davies, 2000, Gould, 2003).

The ascorbate-glutathione cycle (Fig. 1.3a) is the fundamental metabolic pathway that involves the interconnected cycling of antioxidants and enzymes to detoxify cells of H_2O_2 before the formation of the highly reactive $\text{OH}\cdot$ radical (Mittler, 2002). After the initial reduction of $\text{O}_2^{\cdot -}$ to H_2O_2 by superoxide dismutase (SOD), H_2O_2 is scavenged by either catalase (CAT) (Fig. 1.3c) or peroxidases (Mittler, 2002). Ascorbate peroxidase (APX) scavenges H_2O_2 using ascorbate (AsA) as a substrate and producing monodehydroascorbate (MDA). Having eliminated H_2O_2 AsA is now recovered from MDA using the enzyme monodehydroascorbate reductase (MDAR). MDA may also spontaneously dissociate into AsA and dehydroascorbate (DHA), in which case the reduction agent dehydroascorbate reductase (DHAR) is employed, using glutathione (GSH) as a reducing substrate and producing oxidised glutathione (GSSG) in the process. The regeneration of GSH from GSSG by glutathione reductase (GR) completes the cycle (Mittler, 2002). Refer to Table 1.3 for more detailed enzymatic reactions.

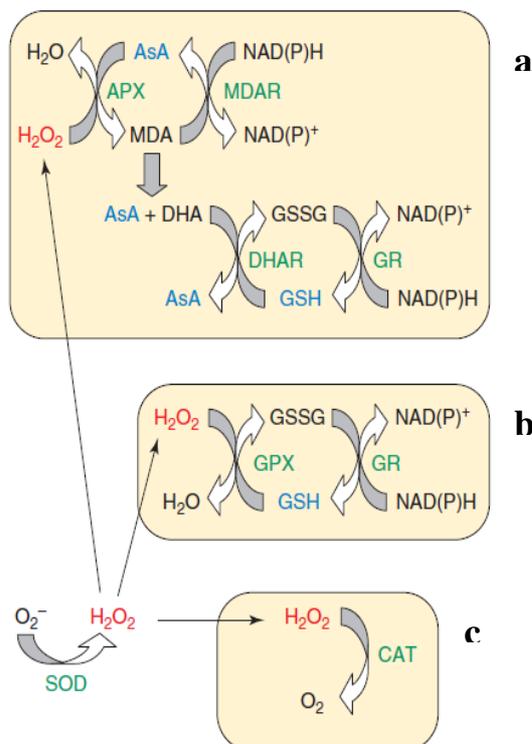


Figure 1.3: Cellular detoxification of ROS via the ascorbate-glutathione cycle (a), the glutathione peroxidase (GPX) cycle (b) and catalase (c). ROS are indicated in red, antioxidants in blue and antioxidant enzymes in green. Important abbreviations: DHAR=dehydroascorbate reductase, GR=glutathione reductase, GSSG=oxidized glutathione and MDAR=monodehydroascorbate reductase. Modified from (Mittler, 2002).

The glutathione peroxide cycle (Fig. 1.3b) works independently and alongside the ascorbate-glutathione cycle to eliminate H_2O_2 with the use of the catalysing enzyme glutathione peroxidase (GPX). The high ratio of reduced to oxidised glutathione (GSH:GSSG) is maintained in this pathway by the recycling of GSSG by GR back to GSH (Davies, 2000, Ahmad, 1995).

Enzymatic Antioxidants

Table 1.3: The key enzymatic antioxidants including a description of their function and the reactions they are involved in and associated Enzyme Commission numerical classification (EC number). A composite developed from information found in (Gould, 2003) and (Ahmad, 1995).

SOD (superoxide dismutase) EC 1.15.1.1	A rapid scavenger of $\text{O}_2^{\cdot-}$, reducing it to H_2O_2 $\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$
CAT (catalase) EC 1.11.1.6	A peroxisomal and mitochondrial scavenger of H_2O_2 $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$
APX (ascorbate peroxidase) EC 1.11.1.11	Key H_2O_2 scavenging enzyme in chloroplasts $2 \text{AsA} + \text{H}_2\text{O}_2 \rightarrow 2\text{MDA} + 2\text{H}_2\text{O}$
MDAR (monodehydroascorbate reductase) EC 1.6.5.4	Reduces MDA to AsA in the stroma $\text{MDA} + \text{NADPH} \rightarrow 2 \text{AsA} + \text{NADP}^+$
DHAR (dehydroascorbate reductase) EC 1.8.5.1	Reduces DHA to AsA producing GSSG $\text{DHA} + 2\text{GSH} \rightarrow \text{AsA} + \text{GSSG}$
GR (glutathione reductase) EC 1.6.4.2	Regenerates GSH from GSSG $\text{GSSG} + \text{NADPH} \rightarrow 2\text{GSH} + \text{NADP}^+$
GPX (glutathione peroxidase) EC 1.11.1.9	Catalyses the oxidation of glutathione with H_2O_2 $\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow 2\text{H}_2\text{O} + \text{GSSG}$

Non-enzymatic antioxidants

Ascorbate (AsA) – Also known as ascorbic acid or vitamin C, AsA is present in animals and plants. It functions as a reductant for ROS and as a substrate for detoxification reactions of H_2O_2 . In addition it can quench $^1\text{O}_2$ and scavenges $\text{O}_2^{\cdot-}$, $\text{HO}\cdot$ and lipid peroxides as well (Lesser, 2006).

Glutathione (GSH) – GSH is a tripeptide existing in both plants and animals. When it is oxidised, forming GSSG, it forms a thiyl radical that reacts with another oxidised glutathione to form a disulfide bond (Lesser, 2006). GSH can reduce H_2O_2 (Davies, 2000) and reacts with $^1\text{O}_2$, $\text{O}_2^{\cdot-}$, and $\text{HO}\cdot$ (Lesser, 2006). Additionally it acts as a substrate for GPX and is also a chain-breaker for free radical reactions (Lesser, 2006).

α -tocopherol – Also known as vitamin E, α -tocopherol is a lipid soluble, phenolic antioxidant with the ability to scavenge ROS, quenching both $^1\text{O}_2$ and peroxides (Lesser, 2006). Animals acquire α -tocopherol through their food while photosynthetic organisms synthesise it in chloroplasts (Lesser, 2006). A marine constituent of this antioxidant (α -tocomonoenol) provides enhanced protection as it can diffuse in viscous lipids and prevent lipid peroxidation (Yamamoto et al., 2001).

Glutathione S-transferase (GST) (EC 2.5.1.13)

This enzyme differs from the other enzymes and antioxidants in that it catalyses the direct conjugation of GSH with an offending toxicant (Ahmad, 1995). When severe oxidative damage prevents the primary antioxidants (mentioned above) from functioning GST can still remove the harmful substance, allowing the cell to regain homeostasis (Perl-Treves and Perl, 2002). In animals the toxicant conjugate is marked for excretion, while in plants it is rendered harmless by sequestration in the apoplast or vacuole, in a method known as ‘storage excretion’ (Marrs, 1996). GST is therefore considered a ‘detoxification enzyme’ rather than a traditional antioxidant (Ahmad, 1995).

Objectives and Rationale

PAHs are a ubiquitous and toxic contaminant of the coastal marine system. They exist in low background concentrations and have the ability to accumulate in sediments, marine invertebrates and algae. Marine species often exhibit oxidative stress during exposure to harmful xenobiotics like PAHs (Cheung et al., 2001, Pavoni et al., 2003, Livingstone, 1998). Little is known about the trophic transfer and possible biomagnification of marine PAH contaminants.

This study investigates the toxicity of the PAH contaminant fluoranthene (FLA), on the cosmopolitan macroalga *Ulva pertusa* and the associated invertebrate grazer *Micrelenchus tenebrosus* exposed to high and low input levels relevant to those *in situ*. These species can be used as low trophic level models of the coastal marine system, to observe the biochemical responses of oxidative damage and antioxidant metabolism as potential monitors of future PAH contamination.

Aims

- 1. To investigate the biochemical responses of *U. pertusa* exposed to a range of environmentally relevant FLA concentrations over time.**
- 2. To investigate the biochemical responses of *M. tenebrosus* after exposure to FLA contaminated seawater.**
- 3. To determine if the grazer *M. tenebrosus* is affected by consuming FLA contaminated food source (*U. pertusa*).**
- 4. To determine which of:**
 - a) contact with FLA contaminated seawater**
 - OR**
 - b) grazing an FLA contaminated food source****is more damaging to the marine invertebrate *M. tenebrosus*.**

Chapter 2 – Macroalgae

Introduction

Macroalgae are a diverse group including members of the rhodophyta (reds), chlorophyta (greens) and phaeophyta (browns) and are commonly known as seaweed (Adams, 1997). Seaweeds are defined as macroscopic, multicellular, photosynthetic organisms. They are the dominant primary producers of coastal sub-tidal marine systems and as such are key species in the energy transfer of this trophic system (Lobban and Harrison, 1997). *Ulva pertusa* (Kjellman, 1897), from the

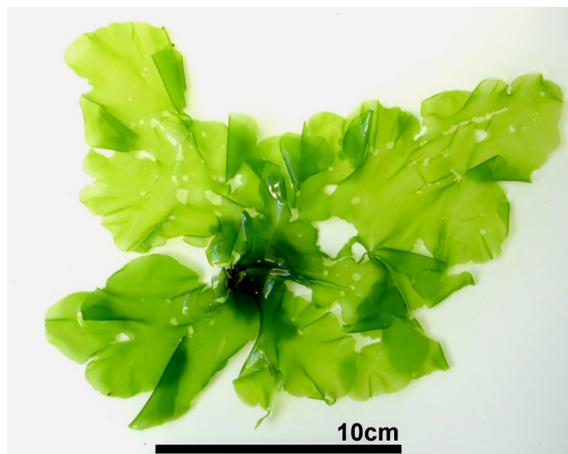


Figure 2.1: Common morphology of an individual *Ulva pertusa* specimen (www.algaebase.org, 2010)

cosmopolitan *Ulva* genus (family: Ulvaceae), and a member of the chlorophycophyta, is green in colour and consists of large rounded blades perforated by irregularly shaped holes, joined at a central thallus (Fig 2.1) (Heesch et al., 2007). *U. pertusa* as one of the most common *Ulva* species in New Zealand, occurring both intertidally and subtidally in harbours and open coasts, is commonly found free-floating or attached to substrates (Heesch et al., 2007).

Ulva and Oxidative Stress

Algal growth and development is heavily influenced by abiotic environmental factors including temperature, salinity, UV, pH and desiccation. When any such factor increases above the tolerable level of the alga stress occurs, influencing development, structure, physiology and biochemical processes (Lobban and Harrison, 1997, Ahmad et al., 2010). In addition to these naturally occurring abiotic stressors algae also face chemical abiotics in the form of xenobiotic pollutants (Torres et al., 2008). During abiotic stress high-energy state electrons are transferred to molecular O₂ forming toxic ROS molecules (Mittler, 2002). In response to oxidative stress caused by the resulting ROS, antioxidant mechanisms are activated to re-establish homeostasis of cells (Ahmad et al., 2010). H₂O₂ is a particularly harmful and persistent ROS in algae as it readily diffuses across membranes and is not restricted in the cell (Collen and Pedersen, 1996, Lesser, 2006) and is a known inhibitor of

photosynthetic enzymes, with the potential to reduce photosynthetic rate (Dummermuth et al., 2003). In addition H_2O_2 can directly inhibit enzymatic antioxidants (e.g. SOD), affecting the antioxidant metabolism and promoting oxidative damage (Forti and Gerola, 1997). If the balance between ROS producing stressors (e.g. pollutants and heavy metals) and stress responsive antioxidants exceeds equilibrium the destruction of lipids, proteins and nucleic acids will lead to permanent damage of the photosynthetic apparatus and eventual cell and organism death (Halliwell and Gutteridge, 2007, Dummermuth et al., 2003). Seaweeds are particularly susceptible to H_2O_2 stress as their carbon concentration mechanisms increase intracellular oxygen concentrations up to 5 times that of air (Raven et al., 1994). With more O_2 available for reduction the potential for ROS production is increased (Fridovich, 1978). In addition to the resulting H_2O_2 production from oxidative stress, photosynthetic organisms regularly produce H_2O_2 as a by-product of photosynthesis during the Mehler reaction (Collen et al., 1995). The most notable effect of H_2O_2 on photosynthetic organisms, like algae, is its disruption of the photosynthetic apparatus and consequent reduction in photosynthetic efficiency and growth (Pinto et al., 2003, Ross and van Alstyne, 2007, Mal et al., 2002, Huang et al., 1997).

Seaweeds and Pollutant Monitoring

Coastal marine zones are targets for anthropogenic pollution resulting from urban and industrial outfall (Lobban and Harrison, 1997). Common inputs from these sources include trace metals and crude oil (Lobban and Harrison, 1997). Biological indicator species have been used globally to monitor the effects of such xenobiotics (Rice, 2003); in the past presence/absence studies of aquatic plants were frequently used to evaluate contamination of waterways (Bat et al., 2001, Samecka-Cymerman and Kempers, 2002). This method is seldom used as the frequency and distribution of aquatic plants varies seasonally, making presence/absence an unreliable parameter for identifying aquatic pollution (Kim et al., 2004). Since presence/absence studies are unreliable, using intracellular xenobiotic levels of bioindicator species became common practice, particularly for evaluating heavy metal contamination (Lobban and Harrison, 1997). Macroalgae are frequently used as bioindicator species as they inhabit the highly polluted coastal environment, accumulating and subsequently reflecting (in their cells) metal contaminant concentrations (Torres et al., 2008). This method of pollutant monitoring has been deemed insufficient as internal metal concentrations of algae can be influenced by other factors including: algal growth rate, season, temperature, salinity and the presence of other pollutants (Ho, 1990, Lobban and Harrison,

1997). The method of observing reduced growth and photosynthetic rate of macroalgae is considered superior in determining heavy metal inputs than simply measuring cellular contamination levels (Pinto et al., 2003). *Ulva* spp meet Phillips (1990) prerequisites for a suitable bioindicator species as it is sessile, abundant, cosmopolitan, able to tolerate high levels of contamination and is a known bioaccumulator. Additionally *Ulva* is coastally ubiquitous, easy to collect and its tissue is uniform, making it an ideal monitor for anthropogenic contaminants (Ho, 1990, Villares et al., 2001). This perhaps explains why *Ulva* species have been and are still used to monitor heavy and trace metal contaminants globally (Ho, 1990, Barraza and Carballeira, 1999, Villares et al., 2001, Wu and Lee, 2008, Han et al., 2008, Pereira et al., 2009, Wu et al., 2009, Gonzalez et al., 2010, Kumar et al., 2010). In particular Barraza and Carballeira (1999) used bioassays to assess physiological stress in *Ulva rigida* exposed to cadmium (Cd) and copper (Cu) contaminated seawater, and found that Cd disturbed photosystem II and Cu diminished photosynthetic efficiency. Exposure to high concentrations of metal contaminants correlates with higher levels of accumulation in *U. rigida* cells, resulting in: photochemical and cell membrane disturbance, leading to electrochemical imbalance of cells and potassium (K^+) loss, and causing severe ionic imbalance and gradual cell death (Barraza and Carballeira, 1999). The observed imbalance of cellular metabolism confirms Pinto's (2003) suggestion that heavy metal contamination results in the production of toxic ROS and consequently oxidative damage.

PAH contamination has similarities with that of heavy metals as both are anthropogenic contaminants, existing in low background concentrations and present in higher concentrations at biologically productive coastal sites associated with urban and industrial centres (Lobban and Harrison, 1997, Pinto et al., 2003). The petroleum derived PAH products constitute up to 20% of crude oil wastes regularly discharged into the coastal marine environment and, as PAHs are lipophilic, they easily penetrate *Ulva* thallus, resulting in cellular disruption (Lobban and Harrison, 1997). This metabolic disruption is commonly observed as a reduction in photosynthetic rate and growth of *Ulva*, similar to that observed during exposure to heavy metal contaminants (Lobban and Harrison, 1997, Zambrano and Carballeira, 1999, Han et al., 2007, Han et al., 2009, Lage-Yusty et al., 2009). However, because photosynthesis is also reduced by oil directly coating *Ulva* thallus, reducing CO_2 diffusion and light penetration, photosynthetic rate alone is not a sufficient indicator of hydrocarbon induced stress (Zambrano and Carballeira, 1999). The production of ROS resulting in oxidative stress and consequently damage to lipids, proteins and DNA as a result of PAH exposure has been explored in higher plants (including *Arabidopsis*) (Alkio et al., 2005, Paskova et al., 2006), briefly in the aquatic plant *Lemna gibba* (duckweed) and in a

marine diatom (Wang et al., 2008), yet there has been no investigation into such effects from direct PAH exposure on important coastal macroalgal species like *Ulva*. Of all 16 priority PAH pollutants, FLA consistently comprises one of the highest fractions in aquatic systems associated with developed areas (Southerland and Lewitus, 2004) and is also one of the most toxic, non-carcinogenic PAHs known (Wang et al., 2008). For these reasons FLA was chosen as the representative PAH for this study.

Chapter Objective

The current chapter investigates the oxidative stress, physiological damage and antioxidant responses of *Ulva pertusa*, exposed to environmentally relevant concentrations of the petroleum derived PAH fluoranthene (FLA), in order to establish a better understanding of the metabolic processes involved and use this information to gain insight on monitoring such ubiquitous pollutants.

Methods

Collection

Ulva pertusa was collected subtidally from Wellers Rock, Otago Harbour (map Fig. 3.2 pg. 45), at approximately 1.0-2.0m depth during low tide, on 24th June 2009. Enough large healthy (dark green coloured thallus) individuals were collected to fill a large chilly bin. Seawater with minimal sediment from the collection site was added to the chilly bin before specimens were transported back to the lab. PAH concentrations in the collected seawater were determined to be below the detectable limit by GC-MS analysis (pers. com. David Burritt). The *Ulva* species was confirmed as *U. pertusa* by genetic analysis prior to collection, though not all *Ulva* samples were tested, the assumption was made that all *Ulva* of a similar morphology collected at this site was *U. pertusa* (Heesch et al., 2007).

Preparation

Newly collected *U. pertusa* individuals deemed healthy by thallus colour were picked clean of any visible epifauna and rinsed briefly under running filtered (Whatman GF/C50) seawater (previously enriched with nutrients, refer to appendix I), referred to as filtered enriched seawater (FESW). These *U. pertusa* thalli were then cut into 360 uniform 5x5cm squares (approx. 200-250mg FW), with the aid of a 5x5cm Perspex template and scalpel. A maximum of six thallus squares were cut from each individual *U. pertusa* thallus, in order to maintain natural variation among the sample population. Once cut all 360 thallus squares were eradicated of any remaining epifauna, placed in an open 21L clear plastic tank containing FESW and acclimated overnight in a growth cabinet (Contherm 620) set at 12°C on a 12hr light/dark cycle.

Treatment and Harvesting

Following overnight acclimation fourteen *U. pertusa* thallus squares were added to each of twenty 1.8L tanks containing 700ml of FESW and one of four FLA treatment concentrations (0.01, 0.1, 1 and 10nmol/l) or, in the case of the control, no FLA just a small amount of ethanol (as ethanol was used to make the initial FLA solutions and dilutions). Four replicate

tanks were allocated for each treatment and the control. The treatment tanks were then placed back in the growth cabinet, this time on a shaker table to keep the solutions mixed and aerated. The remaining thallus squares were harvested and frozen for use as day 0 (basal reference) samples. For each sampling day following experimental setup (day 1, 2, 3 and 5) three thallus squares were harvested from each treatment tank, briefly dabbed on absorbent paper towel to remove excess moisture, wrapped in individual packets of labelled tinfoil, snap frozen in liquid nitrogen and stored in a -80°C freezer. In preparation for extractions sample tissue was ground to a fine powder using a mortar and pestle and liquid nitrogen, except for uptake extractions where the tissue was first rinsed briefly in hexane before grinding.

Determining FLA Tissue Concentration

Tissue concentrations of FLA were then determined by high-performance liquid chromatography (HPLC) as per Gao and Zhu (2004) with minor modifications. FLA was extracted by adding 500µl of hexane:dichloromethane (85:15, v/v) to 100mg (FW) of frozen powdered *U. pertusa* tissue in a glass centrifuge tube, this was then centrifuged at 5,000g for 1min. The supernatant was passed through a Na₂SO₄ column, evaporated to dryness under a stream of nitrogen and redissolved in 2ml of hexane. Samples were then filtered through a silica column (2g of silica) using 1:1(v/v) hexane and dichloromethane as an eluant. Samples were then evaporated to dryness and redissolved in 2ml of methanol. Extracts were separated using a PerkinElmer liquid chromatography system (Perkin Elmer, San Jose, CA, U.S.A.) on a LiChrosphere 100RP-18 column (Phenomenix, Torrence, CA, USA), using methanol/water (83:17). Chromatography was conducted at 30°C, and FLA was detected at 245nm (PerkinElmer series 200 diode array detector). FLA standards, run under the same conditions, were used for identification and quantification. Losses during extraction were determined by the addition of known concentrations of FLA on tissue extracts, and all results were corrected, based on the percentage recovery.

Determining Hydrogen Peroxide

H₂O₂ was extracted by adding 500µl of ice-cold 100mM potassium phosphate buffer (pH 6.4), containing 5mM KCN and the catalase inhibitor hydroxylamine (0.5mM), to 50mg FW of powdered *U. pertusa* tissue in a microcentrifuge tube. This was then centrifuged at 14,000rpm for 15min at 4°C. 50µl of the supernatant was transferred to a microtitre plate well and H₂O₂

levels were then determined colorimetrically as described by Cheeseman (2006). The assay solution contained 50µL of *U. pertusa* extract, 250µM ferrous ammonium sulphate, 100µM sorbitol, 100µM xylenol orange in 25mM H₂SO₄ and 1% ethanol. Absorbance was measured at 550 and 800nm and the difference in absorbance between the two values calculated. A standard curve was generated using standards prepared from 30% H₂O₂ (Merck). The concentration of H₂O₂ in all standards was checked by measuring the absorbance at 240nm and calculating the actual H₂O₂ concentration using an extinction coefficient of 43.6 M⁻¹ cm⁻¹.

Determining Damage

Lipid Peroxides

Lipid peroxides were extracted by adding 300µl of methanol:chloroform (2:1v/v) to 50mg FW of powdered *U. pertusa* tissue left to stand for 1min, then 200µl of chloroform was added and mixed using a vortex mixer for 30sec then 200µl of deionised water (DI H₂O) was added and mixed again with the vortex mixer for 30sec. The resulting mixture was briefly placed in a centrifuge at 10,000rpm to separate the phases. 50µl of the bottom phase (lipid phase) was transferred to a microtitre plate well and lipid hydroperoxides were determined using the ferric thiocyanate method (Mihaljevic et al., 1996) adapted for measurement in a microtitre plate reader. Levels were determined by measuring the absorbance at 500nm. A calibration curve with t-butyl hydroperoxide was used and lipid hydroperoxide peroxide content calculated as nmol lipid hydroperoxide/g FW.

Protein Carbonyls

Protein carbonyls were extracted by adding 500µl of ice-cold enzyme buffer II (EBII) (appendix I) to 50mg FW of powdered *U. pertusa* tissue and mixed by vortex mixer until homogenized. The homogenate was then centrifuged for 5min on 14,000rpm at 4°C. 50µl of the supernatant was transferred to a microtitre plate well and protein carbonyls were determined via reaction with 2,4-dinitrophenylhydrazine (DNPH) (Reznick and Packer, 1994) adapted for measurement in a microplate reader. Levels were determined by measuring the absorbance at 370nm using a Lil420 multilabel counter (Perkin Elmer, San Jose, California, U.S.A.), controlled by a PC, and fitted with temperature control cell and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software package (Perkin Elmer, San Jose, California, U.S.A.). Protein carbonyl content (nmol) was determined using the extinction coefficient of DNPH at 370nm (0.022/µM/cm), corrected for the calculated path-length of the solution (0.6cm). The protein content of the extracts was determined using a

Lowry protein assay (Fryer et al., 1986) and protein carbonyl content expressed as nmol protein carbonyl/mg protein.

DNA

DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with the following modifications (Burritt; pers. com.). The AP1 lysis buffer was supplemented with 5mM deferoxamine and 20mM EDTA, the DNA purification columns were pre-treated with 250µl of a solution containing 5mM deferoxamine, 20mM EDTA, 5mM o-phenanthroline, 20mM Tris-HCl (pH8.0) and then washed with 500µl of sterile deionized water prior to use (Dany et al., 1999). The rest of the protocol followed the manufacturer's instructions. The quantity of DNA in each sample and its relative purity were determined by measuring the absorbance at 260 and 280nm using a PerkinElmer VICTOR³ multilabel plate reader. DNA purity was determined by the A_{260}/A_{280} ratio, with all values being within the range: 1.7–1.9 (Sambrook et al., 1989).

The levels of 8-OHdG were determined by ELISA using mouse monoclonal antibodies N45.1, (Japan Institute for the Control of Aging, Shizuoka, Japan). A sample of extracted DNA was precipitated by the addition of 0.1 volume of 4M NaCl and 2.5 volumes of cold ethanol and digested as per Shigenaga et al (1994) with modifications. Briefly, the precipitated DNA was re-dissolved in 200µl of sterile DNA hydrolysis buffer (1mM deferoxamine, 20mM sodium acetate, pH 5). Nuclease P1 was added (4µL; 3.3mg/ml⁻¹) and samples were incubated at 65°C for 15mins. Alkaline phosphatase (4 U in 1 M Tris-HCl (pH 8)) was added and the samples were incubated at 37°C for 60mins. Finally, 20µL 3M sodium acetate was added to each sample, followed by 20µL of chelating solution (50mM EDTA, 10mM deferoxamine). The solutions were filtered through a 30 kDa cut-off filter-membrane and the filtered solutions, containing the nucleotides, were collected for 8-OHdG analysis.

For the ELISA, 50µL of primary monoclonal antibody and 50µL of DNA sample (50ug/mL) or standard were added to the wells of a microtitre plate, which had been pre-coated with the 8-OHdG. The plates were tightly sealed and incubated at 37°C for 1h. After three washes with 250µL of phosphate-buffered saline (PBS), 100µL of secondary antibody conjugated to horse radish peroxidase was added to each well and the plates were incubated at 37°C for a further 1 h. Following three washes with 250µL of PBS, 100µL of enzyme substrate was added to each well and the plates were incubated at room temperature for 15 minutes with continuous shaking. The reaction was terminated by addition of 100µL 1M phosphoric acid. Absorbance readings were taken 3min later at 450nm. The amount of 8-OHdG in each subject was calculated by comparison with a standard curve.

Determining Enzymatic Antioxidants

All enzymatic antioxidant assays were carried out using a PerkinElmer (Wallac) 1420 multilabel counter (Perkin Elmer, San Jose, California, U.S.A.), controlled by a PC, and fitted with a temperature control cell, set to 25°C, and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software package (Perkin Elmer, San Jose, California, U.S.A.).

Superoxide Dismutase

SOD was extracted with EBII as for protein carbonyls. 50µl of the supernatant was transferred to a microtitre plate well and SOD was determined via the microplate assay described by Banowetz et al (2004) with minor modifications. Briefly, 50µl of extract, diluted extract or standard (prepared from bovine liver SOD (Sigma–Aldrich, St. Louis, MO, U.S.A.) where one unit of SOD corresponded to the amount of enzyme that inhibited the reduction of cytochrome c by 50% in a coupled system with xanthine oxidase at pH 7.8 and 25°C) was mixed with 125µl of freshly prepared reaction solution containing piperazine-1,4-bis(2-ethanesulfonic acid) (Pipes) buffer, pH 7.8, 0.4mM o-dianisidine, 0.5mM diethylenetriaminepentaacetic acid (DTPA), and 26µM riboflavin. The absorbance at 450nm (A_{450}) was measured immediately ($t=0$ min) and samples were illuminated with an 18W fluorescent lamp placed 12cm above the plate for 30min ($t=30$ min) and the A_{450} was measured again. A regression analysis was used to prepare a standard line relating SOD activity to the change in A_{450} and SOD activities in the extracts, calculated with reference to the standard line, were expressed as units SOD per milligram of total protein.

Catalase

CAT was extracted with EBII as for protein carbonyls. 50µl of the supernatant was transferred to a microtitre plate well and CAT was assayed using the chemiluminescent method of Maral et al (1977), as adapted by Janessens et al (2000) for 96-well microplates. Briefly, 50µl of extract, diluted extract or standard (purified bovine liver CAT (Sigma–Aldrich, St. Louis, MO, U.S.A.) in homogenization buffer) was mixed with 100µl of 100mM phosphate buffer (pH 7.0) containing 100mM NaEDTA and 10^{-6} M H_2O_2 . Samples were then incubated at 25°C for 30 minutes, after which 50µl of a solution containing 20mM luminol and 11.6units/ml⁻¹ of horseradish peroxidase (Sigma–Aldrich, St. Louis, MO, U.S.A.) was injected into each well and light emission, the intensity of which was proportional to the amount of H_2O_2 remaining in the mixture, was measured. A regression analysis was used to

prepare a standard line relating standard CAT activities to the intensity of light emission. CAT activities in the extracts were calculated with reference to the standard line and expressed as μM of H_2O_2 consumed per min per milligram of total protein.

Ascorbate Peroxidase

APX was extracted with extraction buffer I (EBI) (appendix I) as for protein carbonyls. 50 μl of the supernatant was transferred to a microtitre plate well and APX activity was assayed by following the decrease in absorbance at 290nm as ascorbate disappeared (Rao et al., 1996). The reaction mixture (1mL) contained 100mM potassium phosphate (pH 7.0), 0.5mM ascorbate, 0.2mM H_2O_2 , and up to 50 μL extract. APX activity ($\mu\text{mol}/\text{min}$) was calculated using an extinction coefficient of 2.8/mM/cm. The assay was conducted using a Pharmacia Ultraspec 3000 spectrophotometer fitted with a temperature controlled cell. The instrument was controlled, and data acquired, by a PC using the Swift (enzyme kinetics) software package (Pharmacia).

Monodehydroascorbate Reductase

MDAR was extracted with EBII as for protein carbonyls. 50 μl of the supernatant was transferred to a microtitre plate well and MDAR was assayed by monitoring the change in absorbance at 340nm, as describe by Foyer et al (1989). The reaction mixture (200 μL) contained 0.4units of ascorbate oxidase, 100mM Hepes/KOH (pH 7.6), 2.5mM ascorbate, 25 μM NADPH and up to 50 μL of enzyme extract. MDAR activity ($\mu\text{mol}/\text{min}$) was calculated using the extinction coefficient (6.2/mM/cm), corrected for the calculated path-length of the solution (0.6cm).

Dehydroascorbate Reductase

DHAR was extracted with EBII as for protein carbonyls. 50 μl of the supernatant was transferred to a microtitre plate well and DHAR activity was assayed by following the decrease in absorbance at 260nm, as described by (Miyake and Asada, 1992). The reaction mixture (200 μL) contained 50mM Hepes/KOH (pH 7.0), 2.5mM GSH, 0.2mM DHA, 0.1mM ETDA and up to 50 μL of enzyme extract. DHAR activity ($\mu\text{mol}/\text{min}$) was calculated using an extinction coefficient of 7.0/mM/cm with corrections made for path-length as above.

Glutathione Reductase

GR was extracted with EBII as for protein carbonyls. 50 μl of the supernatant was transferred to a microtitre plate well and GR was assayed using the method of Cribb et al (1989) with minor modifications. Briefly, 50 μl of extract, diluted extract or standard (GR from wheat germ, Sigma–Aldrich, St. Louis, MO, U.S.A., in homogenization buffer) was mixed with

150µl of 100mM sodium phosphate buffer (pH 7.6) containing 0.1mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 10µl of NADPH (10mg/ml; 12mM). The reaction was initiated by the injection of 10µl of oxidized glutathione (GSSG) (1mg/ml; 3.25mM) and the absorbance at 415nm (A_{415}) was measured every 30 seconds for 3min, with the plate shaken automatically before each reading. The rate of increase in A_{415} per minute was calculated and a regression analysis was used to prepare a standard line relating standard GR activities to the change in A_{415} . GR activities in the extracts, calculated with reference to the standard line, were expressed as µmol or nmol of oxidized glutathione reduced per min per milligram of total protein.

Glutathione Peroxidase

GPX was extracted with EBII as for protein carbonyls. 50µl of the supernatant was transferred to a microtitre plate well and GPX was assayed using a PerkinElmer (Wallac) 1420 multilabel counter (Perkin Elmer, San Jose, California, U.S.A.), controlled by a PC, and fitted with a temperature control cell, set to 25°C, and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software package (Perkin Elmer, San Jose, California, U.S.A.).

Glutathione S-transferase

GST was extracted with EBII as for protein carbonyls. 50µl of the supernatant was transferred to a microtitre plate well and GST was assayed using the photometric 1-chloro-2,4-dinitrobenzene (CDNB) method of Habig et al (1974) with minor modifications. The absorbance at 340 nm (A_{340}) was measured every 30 seconds for 3min, with the plate shaken automatically before each reading. The change in A_{340} per minute was calculated and converted into nmol CDNB conjugated to GSH/min/mg protein using the extinction coefficient of the resulting S-2,4-dinitrophenylglutathione (DNPG): E_{340} 9.6mM⁻¹ cm⁻¹.

Determining Non-enzymatic Antioxidants

Ascorbate

Ascorbate was extracted by adding 500µl of 5% metaphosphoric acid to 50µg of powdered *U. pertusa* tissue in a microcentrifuge tube and mixed by vortex mixer until homogenized. The homogenate was then centrifuged at 14,000rpm for 15 minutes at 4°C. Oxidized and total ascorbate levels were then measured using the microtitre plate based assay described by Gillespie and Ainsworth (2007).

Glutathione

Glutathione was extracted by adding 500µl of 5% sulfosalicylic acid to 50µg of powdered *U. pertusa* tissue in a microcentrifuge tube and mixed by vortex mixer until homogenized. The homogenate was then centrifuged at 14,000rpm for 15 minutes at 4°C. Glutathione and glutathione disulfide levels were measured using the enzymatic recycling method, using the microtitre plate based assay described by Rahman et al (2006).

Data Analysis

Output data from each of the ROS, damage, enzymatic and non-enzymatic antioxidant assays run was collated into excel spreadsheets, where means, standard deviations and standard errors were calculated for each treatment at each time point, from which figures were constructed. Assumptions of normality and equal variance were calculated for data from each of the assays. Having met the assumptions an individual two-way ANOVA and non-parametric Tukeys comparisons were calculated for each assay, using the programme SigmaStat 2.03 Network. For data that did not meet either assumption only results from multiple pairwise comparisons of the non-parametric Tukeys Test were used. In addition, individual one-way ANOVA tests were calculated for each assay to determine if there was an overall time effect for each concentration of that particular assay.

FLA uptake data was treated differently in that a multiple linear regression was calculated (using Sigma stat 2.03) to determine if time and/or FLA concentration were contributing factors. This data was also collated in an Excel spreadsheet where mean, standard deviation and standard error were calculated and graphed. Note: data values that showed up as below the detectable level (bdl) were given a zero value for the purpose of statistical analysis.

Results

Fluoranthene Uptake

Internal FLA concentrations increased in *U. pertusa* tissue with length of exposure ($p < 0.001$). Exposure concentration had an effect on tissue concentration of FLA, $p < 0.001$ (Fig. 2.2). The difference in FLA tissue concentration, between each treatment, was significant for each of days 1-5 (Fig. 2.2).

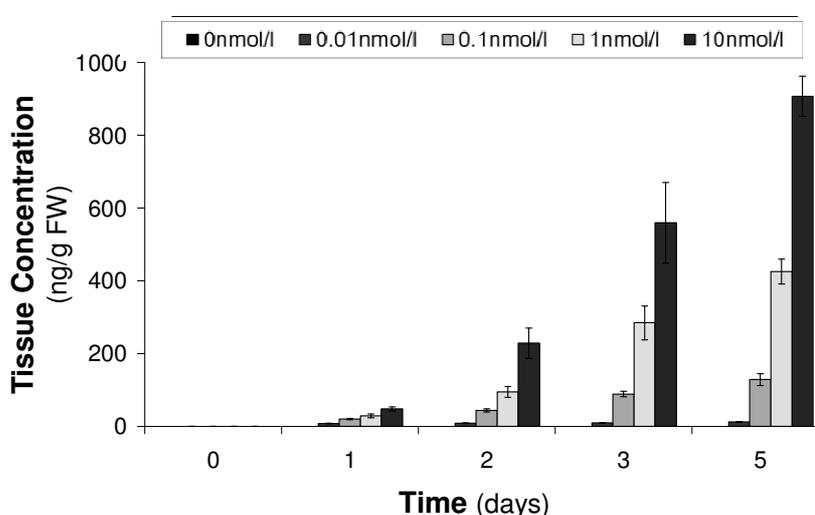


Figure 2.2: Levels of FLA in *U. pertusa* tissue following exposure to four treatment concentrations of FLA (0.01, 0.1, 1, 10nmol/l) and a control (0nmol/l), and the amount taken up over a period of five days, \pm s.e.m. ($n = 4$).

Reactive Oxygen Species

Increased levels of H_2O_2 were observed in *U. pertusa* tissue exposed to FLA (Fig. 2.3). At day 5 *U. pertusa* tissue exposed to 1 and 10nmol/l FLA treatments showed a significantly higher level of H_2O_2 compared to the control ($p = 0.001$ and $p < 0.001$ respectively) (Fig. 2.3). When compared to the control 0.01 and 0.1nmol/l FLA showed no significant difference in levels of H_2O_2 in *U. pertusa* tissue ($p > 0.05$, for all days) (Fig. 2.3). *U. pertusa* demonstrated a steady increase in internal H_2O_2 levels throughout the 5 days of exposure to 10nmol/l FLA, while the levels in tissue exposed to 1nmol/l FLA plateaued after day 3 and exposure to 0.01 and 0.1nmol/l FLA increased internal H_2O_2 initially but this decreased after days 2 and 1 respectively (Fig. 2.3).

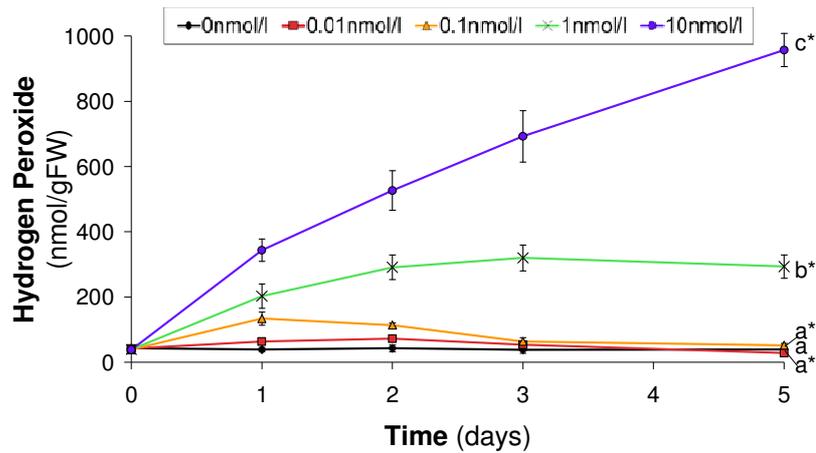


Figure 2.3: Levels of hydrogen peroxide (H_2O_2) found in *Ulva pertusa* tissue following exposure to four treatment concentrations of FLA (0.01, 0.1, 1, 10nmol/l) and a control (0nmol/l), over a period of five days, \pm s.e.m. ($n = 4$). Letters that are the same indicate no significant difference between treatments at the end of the time course (day 5) and treatments marked with an asterisk (*) have a significant effect of time.

Oxidative Damage

Protein carbonyls increased steadily in *U. pertusa* tissue throughout the 5 days of exposure to 10nmol/l FLA, while *U. pertusa* exposed to 1nmol/l FLA showed an increase in protein carbonyls up until day 3 when levels began to decrease, indicating a significance of exposure time ($p < 0.001$, for both treatments) (Fig. 2.4a). *U. pertusa* exposed to 0.01 FLA showed no significant increase in protein carbonyls over time ($p > 0.05$), in contrast *U. pertusa* exposed to 0.1nmol/l showed a slight increase from day 0 observed at day 1 and subsequently incurred a significant effect of time ($p = 0.002$) (Fig. 2.4a). All FLA treatments resulted in an increased level of lipid peroxides in *U. pertusa* tissue throughout the 5 days of exposure ($p < 0.001$, for all treatments) (Fig. 2.4b). Additionally lipid peroxide levels in *U. pertusa* tissue at day 5, for each treatment, was significantly different from each other treatment and the control ($p < 0.001$) (Fig. 2.4b). Increased levels of oxidised DNA were observed in *U. pertusa* throughout the length of exposure to all four treatments, where $p = 0.019$ for 0.01nmol/l, $p = 0.001$ for 1nmol/l and $p < 0.001$ for both 0.1 and 10nmol/l FLA treatments (Fig. 2.4c). *U. pertusa* tissue exposed to 0.1, 1 and 10nmol/l FLA had significantly more oxidised DNA at day 5 compared to the control ($p < 0.001$).

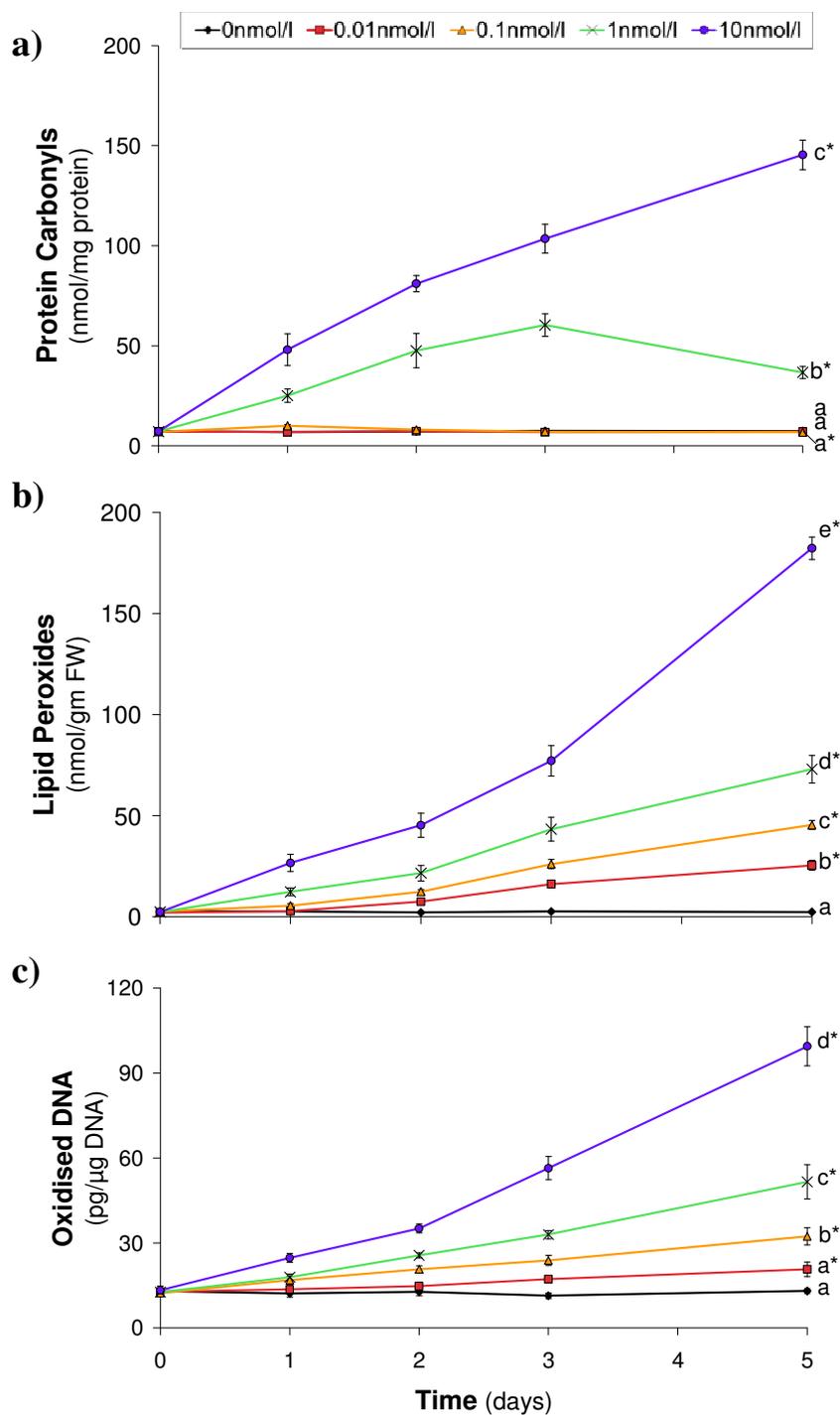


Figure 2.4: Oxidative damage of *Ulva pertusa* proteins (a) lipids (b) and DNA (c) after exposure to each of four treatment concentrations of fluoranthene (0.01, 0.1, 1 and 10nmol/l) and a control (0nmol/l), over a period of five days, \pm s.e.m (n = 4). Letters that are the same indicate no significant difference between treatments at the end of the time course (day 5) and treatments marked with an asterisk (*) have a significant effect of time.

Enzymatic Antioxidants

All four treatments had an effect on both SOD and CAT in *U. pertusa* over time (Fig. 2.5a and 2.5b). The activity of both SOD and CAT increased over time in *U. pertusa* exposed to 0.01, 0.1 and 1nmol/l FLA, where $p < 0.001$ for all three SOD treatments (Fig. 2.5a) and $p = 0.005$ for the 0.01nmol/l CAT treatment and $p < 0.001$ for 0.1 and 1nmol/l CAT treatments (Fig. 2.5b). The activity of SOD and CAT increased from day 0 to day 2 in *U. pertusa* exposed to the highest FLA treatment concentration (10nmol/l) after which both treatments decreased to day 5. At day 2 10nmol/l FLA was significantly different from the control, with $p < 0.001$ for both SOD and CAT. By day 5 SOD was still significantly different from the control for this treatment ($p < 0.001$), and CAT was no longer significantly different from the control with $p = 0.990$ (Fig. 2.5b).

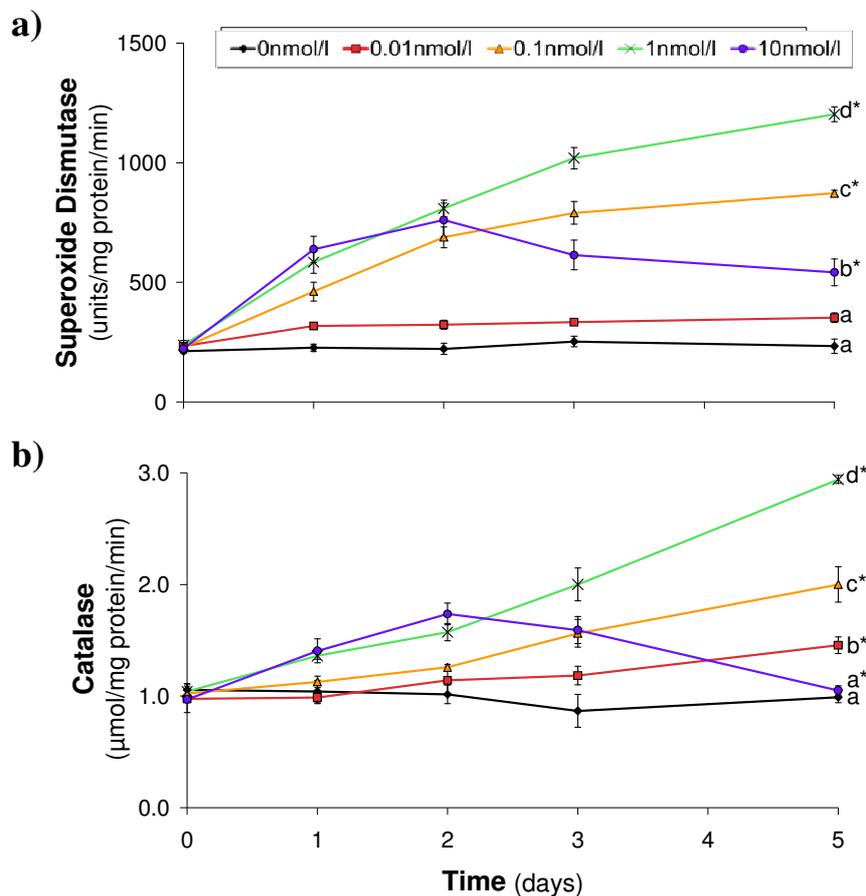


Figure 2.5: Levels of superoxide dismutase (SOD) (a) and Catalase (CAT) (b) present in *Ulva pertusa* tissue following exposure to four treatment concentrations of fluoranthene (0.01, 0.1, 1 and 10nmol/l) and a control (0nmol/l), \pm s.e.m. (n = 4), over a period of five days. Letters that are the same indicate no significant difference between treatments at the end of the time course (day 5) and treatments marked with an asterisk (*) have a significant effect of time.

There was a significant effect on the activity of APX, DHAR, GR and MHAR produced in *U. pertusa* tissue exposed to the three highest FLA concentrations (0.1, 1 and 10nmol/l) (Fig. 2.6a-d). For GR, DHAR and MDAR 0.1 and 1nmol/l FLA treatments generated a steady increase of the respective antioxidants in *U. pertusa* tissue. The activity of APX increased from day 0 to day 3, in *U. pertusa* tissue exposed to 1 and 10nmol/l FLA ($p<0.001$ for both, when compared to the control), from day 3 to day 5 the activity of APX in *U. pertusa* decreased when exposed to these two treatments ($p<0.001$ for both at day 5), when compared to the control (Fig. 2.6a). DHAR and GR increased from day 0 to day 2, in *U. pertusa* tissue exposed to 10nmol/l FLA ($p=0.013$ and $p=0.008$ respectively, when compared to the control) (Fig. 2.6b and c). Glutathione reductase decreased in *U. pertusa* tissue exposed to 10nmol/l FLA from day 2 to day 5, where it was no longer significantly different from the control ($p=0.998$) (Fig. 2.6c). The activity of DHAR decreased in *U. pertusa* after day 2 of exposure to 10nmol/l FLA and was lower than the control at day 3 ($p=0.072$), it then increased again to day 5 ($p=0.138$) (Fig. 2.6b). The activity of MDAR was steady from day 0 to day 2 in *U. pertusa* exposed to 10nmol/l FLA; it decreased from day 2 to day 3 and was then steady till day 5, throughout the length of 10nmol/l FLA exposure MDAR showed no significant difference from the control (Fig. 2.6d).

The activity of glutathione S-transferase (GST) increased over time from day 0 to day 5 in *U. pertusa* exposed to 0.1 and 1nmol/l FLA ($p<0.001$ for both), and GST increased from day 0 to day 3 in *U. pertusa* exposed to 10nmol/l FLA and decreased from day 3 to day 5 (Fig. 2.7a). There was an increase in GPX activity over time in *U. pertusa* tissue exposed to 0.01, 0.1 and 1nmol/l FLA ($p=0.001$ and $p<0.001$ respectively). There was no significant difference, in the activity of GPX present in *U. pertusa*, between the highest FLA concentration treatment (10nmol/l) and the control, at any stage during the course of exposure ($p=0.769$) (Fig. 2.7b).

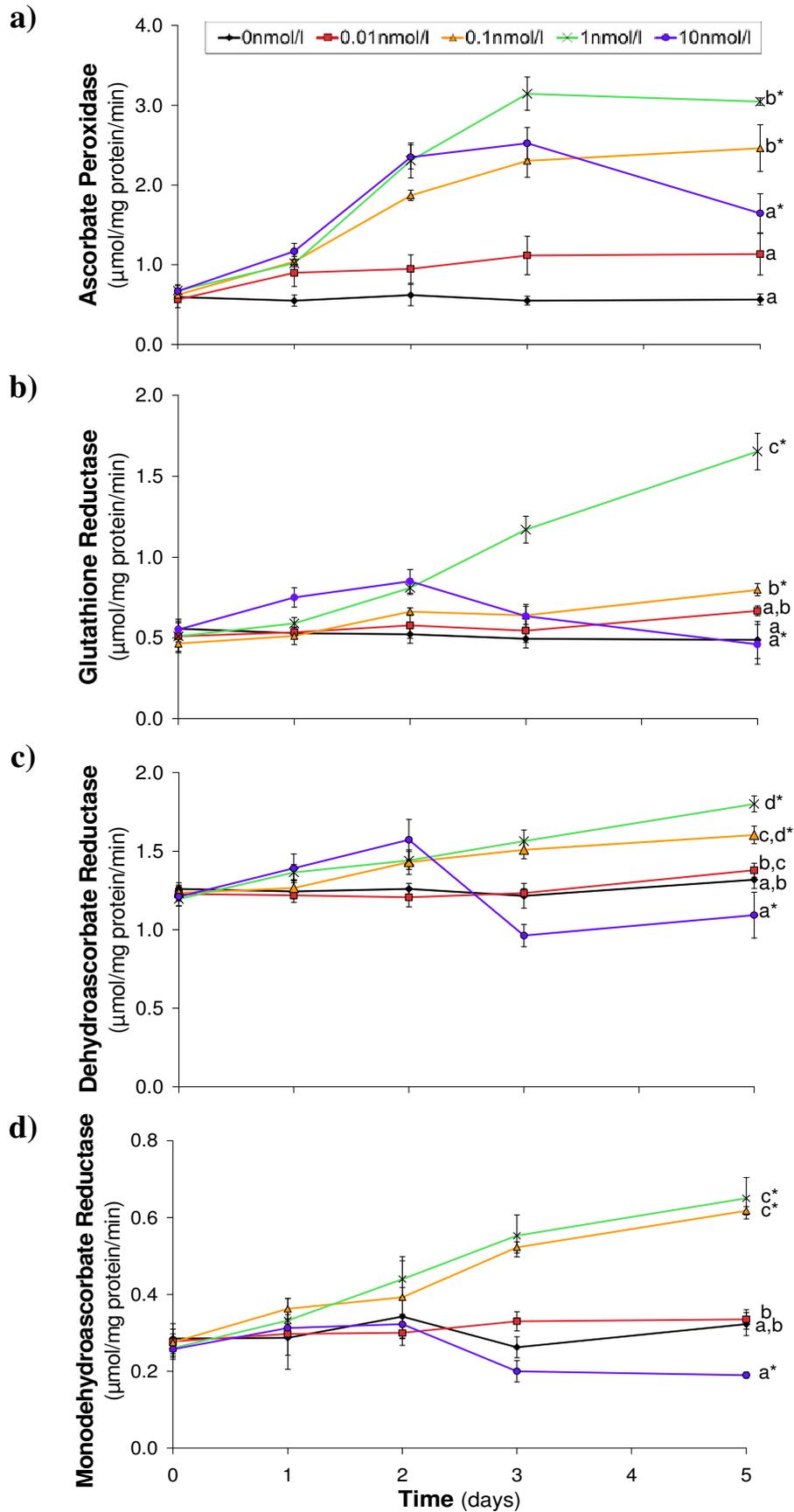


Figure 2.6: Levels of enzymatic antioxidants: ascorbate peroxidase (APX) (a), glutathione reductase (GR) (b), dehydroascorbate reductase (DHAR) (c) and mono-dehydroascorbate reductase (MDAR) (d), present in *Ulva pertusa* tissue following exposure to four treatment concentrations of fluoranthene (0.01, 0.1, 1 and 10 nmol/l) and a control (0 nmol/l), \pm s.e.m. (n = 4), over a period of five days. Letters that are the same indicate no significant difference between treatments at the end of the time course (day 5) and treatments marked with an asterisk (*) have a significant effect of time.

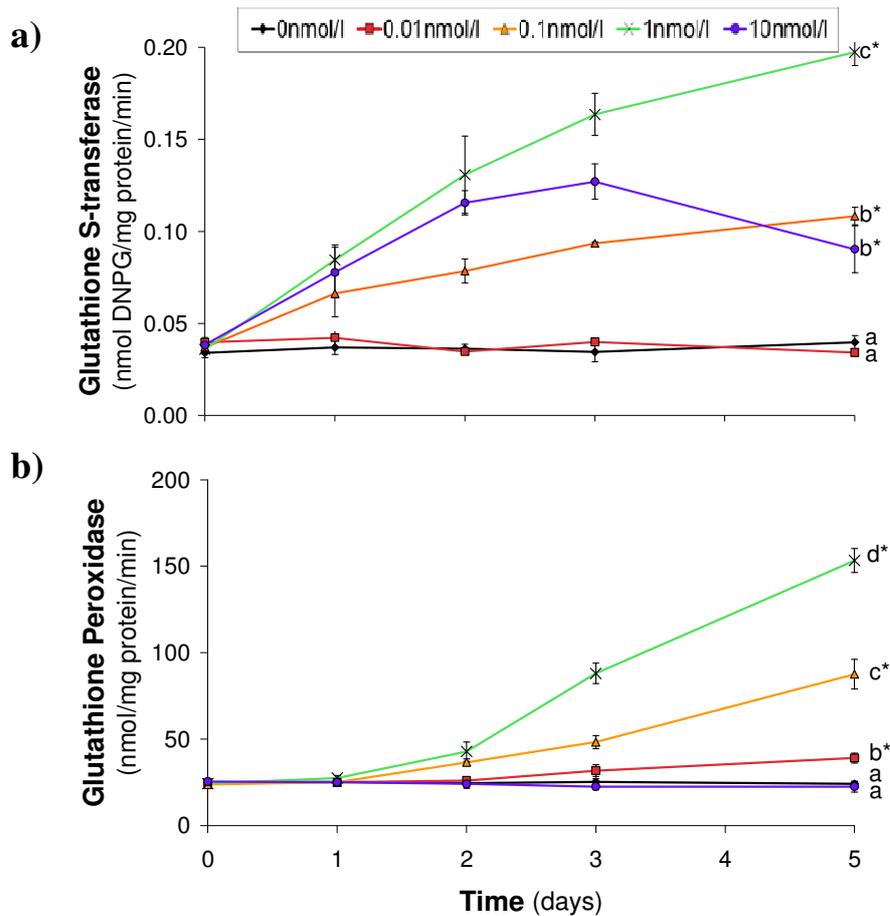


Figure 2.7: Levels of glutathione S-transferase (GST) (a) and glutathione peroxidase (GPX) (b) present in *Ulva pertusa* tissue following exposure to four treatment concentrations of fluoranthene (0.01, 0.1, 1 and 10nmol/l) and a control (0nmol/l), \pm s.e.m. (n = 4), over a period of five days. Letters that are the same indicate no significant difference between treatments at the end of the time course (day 5) and treatments marked with an asterisk (*) have a significant effect of time.

Non-enzymatic Antioxidants

The level of total glutathione changed over time in *U. pertusa* exposed to each of the four treatment concentrations, in particular there was an increase during exposure to 0.01, 0.1 and 1nmol/l ($p=0.002$, $p<0.001$ and $p=0.002$, respectively) and a decrease over time during exposure to 10nmol/l ($p=0.002$) (Fig. 2.8c). Exposure of *U. pertusa* to 0.1 and 1nmol/l FLA produced an increase in total ascorbate over time ($p=0.001$ and $p=0.002$, respectively) and a decrease in total ascorbate after prolonged exposure to 10nmol/l FLA ($p<0.001$) (Fig. 2.8a). The percentage of reduced glutathione (%GSH) decreased from day 0 to day 2 in *U. pertusa* exposed to 0.1 and 1nmol/l FLA (52-32% and 53-26.5%, respectively) and then increased

from day 2 to day 5 (47% and 50% at day 5, respectively) (Fig. 2.8d). *U. pertusa* exposed to 10nmol/l FLA showed a general decrease in %GSH over time ($p<0.001$), starting with 54% (day 0) and ending with 20% (day 5) (Fig. 2.8d). The three lowest treatment concentrations (0.01, 0.1 and 1nmol/l FLA) showed no significant difference in %GSH from the control at day 5 ($p=0.867$, $p=0.718$ and $p=0.979$, respectively) (Fig. 2.8d). The percentage of reduced ascorbate (%AsA) decreased from day 0 to day 3 in *U. pertusa* exposed to 0.01 and 0.1nmol/l FLA, and from day 0 to day 2 during exposure to 1nmol/l FLA (82.5-70.5%, 82.5-60% and 80.5-54% respectively), %AsA then increased back to day 5 (73%, 69% and 66.5% respectively) (Fig. 2.8b). *U. pertusa* exposed to 10nmol/l FLA showed a general decrease in %AsA over time ($p<0.001$), starting with 80.5% (day 0) and ending with 27% (day 5) (Fig. 2.8b). The two lowest concentration treatments (0.01 and 0.1nmol/l FLA) showed no significant difference (from the control at day 5) in the percentage of ascorbate present in *U. pertusa* and these two treatment concentrations, along with the 1nmol/l FLA treatment showed no significant difference from each other at day 5 (Fig. 2.8b).

Summary

There was a significant time, concentration and interaction effect for all ROS, oxidative damage, enzymatic antioxidant and non-enzymatic antioxidant assays conducted on *U. pertusa* after exposure to fluoranthene (FLA) contaminated seawater ($p<0.001$ for all) (Table 2.1).

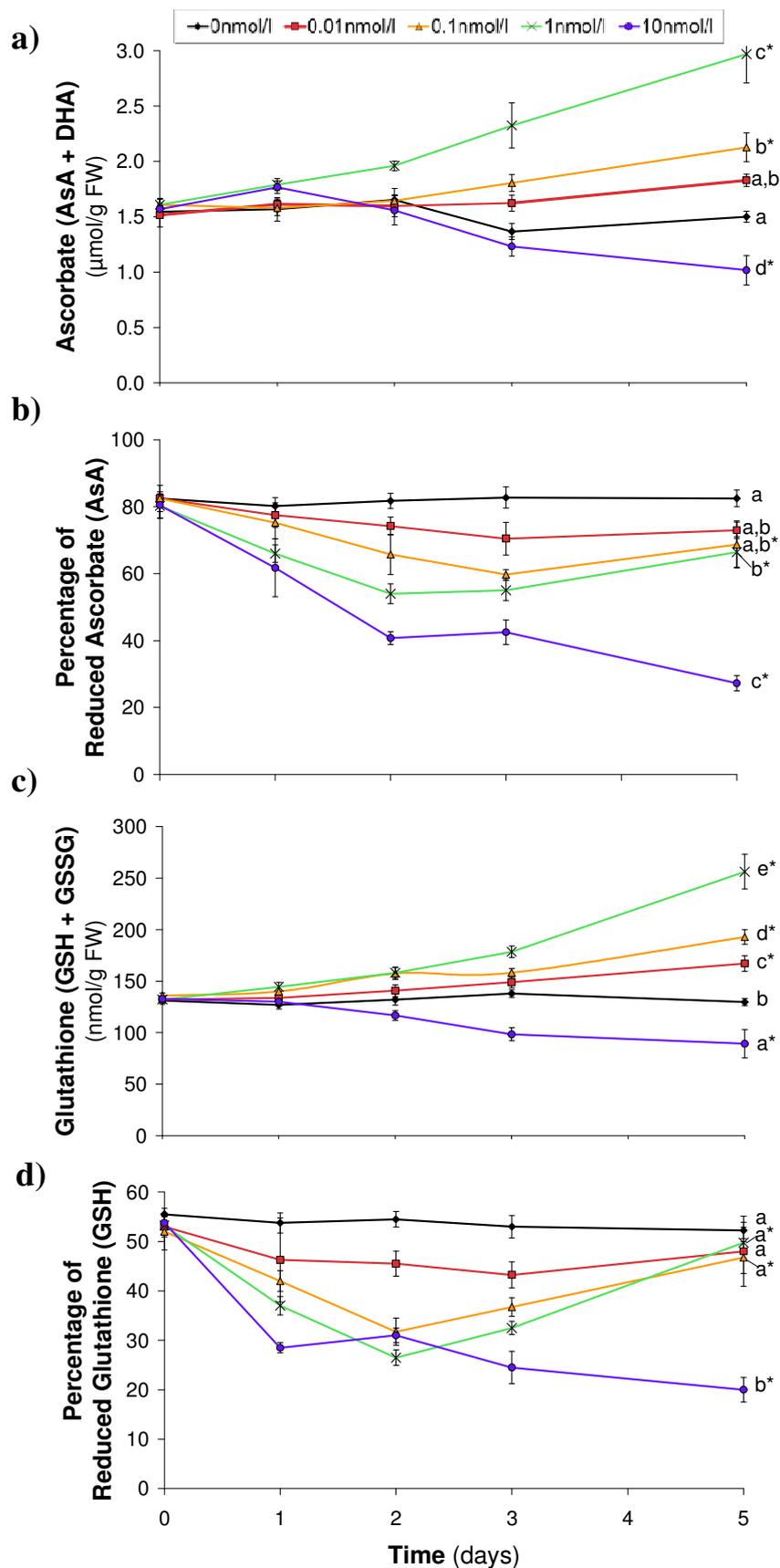


Figure 2.8: Levels of total ascorbate (AsA + DHA) (a), percentage of reduced ascorbate (% AsA) (b), total glutathione (GSH + GSSG) (c) and percentage of reduced glutathione (% GSH) (d) present in *Ulva pertusa* tissue after exposure to four treatment concentrations of fluoranthene (0.01, 0.1, 1 and 10nmol/l) and a control (0nmol/l), \pm s.e.m. (n = 4), over a period of five days. Letters that are the same indicate no significant difference between treatments at the end of the end of the time course (day 5) and treatments marked with an asterisk (*) have a significant effect of time.

Table 2.1: A summary of all sixteen assays conducted and their respective two-way ANOVA results. Including the associated degrees of freedom (DF), F-values (F) and *p*-values (P) for each of the two factors (time (days) and concentration (nmol/l of FLA)) and the interaction between the two factors involved (interaction).

CATEGORY	ASSAY	TEST	DF	F	P	
Reactive Oxygen Species (ROS)	H ₂ O ₂	Time	4	47.76	<0.001	
		Concentration	4	239.64	<0.001	
		Interaction	16	29.16	<0.001	
Oxidative Damage	Protein Carbonyls	Time	4	74.19	<0.001	
		Concentration	4	370.04	<0.001	
		Interaction	16	44.78	<0.001	
	Lipid Peroxides	Time	4	298.74	<0.001	
		Concentration	4	300.79	<0.001	
		Interaction	16	71.15	<0.001	
	Oxidised DNA	Time	4	121.91	<0.001	
		Concentration	4	148.59	<0.001	
		Interaction	16	31.29	<0.001	
Enzymatic Antioxidants	Superoxide Dismutase (SOD)	Time	4	110.91	<0.001	
		Concentration	4	196.12	<0.001	
		Interaction	16	22.92	<0.001	
	Catalase (CAT)	Time	4	39.37	<0.001	
		Concentration	4	53.44	<0.001	
		Interaction	16	15.72	<0.001	
	Ascorbate Peroxidase (APX)	Time	4	63.47	<0.001	
		Concentration	4	71.67	<0.001	
		Interaction	16	10.06	<0.001	
	Dehydroascorbate Reductase (DHAR)	Time	4	7.58	<0.001	
		Concentration	4	12.29	<0.001	
		Interaction	16	5.84	<0.001	
	Monodehydroascorbate Reductase (MHAR)	Time	4	8.95	<0.001	
		Concentration	4	20.08	<0.001	
		Interaction	16	4.42	<0.001	
	Glutathione Peroxidase (GPX)	Time	4	129.74	<0.001	
		Concentration	4	150.19	<0.001	
		Interaction	16	50.44	<0.001	
	Glutathione Reductase (GR)	Time	4	14.30	<0.001	
		Concentration	4	31.58	<0.001	
		Interaction	16	11.37	<0.001	
	Glutathione S-transferase (GST)	Time	4	46.02	<0.001	
		Concentration	4	108.10	<0.001	
		Interaction	16	13.16	<0.001	
	Non-enzymatic Antioxidants	Reduced Glutathione (GSH) (%)	Time	4	21.44	<0.001
			Concentration	4	36.19	<0.001
			Interaction	16	4.57	<0.001
Reduced Ascorbate (AsA) (%)		Time	4	23.01	<0.001	
		Concentration	4	47.44	<0.001	
		Interaction	16	4.91	<0.001	
Total Glutathione (GSH + GSSG)		Time	4	23.62	<0.001	
		Concentration	4	68.33	<0.001	
		Interaction	16	16.41	<0.001	
Total Ascorbate (AsA + DHA)		Time	4	6.59	<0.001	
		Concentration	4	35.35	<0.001	
		Interaction	16	9.25	<0.001	

Discussion

Uptake

Bio-available PAHs like FLA are taken up by aquatic animals and plants, and have been shown to bioaccumulate in terrestrial plants and marine invertebrates exposed to continuous low level contamination (Greenberg, 2003, Meador, 2003). While there are many studies available that show algae, particularly *Ulva* spp, bioaccumulate trace metals from contaminated coasts (Barraza and Carballeira, 1999, Villares et al., 2001, Torres et al., 2008), the literature lacks studies investigating *Ulva* spp as accumulators of PAH contaminants. This could be important as *Ulva* spp are ideal candidates for biomonitoring such contaminants (Ho, 1990). The current study found that *U. pertusa* takes up available FLA from surrounding seawater and accumulates at varying levels according to duration of exposure. PAHs are hydrophobic compounds and therefore have a tendency towards substances with higher lipid content (Douben, 2003). It is likely that the accumulation of FLA seen in *U. pertusa* is by passive diffusion due to the high lipid content of the algal cells, as demonstrated by Floreto (1993) who found *U. pertusa* has 0.23 ± 0.07 (% of FW) and 1.78 ± 0.44 (%DW) lipid content with PUFAs making up 36.3 to 54.4% of the total fatty acid composition. Compounds that penetrate the thallus most easily, and hence are the most toxic, are lower-molecular weight, lipophilic compounds such as FLA. These compounds exert toxicity by entering the lipophilic layer of the cell membrane, disrupting its spacing, and as a result the membrane ceases to properly control the transport of ions in and out of the cell (Lobban and Harrison, 1997).

The current study demonstrates that FLA concentration and duration of exposure are the key contributing factors to the uptake of PAHs by seaweed, as *U. pertusa* exposed to 10nmol/l FLA comprises higher internal FLA levels than any other treatment, and a 2 to 3-fold increase at consecutive time-points, indicating that continuous exposure to FLA results in the cellular accumulation of the contaminant. As the current study was of short duration no evidence of an uptake threshold level was observed, yet it is plausible that, particularly at the highest FLA exposure, intracellular levels would reach a threshold of accumulation and level off, ultimately causing cell death.

Oxidative Stress and Damage

Uptake of xenobiotic pollutants like PAHs increases the production of ROS, and subsequently oxidative stress, in many photosynthetic organisms including aquatic plants and algae. Previous studies indicate PAHs are inducers of oxidative stress in humans (Burczynski et al., 1999), animals (Sun et al., 2006), terrestrial plants (Paskova et al., 2006), *Arabidopsis* (Alkio et al., 2005) and the aquatic liverwort *Riccia fluitans* (Burrill, 2008), yet the effects of coastal PAH inputs on marine algae have not been previously investigated in depth. Hydrogen peroxide (H_2O_2) is commonly produced under stress conditions (Dummermuth et al., 2003). While other ROS molecules have a comparatively short lifetime, due to their rapid reactivity, H_2O_2 itself is not particularly reactive to most biologically important molecules but has the ability to diffuse through membranes rapidly, making it a likely precursor for more reactive oxidants (Asada, 1994, Apostol et al., 1989). H_2O_2 disturbs photosynthesis by inhibiting a number of enzymes in the Calvin cycle, such as fructose biphosphatase, ribulose phosphate kinase and ribulose biphosphate carboxylase/oxygenase (Kaiser, 1979). Therefore monitoring H_2O_2 levels in *U. pertusa* is important in gaining information on the level of stress occurring in the organism. The current study observed high levels of H_2O_2 in response to high intracellular levels of FLA in *U. pertusa* indicating that FLA contamination can cause oxidative stress in seaweeds, similar to that observed during heavy metal exposure (Barraza and Carballeira, 1999) (Pinto et al., 2003). The observed increase in ROS is attributable to the aforementioned uptake and accumulation of FLA in *U. pertusa* tissue, as fluctuations in H_2O_2 levels follow that of internal FLA levels, though it is still unclear how PAHs produce ROS in seaweeds it has been suggested that the process of metabolising such xenobiotics may play a key role (Alkio et al., 2005).

H_2O_2 is known to cause substantial damage to organisms via oxidation of lipids, proteins and nucleic acids (Fridovich, 1978) (Halliwell and Gutteridge, 2007) and the current study was no exception. The production of H_2O_2 resulted in significant damage to lipids and DNA of *U. pertusa* during FLA exposure, damage increasing with duration, yet protein damage only occurred at 1 and 10nmol/l FLA exposures. The high PUFA content of *U. pertusa* tissue (Floreto et al., 1993) combined with the high reactivity of H_2O_2 with lipids, likely proliferated the production of lipid peroxides (Moller et al., 2007), resulting in the increased level of lipid peroxides observed in *U. pertusa*. The degradation of these peroxides and the resulting production of aldehydes that conjugate with DNA (Moller et al., 2007) is the likely mechanism by which DNA damage to *U. pertusa* occurred.

Protein damage has been observed as a result of increased ROS production, negatively affecting protein function in maize (Pastori and Trippi, 1993) and spinach (Wang et al., 2011).

The current study observed an increase in protein carbonyl production in *U. pertusa* in the presence of high internal FLA, where damage increased as a function of time and concentration, and specimens exposed to 10nmol/l FLA did not recover. Protein oxidation occurs rapidly in the presence of OH· which is produced by the reduction of H₂O₂, therefore the availability of H₂O₂-scavenging antioxidants is important to prevent further damage to macromolecules by the highly reactive OH· molecule (Lesser, 2006, Moller et al., 2007). The antioxidant enzyme CAT actively scavenges H₂O₂ and reduces it to water and O₂, preventing the production of the highly reactive OH· (Mittler, 2002). Therefore the up-regulation of CAT observed in response to FLA contamination, is the likely explanation for the lack of protein carbonyl production seen for 0.01 and 0.1nmol/l FLA, and the recovery of proteins after the third day of 1nmol/l FLA exposure. Additionally the rapid decrease in CAT activity with 10nmol/l FLA indicates the enzyme defences are being inhibited by the overwhelming production of ROS (Halliwell and Gutteridge, 2007). This is dangerous for the organism as it can lead to damage of the photosynthetic apparatus (Asada and Takahashi, 1987, Fridovich, 1978, Forti and Gerola, 1997), causing a change in *Ulva* thallus colour, reduction in growth and possible eventual death (Han et al., 2007). In the current study no photosynthetic measurements were taken, however in a previous study photosynthetic damage occurred in *Ulva* under FLA exposure ($F_v/F_m = 0.78, 0.76, 0.70, 0.61$ and 0.41 for 0, 0.01, 0.1, 1 and 10nmol/l respectively) (pers. com David Burritt, unpublished data). Irreparable photosynthetic damage to *Lemna gibba* (duckweed) exposed to the PAH anthracene (Huang et al., 1997) and pigment bleaching in *U. pertusa* exposed to a mixture of aquatic toxicants, including diesel fuel (Han et al., 2007) has also been recorded. Therefore the photosynthetic apparatus of *U. pertusa* is likely to have been compromised during the current study, particularly at the highest FLA exposures, and with increased duration changes in pigment colour may have eventually become visible.

Antioxidant Metabolism

Increased enzymatic antioxidant activity occurred in *U. pertusa* exposed to FLA, indicating oxidative stress resulting from FLA contamination, induces the up-regulation of antioxidant defences in macroalgae. It should also be noted that the impaired activity of enzymatic antioxidants observed in response to extended 10nmol/l FLA corresponds well with the rapid and continuous increase in protein carbonyls, lipid peroxides and oxidised DNA. In response to lower FLA levels the up-regulation of antioxidant enzymes appears to be successful in

scavenging H₂O₂ as indicated by the observed inactivity, plateau and decrease of H₂O₂ levels for respective FLA concentration treatments.

In addition to the aforementioned CAT, APX also actively scavenges H₂O₂ and is primarily executed in the chloroplasts (CAT occupies peroxisomes) (Halliwell and Gutteridge, 2007). APX is said to have a higher affinity for H₂O₂ than CAT, scavenging it through the oxidation of AsA to MDA (Halliwell and Gutteridge, 2007). This may explain the high activity of APX observed in the current study. In comparison to CAT, APX levels were generally higher during FLA exposure and, under 10nmol/l concentrations, APX activity continued to increase for an extra day after the depletion of CAT. Similarly, Dummermuth et al (2003) reported stimulation of APX and CAT activity in the marine macroalgae *Polysiphonia arctica* in response to high H₂O₂ concentrations and with increasing duration of exposure.

APX exists as part of the ascorbate-glutathione cycle and detoxifies H₂O₂, using AsA as a substrate for the reaction, and catalysing the transfer of electrons from ascorbate to the peroxide, producing 2H₂O and 2MDA (Raven, 2000). AsA is a particularly important antioxidant in the prevention of oxidative damage in higher plants (Larson, 1988) and is also found in marine algae (Dummermuth et al., 2003). As a critical antioxidant of the ascorbate-glutathione cycle AsA must be replenished after detoxification of H₂O₂ and this is achieved when MDAR catalyses the reduction of MDA and H⁺ donated by NADPH to 2AsA (Eltner, 1982). The response of MDAR activity to decreasing levels of AsA in *U. pertusa* at 10nmol/l FLA indicates that the detoxification of increasing H₂O₂ produced high levels of MDA. In contrast 0.1 and 1nmol/l FLA showed increased MDAR activity after a few days and subsequently the percentage of AsA increased, reflecting the reduction of MDA to AsA. This corresponds to the observed levels of H₂O₂ in *U. pertusa* under varied concentration of FLA contamination. Similar results were recorded in *Ulva* in the presence of excess H₂O₂ and as a result AsA was depleted to such low levels it caused irreversible inactivation of APX (Asada and Takahashi, 1987). An organism is unlikely to synthesise more APX once it has been depleted because its *de nova* synthesis is energetically expensive (Dummermuth et al., 2003). AsA is less expensive to synthesise, but if ROS levels are too high the reduction of MDA may not be enough to keep up sufficient levels of AsA required for APX to detoxify H₂O₂, in which case APX becomes inactive (Ahmad, 1995). *U. pertusa* exhibited a rapid decrease in APX activity in conjunction with an observed drop in AsA below 40%, indicating that AsA levels were no longer sufficient for APX to function properly. MDA can also spontaneously dissociate into AsA and DHA (Gould, 2003). DHA is then reduced to AsA in a reaction with

2GSH, catalysed by DHAR, and producing GSSG in the process (Foyer and Halliwell, 1976) (Gould, 2003). GSH is then regenerated by a GR catalysed reaction of GSSG and H⁺ donated by NADPH (Gould, 2003, Ahmad, 1995). The present study demonstrates the relationship between DHAR, GSH and GR during times of FLA induced oxidative stress. DHAR activity increased in *U. pertusa* exposed to 0.1 and 1nmol/l FLA and the percentage of GSH decreased for the first few days of exposure. This suggests DHA was being reduced back to AsA, likely contributing to the observed increases in AsA mentioned previously. GR activity also increased in *U. pertusa* exposed to 0.1 and 1nmol/l FLA as did the percentage of GSH, indicating GSSG from the previous reaction was being reduced back to GSH. GSH decreased continuously in *U. pertusa* exposed to 10nmol/l FLA, eventually dropping to unrecoverable levels. In addition DHAR and GR activity declined after a few days suggesting the system was overwhelmed under such high levels of oxidative stress. In *U. pertusa* glutathione pools (GSH + GSSG) were approximately ten times lower overall (100 to 250nmol/g) than ascorbate pools (1.5 to 3.0µmol/g). While both are important components of the ascorbate-glutathione cycle it has been suggested that glutathione plays a more critical role in animals than in photosynthetic organisms (Dummermuth et al., 2003). This may explain the low activity of GSH observed in *U. pertusa*.

Glutathione peroxidase (GPX) acts independently of the ascorbate-glutathione cycle to quench H₂O₂ using GSH as a substrate and consequently producing GSSG, which is again recycled back to GSH by GR. The activity of GPX is less pronounced than DHAR, likely because there is a lack of GSH as it is in use in the ascorbate-glutathione cycle, this is particularly prominent during exposure to 10nmol/l FLA, where GPX was completely inactive and unvarying from the control for the entire duration of exposure. GPXs are present in a wide variety of plants, but are much more common in animals, and may not be as strongly induced during oxidative stress as those of animal origin (Smirnov, 2005). There is little knowledge of GPXs in *Ulva* and other seaweeds, but it is safe to assume that GPXs in algae are more similar to those of plants than animals, therefore the limited presence and activity of GPX observed in the current study was not unexpected.

Glutathione S-transferase (GST)

Xenobiotic metabolism provides plants and animals with a chance to avoid pollutant induced oxidative stress (Pflugmacher et al., 2000). GST is a pivotal enzyme of the xenobiotic metabolism, detoxifying hydrophobic compounds, like PAHs, by catalysing their conjugation with GSH (Marrs, 1996, Lei et al., 2003). In photosynthetic organisms xenobiotic conjugates are compartmentalised in the vacuole while in aquatic animals they are typically excreted

from the cell (Torres et al., 2008). This presents an alternative explanation for the reduction in GSH levels of *U. pertusa* during FLA contamination, as GSH was likely bound to FLA in the vacuole. Xenobiotics are more water soluble as conjugates and therefore less reactive, preventing additional ROS production and increasing the organisms' xenobiotic tolerance (Ahmad, 1995, Marrs, 1996). Increased GST activity has been demonstrated in multiple aquatic plants and freshwater microalgae species (chlorophyta) in response to BaP (Roy et al., 1994), PAH mixture (Schrenk et al., 1998) and pyrene (Lei et al., 2003) contaminants. GST activity was both time and FLA dependant in *U. pertusa*, reflecting the results of pyrene induced GST activity observed by Lei et al (2003) in three freshwater microalgal species. The previously neglected detoxification mechanisms of macroalgae under xenobiotic stress were recently addressed by Cairrao et al (2004) and Dere et al (2007). Maximum GST activity in *Fucus* sp., from sites contaminated by petroleum derived products, range from 130.2 to 789.8 nmol/mg protein/min (Cairrao et al., 2004), while that of *U. pertusa* reached a maximum of 0.20 nmol/mg protein/min after 5 days at 1nmol/l FLA. This emphasises the wide taxonomic distribution and species dependence of GST activity in macroalgae (Pflugmacher et al., 2000). GST activity declined in *U. pertusa* after three days of 10nmol/l FLA exposure, a similar response to that observed by Lei et al (2003) for freshwater microalgae under high pyrene concentrations. The most likely cause of declining GST activity for *U. pertusa* under 10nmol/l FLA is insufficient GSH for conjugation, as it had dropped below 25% by day 3. GST and GSH are clearly important components of the xenobiotic metabolism in *U. pertusa*, aiding the macroalgas' tolerance to FLA induced oxidative stress, therefore changes in GST activity in *U. pertusa* has a potential use as a biological marker for aquatic PAH contamination (Rees, 1993, Dere et al., 2007).

Summary and Conclusions

The aquatic macroalgae *U. pertusa* readily takes up available FLA from the surrounding aqueous environment and accumulates it in the tissue. Bioaccumulation of FLA increases with the level and duration of exposure. The accumulation of FLA results in the intracellular production of ROS, indicated by the increased level of H₂O₂ and its association with the levels of FLA present in the tissue of *U. pertusa*. This increase in ROS resulted in the oxidation and damage of important macromolecules including: nucleic acids, proteins and lipids. In response to ROS production, antioxidants and enzymes were up-regulated in order to maintain homeostasis of the cells and prevent lasting damage to the photosynthetic apparatus. At extreme levels of FLA exposure all antioxidant responses eventually shut-down,

unable to contend with such high levels of ROS production. AsA and the ascorbate-glutathione cycle appears to be a particularly important oxidative stress defence system in macroalgae as does the detoxification enzyme GST.

Chapter 3 – Macroalgal Grazer

Introduction

Xenobiotic Monitoring

The continuous input of xenobiotics, such as PAHs, into the coastal marine environment is a concern and as such measures have been adopted to monitor the effect of these inputs on the surrounding environment. Early monitoring of coastal pollutants used physical and chemical measurements, providing information on contamination levels in water and sediment, but this lacks understanding on how biota are affected by xenobiotic pollutants (Lam and Gray, 2003). In addition to the lack of biological data, measurements of PAH contamination taken from water and sediment are unreliable as PAHs often exist below detectable levels in water and sediment levels are not representative of the bioavailable fraction for biota (Wolfe et al., 1999) (Binelli and Provini, 2003). The low vapour pressure of PAHs means they are easily and rapidly absorbed by living organisms (Nielson et al., 1997). As a result Torres et al (2008) suggests that the relationship between abiotic and biotic pollution levels must be thoroughly understood in order to develop early and realistic environmental risk assessments (ERA). As such the identification and quantification of pollutants in basal-level trophic organisms, such as algae, is an essential diagnostic tool for successfully monitoring xenobiotics (Handy et al., 2003).

As discussed in chapters 1 and 2, macroalgal species like *Ulva* are an ideal biomonitor for coastal pollution and are often used as such, yet PAH concentrations in algal tissue alone is of little use because algae bioaccumulate these hydrophobic compounds and do not assume equilibrium with water (Dachs et al., 1999). Additionally the use of biochemical biomarkers, like oxidative damage and the activity of antioxidants, in macroalgae could provide an early warning biomonitoring system for coastal marine environments (Torres et al., 2008) (as suggested in chapter 2). Biomonitoring is defined as: “the systematic use of biological responses to evaluate changes in the environment, with the intent of establishing a quality control program, offering the opportunity to assess the impact of pollutants on the aquatic environment more realistically” (Cairns Jr and van der Schalie, 1980). In aquatic systems bivalve molluscs, particularly the freshwater zebra mussel (*Dreissena polymorpha*) are used globally as biomonitors for xenobiotic pollutants (Becker et al., 1992, Mersch et al., 1992, Bruner, 1994) including trace metal contaminants (Gundacker, 1999) and PAHs (Hendriks et

al., 1998). The use of basal trophic level monitors, such as *Ulva* spp, does not however give any indication of the effect of marine xenobiotics to higher trophic level species (Binelli and Provini, 2003), and bioaccumulation, magnification and food chain transfer are important in evaluating ecosystem impact (Torres et al., 2008).

Bioaccumulation and Trophic Transfer

Knowledge of trophic transfer of PAHs is important as bioavailability of these pollutants may increase through solubilisation and emulsification in the water column, and interactions with biological membranes may increase bioaccumulation, resulting in increased toxicity through the food chain (Wolfe et al., 1999). PAHs have been shown to induce xenobiotic-metabolising enzymes, such as cytochrome P-450, which can activate toxicity and carcinogenesis (Shimada and Fujii-Kuriyama, 2004), potentially increasing the toxicity of these compounds to higher trophic species via the food chain. Additionally it has been well documented that the primary source of accumulation of contaminants in aquatic biota is through the food chain, not via direct uptake from water (Evans et al., 1982, Thoman and Connolly, 1984, Van der Oost et al., 1988, Wang et al., 1998). PCB is an example of a hydrophobic xenobiotic that bioaccumulates and has been shown to exist up to thousands of times higher in biota relative to surrounding aquatic concentrations (Van der Oost et al., 1988). Similarly to PCBs, PAHs are highly lipid soluble giving them an enhanced ability to bioaccumulate in organisms and biomagnify up the food chain (Clark and Mackay, 1991). Binelli and Pavoni (2003) demonstrated that algae alone are insufficient models for biomagnification of pollutants, as they incur great seasonal variation in density, yet biomagnification of PCBs was present in the consumers (filter feeding zebra mussels) of contaminated algae. The consumers of these zebra mussels (secondary consumers) were observed to take up PCBs directly from their food source (Binelli and Provini, 2003). Studies that have shown PCBs concentrate up each level of the marine trophic system, indicate that the final concentration for biota is highly dependant on seawater concentration being amplified in a step-wise fashion up the food chain (Scura and Theilacker, 1977, Wang et al., 1998). Other factors including, biomass, length of exposure and contamination conditions may also influence the accumulation of such xenobiotics (Scura and Theilacker, 1977). Dietary uptake of PAHs can also significantly contribute to the overall tissue residues in consumers (Filipowicz et al., 2007). A study exposing mussels to the PAH phenanthrene (PHE) and various concentrations of algal cells (as a food source), concluded that uptake of dissolved PHE increased in mussels when a high concentration of algal cells was consumed (Bjork and Gilek, 1996). A similar study

conducted by Okay et al (2000) investigated transfer of the PAH benzo[a]pyrene (BAP) using phytoplankton and a consuming mussel species, and produced similar results to Bjork and Gilek (1996). This second study showed that the difference in PAH accumulation between mussels fed with low and high algal concentrations is due to the higher uptake of algal associated PAH (Okay et al., 2000). Additionally, FLA has been shown to be dietarily transferred from estuarine sediments to benthic infauna and then to shrimp consumers, bioaccumulating in the shrimp tissues (Filipowicz et al., 2007).

There is evidence that the uptake of contaminants like PAHs, from sediments, the water-column and food-sources, can stimulate increased production of ROS resulting in oxidative damage in marine invertebrates (Livingstone, 2001). In this situation xenobiotics are metabolically reduced to what are known as reactive intermediates and, at this stage, individual electrons may be rapidly lost to O₂ and superoxide radicals are produced (Kappus, 1987). One of the most important defences against this type of oxidative stress is glutathione (GSH), a widely distributed tripeptide-thiol synthesised in most animal cells (Meister, 1994). GSH plays a variety of roles in animals including use as a substrate for the production of antioxidant enzymes, scavenging of hydroxyl radicals and the reactivation of some enzymes inhibited by oxidative stress (Halliwell and Gutteridge, 2007). The recycling of GSH and its associated enzymes (GR, GPX and GST) with its oxidised form GSSG, completes a complex and important cycle in animals. In the first instance GPX degrades H₂O₂ and hydroperoxides and the secondary enzyme GST protects the cell from oxidative damage by catalysing the conjugation of GSH with nucleophilic xenobiotics and damaged cellular components (Storey, 1996). GR then catalyses the reduction of GSSG replenishing levels of GSH, in this way the ratio of cellular GSH/GSSG is a good indicator of oxidative stress in an organism. When ROS levels are high and the formation of GSSG (the oxidized form of glutathione) exceeds its reduced form (GSH) the ratio decreases, indicating that the organisms defences are struggling to cope with the level of oxidation (Storey, 1996, Halliwell and Gutteridge, 2007).

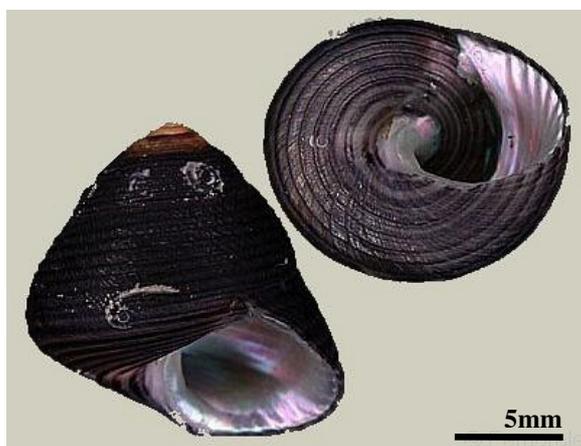


Figure 3.1: Indication of the common morphology of the New Zealand marine mollusc *Micrelenchus tenebrosus*.

Study Species

For this study the gastropod mollusc *Micrelenchus tenebrosus* (Adams, 1853), (family: trochidae) was chosen as a model for trophic transfer of the PAH fluoranthene because it has a close association with *Ulva*

beds and readily grazes on the *Ulva* directly (personal observation). Based on the idea by Filipowicz et al (2007) that primary consumers have a high capacity for removing tissue contaminants via dietary transfer, *M. tenebrosus* is an ideal species to investigate bioaccumulation and trophic transfer of FLA from contaminated *Ulva* tissue. This species also exists in relative abundance and was easy to collect and identify from other marine gastropods inhabiting the same environment. Little information on this species is available in the literature, but it is known to be restricted to New Zealand, with a range covering most of the country (Powell, 1979). *M. tenebrosus* is a micro-grazer inhabiting open water seaweed beds and is mainly southern in distribution (Powell, 1979). A proposed ecotype of this species (*M. huttoni*) exists in sheltered mudflat areas and *Zostera* (seagrass) beds, yet it is difficult to distinguish between the two when these habitats overlap (Powell, 1979), therefore to save confusion they are commonly both referred to under *M. tenebrosus*. This species is 9.0 – 9.5mm high and 8.5 – 10.0mm wide and can be identified by its turbate shell of dark-bluish to dark-greenish grey, with a greenish or bluish iridescent interior of the aperture (Fig. 3.1) (Powell, 1979).

Chapter Objective

From the previous chapter it became clear that the coastal marine macroalga *Ulva pertusa* takes up and accumulates FLA directly from seawater. However, because little is known about the ability of PAHs to be transferred trophically, therefore the research conducted in this chapter aimed to begin filling this knowledge gap. To accomplish this, two independent studies were conducted. The first aimed to establish the relationship between FLA contaminated seawater and the invertebrate species living in such an environment by observing the uptake, oxidative damage and antioxidant metabolism of the marine gastropod *Micrelenchus tenebrosus*. The second was designed to gather information on the ability of invertebrate grazers to accumulate PAH contaminants, like FLA, from a contaminated food source. *M. tenebrosus* was used as the model marine invertebrate grazer and coupled with the knowledge that *U. pertusa* takes up and accumulates FLA, the marine snail was allowed to graze on the contaminated food source for four days.

Methods

For this section two separate ‘contamination regime’ studies were conducted back to back but not concurrently. The first used FLA contaminated seawater and aimed to investigate the effect of FLA contaminated seawater on an actively grazing marine gastropod, *M. tenebrosus*, by simulating its natural environment on a small scale in the lab. The second used an FLA contaminated food source (*U. pertusa*) and aimed to investigate the effect of its consumption on the associated grazer (*M. tenebrosus*).

Collection

Fifteen *U. pertusa* individuals were collected subtidally from Wellers Rock, Otago Harbour (Fig. 3.2), at approximately 1.0m depth during low tide, for each experiment. These were placed in a small chilli bin containing seawater from the collection site and transported back to the lab. Three hundred *M. tenebrosus* snails were collected from Papanui inlet, on the Otago peninsula (Fig. 3.2) for each experiment. These were placed in a container containing seawater from the collection site and a small amount of *Ulva*, as a food source, for the journey back to the lab. It is important to note that GCMS analysis of existing PAH levels ($\Sigma 16\text{PAH}$)

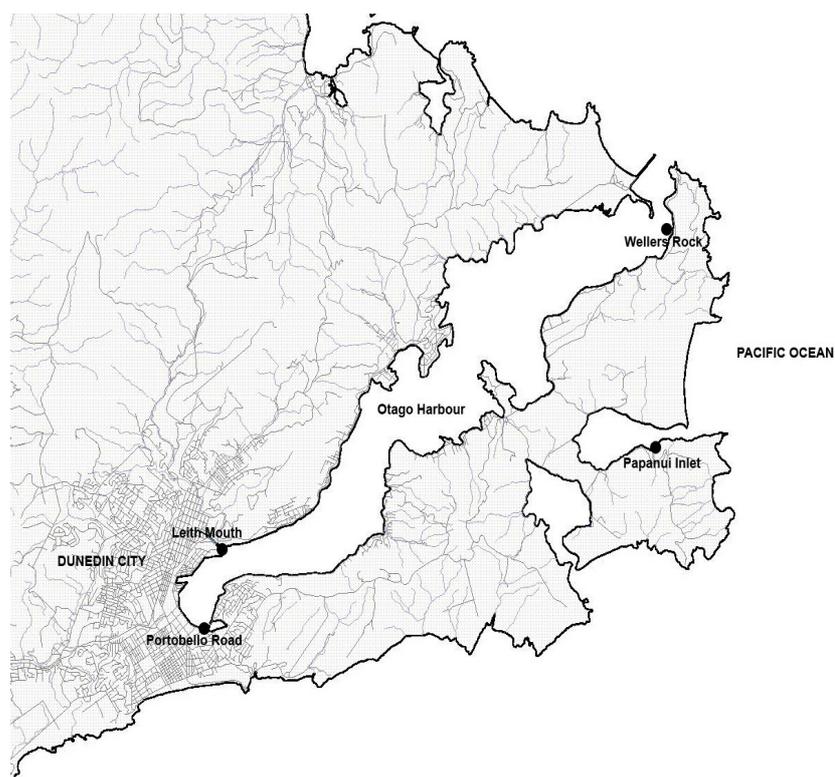


Figure 3.2: Illustrates the collection sites of *U. pertusa* (Wellers Rock) and *M. tenebrosus* (Papanui Inlet), including points of reference in the Otago Harbour. Map created and supplied by D. Burritt.

at Papanui Inlet were below the detectable level (bdl) in suspended sediment, water and Ulva. At the Wellers Rock site $\Sigma 16\text{PAH}$ was bdl in suspended sediment and water, yet $3.75 \pm 1.34 \text{ ng/g DW}$ was found in Ulva at this site. A tourist boat operates daily from a dock within close proximity to the Wellers Rock site and it is likely that trace levels of boat fuel attaching directly to the surface of the Ulva was the source of the detected PAH levels in the seaweed.

Preparation

Newly collected *U. pertusa* was cleaned and prepared as in chapter 2, though this time individuals were kept whole, before being left overnight in a growth cabinet (Contherm 620), set at 12°C on a 12hr light/dark cycle. Once back in the lab *M. tenebrosus* were divided between four 1.8L tanks, containing 1000ml FESW, and were left to evacuate their guts over a period of 40hrs.

Treatment

Contaminated Seawater Regime Following overnight acclimation 12 healthy *U. pertusa* individuals, weighing approximately 10g wet weight (WW) each, were rinsed in running FESW, blotted dry and placed in each of 12 1.8L tanks containing 700ml of the FESW and one of three FLA concentrations (0.01, 0.1 and 1 nmol/l) or, in the case of the control, no FLA just a small amount of ethanol. Three replicate tanks were allocated for each treatment and the control. Having evacuated their guts, 24 snails (averaging 7-8mm in length) were added to each of the 12 tanks, which were then placed onto a shaker table in the aforementioned growth cabinet. Each tank was covered with a piece of light mesh to ensure snails would not escape (mesh holes were approximately 3mm in diameter).

Contaminated Food-source Regime: Again following overnight acclimation 12 healthy *U. pertusa* individuals (11g each) were chosen and placed in 12 separate tanks containing the 700ml FESW and either the FLA mixtures or ethanol, though no snails were added at this stage. The *U. pertusa* was left to take up the FLA over a period of four days equating to levels of 0, 12.5, 122 and 402 ng/g DW in the tissue. At the end of the accumulation period Ulva was removed from the FLA contaminated FESW, briefly dabbed dry on a paper towel and placed straight back into 12 clean tanks containing 700ml of clean FESW (no FLA added). A 1g

tissue sample was removed from each of the FLA treated *U. pertusa* specimens, snap frozen and stored in a -80°C freezer to be used in FLA uptake assays at a later date. To each of the 12 treatment tanks 24 snails (having previously evacuated their gut) were added along with the pre-treated *U. pertusa*. Tanks were then placed on a shaker table in the growth cabinet and again each tank was covered with a light mesh (as above).

Harvesting

The snails remaining after experimental setup were harvested and frozen in liquid nitrogen for use as day 0 (basal reference) samples. For each sampling day following experimental setup (day 1, 2 and 4) eight individual snails were harvested from each treatment tank, placed into tubs containing clean FESW and left for 24hrs to once again evacuate their guts. The following day the snails were removed, dabbed dry, snap frozen in liquid nitrogen and stored in a -80°C freezer. In preparation for extractions the shell of each snail was removed and the remaining tissues from eight individual snails were pooled together for each sample and, with a sterile mortar and pestle, were ground to a fine powder in liquid nitrogen. Only day 0, 2 and 4 samples were used for the food source contamination regime as day 1 samples were discarded after accidental defrosting.

Determining FLA Tissue Concentration

Tissue concentrations of FLA were then determined by high-performance liquid chromatography (HPLC) as per Gao and Zhu (2004) with minor modifications. FLA was extracted by adding 500µl of hexane:dichloromethane (85:15 v/v) to 100mg (FW) of frozen powdered *M. tenebrosus* tissue in a glass centrifuge tube, this was then centrifuged at 5,000g for 1min. The supernatant was passed through a Na₂SO₄ column, evaporated to dryness under a stream of nitrogen and redissolved in 2ml of hexane. Samples were then filtered through a silica column (2g of silica) using 1:1(v/v) hexane and dichloromethane as an eluant. Samples were then evaporated to dryness and redissolved in 2ml of methanol. Extracts were separated using a PerkinElmer liquid chromatography system (Perkin Elmer, San Jose, CA, U.S.A.) on a LiChrosphere 100RP-18 column (Phenomenix, Torrence, CA, USA), using methanol/water (83:17). Chromatography was conducted at 30°C, and FLA was detected at 245nm (PerkinElmer series 200 diode array detector). FLA standards, run under the same conditions, were used for identification and quantification. Losses during extraction were determined by

the addition of known concentrations of FLA to tissue extracts, and all results were corrected, based on the percentage recovery.

Determining Damage

Lipid Peroxides

Lipids were extracted by adding 300µl of methanol:chloroform (2:1v/v) to 50mg FW of powdered *M. tenebrosus* tissue, left to stand for 1min, then 200µl of chloroform was added, the sample mixed using a vortex mixer for 30sec, then 200µl of deionised water (DI H₂O) was added and mixed again with the vortex mixer for 30sec. The resulting mixture was briefly placed in a centrifuge at 10,000rpm to separate the phases. 50µl of the bottom phase (lipid phase) was transferred to a glass microtitre plate well and lipid hydroperoxides were determined using the ferric thiocyanate method of Mihaljevic et al (1996) adapted for measurement in a microtitre plate reader. Levels were determined by measuring the absorbance at 500nm. A calibration curve with t-butyl hydroperoxide was used and lipid hydroperoxide peroxide content calculated as nmol lipid hydroperoxide/g FW.

Protein Carbonyls

Protein carbonyls were extracted by adding 500µl of ice-cold Animal Buffer (AB) (appendix I) to 50mg FW of powdered *M. tenebrosus* tissue and mixed by vortex mixer until homogenized. The homogenate was then centrifuged for 5min on 14,000rpm at 4°C. 50µl of the supernatant was transferred to a microtitre plate well and protein carbonyls were determined via reaction with 2,4-dinitrophenylhydrazine (DNPH) (Reznick and Packer, 1994) adapted for measurement in a microplate reader. Levels were determined by measuring the absorbance at 370nm using a Lil420 multilabel counter (Perkin Elmer, San Jose, California, U.S.A.), controlled by a PC, and fitted with temperature control cell and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software package (Perkin Elmer, San Jose, California, U.S.A.). Protein carbonyl content (nmol) was determined using the extinction coefficient of DNPH at 370nm (0.022/µM/cm), corrected for the calculated path-length of the solution (0.6cm). The protein content of the extracts was determined using a Lowry protein assay (Fryer et al., 1986) and protein carbonyl content expressed as nmol protein carbonyl/mg protein.

DNA

DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the protocol for 'Purification of total DNA from animal blood or cells' and following the procedure for cultured cells, including the optional addition of 4µl RNase A at

step 1c, and with the following modifications (Burritt; pers. com.). The API lysis buffer was supplemented with 5mM deferoxamine and 20mM EDTA, the DNA purification columns were pre-treated with 250µl of a solution containing 5mM deferoxamine, 20mM EDTA, 5mM o-phenanthroline, 20mM Tris-HCl (pH8.0) and then washed with 500µl of sterile deionized water prior to use (Dany et al., 1999). The rest of the protocol followed the manufacturer's instructions. The quantity of DNA in each sample and its relative purity were determined by measuring the absorbance at 260 and 280nm using a PerkinElmer VICTOR³ multilabel plate reader. DNA purity was determined by the A_{260}/A_{280} ratio, with all values being within the range: 1.7–1.9 (Sambrook et al., 1989). The amount of oxidised DNA was determined following the method of Shigenaga et al (1994), with slight modifications (refer to Chapter 1 methods pg. 19 for full methodology).

Determining Antioxidants and Enzymes

All enzymatic antioxidant assays were carried out using a PerkinElmer (Wallac) 1420 multilabel counter (Perkin Elmer, San Jose, California, U.S.A.), controlled by a PC, and fitted with a temperature control cell, set to 25°C, and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software package (Perkin Elmer, San Jose, California, U.S.A.).

Superoxide Dismutase

SOD was extracted with AB as for protein carbonyls. 50µl of the supernatant was transferred to a microtitre plate well and SOD was determined via the microplate assay described by Banowetz et al (2004) with minor modifications. Briefly, 50µl of extract, diluted extract or standard (prepared from bovine liver SOD (Sigma–Aldrich, St. Louis, MO, U.S.A.) where one unit of SOD corresponded to the amount of enzyme that inhibited the reduction of cytochrome c by 50% in a coupled system with xanthine oxidase at pH 7.4 and 25°C) was mixed with 125µl of freshly prepared reaction solution containing piperazine-1,4-bis(2-ethanesulfonic acid) (Pipes) buffer, pH 7.4, 0.4mM o-dianisidine, 0.5mM diethylenetriaminepentaacetic acid (DTPA), and 26µM riboflavin. The absorbance at 450nm (A_{450}) was measured immediately ($t=0$ min) and samples were illuminated with an 18W fluorescent lamp placed 12cm above the plate for 30min ($t=30$ min) and the A_{450} was measured again. A regression analysis was used to prepare a standard line relating SOD activity to the change in A_{450} and SOD activities in the extracts, calculated with reference to the standard line, were expressed as units SOD per milligram of total protein.

Catalase

CAT was extracted with AB as for protein carbonyls. 50µl of the supernatant was transferred to a microtitre plate well and CAT was assayed using the chemiluminescent method of Maral et al (1977), as adapted by (Janssens et al., 2000) for 96-well microplates. Briefly, 50µl of extract, diluted extract or standard (purified bovine liver CAT (Sigma–Aldrich, St. Louis, MO, U.S.A.) in homogenization buffer) was mixed with 100µl of 100mM phosphate buffer (pH 7.4) containing 100mM NaEDTA and 10^{-6} M H₂O₂. Samples were then incubated at 25°C for 30 minutes, after which 50µl of a solution containing 20mM luminol and 11.6units/ml⁻¹ of horseradish peroxidase (Sigma–Aldrich, St. Louis, MO, U.S.A.) was injected into each well and light emission, the intensity of which was proportional to the amount of H₂O₂ remaining in the mixture, was measured. A regression analysis was used to prepare a standard line relating standard CAT activities to the intensity of light emission. CAT activities in the extracts were calculated with reference to the standard line and expressed as µM of H₂O₂ consumed per min per milligram of total protein.

Glutathione Reductase

GR was extracted with AB as for protein carbonyls. 50µl of the supernatant was transferred to a microtitre plate well and GR was assayed using the method of Cribb et al (1989) with minor modifications. Briefly, 50µl of extract, diluted extract or standard (GR from wheat germ, Sigma–Aldrich, St. Louis, MO, U.S.A., in homogenization buffer) was mixed with 150µl of 100mM sodium phosphate buffer (pH 7.4) containing 0.1mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 10µl of NADPH (10mg/ml; 12mM). The reaction was initiated by the injection of 10µl of oxidized glutathione (GSSG) (1mg/ml; 3.25mM) and the absorbance at 415nm (A₄₁₅) was measured every 30 seconds for 3min, with the plate shaken automatically before each reading. The rate of increase in A₄₁₅ per minute was calculated and a regression analysis was used to prepare a standard line relating standard GR activities to the change in A₄₁₅. GR activities in the extracts, calculated with reference to the standard line, were expressed as µmol or nmol of oxidized glutathione reduced per min per milligram of total protein.

Glutathione Peroxidase

GPX was extracted with AB as for protein carbonyls. 50µl of the supernatant was transferred to a microtitre plate well and GPX was assayed using a PerkinElmer (Wallac) 1420 multilabel counter (Perkin Elmer, San Jose, California, U.S.A.), controlled by a PC, and fitted with a temperature control cell, set to 25°C, and an auto-dispenser. Data were acquired and

processed using the WorkOut 2.0 software package (Perkin Elmer, San Jose, California, U.S.A.).

Glutathione S-transferase

GST was extracted with AB as for protein carbonyls. 50µl of the supernatant was transferred to a microtitre plate well and GST was assayed using the photometric 1-chloro-2,4-dinitrobenzene (CDNB) method of Habig et al (1974) with minor modifications. The absorbance at 340nm (A_{340}) was measured every 30 seconds for 3min, with the plate shaken automatically before each reading. The change in A_{340} per minute was calculated and converted into nmol CDNB conjugated to GSH/min/mg protein using the extinction coefficient of the resulting S-2,4-dinitrophenylglutathione (DNPG): $E_{415} 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

Glutathione

Glutathione was extracted by adding 500µl of 5% sulfosalicylic acid to 50µg of powdered *M. tenebrosus* tissue in a microcentrifuge tube and mixed by vortex mixer until homogenized. The homogenate was then centrifuged at 14,000rpm for 15 minutes at 4°C. Glutathione and glutathione disulfide levels were measured using the enzymatic recycling method, using the microtitre plate based assay described by Rahman et al (2006).

Data Analysis

Output data from each of the uptake, damage and antioxidant assays was collated into excel spreadsheets, where means, standard deviations and standard errors were calculated for each treatment at each time point, from which figures were constructed. Assumptions of normality and equal variance were calculated for data from each of the assays. Having met the assumptions an individual two-way ANOVA and non-parametric Tukeys comparisons were calculated for each assay, using the programme SigmaStat 2.03 Network. For data that did not meet either assumption only results from multiple pairwise comparisons of the non-parametric Tukeys test were used. In addition, individual one-way ANOVA tests were calculated for each assay to determine if there was an overall time effect for each concentration treatment of that particular assay.

FLA uptake data was treated differently in that a multiple linear regression was calculated (using SigmaStat 2.03) to determine if time and/or FLA concentration were contributing factors. This data was also collated in an Excel spreadsheet where mean, standard deviation and standard error were calculated and graphed. Note: data values that showed up as below the detectable level (bdl) were given a zero value for the purpose of statistical analysis.

The two experiments are not statistically comparable as they were conducted independently (due to logistical and resource restrictions), therefore observational comparisons between graphically presented data and values obtained were used compare the two contamination regimes.

Results

Exposure to FLA Contaminated Seawater

Fluoranthene Uptake

Internal FLA concentrations increased in *M. tenebrosus* tissues with length of exposure to FLA contaminated seawater ($p < 0.001$). FLA exposure concentration had an effect on internal tissue concentration of FLA, $p < 0.001$ (Fig. 3.3). The difference in FLA concentrations of tissues was significant between each treatment on each of the days measured (Fig. 3.3).

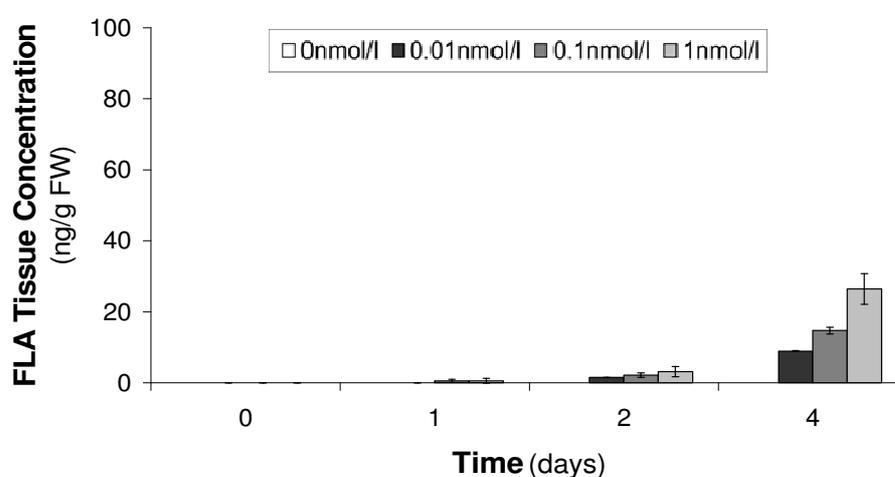


Figure 3.3: Levels of FLA in *M. tenebrosus* tissues following exposure to three treatment concentrations of FLA (0.01, 0.1 and 1nmol/l), a control (0nmol/l) and a food source (*U. pertusa*). Indicates the amount of FLA taken up over a period of four days, \pm s.e.m. ($n = 3$).

Oxidative Damage

Protein carbonyls and lipid peroxide levels both increased from day 0 to day 2 in *M. tenebrosus* exposed to 1nmol/l FLA contaminated seawater, and also from day 0 to day 1 in *M. tenebrosus* exposed to 0.1nmol/l FLA contaminated seawater. After these days a steady decrease was observed in the respective carbonyls and peroxides until the termination of the experiment (day 4) (Fig. 3.4a and 3.4b). An increase in oxidised DNA was also observed in *M. tenebrosus* exposed to 0.1 and 1nmol/l treatments (Fig. 3.4c), though these elevated levels began to decrease after days 2 and 1 respectively, and continued to decrease for the remainder of the experimental period. All three measures of damage were significantly influenced by the length of exposure with respect to the 0.1 and 1nmol/l FLA treatments for protein carbonyls and lipid peroxides, and the 1nmol/l treatment for oxidised DNA, where $p < 0.01$ for each. The 0.1nmol/l treatment for oxidised DNA did not show a significant time effect, $p = 0.132$, though, and neither did any of the 0.01nmol/l treatments (Fig. 3.4c).

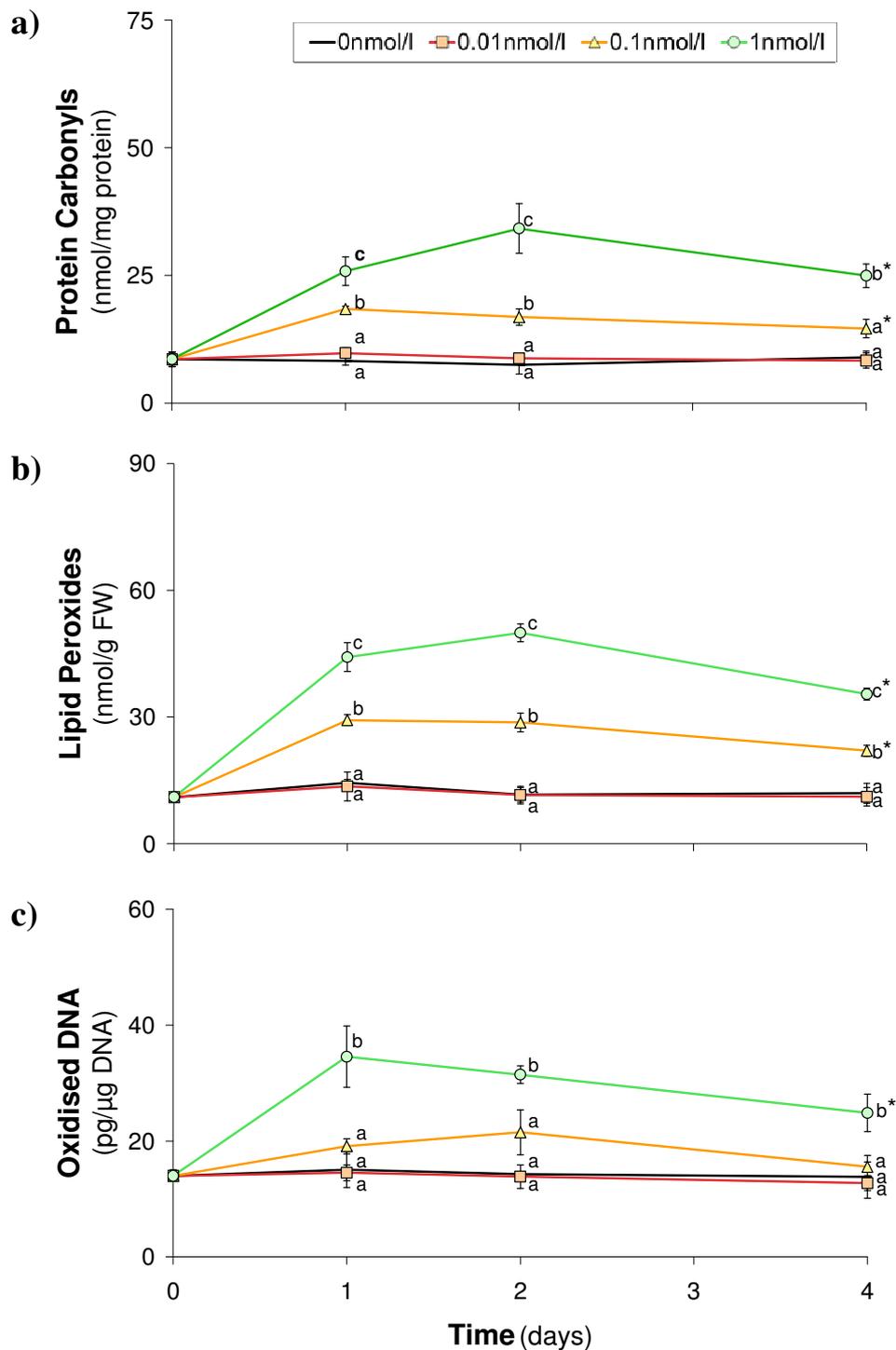


Figure 3.4: Level of damage to *M. tenebrosus* proteins (a), lipids (b), and DNA (c) after exposure to each of three treatment concentrations of FLA (0.01, 0.1 and 1nmol/l), a control (0nmol/l) and a food source (*Ulva pertusa*), over a period of four days, \pm s.e.m (n = 3). Letters that are the same indicate no significant difference between treatments at the end of the time course (day 4) and treatments marked with an asterisk (*) have a significant effect of time.

Enzymatic Antioxidants

SOD levels steadily increased in *M. tenebrosus* exposed to 0.1 and 1nmol/l FLA contaminated seawater, throughout the duration of exposure, $p=0.002$ and 0.004 respectively, and were significantly increased from the control by day 4 ($p<0.001$) (Fig 3.5a). The lowest FLA treatment (0.01nmol/l) was not significantly different from the control and showed no effect of time ($p=0.904$). CAT increased steadily in *M. tenebrosus* during exposure to 1nmol/l FLA where $p=0.021$ (Fig 3.5b). The 0.01 and 0.1nmol/l FLA treatments produced no significant difference in CAT levels compared to the control and showed no significant time effect (Fig 3.5b).

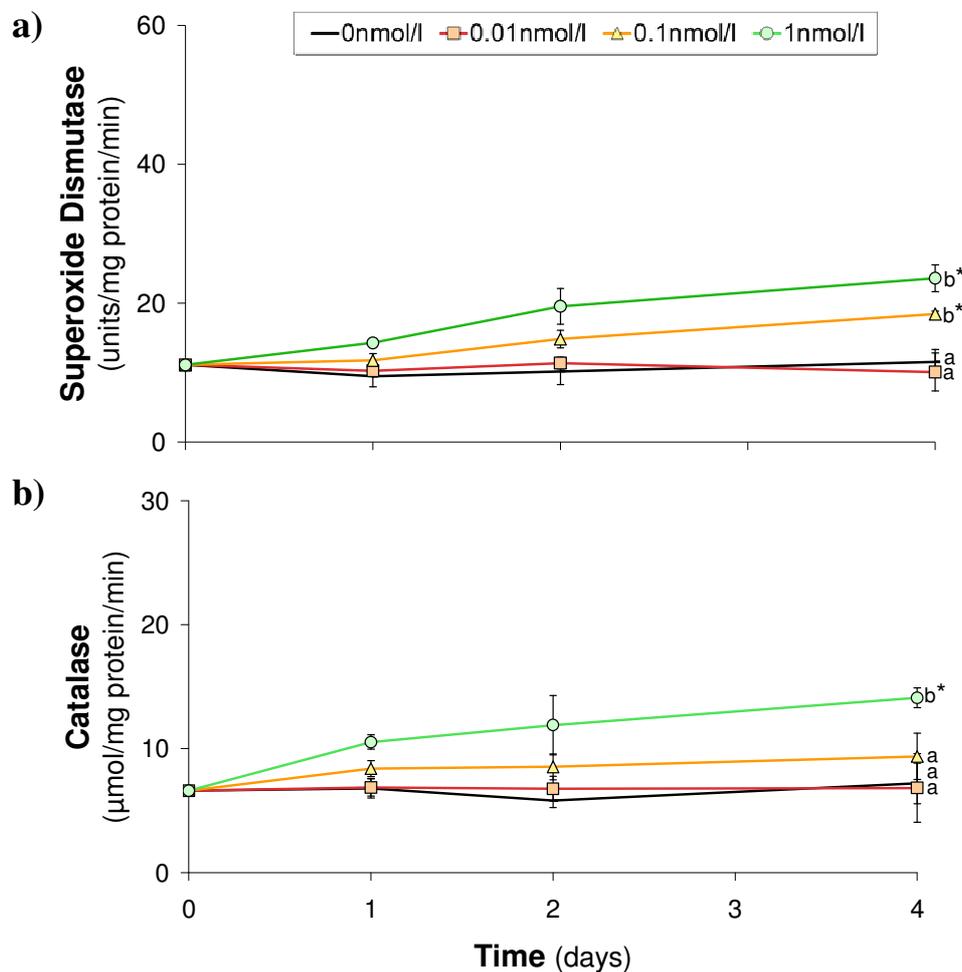


Figure 3.5: Level of SOD (a) and CAT (b) present in *M. tenebrosus* tissues following exposure to three treatment concentrations of FLA (0.01, 0.1 and 1nmol/l), a control (0nmol/l) and a food source (*U. pertusa*), \pm s.e.m. ($n = 3$), over a period of four days. Letters that are the same indicate no significant difference between treatments at the end of the time course (day 4) and treatments marked with an asterisk (*) have a significant time effect.

A steady increase in GR occurred in *M. tenebrosus* exposed to 1nmol/l FLA with $p<0.001$, indicating length of exposure had a significant effect. The GR levels increased slightly in *M. tenebrosus* exposed to 0.1nmol/l FLA from the basal level of 7.5nmol/mg protein/min to 9.8nmol/mg protein/min at day 2 ($p=0.049$) and then levelled off to 10.1nmol/mg protein/min at day 4, with a significant time effect of $p<0.001$ (Fig 3.6a). The lowest FLA treatment showed no significant difference in GR levels from the control and levels were not significantly affected by time ($p=0.315$) (Fig. 3.6a).

There was a steady increase in the level of GPX observed in *M. tenebrosus* exposed to 1nmol/l FLA, throughout the duration of exposure ($p<0.001$). 0.1nmol/l FLA also showed an increase in GPX levels over time, $p=0.001$, though this treatment showed an initial increase from the basal level of 4.1nmol/mg protein/min to 5.2nmol/mg protein/min at day 2 and then up to 6.3nmol/mg protein/min at day 4 (Fig. 3.6b). The lowest FLA treatment (0.01nmol/l) showed no significant difference in GPX levels from the control, for the duration exposure and consequently no time effect was seen ($p=0.594$).

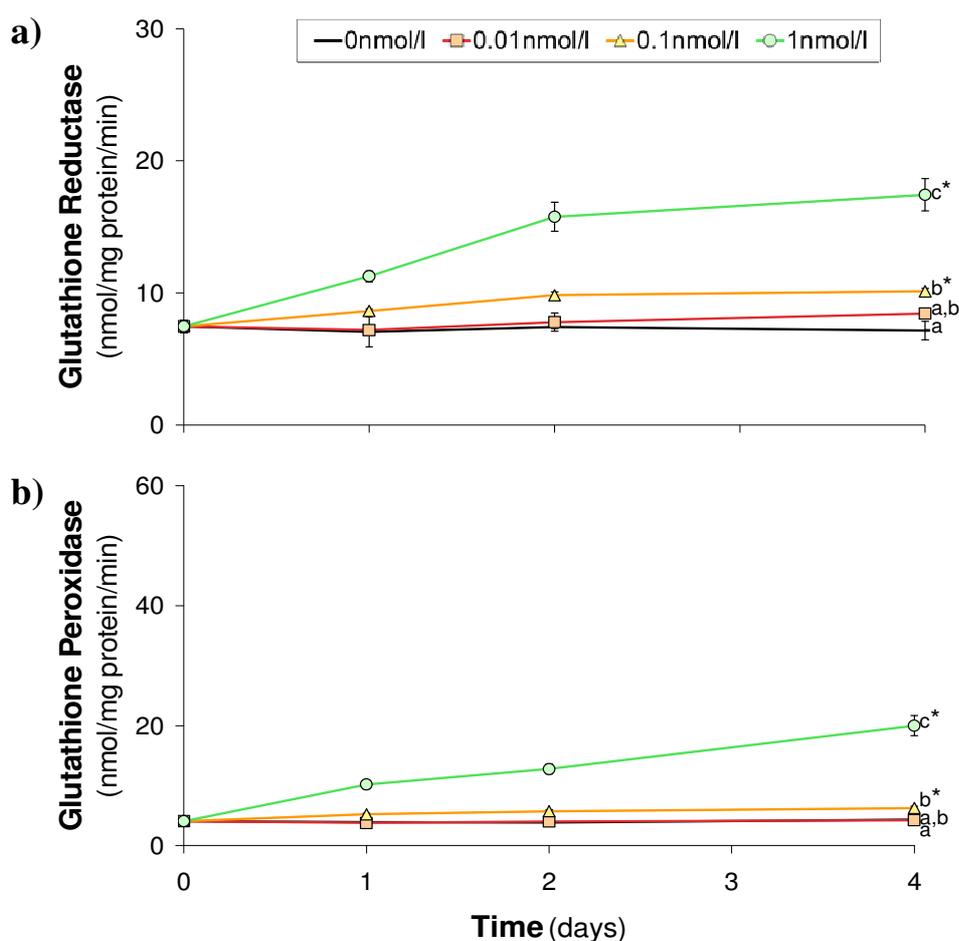


Figure 3.6: Level of GR (a) and GPX (b) present in *M. tenebrosus* tissues following exposure to three treatment concentrations of FLA (0.01, 0.1 and 1nmol/l), a control (0nmol/l) and a food source (*U. pertusa*), \pm s.e.m. (n = 3), over a period of four days. Letters that are the same indicate no significant difference between treatments at the end of the time course (day 4) and treatments marked with an asterisk (*) have a significant time effect

There was a significant increase in the level of GST in *M. tenebrosus* throughout the duration of exposure to 0.01, 0.1 and 1nmol/l FLA ($p=0.021$, $p=0.036$ and $p<0.001$ respectively). Treatments of 0.1 and 1nmol/l FLA produced an overall increase in GST from the basal level of 105nmol DNPG/mg protein/min to 142 and 191nmol DNPG/mg protein/min respectively, with $p<0.001$ for both at day 4 (Fig. 3.7). The GST levels from the lowest FLA treatment were not significantly different from the control at days 1, 2 or 4.

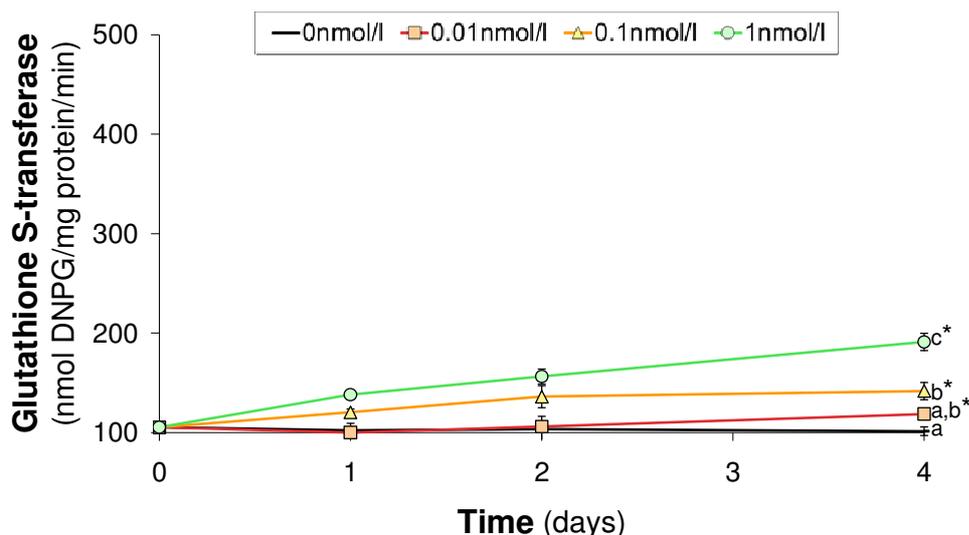


Figure 3.7: Level of GST present in *M. tenebrosus* tissues following exposure to three treatment concentrations of FLA (0.01, 0.1 and 1nmol/l), a control (0nmol/l) and a food source (*U. pertusa*), \pm s.e.m. ($n = 3$), over a period of four days. Letters that are the same indicate no significant difference between treatments at the end of the time course (day 4) and treatments marked with an asterisk (*) have a significant time effect.

Non-enzymatic Antioxidants

The amount of total glutathione changed over time in *M. tenebrosus* exposed to 0.1 and 1nmol/l FLA ($p=0.027$ and $p=0.003$, respectively). In particular the level of total glutathione decreased continuously for the duration of exposure to 0.1nmol/l, while glutathione from the 1nmol/l treatment decreased from the basal level up till day 2 and then there was an increase from day 2 to day 4 (Fig. 3.8a). The percentage of reduced glutathione (%GSH) decreased in *M. tenebrosus* over time in all three FLA treatments from the basal level of 84% to 70, 63 and 61% at day 4, respectively (Fig. 3.8b).

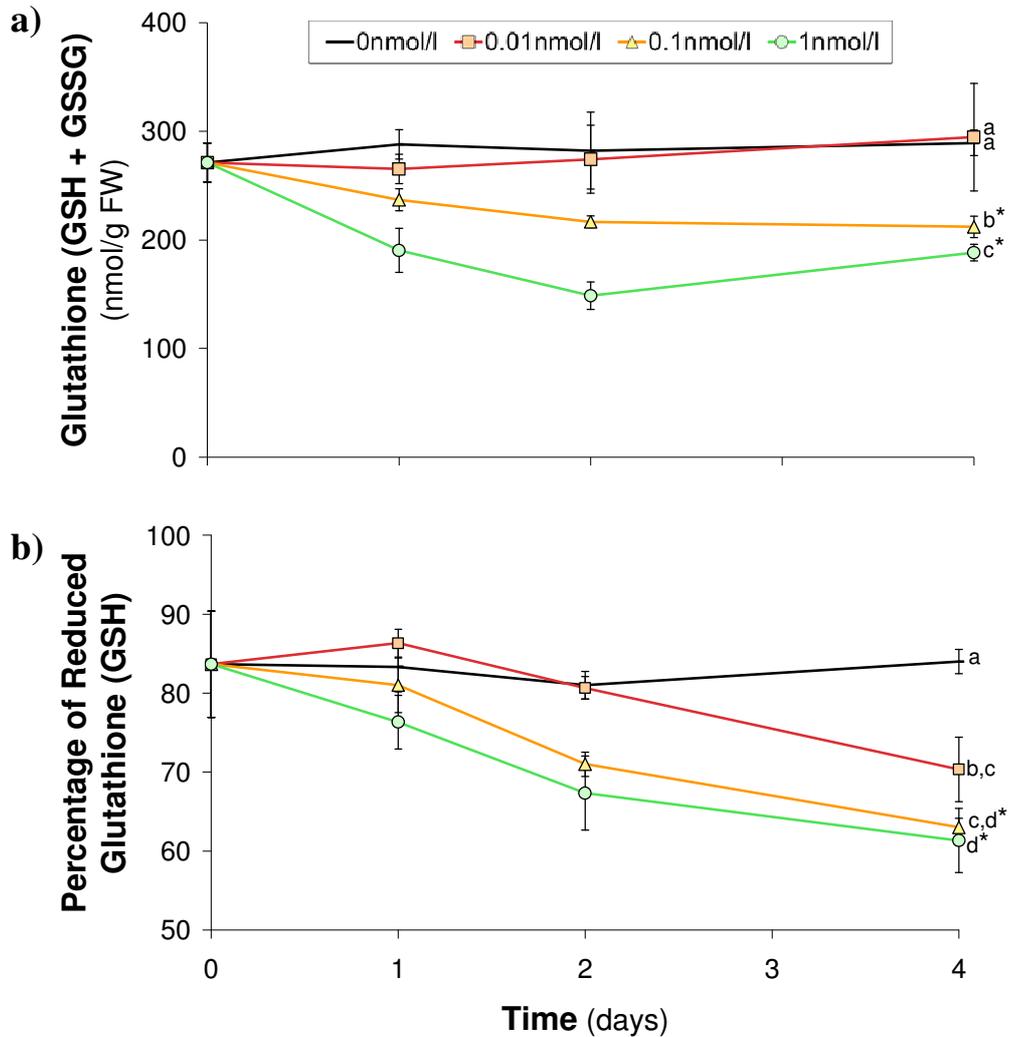


Figure 3.8: Level of total glutathione (GSH + GSSG) (a) and percentage of reduced glutathione (% GSH) (b) present in *M. tenebrosus* tissues following exposure to three treatment concentrations of FLA (0.01, 0.1 and 1nmol/l), a control (0nmol/l) and a food source (*U. pertusa*), \pm s.e.m. (n=3), over a period of four days. Letters that are the same indicate no significant difference between treatments at the end of the time course (day 4) and treatments marked with an asterisk (*) have a significant time effect.

Grazing on FLA Contaminated Seaweed

Ulva FLA Uptake

Ulva exposed to FLA contaminated seawater for four days prior to grazing by the snails had levels of 12.5ng/g DW, 122ng/g DW and 402ng/g DW, for the 0.01, 0.1 and 1nmol/l treatments respectively.

Fluoranthene Uptake:

Internal FLA concentrations increased in *M. tenebrosus* tissues with length of exposure to FLA contaminated food source ($p < 0.001$). FLA contaminated *U. pertusa* exposure had an effect on FLA tissue concentration of *M. tenebrosus*, $p < 0.001$ (Fig. 3.9). FLA tissue concentrations of *M. tenebrosus* were significantly different between each treatment on each of the days (Fig. 3.9).

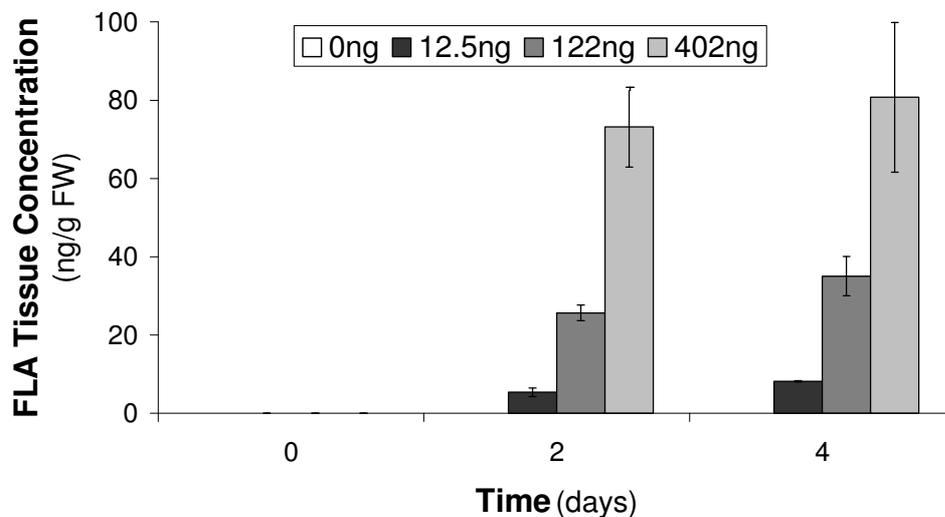


Figure 3.9: Levels of FLA present in *M. tenebrosus* tissues following consumption of a food source (*U. pertusa*) contaminated with three treatment concentrations of FLA (12.5, 122 and 402ng/g DW) and a control (0ng/g DW). Indicates the amount of FLA taken up by *M. tenebrosus* over four days of FLA contaminated Ulva consumption, \pm s.e.m. (n = 3).

Oxidative Damage

Damage to proteins was elevated in *M. tenebrosus* exposed to 122 and 402ng FLA contaminated *U. pertusa*, and both treatments had a significant effect over time, $p < 0.01$ (Fig. 3.10a). In particular protein damage increased from the basal level of 8.6nmol/mg protein to 53nmol/mg protein at day 2 and levelled off at day 4 with 54nmol/mg protein for the 402ng FLA treatment (Fig. 3.10a). By contrast protein carbonyls for the 122ng FLA treatment continued to increase from day 2 (30nmol/mg protein) to day 4 (35nmol/mg protein). *M. tenebrosus* exposed to 12.5ng contaminated *U. pertusa* showed no significant difference in protein carbonyl production from the control and produced no significant effect over time ($p = 0.106$) (Fig. 3.10a).

The lipid peroxide content in the tissues of *M. tenebrosus* was significantly affected by length of exposure to 12.5, 122 and 402ng FLA contaminated *U. pertusa* ($p < 0.001$), with increased levels seen throughout the length of exposure to 12.5 and 402ng treatments (Fig. 3.10b). The 122ng treatment however showed an increased level of LPX from the basal level of 11nmol/g to 39nmol/g at day 2 and a decrease to 35nmol/g at day 4.

Oxidised DNA levels increased in *M. tenebrosus* throughout the length of exposure to 12.5, 122 and 402ng FLA contaminated *U. pertusa* ($p = 0.025$, $p = 0.013$ and $p = 0.002$, respectively) (Fig. 3.10c). In particular, oxidised DNA levels increased steadily during the 402ng treatment while the 122ng treatment showed an initial increase in Oxidised DNA from the basal levels to day 2 followed by a decrease to the termination of exposure at day 4 (Fig. 3.10c).

Enzymatic Antioxidants

The level of SOD increased steadily in *M. tenebrosus* exposed to 12.5, 122ng and 402ng contaminated *U. pertusa* and the levels increased throughout the length of exposure ($p = 0.02$, $p < 0.001$ and $p = 0.001$, respectively) (Fig. 3.11a). The internal SOD levels of the 402ng treatment significantly increased from the basal level of 11units/mg protein to 38units/mg protein at day 2 ($p < 0.001$) and 48.6units/mg protein at day 4 ($p < 0.001$). Similarly but less extreme was the increase in SOD levels for the 122ng treatment, with respective p -values of 0.01 and 0.013 for days 2 and 4.

CAT levels increased over time in *M. tenebrosus* with exposure to 12.5, 122 and 402ng contaminated *U. pertusa* ($p = 0.01$, $p < 0.001$ and $p < 0.001$, respectively) (Fig. 3.11b). CAT levels increased from the basal level of 6.6 μ mol/mg protein to 20 μ mol/mg protein at day 2 and 25 μ mol/mg ($p < 0.001$) protein at day 4 ($p < 0.001$). Similarly CAT levels had increased significantly by day 4, compared to basal levels, in *M. tenebrosus* exposed to 12.5 and 122ng contaminated *U. pertusa* ($p = 0.002$ and $p < 0.001$, respectively).

Glutathione reductase levels increased in *M. tenebrosus* consuming 12.5, 122 and 402ng FLA contaminated *U. pertusa*, and GR further increased with time for these treatments ($p=0.045$, $p<0.001$ and $p<0.001$, respectively) (Fig. 3.12a). The 122 and 402ng treatments showed a significant increase from the basal levels of GR at days 2 and 4 ($p<0.001$), while the 12.5ng treatment showed no significant difference from basal GR levels throughout exposure ($p=0.527$ and $p=0.284$, for days 2 and 4 respectively) (Fig. 3.12a).

All three FLA contaminated *U. pertusa* treatments produced a significant increase in GPX levels of *M. tenebrosus* over time ($p<0.001$ for all) (Fig. 3.12b). In particular the 402ng treatment produced an increase in GPX from the basal level of 4nmol/mg protein to 48nmol/mg protein at day 4 ($p<0.001$), while the 122ng treatment produced an increase from the basal level to 19nmol/mg protein at day 4 ($p=0.002$) (Fig. 3.12b).

GST levels increased in *M. tenebrosus* consuming 012.5, 122 and 402ng FLA contaminated *U. pertusa* with time ($p=0.027$, $p=0.002$ and $p=0.004$, respectively) (Fig. 3.13). In particular 122 and 402ng treatments increased from the basal GST level of 105nmol DNPG/mg protein/min to 188 ($p=0.001$) and 400nmol DNPG/mg protein/min ($p<0.001$) at day 4 (Fig. 3.13).

Non-enzymatic Antioxidants

Overall, there was a decrease in total glutathione of *M. tenebrosus* consuming 122 and 402ng FLA contaminated *U. pertusa* ($p=0.003$ and $p<0.001$, respectively) (Fig. 3.14a). In particular the 122ng treatment showed a continuous decrease in total glutathione from the basal level of 271nmol/g to 176nmol/g at day 4 ($p=0.004$), while the 402ng treatment showed a decrease to 126nmol/g at day 2 ($p<0.001$) followed by a slight increase to 147nmol/g at day 4 ($p<0.001$). All three treatments showed a reduction in the percentage of GSH from the basal level in *M. tenebrosus* (84%) with time ($p<0.001$ for each) (Fig. 3.14b). Both the 12.5ng and 402ng treatments showed a decrease all the way through from day 0 to day 4 (64 and 53%, respectively) while the 122ng treatment showed a decrease to 60% at day 2 and then GSH levelled off by day 4 (Fig. 3.14b).

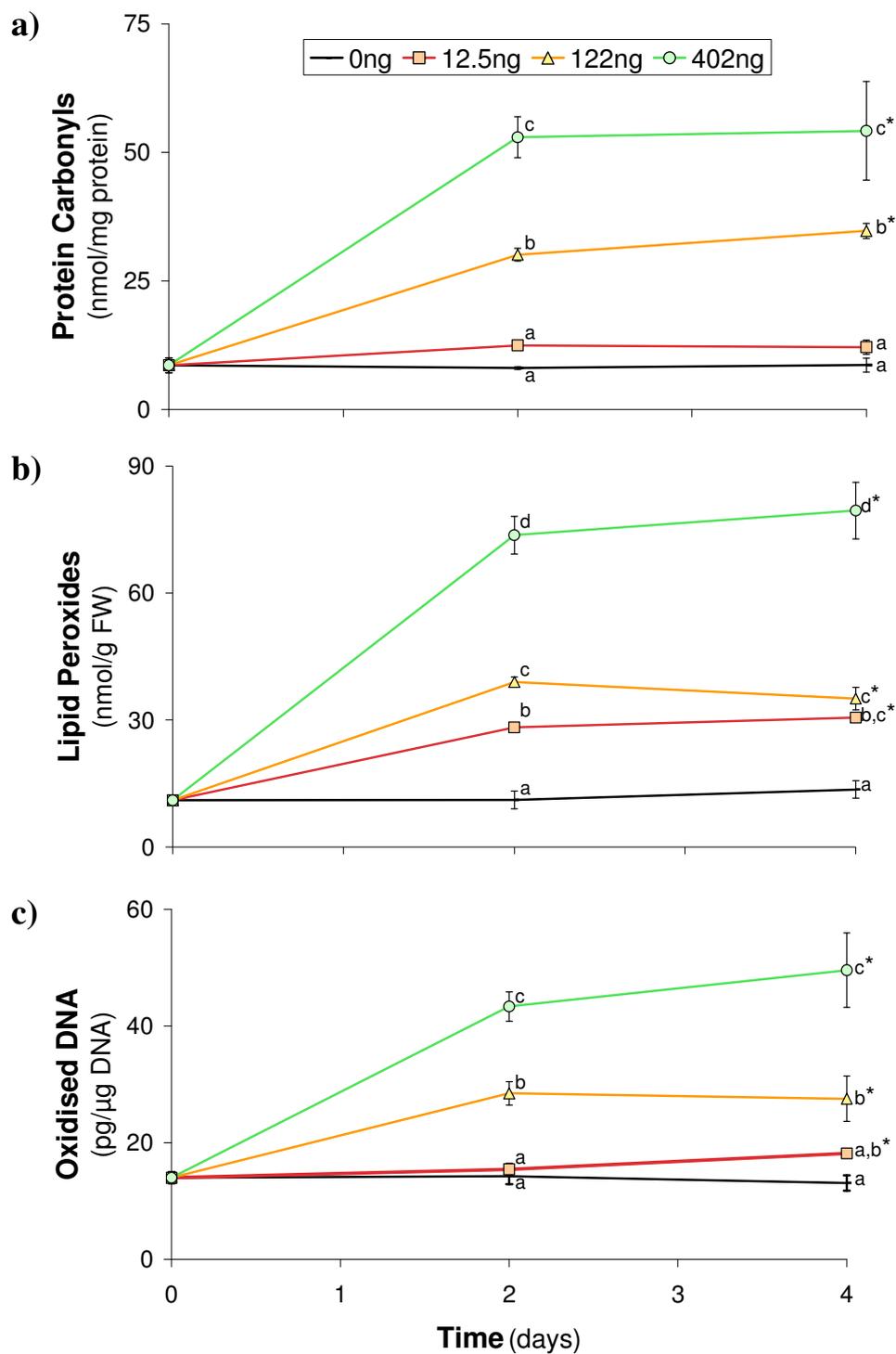


Figure 3.10: Level of damage to *M. tenebrosus* proteins (a), lipids (b), and DNA (c) following consumption of a food source (*U. pertusa*) contaminated with three treatment concentrations of FLA (12.5, 122 and 402ng/g DW) and a control (0ng/g DW), over a period of four days, \pm s.e.m. (n = 3). Letters that are the same indicate no significant difference between treatments at the end of the time course (day 4) and treatments marked with an asterisk (*) have a significant time effect.

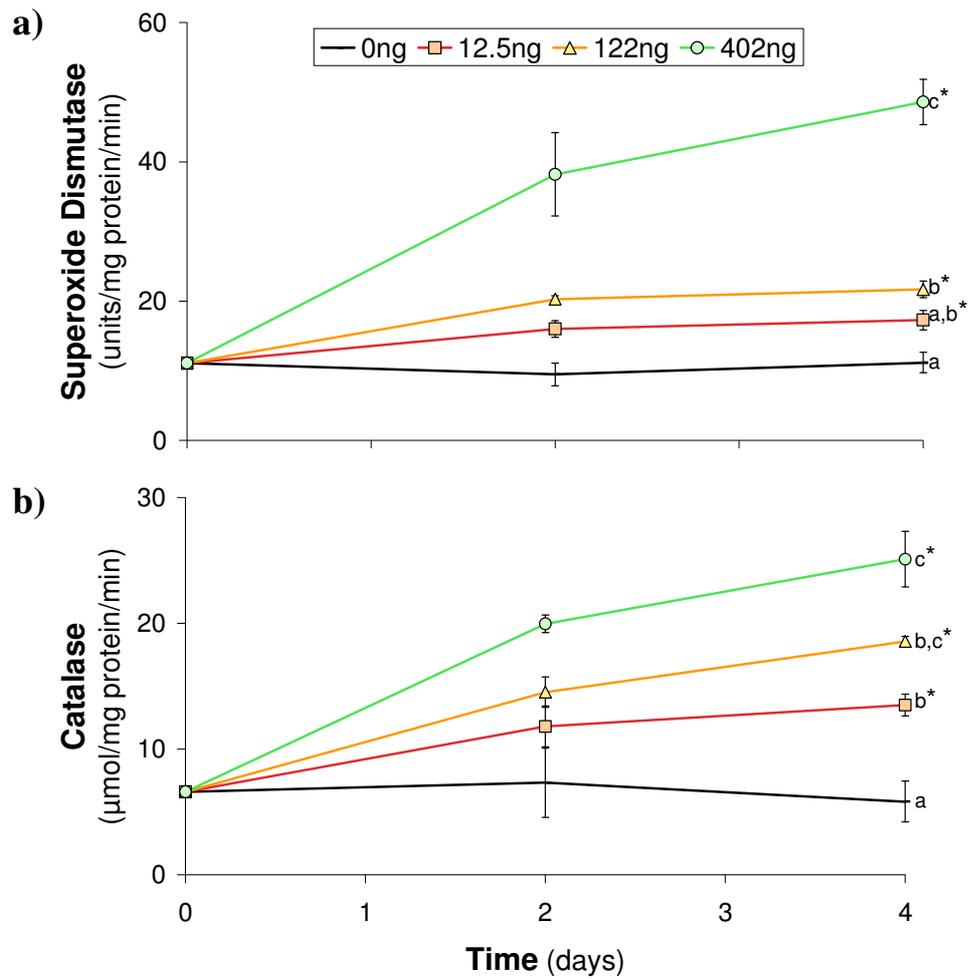


Figure 3.11: Level of SOD (a) and CAT (b) present in *M. tenebrosus* tissues following consumption of a food source (*U. pertusa*) contaminated with three treatment concentrations of FLA (12.5, 122 and 402ng/g DW) and a control (0ng/g DW), over a period of four days, \pm s.e.m. (n = 3). Letters that are the same indicate no significant difference between treatments at the end of the time course (day 4) and treatments marked with an asterisk (*) have a significant time effect.

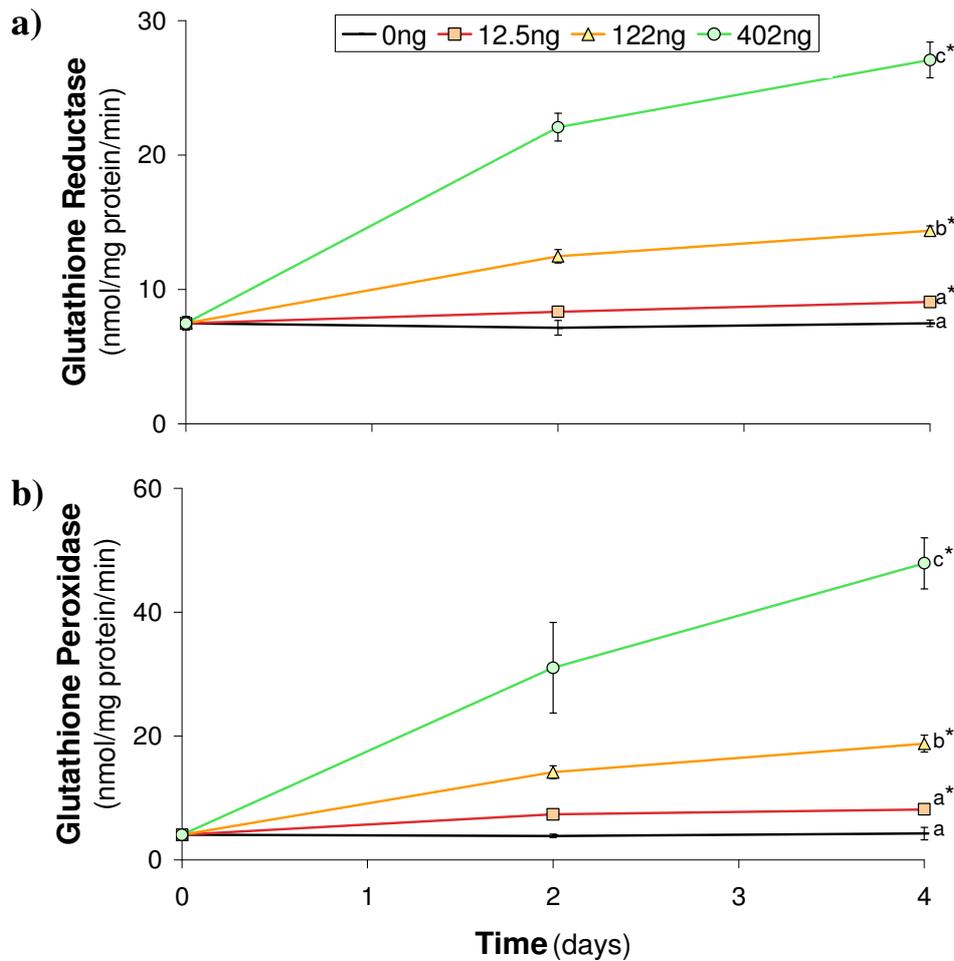


Figure 3.12: Levels of GR (a) and GPX (b) present in *M. tenebrosus* tissues following consumption of a food source (*U. pertusa*) contaminated with three treatment concentrations of FLA (12.5, 122 and 402ng/g DW) and a control (0ng/g DW), over a period of four days, \pm s.e.m. ($n = 3$). Letters that are the same indicate no significant difference between treatments at the end of the experimental time course (day 4) and treatments marked with an asterisk (*) have a significant time effect.

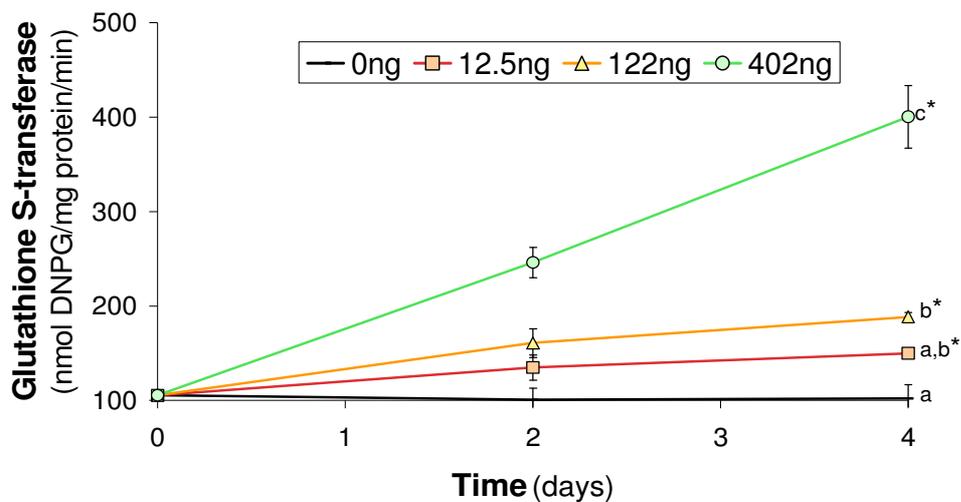


Figure 3.13: Level of GST present in *M. tenebrosus* tissues following consumption of a food source (*U. pertusa*) contaminated with three treatment concentrations of FLA (12.5, 122 and 402ng/g DW) and a control (0ng/g DW), over a period of four days, \pm s.e.m. ($n = 3$). Letters that are the same indicate no significant difference between treatments at the end of the time course (day 4) and treatments marked with an asterisk (*) have a significant time effect.

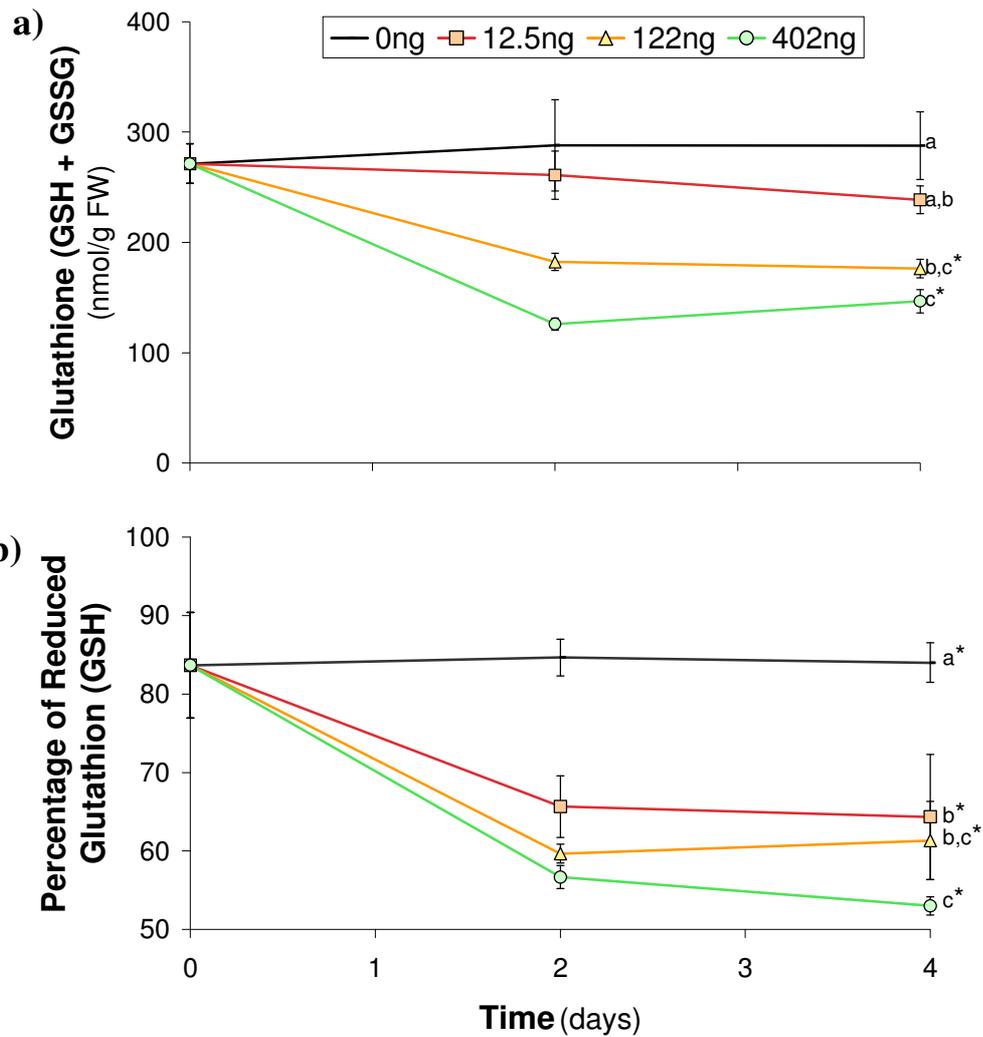


Figure 3.14: Level of total glutathione (GSH + GSSG) (a) and percentage of reduced glutathione (GSH) (b) present in *M. tenebrosus* tissues following consumption of a food source (*U. pertusa*) contaminated with three treatment concentrations of FLA (12.5, 122 and 402ng/g DW) and a control (0ng/g DW), over a period of four days, \pm s.e.m. (n = 3). Letters that are the same indicate no significant difference between treatments at the end of the time course (day 4) and treatments marked with an asterisk (*) have a significant time effect.

Discussion

Uptake

The literature related to the uptake and trophic transfer of PAHs is limited and varies in its conclusions, although uptake by aquatic animals is generally considered to be a passive process of diffusion through the integument or via ventilation over the gill surface (Landrum and Stubblefield, 1991, Meador, 2003). It is important to note that the levels of FLA in the loaded *U. pertusa* after 4 days of exposure are similar to the levels found in *U. pertusa* after 3 and 5 days (chapter 2).

M. tenebrosus having actively ingested FLA by grazing contaminated *U. pertusa* had considerably more FLA in the tissues than specimens taking up FLA passively via contaminated seawater, FLA tissue concentrations in *M. tenebrosus* consuming the highest treatment seaweed (402ng/g) were more than 3-fold that of the tissue levels in specimens exposed to seawater contaminated with the same FLA concentration on the fourth day of exposure (81 and 26.5ng/g FW, respectively). This difference was less pronounced for the lesser concentration treatment (35 and 15ng/g FW) and at the lowest level of FLA contamination almost no difference between experiments was evident (8.2 and 9ng/g FW, respectively on day four). The likely explanation of such an observation is that ingestion of a contaminant from a food source increases the bioavailable fraction of that contaminant (Torres et al., 2008). It could also simply be that as the gut is high in lipids (Arakelova, 2008), and with FLA being highly lipophilic, more of the contaminant is absorbed in this way than by passive diffusion through the integument from the water column. The inflow of contaminants into marine invertebrates comes from both water and food and so is dependant on the bioavailable fraction of the contaminant (Hendriks, 1995). Accumulation of pollutants often depends on the amount which passes over the gills (ventilation) or feeding rate, as well as assimilation efficiency of *M. tenebrosus* (Palmqvist et al., 2006). Unfortunately in the present studies snails were small in size (<10mm) so it was not possible to analyse the gill and gut tissues separately, though gut contents were evacuated before analysis.

The dose related increases in FLA found in this study are similar to those found in other PAH studies where intestinal levels of FLA found in the predatory polychaete *Nereis virens* are directly related to the dose levels of their prey *Capitella* sp. (Palmqvist et al., 2006). Tissue concentrations in terrestrial snails exposed to PAH contaminated soil also present a concentration-dependant dose response, with highest internal concentrations of 31, 6.9 and 1.4µg/g WW of Dibenzothiophene, Carbazole and Acridine, respectively (Sverdrup et al., 2006). FLA also has a log K_{ow} value of 5.22 and PAHs ≤ 5.5 are considered relatively water-

soluble while those with values ≥ 5.5 are more hydrophobic and so these are more likely to be associated with lipids and organic carbon (Meador, 2003). As FLA falls almost on the cusp of the defining octanol/water partition coefficient (K_{ow}) value it can be assumed that FLA would be taken up in organisms both by ingestion and passive diffusion. Though FLA may be more water soluble than other PAHs, it is important to note that it is still more soluble in lipids than water in general (Douben, 2003). Therefore the key factors affecting tissue residues are duration and concentration of exposure, organism physiology and its ability to metabolise the contaminant.

Oxidative Stress and Damage

The production of ROS in aquatic organisms, as with other organisms, occurs continuously through the partial reduction of O_2 which produces potentially damaging radicals such as $O_2^{\cdot-}$, H_2O_2 and OH . These radicals exist in manageable amounts under normal conditions, only 1-3% of O_2 consumed is converted to ROS (Halliwell and Gutteridge, 2007), yet recent research emphasised the importance of increased ROS production in the presence of xenobiotics including PAHs (Livingstone, 2001). However, ROS levels were not assayed for the current study due to the technical difficulties of such a measurement in animals (de Almeida et al., 2007). As an alternative, oxidative damage in the form of changes to protein carbonyl, lipid peroxide and oxidised DNA levels, were measured as indirect evidence of FLA-stimulated ROS production.

Oxidative damage to all three of these macromolecules occurs in a range of aquatic organisms including invertebrates (molluscs) and fish, as a result of contaminant-stimulated ROS production (refer to (Livingstone, 2001) for examples in the literature). The current study confirms that protein carbonyl production, lipid peroxidation and DNA oxidation occurs as a result of FLA contamination. Both seawater and food source contamination increased damage to *M. tenebrosus* macromolecules, particularly during exposure to the highest FLA treatment (1nmol/l). This suggests that the higher the level of FLA exposure and the more taken up in the tissues increases the level of oxidative damage to proteins, lipids and DNA. Interestingly the level of damage in *M. tenebrosus* having consumed contaminated seaweed was much higher and continued to increase over the length of exposure, compared to specimens exposed to FLA contaminated seawater, which showed an increase in oxidative damage at the highest levels of exposure, but this increase in damage decreased not long into the exposure period. As there was more FLA present in tissues of *M. tenebrosus* that consumed the contaminant it follows that those specimens also have higher levels of oxidative

damage than snails exposed to contaminated seawater. It is also important to note that in both experiments lipid damage in the form of lipid peroxide production was higher than damage to either proteins or DNA. This is a likely result because ROS, in particular superoxide and H_2O_2 , react more readily with lipids than DNA (Moller et al., 2007). Damage to an organisms DNA results in mutations and strand-breaks, preventing replication, cell division and ultimately interfering with cell signalling (Halliwell and Gutteridge, 2007). Oxidised DNA must therefore be removed quickly before further damage occurs as genetic mutations are difficult and energetically expensive to repair (Halliwell and Gutteridge, 2007). If mutations are unable to be repaired or removed they have the potential to be passed on as adverse genetic mutations to offspring or result in death of the individual (Halliwell and Gutteridge, 2007). DNA is oxidised rapidly by the $\text{OH}\cdot$ radical therefore the elimination of superoxide and H_2O_2 by SOD and CAT observed in snails likely reduced the amount of $\text{OH}\cdot$ produced and consequently prevented detrimental DNA damage. In contrast lipids are highly reactive with most ROS, and lipid peroxidation propagates the production of further lipid peroxides (refer to chapter 1) (Halliwell and Gutteridge, 2007). Lipids make up an integral part of most animal cells and organelles, therefore damage to lipids can result in any number of failures to cell function, the worst being loss of membrane integrity, damaging proteins and inactivating enzymes (Halliwell and Gutteridge, 2007). Under these conditions ion channels are shut down and cell signalling becomes compromised (Halliwell and Gutteridge, 2007). Therefore the up-regulation of antioxidants observed in snails under both FLA contaminant regimes was important in preventing genetic and cellular damage and maintaining homeostasis and function of the organisms.

Antioxidant Response

Recent monitoring studies have focussed on the use of antioxidants in marine molluscs as potential biomarkers for aquatic pollutants (Cheung et al., 2001, de Almeida et al., 2007, Richardson et al., 2008). Antioxidant defences exist as enzymes (SOD, CAT, GR, GPX), reductants (GSH and AsA) and vitamins (α -tocopherol) (Kappus, 1987). These antioxidants are induced in response to oxidative stress and are particularly important in combating pollutant induced stress because their production is dependant upon duration and intensity of contaminant-stimulated stress applied to the organism (Sole, 2000). Maintaining the balance between ROS production and antioxidant defences is crucial in preventing oxidative damage to macromolecules (Halliwell and Gutteridge, 2007) and it is therefore important to monitor

antioxidant activity in response to contaminant exposure, in order to gather insight into how the organism is coping with such stress.

Changes in GSH activity are particularly useful in monitoring the detoxification ability of animals as it regulates the balance of the antioxidant metabolism and ROS by both scavenging radicals directly and aiding in the conjugation and removal of harmful substances. GSH was reduced in *M. tenebrosus* exposed to FLA contaminated seawater yet at the highest treatment concentration it increases again after day 2. This indicates that glutathione has become depleted beyond the point of regeneration. This is backed up by the observed and continuous reduction in the percentage of reduced glutathione, indicating that GSSG is no longer being reduced by GR to GSH or that available GSH is bound to FLA. A similar pattern of total glutathione reduction and decreased percentage of reduced glutathione appears in snails that ingested FLA contaminated food. The obvious difference of the two contaminant regimes is that glutathione reduction is more pronounced in the consumption experiment, no doubt in direct relation to the amount of contaminant present in the snail tissues. Increased levels of GSH have been reported in other invertebrate species exposed to PAHs, in particular Cheung et al (2001) found that GSH levels increased in the mussel *Perna viridis* exposed to a mixture of five PAHs and showed that GSH had the strongest response (out of all the antioxidants measured) and was correlated with total PAH tissue concentrations. In the current study it appears that increased GSH activity is sufficient to prevent damage to lipids in *M. tenebrosus* at the lower levels of FLA contamination in both seawater and a food source. Yet for snails ingesting highly contaminated *U. pertusa* (402ng/g DW) GSH levels became diminished resulting in increased lipid peroxidation due to high levels of ROS, indicating that the antioxidant is unable to cope with such a high level of contaminant uptake.

Activity of all enzymatic antioxidants (SOD, CAT, GR and GPX) in *M. tenebrosus* increased in response to both FLA contaminant regimes. In particular, the highest levels of contamination induced the highest increases in enzymatic antioxidants and increases continued throughout the duration of exposure, supporting the theory that production of defences is related to the duration and intensity of contamination. Interestingly the lowest level of contaminated seawater did not induce a significant change in any of the enzymatic antioxidants, while that of the contaminated food-source did, this may be due to the much higher internal uptake of FLA found in *M. tenebrosus* after consumption of contaminated *Ulva*. The reduction in percentage of GSH indicates increased detoxification activity and this parallels the observed increase of GR in response, particularly at the highest contaminant concentrations, likely explained by the utilisation of GR to catalyse the regeneration of GSH from GSSG. Thus, the increased activity of GR observed in response to high FLA

contamination for both exposure regimes, indicates the difficulty in regenerating GSH under high oxidative stress.

SOD and CAT are often the first two antioxidants to be induced in response to ROS, as they act independently of the ascorbate-glutathione cycle, and as such could be useful contaminant biomarkers (Cossu et al., 1997). In the present study CAT activity increased with increasing concentration and duration of FLA exposure for both exposure regimes, with higher overall CAT levels found in *M. tenebrosus* having ingested the contaminant compared to those having taken it up via diffusion from seawater. Increases in CAT activity have previously showed positive correlations with PAH tissue concentrations in mussels from both field (Cheung et al., 2001) and laboratory studies (Richardson et al., 2008). Interestingly Cheung et al (2001) found that CAT only showed a positive correlation to internal BaP concentrations in gills, while CAT from the hepatopancreas (digestive gland) showed a negative correlation. The main function of CAT is to transform H_2O_2 to molecular H_2O and as this is also a function of GPX, the two enzymes may be 'competing' for the same substrates (Kappus, 1987). In the current study GPX levels increased to twice that of CAT under both exposure regimes, and was most pronounced at high exposure concentrations, yet CAT activity was dominant in snails ingesting low level FLA contaminated *U. pertusa*. This is an indication of the locations of GPX and CAT activity in the animal, it is possible that CAT is more active in the digestive gland (where the first oxyradicals would appear when FLA is ingested) while GPX may dominate other tissue locations in the marine snail, opposing the findings of Cheung et al (2001). SOD is the first antioxidant enzyme to act on oxidative radicals, transforming $O_2^{\cdot-}$ to H_2O_2 and O_2 (Halliwell and Gutteridge, 2007). Therefore an increase in SOD activity will ultimately result in increased production of H_2O_2 and observed increases in CAT or GPX activity. In addition to CAT animals use GPX as a first line of defence, reducing H_2O_2 to H_2O using GSH as a substrate and subsequently oxidising glutathione (produces more GSSG), separate from the ascorbate-glutathione cycle (refer to chapter 1) (Mittler, 2002). The current study shows a response, where increased SOD activity is observed and there is also an increase in CAT activity for both exposure regimes. SOD activity is again higher in snails that have directly ingested FLA contaminated *U. pertusa*, and activity is more pronounced at lower concentrations than in snails exposed to contaminated seawater. This response follows the pattern of elevated tissue FLA concentrations, as with the other antioxidant responses. Positive correlations between SOD and CAT have been reported in mussels from two independent studies (Cheung et al., 2001) (Richardson et al., 2008), and both also made correlations between internal PAH levels and increased SOD activity. Increases in GPX activity was also observed, producing an increase in GSSG, which in turn

increases the activity of GR in order to maintain the ratio of GSH to GSSG by reducing the latter back to the former. This process likely contributed to the reduction of GSH observed in the current study, in addition to the aforementioned detoxification.

Glutathione S-transferase

The role of the enzyme GST in combating oxidative damage is very different to that of the antioxidants and other enzymes, it functions by directly metabolising molecules carrying hydroxyl groups (including but not limited to lipid peroxides) and also by catalysing the conjugation of xenobiotics with glutathione molecules (Livingstone, 2001, Le Pennec and Le Pennec, 2003, Halliwell and Gutteridge, 2007). The conjugation of PAHs to glutathione has been shown to increase the water solubility of such xenobiotics, making them easier for the organism to excrete, as seen in the crab *Macrophthalmus hirtipes* (Koenig et al., 2008). The current study produced increased GST activity in *M. tenebrosus* in response to the two highest FLA contaminant levels of both regimes, and GST levels during consumption of FLA contaminated *U. pertusa* increased to almost twice that of *M. tenebrosus* exposed to contaminated seawater. GST activity in bivalve molluscs has been shown to be both negatively and positively correlated with PAH tissue concentration (Cheung et al., 2001, Richardson et al., 2008). In addition the current studies observed decreases in available GSH, suggesting GST was catalysing its conjugation with FLA and reducing its availability in the glutathione peroxide cycle (refer to chapter 1). The contradiction existing around GST activity in response to PAH contamination suggests it is an unreliable marker of xenobiotic contamination and that much more is to be learnt about the complex interactions of this enzyme.

Summary and Conclusions

In conclusion, the consumption of an FLA contaminated food source produces an increased amount of the contaminant taken up by the consuming organism, when compared to organisms exposed to FLA contaminated seawater. This increased uptake of the harmful pollutant is the likely cause of observed oxidative stress to macromolecules of the organism and in turn an increase in antioxidant defence activity. A comparison of gill and gut tissue under the two contamination regimes tested here would provide a better understanding of FLA toxicity from the water column and food-sources, this would require a larger grazing invertebrate where tissue types could be separated and examined individually.

Chapter 4 – General Discussion

Overview

Bioavailable FLA is taken up by the green macroalgae *U. pertusa* and the marine gastropod *M. tenebrosus* from contaminated seawater. Both *Ulva* and *M. tenebrosus* bioaccumulate FLA in their tissues and the resulting level of tissue FLA is a function of concentration and duration of exposure to the contaminant. Animals and photosynthetic organisms differ physiologically, yet the biochemical responses of *U. pertusa* and *M. tenebrosus* under oxidative stress are very similar, both exhibiting similar antioxidant responses to FLA contamination. The ascorbate-glutathione cycle is an important defence system in seaweeds that is activated in response to the abiotic stressors of: desiccation, changes in salinity, heavy metal contamination and excess H₂O₂ (Collen and Pedersen, 1996, Collen and Davison, 1999, Burritt et al., 2002, Collen et al., 2003, Lu et al., 2006), but knowledge of the biochemical processes of seaweeds under xenobiotic induced stress was limited (Zambrano and Carballeira, 1999). This study demonstrates that ROS production is increased by FLA contamination in *U. pertusa*, resulting in similar antioxidant defence activity to that observed in terrestrial plants under oxidative stress (Paskova et al., 2006). Knowledge of the trophic transfer ability of PAHs has been the subject of much debate as studies vary in their results, some showing trophic transfer and others not (Dobroski and Epifanio, 1980, Clements et al., 1994). Existing studies on trophic transfer of PAHs lack information on the consumption of contaminated primary producers, instead focussing on higher trophic level species (Wolfe et al., 1999, Palmqvist et al., 2006, Filipowicz et al., 2007). The present study shows that dietary transfer is an important source of contamination for an invertebrate grazer in the marine system.

Monitoring

Coastal marine systems associated with urbanised centres are an environmental depository for many pollutants, including PAHs (Douben, 2003). Often such contaminants exist as trace levels in water but bioaccumulate in organisms, as is the case with *U. pertusa*, making chemical analysis of water an insufficient method of risk assessment (Torres et al., 2008). Gaining ecosystem level information on anthropogenic inputs is therefore important in assessing and managing environmental risk, particularly in coastal systems where low chronic concentrations often exist for extended periods of time (Torres et al., 2008). As PAHs are highly persistent and rapidly absorbed by organic matter (Nielson et al., 1997) basal-level

trophic organisms, like macroalgae, are essential diagnostic tools for early and accurate ERA (Handy et al., 2003, Torres et al., 2008). Physiological changes to such organisms have been widely used for monitoring over the past twenty years (Kirso and Irha, 1998, Pinto et al., 2003, Lei et al., 2007), yet antioxidant defences may be sufficient in preventing serious physiological damage, rendering biomonitoring unrepresentative of damage at a cellular and genetic level (Torres et al., 2008). In *U. pertusa* the up-regulation of antioxidants was sufficient to prevent debilitating damage to macromolecules during persistent exposure to environmentally relevant FLA concentrations, yet tissue concentrations continued to increase, suggesting FLA is retained and bioconcentrated by the organism. Therefore, PAH contamination presents a concern at the ecosystem level, as bioavailability is likely increased via dietary transfer, enhancing the potential impact to higher trophic species (Torres et al., 2008). To combat this concern, biochemical changes in the basal level species should be measured as part of an ERA, yet measuring such changes is complex and frequently omitted due to a lack of existing knowledge for use as reference points for such species (Depledge et al., 1993). Biochemical responses used in monitoring are known as biomarkers, and are defined as “quantitative measures of changes in the biological system that can be related to the toxic effects of environmental chemicals” (WHO, 1993, Peakall and Walker, 1994). Biomarkers are typically antioxidants or antioxidant enzymes, in particular GSH, AsA, CAT, APX and SOD (including its isoforms FeSOD, MnSOD and CuZnSOD) (Torres et al., 2008). In addition to the above biomarkers cytochrome P-450 may also be used as a biomarker.

Trophic Transfer

Seaweeds possess similar detoxification enzymes to that of the mammalian liver (cytochrome P-450 and GSTs), and are considered the ‘green liver’ (Pflugmacher et al., 1999). Due to their xenobiotic metabolism properties and large biomass marine plants are therefore an important sink for marine contaminants like PAHs (Pflugmacher et al., 1999). Plants metabolise xenobiotics in three phases: Phase I (transformation) carried out by cytochrome P-450, phase II (conjugation) carried out by GST and phase III (compartmentation) taking place in cell wall fractions or the vacuole (Pflugmacher et al., 1999). Marine macroalgae have the same set of detoxification enzymes as those documented for the animal kingdom and terrestrial plants (Pflugmacher et al., 1999). These enzymes are important for the quantification of the metabolic sink potency and the detoxification potential of these marine plants (Pflugmacher et al., 1999).

Though biomonitors are a good idea, low levels of PAH may not be sufficient to induce significant biochemical responses in the algae, whereas bioconcentration through the

food web may cause measurable impacts on higher trophic organisms (Torres et al., 2008). Trophic transfer and biomagnification of contaminants up the food-chain is the subject of much debate, and the importance of dietary accumulation versus uptake from water is a controversial topic (Clements et al., 1994). The present study provides evidence that FLA taken up by *U. pertusa* is transferred trophically into the grazing *M. tenebrosus*, potentially amplifying the effect in the consumer. In contrast levels of FLA in *M. tenebrosus* tissues under the water contamination regime were 2-3-fold lower. Therefore, in the case of FLA contamination, dietary uptake from a contaminated food-source is important at low trophic levels. The most influential factors contributing to dietary uptake of FLA are bioavailability and absorption efficiency of the consumer (McElroy and Sisson, 1989). Metabolic transformation of PAHs into intermediate metabolites by cytochrome P-450 can affect both these factors (Neff, 1979, McElroy and Sisson, 1989). Biotransformation of xenobiotics by cytochrome P-450 is an additional defence against oxidative stress in both animals and plants (Sandermann Jr, 1992) and recently cytochrome P-450 was detected in a marine seagrass (Hamoutene et al., 1995) marine algae (Pflugmacher and Sandermann Jr, 1998) and macroalgae, including *Ulva lactuca* (Pflugmacher et al., 1999). FLA can be easily transformed by marine organisms (Palmqvist et al., 2006) and as such it has the ability to be both acutely toxic and genotoxic to other marine organisms (Sepic et al., 2003). The metabolites produced by cytochrome P-450 in the food-source are often easily absorbed, increasing contaminant availability to the consumer and resulting in increased risk of toxicity for the consumer (McElroy and Sisson, 1989). Cytochrome P-450 was not measured in *U. pertusa*, yet the transformation of FLA into its metabolites by cytochrome P-450 existing in the seaweed is a plausible explanation for the 2-3-fold difference observed in *M. tenebrosus* under the consumption regime. The only study available for comparison to this one does not include a primary producer, however it does investigate trophic transfer of FLA between low trophic level species. The polychaete *Capitella* sp. consumed FLA contaminated sediment, and was then fed to the predatory polychaete *Nereis virens* (Palmqvist et al., 2006). As a result FLA metabolites of *Capitella* were taken up more easily by *N. virens* than the parent compound, producing a higher rate of toxicity in consumers of FLA metabolites (Palmqvist et al., 2006), similar to that observed in consuming snails of the present study. In conjunction with biotransformation by cytochrome P-450, enzymatic conjugation processes involving GST and GSH render the metabolites water-soluble and thereby more reactive, potentially further aiding the production of ROS (Lee and Singer, 1980). While acute toxicity was much more pronounced in snails consuming FLA and genetic toxicity in the form of DNA oxidation increased, measurements of DNA adducts under these conditions were not recorded, yet the

risk of macromolecular adducts has been shown to increase in animals as a result of phase I metabolism products (McElroy and Sisson, 1989).

In order for biomarkers to be used successfully a detailed reference of their activity under various stress conditions must be gathered for the intended biomonitoring species. The aim of this study was to gather detailed information on antioxidant activity of the cosmopolitan macroalgal species *Ulva* (using *U. pertusa* as a model) in response to PAH induced stress. *U. pertusa* is negatively affected by low and extreme environmental concentrations of FLA at a biochemical level, when physical changes are not apparent, and importantly antioxidant enzyme levels increased, in particular SOD and CAT. With more research biomarkers in *Ulva* spp could make them ideal basal-level models for monitoring anthropogenic inputs of PAHs to the coastal marine system. In addition this study has shown the need for a more ecological approach to monitoring, gathering information from multiple species of varying and interacting trophic level species, to gain a more comprehensive risk assessment (Wu et al., 2008).

Overall Summary

As one of the most common PAHs in the coastal marine system FLA presents a great threat to coastal marine organisms (Gao et al., 1998). The present study investigated the use of the cosmopolitan macroalgae *U. pertusa* and an associated grazer to research their use as biomonitor species for PAHs in the coastal marine system. This study demonstrates that exposure to FLA contaminated seawater induces uptake of the pollutant in macroalgae and molluscs, resulting in biochemical changes including increases in: ROS production, oxidative stress, damage to macromolecules and antioxidant activity. Similar responses are present throughout the scientific literature on PAH contamination in species of plants and animals, though examples of macroalgae and non-benthic invertebrates were lacking and many just describe visible of physiological effects without details of the underlying biochemical changes. FLA is acutely toxic to marine macroalgae and invertebrates, damaging macromolecules at low and extreme inputs relevant to those *in situ*. Toxicity to each organism is proportional to duration and concentration of FLA exposure and antioxidant metabolism is activated even in response to low FLA concentrations. With further research into the biochemical responses of *Ulva* spp this cosmopolitan seaweed would make the ideal global monitor for coastal PAH pollution. Dietary transfer of FLA in low trophic level species is biomagnified from the primary producer to the consumer, resulting in higher toxicity from contaminated food-sources than contaminated seawater.

Conclusions

Macroalgal species, in particular *Ulva* spp, could make an ideal biomonitor for coastal anthropogenic PAH pollution, as they incur physiological and biochemical changes proportional to the concentration and duration of exposure. The trophic transfer of FLA occurs between macroalgae and grazing invertebrates, causing dramatic biomagnification of the contaminant in the consumer. This indicates that trophic interactions should be taken into account when monitoring the environmental risk of PAHs in the marine environment. Ideally future monitoring of PAHs should include data on biochemical responses of a basal trophic level species, like seaweed, in addition to higher trophic species and chemical analysis of water and sediments. Additional research on cytochrome P-450 in *Ulva* spp under contaminant stress would be particularly useful for the development of biomarkers as a monitoring tool for the global future.

Appendix I

Filtered Enriched Seawater (FESW)

Natural seawater filtered (Whatman GF/C50) and enriched with nutrients (1.0mM KNO₃, 0.05mM FeCl₃, 0.1mM K₂HPO₄, 1.0μM thiamine, 0.1μM cobalamin, 10μM biotin and vitamins)

Enzyme Buffer I (EBI)

100mM potassium phosphate (pH 7.5), 1mM K₂EDTA, 5mM ascorbate, 0.1% (v/v) Triton X-100 and 1% (w/v) Polyclar AT (SERVA Chemicals Ltd).

Enzyme Buffer II (EBII)

100mM potassium phosphate (pH 7.5), 1mM K₂EDTA, 0.1% (v/v) Triton X-100 and 1% (w/v) Polyclar AT (SERVA Chemicals Ltd).

Animal Enzyme Buffer (AB)

50mM potassium phosphate (pH 7.4), 1mM EDTA, 10μmol phenylmethylsulfonyl fluoride (stock solution was 1mMol in ethanol).

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