Indicators of Petroleum Product Exposure in Benthic Cold-Water Marine Invertebrates

By

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Abstract

The ubiquity of petroleum in society has led to widespread pollution of the marine environment resulting in a need to identify suitable biomarkers. The aim of this study was two-fold: (1) To measure variables that influence baseline biomarker activity and (2) to determine their response to the water-accommodating fraction of used engine lubricating oil. The biomarkers glutathione peroxidase (GPx) and ethoxyresorufin-*O*-deethylase (EROD) were studied across three stages of the breeding cycle within the reproductive and digestive tissues of the common sea star, the orange-footed sea cucumber, the daisy brittle star, and the green sea urchin. Overall, sea stars were identified as the most promising cold-water biomonitors of oil contamination among the species studied, and GPx was found to be the most suitable biomarker due to its widespread activity, lack of seasonal baseline variation, and the simple separation of sexes during analyses. In comparison, the baseline activity of EROD was influenced by both season and sex.

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- CYP450 – Cytochrome P450
- EC_{50} – Effective concentration on 50 % of the population
- EROD – Ethoxyresorufin-*O*-deethylase
- GPx – Glutathione peroxidase
- MFO – Mixed-function oxidase
- PAH – Poly-aromatic hydrocarbons
- pdu – Procedure defined units
- ULO – Used engine lubricating oil
- WAF – Water accommodated fraction
- i.e. – id est (that is)
- e.g. – exempli gratia (for example)

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Co-authorship Statement

The work described in the present thesis was carried out by Matthew Taylor Osse with guidance from Annie Mercier, Jean-François Hamel, Juan C. Pérez Casanova, and Matthew Rise. Matthew Taylor Osse was responsible for laboratory data collection and analysis with assistance from Annie Mercier. The main chapter was written by Matthew Taylor Osse as a journal manuscript with intellectual and editorial input by co-authors as follows:

Authorship for **Chapter 2** is M. T. Osse, J.-F. Hamel, A. Mercier

Chapter 1: General Introduction

1.1 Petroleum Pollution in Aquatic Environments

1.1.1 Sources

The latest report from the National Research Council (2003) places annual worldwide estimates of petroleum input into the world's oceans as exceeding 1.44 trillion litres. The single largest source of petroleum pollution emanates from natural seeps, contributing an estimated 681 million litres each year to the world's oceans. Consumption of petroleum comes in at a close second, with an estimated 530 million litres of petroleum released each year (NRC, 2003). Extraction and transportation contribute to the release of 42 million litres and 167 million litres of petroleum in the environment, respectively (NRC, 2003) (Fig. 1.1). Within consumption of petroleum, the major sources are land-based (river and run-off) and operational discharges of large vessels (≥100 gross tonnage) with 155 million litres and 298 million litres, respectively (Table 1.1). Spills, operational discharges of small vessels (<100 gross tonnage), atmospheric deposition, and aircraft dumping make up the remainder.

To help mitigate oil pollution, North America and Western Europe introduced stringent regulations to recycle the majority of spent petroleum products (Farrington, 2013). However, less developed nations do not have these regulations and it is not uncommon for petroleum products, such as used engine oil, to be poured into sewer and storm-water drains (Chukwu and Odunzeh, 2006; Ssempebwa and Carpenter, 2009). Even in developed nations that rely on regulations, urban run-off contributes a significant amount of petroleum pollution (Farrington, 2013).

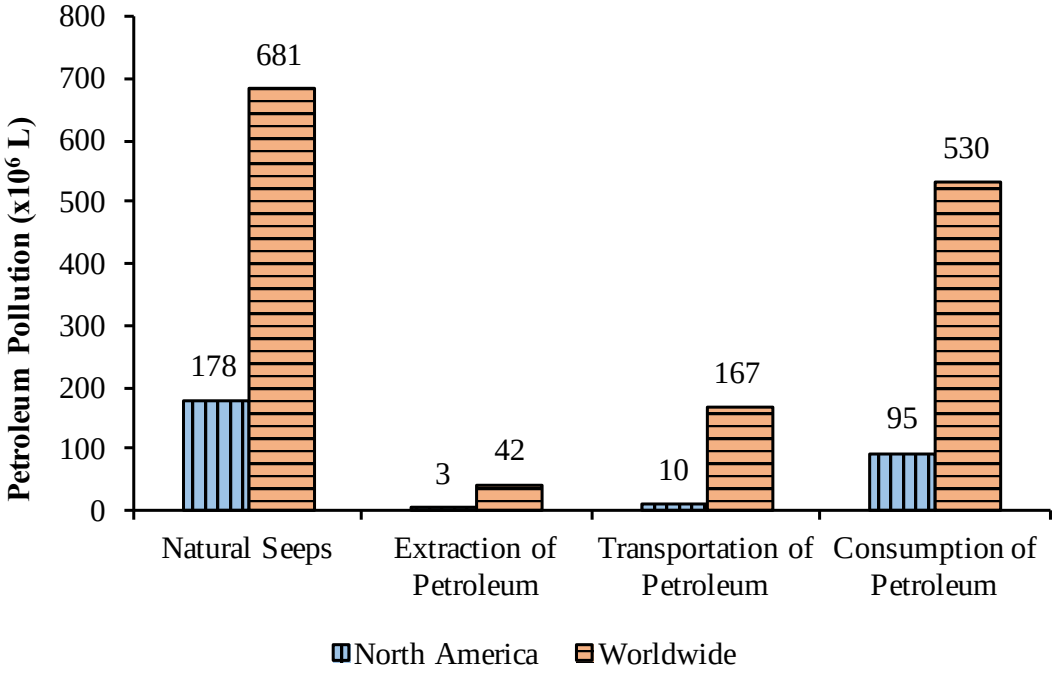

Figure 1.1: Best estimates of petroleum pollution entering oceans from North American and worldwide sources. Adapted from "Oil in the Sea III: Inputs, Fates, and Effects" National Academies Press 2003, Washington, DC.

Table 1.1: Best estimate of worldwide average annual releases (1990-1999) of petroleum by input sources. Adapted from "Oil in the Sea III: Inputs, Fates, and Effects" National Academies Press 2003, Washington, DC.

Input Sources	Annual Release Worldwide (millions of litres)
Natural Seeps	**681**
Extraction of Petroleum	**42**
Platforms	0.94
Atmospheric deposition	1.4
Produced waters	39
Transportation of Petroleum	**167**
Pipeline spills	13
Spills (tank vessels)	111
Operational discharges (cargo oil)	40
Coastal facility spills	5.4
Atmospheric deposition	0.44
Consumption of Petroleum	**530**
Land-based (river and runoff)	155
Recreational vessel discharge	nd
Spills (commercial vessels ≥ 100 GT)	7.8
Operational discharges (vessels ≥ 100 GT)	298
Operational discharges (vessels < 100 GT)	nd
Atmospheric deposition	57
Aircraft dumping	8.3
Total	**1 438**

nd – insufficient data available

GT – vessel gross tonnage

note: Totals may not equal sum of components due to independent rounding.

Expansive research on petroleum pollution has been performed; however, used engine oil remains relatively understudied (Vazquez-Duhalt, 1989). Until 2005, Environment Canada had not made a decision on whether used engine oil (from the consumption of petroleum) had a significant toxic effect on the environment, as well as on terrestrial and aquatic biota, due to a lack of studies (Government of Canada, 1994, 2005). Acute point-source pollution (spills) get the bulk of the attention and media coverage since they generate visually dramatic effects (Farrington, 2013; Hoffman et al., 1982). Good examples of acute pollution include the 2010 Deepwater Horizon oil spill in the Gulf of Mexico, and the Exxon Valdez tanker spill (1989) in Prince William Sound, Alaska. While comparatively less often publicized, chronic inputs of petroleum are a concern and studies of their effects are also crucial (Farrington, 2013; Hoffman et al., 1982; Tanacredi, 1977).

1.1.2 Pollution in a Changing World

Marine environments previously isolated from anthropogenic influence may be increasingly affected by inputs of petroleum, as oil and gas exploration, and shipping activities, intensify in previously unexplored, relatively remote areas. Case in point: the arctic and sub-arctic regions, which contain vast reserves of oil and natural gas (Lee et al., 2011), have become new targets for exploitation. An estimated 400 oil and gas fields lie North of the Arctic Circle, with 70 % of these being offshore (Jonsson et al., 2010). Due to Arctic warming, these previously cost-prohibitive and inaccessible sources are gaining attention as traditional resources have begun to diminish (Lee et al., 2011; Pietri et al., 2008). Moreover, as sea ice cover melts, there is increased interest in establishing arctic

shipping routes; the lengthening and intensification of commercial maritime activity will also lead to increased levels of pollution (Corbett et al., 2010; Pietri et al., 2008). With a surge in oil tanker traffic and production, the probability of petroleum spills will rise (Pietri et al., 2008). Importantly, low temperatures mean that any petroleum spills or discharges that do occur will likely persist for long periods of time (Jonsson et al., 2010). The change in climate is also expected to alter the base of the arctic food webs, migrations, and forestry, and to cause widespread thawing, along with a decrease in biodiversity (Pietri et al., 2008). Currently, there is limited data on the impact of petroleum products on cold-water species, highlighting a necessity to identify biomonitor species, and an associated need for collection of baseline data prior to any incident (Jonsson et al., 2010). Baseline data of biomonitor species will assist in the determination of the effect an incident has on the surrounding ecosystem.

1.2 Response to Contamination in Marine Organisms

1.2.1 General Principles

A xenobiotic is defined as any external foreign chemical compound that an organism may uptake (Iyanagi, 2007; Livingstone, 1991, 1998; Parkinson, 2001). While the rate and route of uptake will differ between species (Livingstone, 1993), benthic organisms can take up xenobiotics through their diet, as well as contact with the contaminated substrate and/or water column (Danis et al., 2004; Livingstone, 1991). Upon exposure, the primary response of an organism is to decrease or eliminate the toxicity of the compound and convert it into an easily excretable form (Livingstone, 1991, 1998; Parkinson, 2001). The transformation of xenobiotics is performed through a

metabolic process that can be broken down into two parts, i.e. phase I metabolism and phase II metabolism (Livingstone, 1991; Parkinson, 2001). There are some arguments towards phase III metabolism being present (Livingstone, 1991); however, it will not be covered here. Within vertebrates, phase I and II metabolic processes are concentrated in the liver (Livingstone, 1991). Since invertebrates do not necessarily possess equivalent organs, these processes primarily concentrate in other tissues, for example the stomachs, pyloric caeca, and gonads of sea stars, or the digestive gland and gonads of sea urchins (Livingstone, 1991).

The phase I metabolic process involves the addition of reactive functional groups to xenobiotics. This allows conjugation with numerous water-soluble compounds to occur in phase II metabolism (Iyanagi, 2007; Livingstone, 1991). The phase I metabolic processes are governed by a wide variety of enzymes that catalyze reactions with xenobiotics, such as oxidation, reduction, hydrolysis, and hydration (Iyanagi, 2007; Livingstone, 1991; Parkinson, 2001). Some of the phase I metabolic processes can result in the production of toxic metabolites, which can be several times more toxic than the original compounds (Livingstone, 1991; Parkinson, 2001). The mixed function oxidases (MFOs) are an example of a phase I metabolic process. MFOs are a family of membrane-bound enzymes that operate by modifying aromatic and lipophilic compounds to increase their water solubility (Hodson et al., 1991). While phase II metabolism often uses the phase I metabolites, it is still possible for phase II to be performed on parent xenobiotic compounds (Iyanagi, 2007; Livingstone, 1991; Parkinson, 2001). Phase II metabolism is the primary source of water-soluble (excretable) products that are inactive (Livingstone, 1991; Parkinson, 2001). While less frequent, it is still possible for phase II metabolites to

be toxic (Livingstone, 1991). Along with normal metabolic process, phase I and II metabolism can result in the production of oxyradicals and superoxides through undesirable side reactions (Livingstone et al., 1990).

Additional defense against cellular damage involves the action of antioxidants to neutralize oxyradicals and superoxides, which are known to cause damage through redox imbalances, enzyme inactivation, protein degradation, lipid peroxidation, DNA damage, and cellular death (Livingstone, 1991; Livingstone et al., 1990). Ecotoxicology makes use of the aforementioned detoxification processes to monitor exposures to xenobiotics.

1.2.2 Monitoring

Environmental monitoring through bioindicators and biomonitors (biomarkers) can provide qualitative and quantitative information about a habitat (Markert et al., 2003). In particular, their use allows the study of anthropogenic effects, including petroleum pollution (Markert et al., 2003). The study of bioindicators may incorporate the ecology of entire communities and ecosystems, potentially over extended periods of time (i.e. months, years), to determine changes elicited by the modification or fragmentation of habitats, pollution, exploitation, and climate change (Lagadic et al., 1994; Markert et al., 2003). Bioindicator species or groups of species are those that reflect the condition of the environment (Lagadic et al., 1994). Examples of bioindicators include changes in species foraging, feeding, and locomotive behaviour, reproductive efficiency and population dynamics (Lagadic et al., 1994). Biomarkers are a form of biomonitors that have been shown to provide indications on the health of individual animals living in areas exposed to pollutants (Morales-Caselles et al., 2008a) (Table 1.2). They are measurable quantities

within an organism such as genetic, enzymatic, behavioural, physical, or morphological variables (Markert et al., 2003; Sarkar et al., 2006). Ideal biomarkers are those with high sensitivity and specificity, that can be applicable to a wide range of animals (Livingstone, 1993). Biomarkers have also been shown to detect early biological effects and have the ability to be monitored over time within organisms (Morales-Caselles et al., 2008a). The downside of using biomarkers is that it may be difficult to extrapolate data gathered from individuals to entire populations, due to the complexity of several stressors acting on genetically heterogeneous organisms in nature (Markert et al., 2003).

Table 1.2: Brief overview of bioindicators and biomonitors (biomarkers).

	Bioindicator	**Biomonitor (Biomarker)**
Type	Qualitative	Quantitative
Scale	Communities and Ecosystems	Individual
Period	Usually long-term	A specific time point or consecutive time points
Examples	Population Dynamics Habitat Changes Habitat Fragmentation Effects of Climate Change	Genetic Enzymatic Behavioural Physical Morphological

1.2.3 Biomonitors

In the search for biomonitoring species, some marine organisms present technical challenges due to the limited information available on their general biology, biochemistry, enzymology, and tissue/cell anatomy. Since they are familiar and central to economic imperatives, commercial species are commonly selected (Bignell et al., 2011; Costa et al., 2013). However, they are not necessarily the most representative or biologically relevant species for biomonitoring. Knowledge of the biology and ecological role of benthic organisms (abundance, morphology, lifespan, feeding mode, trophic level) should drive the choice of optimal biomonitor species that are sensitive, easy to collect, and lend themselves well to biochemical analyses. Ideally, they should have an easily identifiable taxonomy, a wide global distribution, a benthic lifestyle (close contact with sediment), low migratory activity, and a well-documented biology including physiology, feeding strategy, and life cycle (Martínez-Gómez et al., 2010b; Rainbow, 1995).

1.3 The Use of Echinoderms as a Biomonitor in Cold-Ocean Ecosystems

1.3.1 Benefits and Taxonomic Characteristics

Echinoderms are known to occupy ecological niches that are essential to the health and functionality of marine ecosystems (Lawrence, 2001; Mah and Blake, 2012). Like all marine animals, they are threatened by anthropogenic activities, including chemical pollution, which is one of the primary threats to marine environments, along with overfishing, ocean acidification, and climate change in general (Markert et al., 2003;

Morales-Caselles et al., 2008a). The echinoderm species selected for the present study were chosen based on their global abundance, well-studied physiology, ecological importance, and benthic and sedentary lifestyles of the adults. Some of them are also commercially exploited.

Unlike other marine invertebrates, such as annelids, mollusks and arthropods, which belong to the superphylum Protostomia, the phylum Echinodermata belongs to the superphylum Deuterostomia (Pawson, 2007; Ruppert et al., 2004), which also includes the phyla Hemichordata and Chordata. Hence, echinoderms are more closely related to vertebrates than to annelids, mollusks, and crustaceans. The majority of the ~7,000 living species of echinoderms are benthic animals that possess a water vascular system and mildly to heavily calcified tissues forming outward spines and warts (Lawrence, 1987b; Pawson, 2007; Ruppert et al., 2004). The phylum Echinodermata comprises the sub-phyla Asterozoa to which asteroids (sea stars) and ophiuroids (brittle stars) belong, Crinozoa to which crinoids belong, and Echinozoa to which echinoids (sea urchins) and holothuroids (sea cucumbers) belong (WoRMS, 2009). Because of their ecological significance and ubiquitous distribution in marine environments, Asterozoa and Echinozoa are the primary focus of this research.

Many asteroids (sea stars) are scavengers and carnivores that consume mollusks, bivalves, crustaceans, polychaetes, and echinoderms (Jangoux, 1982; Ruppert et al., 2004). Additionally, a few species of asteroids are suspension or deposit feeders that consume plankton and detritus (Jangoux, 1982; Ruppert et al., 2004). Asteroids have the ability to regenerate lost limbs and parts of the central disc (Chia and Walker, 1991; Lawrence, 2013; Ruppert et al., 2004). The majority of species are gonochoric,

possessing distinctive sexes, and release gametes into the surrounding seawater where fertilization occurs (i.e. broadcast spawning) (Mercier and Hamel, 2009, 2013). Several species of brooding asteroids exist, which protect their young by enveloping them in their arms, near the mouth, or carrying them in specialized chambers (Chia and Walker, 1991; Mercier and Hamel, 2009, 2013; Ruppert et al., 2004). To a lesser extent, instances of reproduction through fission, division and regrowth, have been observed in a few species (Chia and Walker, 1991; Mercier and Hamel, 2013). Additionally, reproduction through internal fertilization, parthenogenesis, and the birth of live juveniles (viviparity) are also known to occur (Chia and Walker, 1991; Mercier and Hamel, 2009, 2013). As predators of commercially important species of bivalves (mussels, clams, and oysters), asteroids may locally be considered an economic nuisance (Byrne et al., 2013; Ruppert et al., 2004). In some areas, this has led to their intentional eradication by humans (Barkhouse et al., 2007; Byrne et al., 2013; Ruppert et al., 2004).

Of the echinoderms, echinoids (sea urchins) have quite possibly the greatest range of food sources (Ridder and Lawrence, 1982). Typically, they are considered omnivores that actively graze on seagrasses, seaweeds, and encrusting algae; they are also known to consume soft-bodied animals, and opportunistically scavenge animal matter (Briscoe and Sebens, 1988; Lawrence, 1987a; Ridder and Lawrence, 1982; Ruppert et al., 2004). Most echinoids are gonochoric and release their gametes into the water column to be fertilized, although a few species brood their eggs on their ventral surface near their mouth (Lawrence, 1987b; Pearse and Cameron, 1991; Ruppert et al., 2004). Unlike other echinoderms (asteroids, ophiuroids, and holothuroids), no instances of reproduction through fission (division and re-growth) have been observed in echinoids (Pearse and

Cameron, 1991). However, like most echinoderms, they do possess the ability to regenerate lost spines, pedicellarias, denticles, and podia (Pearse and Cameron, 1991). Echinoids are of commercial importance to humans who collect their roe (gonads) for consumption; therefore overexploitation has led to the destruction of some echinoid populations (Lawrence, 2007; Ruppert et al., 2004).

Holothuroids (sea cucumbers) are deposit or suspension feeders that may gather plankton and/or other organic matter from the sediment or the water column. Deposit feeding species consume sand and silt, filtering out food particles (Ruppert et al., 2004). Suspension feeders dominate rocky shores since they are able to capture and consume a wide variety of suspended particulate matter including phytoplankton, detritus, and bacteria, as well as dissolved nutrients (Ricciardi and Bourget, 1999). Some species possess the ability to eviscerate either special organs called Cuvierian tubules, or their viscera, including gonadal tubules, respiratory tree, and intestines, when threatened by a predator; these organs are later regenerated within a matter of weeks in temperate and tropical environments (García-Arrarás et al., 1998, 1999; Vandenspiegel et al., 2000). Most holothuroids are gonochoric and reproduce through broadcast spawning. Brooding and fission have been observed in a few species (Ruppert et al., 2004; Smiley et al., 1991). Holothuroids are preyed upon by asteroids (So et al., 2010), and to a lesser extent by fishes and crustaceans, due to their soft body and exposed lifestyle (Francour, 1997; Ruppert et al., 2004). However, the greatest predators of holothuroids are humans (Ruppert et al., 2004). Sea cucumbers are a luxury seafood that command high prices in Asian markets, which has led to the over-exploitation and near extinction of some of the most prized species (Gianasi et al., 2015; Purcell et al., 2013, 2014; So et al., 2010).

Like other echinoderms, ophiuroids (brittle stars) are found throughout the world, from coastal waters to the deepest part of the oceans (Stöhr et al., 2012). They may be carnivores, scavengers, deposit feeders or suspension feeders, with most combining several feeding strategies (Lawrence, 2012; Ruppert et al., 2004; Stöhr et al., 2012). Deposit and suspension feeders may consume detritus and plankton, while carnivores may consume small crustaceans (Ruppert et al., 2004; Stöhr et al., 2012). Similar to asteroids, ophiuroids possess the ability to replicate through fission, however the majority are gonochoric and reproduce through several methods: viviparity, brooding, and broadcasting (Hendler, 1991; Ruppert et al., 2004). Like most other members of Echinodermata, ophiuroids are able to regenerate lost limbs (Hendler, 1991; Lawrence, 1987b). Predators of ophiuroids include fish, crustaceans, and echinoderms (Gaymer et al., 2001a; Mooi, 2001)

1.3.2 Focal Species

Four species of echinoderms (Fig. 1.2) were used to meet the goals of this research, including the common sea star *Asterias rubens*, the orange-footed sea cucumber *Cucumaria frondosa*, the daisy brittle star *Ophiopholis aculeata*, and the green sea urchin *Strongylocentrotus droebachiensis*. These cold-water species were chosen based on their well-known biology, wide distribution, abundance, and benthic sedentary lifestyle.

The common sea star *A. rubens* (Fig. 1.2A), synonymous with *A. vulgaris*, occupies a broad range in the eastern and western North Atlantic; in the Northwest Atlantic, it occurs from the Arctic Ocean to the Gulf of Mexico (Byrne et al., 2013; Gaymer et al., 2001a; Mah, 2015). They can be found from the littoral zone down to a

depth of 400 m, in some cases 650 m (Vevers, 1949). Juveniles that occur at shallower depths generally consume a mixture of barnacles and bivalves (Vevers, 1949). The adults are opportunistic generalists consuming living and dead animal matter, with the former including worms, crustaceans, echinoderms, and bivalves (Byrne et al., 2013; Jangoux, 1982; Vevers, 1949). Feeding ecology is typically dependent on prey availability and density within different regions (Byrne et al., 2013). Reproduction in *A. rubens* occurs through a process referred to as broadcast spawning that involves the release of gametes into the water column that develop into planktotrophic (feeding) larvae (Mercier and Hamel, 2010). Along the east coast of Newfoundland, Canada, spawning generally occurs in March and April (Mercier and Hamel, 2010).

The orange-footed sea cucumber *C. frondosa* (Fig. 1.2B) is widely distributed throughout the North Atlantic Ocean, the Arctic Ocean, and the North Pacific Ocean (Hamel and Mercier, 1996; Paulay, 2015). Within its distribution, it can commonly be found from the littoral zone down to a depth of 300 m, and only rarely below 800 m (Hamel and Mercier, 2008). It is a suspension feeder that uses its 10 dendritic tentacles to capture food particles (living and nonliving) within the water column and transfer the food to its oral cavity (Hamel and Mercier, 1998). *C. frondosa* reproduces by broadcasting gametes into the water column that develop into lecithotrophic (non-feeding) pelagic larvae (Mercier and Hamel, 2010). Spawning occurs during the full moon of March and April along the eastern coast of Newfoundland, Canada (Mercier and Hamel, 2010). Along the coast of the Northwest Atlantic they are harvested and sold primarily to Asian markets (Hamel and Mercier, 2008).

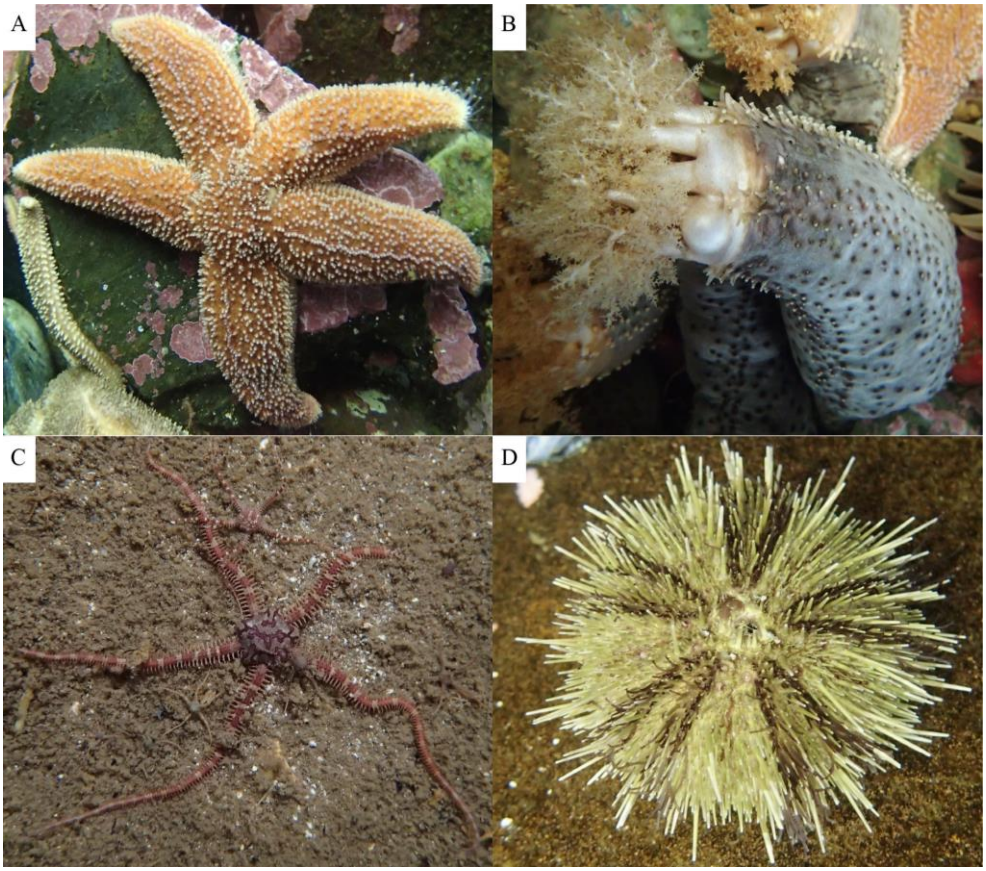

Figure 1.2: Focal species. A) The common sea star *Asterias rubens*. B) The orange-footed sea cucumber *Cucumaria frondosa*. C) The daisy brittle star *Ophiopholis aculeata*. D) The green sea urchin *Strongylocentrotus droebachiensis*. (© M. Osse 2016)

The daisy brittle star *O. aculeata* (Fig. 1.2C) can be found within the Arctic, the North Atlantic, and the North Pacific Oceans (Stöhr, 2015). They occur in the littoral zone down to a depth of at least 100 m (Southward and Campbell, 2006). *O. aculeata* is a suspension feeder and facultative carnivore that uses strong ciliary currents to catch detritus (Roushdy and Hansen, 1960). Reproduction occurs through broadcast spawning, which involves the release of gametes into the water column that develop into planktotrophic (feeding) larvae (Mercier and Hamel, 2010). Daisy brittle star populations along the eastern coast of Newfoundland, Canada, generally spawn during the full moon of April (Mercier and Hamel, 2010).

The green sea urchin *S. droebachiensis* (Fig. 1.2D) has a very wide distribution within the Arctic and North Atlantic Oceans, extending from the Canadian Arctic down to Cape Cod, USA, and across to Northern Europe (Kroh, 2015; Scheibling and Hatcher, 2007). Within their distribution, they are found from the littoral zone down to a depth of 300 m, typically at shallower depths (Scheibling and Hatcher, 2007). The green sea urchin is an omnivore that actively forages for seaweeds and sea grasses but is also known to be an opportunistic scavenger (Briscoe and Sebens, 1988; Scheibling and Hatcher, 2007). During reproduction it broadcast spawns its gametes into the water column, which develop into planktotrophic (feeding) larvae (Mercier and Hamel, 2010). Spawning occurs in spring, mainly during the March full moon in populations along the eastern coast of Newfoundland, Canada (Mercier and Hamel, 2010).

1.4 Thesis Objectives

The aim of my research presented in this thesis was two-fold: (1) To determine variables that influence baseline activity of selected enzyme biomarkers in representatives of the main classes of cold-water echinoderms; and (2) to assess the acute response of the same biomarkers to low levels of oil contamination, in the form of the water accommodating fraction (WAF) of used engine lubricating oil (ULO). Two common and well-known biomarkers were selected for this study: the antioxidant biomarker GPx (glutathione peroxidase) and the phase I biomarker EROD (ethoxyresorufin-*O*-deethylase).

To attain the goals of my thesis, the activity of both biomarkers (GPx and EROD) within focal species of echinoderms (section 1.3.2) were measured in various tissues (digestive, storage, and reproductive organs) and at different times (before, during, and after the reproductive season). Control and exposed (in the presence of ULO WAF) treatments were used to elucidate biomarker activity over a series of 96-h acute exposure trials. In addition to experimental treatment and season, the importance of sex on biomarker activity was also determined.

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Chapter 2: Markers of Oil Exposure in Cold-Water Benthic Environments: Insights and Challenges from a Study with Echinoderms

2.1 Abstract

In spite of increasing naval activities and petroleum exploration in cold environments, there is currently a paucity of tools available to monitor oil contamination in boreal marine life, especially in sedentary (non-fish) species that form the bulk of benthic communities. The present research aimed to identify biotic sources of variation in biomarkers using cold-water subarctic benthic species, and to identify suitable biomarkers of exposure to hydrocarbons. The focal species included the sea star *Asterias rubens*, the brittle star *Ophiopholis aculeata*, the sea urchin *Strongylocentrotus droebachiensis*, and the sea cucumber *Cucumaria frondosa*, which are among the most abundant echinoderms in the North Atlantic and Arctic Oceans. The latter two species are also commercially exploited. A series of 96-h acute exposures of the water-accommodating fraction (WAF) of used lubricating oil (ULO) were performed during different seasons (i.e. reproductive stages). Digestive and reproductive tissues were analyzed for baseline and response levels of glutathione peroxidase (GPx) and ethoxyresorufin-*O*-deethylase (EROD). GPx activity was detected in the pyloric caeca, stomach, and gonad of sea stars, the intestine and gonad of sea cucumbers, and the gonad of brittle stars and sea urchins. No seasonal variation in baseline GPx activity occurred. Upon exposure to the ULO WAF, sex-based differences were elicited in the GPx activity of sea star stomachs, whereby exposed females had significantly less activity than males. EROD activity was present in the pyloric caeca of sea stars, and the gonads of brittle stars and sea urchins. An interaction between season and sex on baseline EROD activity was measured in the gonads of sea urchins. Ovaries exhibited significant seasonal variation in EROD activity and had greater activity than

testes during the spawning and post-spawning seasons. Seasonal variation in EROD activity also occurred in sea star pyloric caeca and brittle star gonads. Furthermore, testes of sea urchins exposed to the ULO WAF exhibited suppressed EROD activity compared to baseline levels. The presence of GPx activity within all species and tissues under study highlights its potential as a universal biomarker, while the presence of EROD activity was more limited. Findings suggest a complex relationship between temporal and biotic factors on both the baseline and response levels of enzymatic activity, emphasizing the need to consider sex and sampling season in studies of biomarkers of hydrocarbon exposure in boreal indicator species that typically display annual reproductive cycles.

2.2 Introduction

Most diagnostic tools developed for assessing the effects of xenobiotics, including hydrocarbons, in the marine environment have focused on fishes and a limited number of invertebrates, such as bivalves and crustaceans. In recent years there has been an increased interest in researching species from polar and arctic environments; however, they still remain understudied in comparison to species originating from tropical and temperate regions (Hansen et al., 2013; Martínez-Gómez et al., 2010a; Payne et al., 2003; Sandrini-Neto et al., 2016; Sundt et al., 2012; Thain et al., 2008). Tools have not been developed to the same extent for assessing the effects of hydrocarbons on other benthic macro-invertebrates, despite their predominance in marine ecosystems. In particular, there is a general shortage of studies on boreal and cold-water invertebrate species (Bechmann et al., 2010; Hannam et al., 2010; Regoli et al., 2002; Sandrini-Neto et al., 2016), in spite

of a growing need for reliable biomarkers of human activity and climate change in arctic and subarctic environments (Nahrgang et al., 2013; Sandrini-Neto et al., 2016).

Where biochemical studies have been carried out on tropical and temperate marine invertebrates (excluding bivalves and crustaceans), direct correlation of responses to hydrocarbons have sometimes been weak or absent (den Besten, 1998; Fossi et al., 2000; Payne and May, 1979; Pérez et al., 2004; Snyder, 2000; Solé and Livingstone, 2005). While bivalves and crustaceans are the most common invertebrate taxa studied and have shown promising results (Baussant et al., 2009; Fossi et al., 2000; Morales-Caselles et al., 2008a; Morales-Caselles et al., 2008b; Nahrgang et al., 2013), their ability to rapidly metabolize some xenobiotics may not be ideal (Koenig et al., 2012). At present, information is scarce on the optimal conditions for sample collection/processing, due to variations in biotic and abiotic factors (Shaw et al., 2004; Sheehan and Power, 1999; Viarengo et al., 1991), and the identification of suitable biomarkers in (cold-water) species necessitates a case-by-case approach (Martínez-Gómez et al., 2010b).

Seasonal changes in animal physiology may occur through variations in nutrient availability, reproductive status, and growth (Sheehan and Power, 1999). Environmental variables such as food availability, photoperiod, and water temperature are important sources of fluctuations (Viarengo et al., 1991). Polar and temperate-cold regions are exposed to particularly marked environmental variations (Sheehan and Power, 1999), making them especially prone to seasonal changes in biological activities (i.e. feeding, growth, and reproduction), which have been shown to influence the expression of biomarkers (Shaw et al., 2004). Hence, there is a need to study changes in baseline

biomarker activity and response to anthropogenic effects separately across multiple seasons in focal species (Nahrgang et al., 2013).

Biomarkers, including glutathione peroxidase (GPx) and ethoxyresorufin-*O*-deethylase (EROD), are commonly used to detect the first signs of biological exposure to adverse compounds (Nahrgang et al., 2013). These assays focus on different enzymatic processes, covering a broad scope of putative responses, and standard techniques are already available to measure them routinely.

Glutathione peroxidase (GPx) is a mitochondrial antioxidant enzyme (Morales-Caselles et al., 2008a) that destroys hydrogen peroxide (H_2O_2) and organic hydroperoxides that may cause oxidative damage (Doyotte et al., 1997). Increases in GPx activity presumably indicates that the organism is under oxidative stress (Reid and MacFarlane, 2003). In the absence of antioxidants, oxidative stress may cause DNA damage, enzymatic inactivation, and lipid peroxidation (Doyotte et al., 1997). GPx activity is measured indirectly by a coupled reaction, whereby glutathione reductase and NADPH are used to reduce oxidized glutathione, which is produced during the reduction of hydroperoxide by GPx. The oxidation of NADPH results in a change in absorbance that can be measured (Ceballos-Picot et al., 1992; Forstrom et al., 1978; Paglia and Valentine, 1967). Significant dose-dependent increase in activity of GPx in the visceral mass of the gastropod mollusk *Austrococchlea porcata* has been observed after exposure to crude oil (Reid and MacFarlane, 2003). Additionally, Gamble et al. (1995) found GPx in the pyloric caeca of the sea star *A. rubens*. Since, the prevention of oxidative damage is beneficial to all types of tissues, it is expected that GPx activity will be widespread within most marine organisms.

Ethoxyresorufin-*O*-deethylase (EROD) is commonly used to measure cytochrome P450 1A (CYP450 1A) activity (Sarkar et al., 2006). The CYP450 1A subfamily is involved in the biotransformation of dioxins, furans, polychlorinated biphenyls (PCBs) and poly-aromatic hydrocarbons (PAHs) (Sarkar et al., 2006). The CYP450 dependent mixed-function oxidase (MFO) enzymatic system is a family of hemoproteins, which includes cytochrome P450, located on the smooth endoplasmic reticulum within cells (Gagné and Blaise, 1993). The primary function of the MFO system appears to be to alter nonpolar lipophilic organic compounds to make them more water-soluble and therefore more available for excretion (Neff, 1985). The assay operates by measuring the rate of conversion of 7-ethoxyresorufin (7-ER) to 7-hydroxyresorufin (7-HR) using fluorescence. The conversion of 7-ER to 7-HR is performed by CYP450 1A and therefore EROD induction is a clear signal of CYP450 1A1 and CYP450 1A2 enzyme activities (Martín-Díaz et al., 2007). EROD has been used to measure the response to persistent organic pollutants in vertebrates (Sarkar et al., 2006). In fish from polluted estuaries, levels of hepatic EROD were increased by three classes of compounds: PAHs, heavy metals, and estrogenic compounds (Sarkar et al., 2006). An increase in EROD activity has been documented in crab and clam species exposed to sediments containing high concentrations of PCBs and PAHs (Martín-Díaz et al., 2007). The cytochrome P450 family has previously been detected by Den Besten (1998) in the pyloric caeca and stomach of the sea star *A. rubens* as well as, the haemal plexus of the sea cucumber *Holothuria forskali*. Therefore, EROD activity is predicted to be the most prevalent in the digestive tissues (pyloric caeca, digestive gland and intestine) of echinoderms.

Used lubricating oil (ULO), sometimes referred to as used/spent crankcase or engine oil, is a significant contributor to petroleum pollution due to consumption, which itself constitutes approximately 70 % of the ~760 million litres of anthropogenic petroleum pollution released worldwide annually (NRC, 2003). Studies in Canada, the United States, and several other countries have found ULO to be a large contributor, if not the single largest source, of saturated and aromatic hydrocarbons in urban runoff (Fam et al., 1987; Government of Canada, 2005; Hoffman et al., 1982; Latimer et al., 1990), a primary input of pollutant into the aquatic environment. While acute sources of pollution (spills) are more publicized, long-term chronic petroleum pollution from human activities may be equally damaging in a more insidious way (Martínez-Gómez et al., 2010b). As oil weathers, the lightweight volatile compounds evaporate, leaving larger molecules behind, including partially water-soluble PAHs (Galt et al., 1991). These compounds are of particular interest to researchers since they can accumulate in animals and cause biological damage (Bechmann et al., 2010). Through the use of water-accommodating fractions (WAFs) the effects of oil, which consists of a complex mixture of thousands of compounds (Singer et al., 2000), can be studied. WAFs contain the partially water-soluble compounds within oil that aquatic marine organisms are most likely to come in contact with (Gagnon and Holdway, 2000).

The present research was designed to develop biomarkers of oil pollution in marine invertebrates from temperate-cold and subarctic regions. Four species among the most common in subtidal areas of the Northwest Atlantic and Arctic Oceans were chosen: the predatory sea star *Asterias rubens*, the suspension-feeding brittle star *Ophiopholis aculeata*, the herbivorous omnivore sea urchin *Strongylocentrotus droebachiensis*, and

the suspension-feeding sea cucumber *Cucumaria frondosa*. These exemplify four classes within the phylum Echinodermata; they also occupy different ecological niches and exhibit distinct reproductive strategies (Giese et al., 1991; Lawrence, 1987c; Mercier and Hamel, 2009). Two of them (*S. droebachiensis* and *C. frondosa*) are commercially exploited (Grabowski and Chen, 2004; Hamel and Mercier, 2008). The aim of the present study was twofold. (1) To ascertain whether there were seasonal and sex-related fluctuations in baseline levels of EROD and GPx activity within various tissues of the focal species, and (2) to test the sensitivity and specificity of standard enzymatic biomarkers through a series of acute exposures to WAF of ULO, by comparing induced responses to sex-dependent and seasonal fluctuations in baseline (control) activity levels.

2.3 Materials and Methods

2.3.1 Species Collection

All species were collected from southeastern Newfoundland (47°18′57″N 52°48′37″W) between 10-20 m depth, at least two weeks before using them for the various trials. Individuals were maintained under flow-through conditions (2.5 L min^{-1}) in two large tanks (500 L) at a temperature of 6 ºC and mirrored ambient photoperiod with a daytime light intensity of ~200 lux within the Joe Brown Aquatic Research Building (JBARB) of the Department of Ocean Sciences (Memorial University). For all species only adult individuals of similar sizes were chosen. Sea stars (n=83 *A. rubens*, 8-11 cm radius) were fed *ad libitum* a diet of blue mussels (*Mytilus edulis*) while sea urchins (n=113 *S. droebachiensis*, 8-10 cm test diameter) were fed kelp (*Laminaria* sp.), up to 1 week prior to experiments. The plankton feeders, such as sea cucumbers (n=117 *C.*

frondosa, 17-25 cm length) and brittle stars (n=540 *O. aculeata*, 1.2-2.0 cm central disc diameter*)*, had access to a minimum level of fine planktonic material available in the natural seawater; no supplementary food was added since natural levels of phytoplankton are minimal during the study period, i.e. full blooms do not generally occur until late April or May along the eastern coast of Newfoundland (Fuentes‐Yaco et al., 2007).

Only healthy individuals that did not exhibit any lesions or signs of stress, such as blistering in sea cucumbers, loss of spines in sea urchins, or missing limbs in brittle stars and sea stars, were selected for the trials.

The study initially aimed to use the sea star species *Leptasterias polaris* but difficulties in obtaining adult, reproductively mature individuals were encountered for the spawning and post-spawning trials; therefore, collections shifted to *A. rubens*. Both species have similar size structures, spatial and temporal distributions, and feeding ecologies (Gaymer et al., 2001a, b), as well as similar reproductive periods (Hamel and Mercier, 1995; Mercier and Hamel, 2010). Nevertheless, in order to avoid any confounding effect of species, *L. polaris* was removed from the analyses, thus keeping only the spawning and post-spawning trials for sea stars.

Sex was not a predetermined factor in this study; individuals were allocated to treatment groups at random. While most species display a 1:1 sex ratio, variances are not uncommon and, therefore, skewed sex ratios were encountered in some instances.

2.3.2 Experimental Design and Procedures

The experimental setup consisted of 8 tanks (45 L), which were randomly disposed in 4 pairs (control/exposed) throughout the laboratory. Each pair of experimental

tanks were submerged in larger flow-through tubs that maintained temperatures at 6 °C throughout the trials with supplementary air provided via a bubbler. Each tank hosted 2 sea stars, 2 sea urchins, 2 sea cucumbers, and 15 brittle stars (in total: 16 *A. rubens, S. droebachiensis,* and *C. frondosa,* and 120 *O. aculeata*). Each trial consisted of a 96-h static replacement in order to minimize fluctuations in hydrocarbon concentration over time and maximize the amount of time individuals were exposed while maintaining oxygen concentration (Benson and Krause, 1984). All tanks were cleaned and maintained under flow-through conditions between trials (and the control and experimental tanks were never crossed). During the trials, 50 % of the seawater and water-accommodating fraction (WAF) of the used lubricating oil (ULO), when applicable, was replaced every 24 h. Individuals were not allowed to feed for 1 week prior to the trials, nor were they fed during the experimental period. It is common for cold-water echinoderms to undergo periods of decreased or null feeding during autumn/winter seasons (Franz, 1986; Hamel and Mercier, 1998; Singh et al., 1999) with short-term starvation not significantly affecting biochemical (lipid, protein, and carbohydrate) stores (Feral, 1985; Jangoux and Impe, 1977). The lighting was adjusted at ~140 lux under ambient photoperiod.

Trial 1 was performed in early January 2014, using the low concentration WAF (0.22 mL of ULO L^{-1} seawater, see section 2.3.3). This trial coincided with the pre-spawning period in all species under study (Mercier and Hamel, 2010). Trial 2 was performed in mid-March 2014, using a higher concentration of pollutant (0.33 mL ULO L^{-1}). This trial coincided with the spawning period of all species tested. Trial 3 was performed in mid-May 2014, also using the higher concentration of toxicant, and it included a depuration period (96-h). For the later, the number of individuals from

each species was doubled such that a sufficient number of individuals remained to cover the depuration period sampling. This trial coincided with the post-spawning season of all species, as reported by Mercier and Hamel (2010). The increase in ULO concentration after trial 1 was performed in the interest of eliciting a response in case the lower concentration was insufficient.

2.3.3 Contaminant and Water Accommodated Fraction (WAF)

Used lubricating oil (ULO) was obtained directly from multiple vehicles by a major garage in St. John's (Newfoundland) and collected over 2 days (for a total of 35 L). ULO was stored in an amber glass bottle with PTFE cap in a cool dark location before and between uses.

A seawater sample (750 mL) was obtained from each of the experimental tanks (to maintain a precise volume of seawater within the tanks) and heated to 20 ºC (to increase oil solubility in seawater) before adding either 10 mL or 15 mL of ULO (final concentration of WAF ULO 0.22 mL L^{-1} or 0.33 mL L^{-1}) and vigorously agitated manually for 3 min in a separatory funnel. The ULO was then allowed to separate from the WAF for a period of 10 min, and the latter was used in the trials. The WAF was prepared daily, within 10 min prior to exposures or replenishment of the concentration after water changes. Each of the 4 control tanks were treated similarly (see above), except ULO was not added to seawater.

A sample of the WAF prepared at the high exposure concentration was analyzed by an external analytical chemistry laboratory for PAHs and heavy metals. The concentration of PAHs within the sample was determined via gas chromatography (GC)

paired with a mass spectrometer (MS) based on EPA method 8207D. Heavy metal concentrations were determined by inductively coupled plasma (ICP) paired with a mass spectrometer (MS) based on EPA method 6020A R1.

2.3.4 Monitoring of Environmental Factors

Temperature, light intensity, dissolved oxygen, and hydrocarbons were monitored in control and exposed tanks. A temperature/light data logger (Onset HOBO Pendant UA-002) was placed within one of the control tanks, and set to measure temperature and lighting at intervals of 5 min. Dissolved oxygen was measured twice a day in one control and one exposed tank, in the morning and in the afternoon, using a CHEMetrics vacu-vials kit (K-7513) combined with a Thermo Spectronic Genesys 10UV spectrophotometer. To rapidly and economically monitor fluctuations of specific PAH equivalents throughout the trials, a modified method (Aas et al., 2000) relying on fixed wavelength fluorometry was used. Samples were taken and measured twice a day, in the morning and in the afternoon. Collected water samples were extracted with HPLC-grade hexane and the fluorescence intensity (F.I.) of naphthalene (290/335 nm), phenanthrene (256/380 nm), pyrene (341/383 nm), general oil (355/430 nm), benzo(α)pyrene (380/430 nm), and total petroleum hydrocarbons (TPH) (255/407 nm) equivalents were measured using a Perkin Elmer LS-5 fluorescence spectrometer.

2.3.5 Behavioural and Physiological Monitoring

Behavioural observations were made up to three times a day, in the morning prior to seawater monitoring, close to midday before replacement of seawater, and in the afternoon 2-4 hours after the seawater replacement. Sea stars, sea urchins, and sea

cucumbers were visually monitored for position within tanks, posture, and interactions among individuals. For the sea cucumbers, instances of lesions on the body wall, evisceration and retraction/deployment of the feeding tentacles were used to determine the level of stress as per Gianasi et al. (2015). For sea stars and brittle stars, individuals were monitored for limb loss and adhesion to surfaces (Jangoux, 1984). Sea urchins were monitored for adhesion to surfaces (Jangoux, 1984; Scheibling and Stephenson, 1984) and spine loss (Scheibling and Stephenson, 1984).

2.3.6 Sample Collection and Processing

At the end of the 96-h trials, all individuals in the control and exposed tanks were collected and processed, except in trial 3, where half the animals were collected (and the remainder were collected at the end of the 96-h depuration). Sea star gonads, pyloric caeca, and stomach were removed, weighed, and then stored at -80 ºC. In the brittle stars, only the gonads were collected; in addition, samples from specimens of the same sex and tank were pooled due to insufficient tissue available per individual. For each sex, all central disks were counted, pooled, and weighed together. For the sea urchins, the digestive gland and gonads were weighed and frozen at -80 ºC. For the sea cucumbers, the gonads, intestines, and respiratory trees were removed, weighed and frozen. In all species and individuals, sex was determined under a light microscope (Nikon eclipse 80i) based on the presence of oocytes or spermatozoa in a gonad smear.

All tissue samples were processed using an S9 homogenization procedure to obtain the supernatant for enzymatic assay analysis (Hodson et al., 1991; Mathieu et al., 2011). Samples were diluted, using a 3:1 volume (mL) to weight (g) ratio, with 50 mM

Tris-HCl buffer containing anti-protease tablets (Sigma S8820) to prevent the proteolytic degradation of cytochrome P450 (Livingstone, 1991). Tissues and buffer were then mechanically homogenized within a glass tube using a Teflon plunger. Homogenate was then centrifuged at 9 000 x *g* and the supernatant removed and frozen at -65 ºC until analysis (Hodson et al., 1991; Mathieu et al., 2011).

2.3.7 Bioassays

2.3.7.1 Glutathione Peroxidase (GPx) Assay

This assay was performed using GPx assay kits from Cayman Chemicals (Cat. 703102) and Trevigen (Cat. 7512-100-K) (Ceballos-Picot et al., 1992; Chu et al., 2004; Forstrom et al., 1978; Mukhopadhyay et al., 2009; Ozdemır et al., 2005; Paglia and Valentine, 1967; Sindhu et al., 2005). Samples were loaded into wells and reacted with NADPH, glutathione, and glutathione reductase and then exposed to cumene hydroperoxide. The kinetic rate of reaction was measured, using absorbance at a wavelength of 340 nm, over a 10 min period and corrected for background activity. A GPx positive control was run simultaneously with samples to ensure assay reliability. GPx activity was standardized among samples using protein concentrations obtained from the Lowry protein assay (Lowry et al., 1951; see below). The final units for GPx activity and standard error were nmol min^{-1} mg of $protein^{-1}$. Procedure defined unit (pdu) will be used in text.

2.3.7.2 Ethoxyresorufin-*O*-Deethylase (EROD) Assay

Supernatants were analyzed using a modified version of the EROD Assay method developed by Pohl and Fouts (1980). Briefly, the supernatant was reacted with a mixture

of 50 mM Tris-HCl, NADPH (Bioshop Canada NAD004), and 7-ethoxyresorufin ethyl ether (Sigma E3763). Production of the fluorescent compound 7-hydroxyresorufin was measured by a Perkin Elmer LS-45 fluorescence spectrometer equipped with a Perkin Elmer S10 auto-sampler, at an excitation wavelength of 550 nm and an emission wavelength of 585 nm. High and low activity positive controls prepared from fish liver were run concurrently with samples to ensure assay reliability. Activity was calculated using a standard curve prepared from 7-hydroxyresorufin dye (Sigma 424455) and standardized between samples using the Lowry protein assay (Hodson et al., 1991; Lowry et al., 1951; see below). The final units for EROD activity and standard error were pmol min^{-1} mg of $protein^{-1}$. Pdu will be used in text.

2.3.7.3 Lowry Protein Assay

Protein concentration was determined following the Lowry protein assay method (Lowry et al., 1951). To perform the assay, S9 supernatant samples were diluted by a factor of 10 to insure values fell within the range of the bovine serum albumin protein standard curve. Diluted samples were loaded into a 96 well microplate. The auto-injector system of the BMG Labtech FLUOstar Optima microplate reader was used to distribute the Lowry reagent and Folin-Ciocalteu reagent. Fish liver was run concurrently with samples to ensure reliability. Results were expressed in mg of protein ml^{-1}.

2.3.8 Statistical Analysis

Normality and homogeneity of variance were confirmed for all statistical tests through quantile-quantile (Q-Q) plots and Levene's test, respectively. One-way analysis of variance (ANOVA) was used to test for the presence of tank effects. In some instances,

the assumption of homogeneity of variance was not met and could not be corrected through data transformation, leading to the use of Welch's ANOVA or the non-parametric Kruskal-Wallis ANOVA on ranks. With one exception (detailed in the results), no significant tank effects were detected (Tables A1, A2) and this factor was thus omitted from further analyses.

Using two-way ANOVAs (type III SS), all baseline (control) groups were analyzed for the influence of season (pre-spawning, spawning, post-spawning) and sex (male, female) on baseline enzymatic levels measured in the various tissues. *Post-hoc* analysis was performed using Games-Howell test. Similarly, a series of two-way ANOVAs were used to determine the effect that treatment, ULO concentration, season, sex, and depuration had on enzyme activity in the tissues of exposed groups, using the control group as the baseline. To determine if a confounding effect between sex and ULO WAF concentration existed both treatments (control and high ULO exposed) and ULO concentrations (0, 0.22, and 0.33 mL L^{-1}) were analyzed independently for an interaction with sex. Analyzing them separately assessed whether a confounding variable was present and increased statistical power. When a significant interaction ($p<0.05$) between factors was detected, independent one-way ANOVAs followed by a pairwise comparison were conducted. All statistical analyses were performed in R (R Development Core Team, 2015) using $\alpha = 0.05$.

2.4 Results

2.4.1 Water-Accommodated Fraction and Experimental Factors

According to the test results, 16 PAHs were detected in the seawater-accommodated fraction (WAF) sample (Table 2.1). The concentrations of naphthalene and its derivatives 1-methylnaphthalene and 2-methylnaphthalene were 2.3, 0.7, and 1.6 μg L^{-1}, respectively. Phenanthrene was 10-20 times less concentrated at 0.1 μg L^{-1}. Low levels of acenaphthene, acenapthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i)perylene, chrysene, fluoranthene, fluorine, indeno(1,2,3-cd)pyrene, and pyrene, were also detected (Table 2.1). Moreover, of the heavy metals detected in the WAF, only zinc was found to be above background seawater levels (63 μg L^{-1}) (Table 2.2).

In the control tanks, the majority of PAHs monitored showed no or negligible fluorescence (Fig. 2.1A). At the low exposure concentration within the tanks (Fig. 2.1B), the equivalents of naphthalene, phenanthrene, and total petroleum hydrocarbons were found to vary between 10 and 17 F.I. Pyrene, benzo(a)pyrene, and general hydrocarbons equivalents were also found to fluoresce to some extent. At the high exposure concentration (Fig. 2.1C) the equivalents of naphthalene, phenanthrene, and total petroleum hydrocarbons were found to vary between 10 and 30 F.I. Pyrene, benzo(a)pyrene and general hydrocarbons equivalents were also found to fluoresce.

Dissolved oxygen (DO) varied between 9.4-8.3 mg L^{-1} O_2 (95-84 % O_2 saturation) in control tanks and 9.6-7.8 mg L^{-1} O_2 (98-79 % O_2 saturation) in exposed tanks. Values did not differ significantly between treatments in trial 1 ($F_{1,14}$=3.23; p=0.094) nor trial 3

($F_{1,14}$=0.56; p=0.468). However, they were significantly higher in the control than exposed tanks in trial 2 ($F_{1,14}$=6.79; p=0.021), with a mean difference of 0.3 mg L^{-1} O_2. These concentrations were all well above the levels (1.22 mg L^{-1} O_2) considered to be detrimental for echinoderms (Vaquer-Sunyer and Duarte, 2008).

Table 2.1: Concentrations of 16 (out of 20 possible) poly-aromatic hydrocarbons present in seawater accommodated fraction (WAF) of used lubricating oil (ULO) measured by an external analytical chemistry laboratory, prepared at high experimental tank concentration (EPA method 8207D).

Poly-aromatic Hydrocarbon (PAH)	**Measured Concentration (µg L^{-1})**
1-Methylnaphthalene	0.68
2-Methylnaphthalene	1.6
Acenaphthene	0.011
Acenaphthylene	0.037
Anthracene	0.036
Benzo(a)anthracene	0.030
Benzo(a)pyrene	0.017
Benzo(b)fluoranthene	0.022
Benzo(g,h,i)perylene	0.032
Benzo(j)fluoranthene	ND
Benzo(k)fluoranthene	ND
Chrysene	0.023
Dibenz(a,h)anthracene	ND
Fluoranthene	0.040
Fluorene	0.034
Indeno(1,2,3-cd)pyrene	0.015
Naphthalene	2.3
Perylene	ND
Phenanthrene	0.12
Pyrene	0.062
Total PAHs	**5.059**

ND - None Detected

Table 2.2: Concentration of heavy metals in seawater accommodated fraction (WAF) of used lubricating oil (ULO) measured by an external laboratory. Prepared at high experimental tank concentrations (EPA method 6020A R1).

Metals (Total)	**Reportable Detection Limit**	**WAF OF ULO ($\mu g\ L^{-1}$)**
Aluminum (Al)	50	ND
Antimony (Sb)	10	ND
Arsenic (As)	10	ND
Barium (Ba)	10	ND
Beryllium (Be)	10	ND
Bismuth (Bi)	20	ND
Boron (B)	500	4300
Cadmium (Cd)	0.10	ND
Calcium (Ca)	1000	380000
Chromium (Cr)	10	ND
Cobalt (Co)	4.0	ND
Copper (Cu)	20	ND
Iron (Fe)	500	ND
Lead (Pb)	5.0	ND
Magnesium (Mg)	10000	1200000
Manganese (Mn)	20	ND
Molybdenum (Mo)	20	ND
Nickel (Ni)	20	ND
Phosphorus (P)	1000	ND
Potassium (K)	1000	340000
Selenium (Se)	10	ND
Silver (Ag)	1.0	ND
Sodium (Na)	1000	9600000
Strontium (Sr)	20	7000
Thallium (Tl)	1.0	ND
Tin (Sn)	20	ND
Titanium (Ti)	20	ND
Uranium (U)	1.0	3.2
Vanadium (V)	20	ND
Zinc (Zn)	50	63

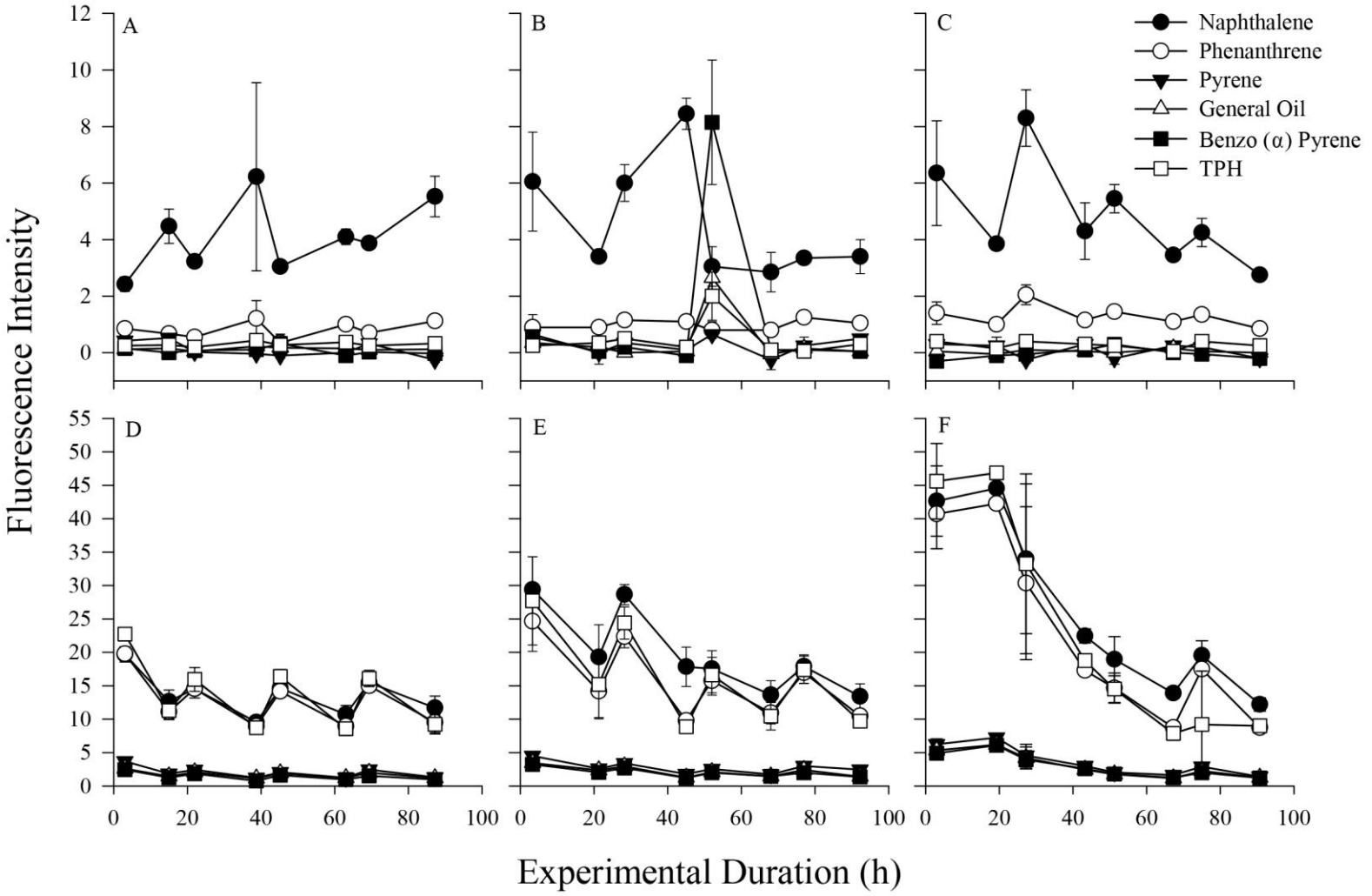
ND - None detected

2.4.2 Behavioural and Physiological Response

Behavioural responses were recorded during trial 2 (spawning period) and trial 3 (post spawning period). In trial 2, full deployment of the sea cucumber tentacles (Fig. 2.2A) occurred most frequently in the exposed treatment groups ($F_{1,34}$=11.99; p=0.002), whereas feeding behaviour (introduction of tentacles in the mouth) was solely observed in control groups ($F_{1,34}$=5.74; p=0.022). In trial 3, control groups exhibited (Fig. 2.2B) higher incidences of full tentacle deployment than the exposed groups ($F_{1,42}$=11.01; p=0.002) and were the only ones to display feeding behaviour. Similar tentacle behaviour was recorded in both control and exposed groups during depuration (Fig. 2.2C). Moreover, after 48-h of depuration, 25 % of exposed sea cucumbers displayed blisters (Fig. 2.3).

During trials 2 and 3, instances of sea stars with arms stretched and inverted under the water surface were observed within both control and exposed tanks. During the depuration period in trial 3, nearly double the incidences of this behaviour were observed in exposed individuals relative to controls.

Figure 2.1 (next page): Mean (± SE) fluorescence intensity of hydrocarbons monitored throughout the experimental trials within treatment group. A – Hydrocarbon fluorescence in baseline tanks during the pre-spawning season trial. B – Hydrocarbon fluorescence in spawning baseline tanks. C – Hydrocarbon fluorescence in post-spawning baseline tanks. D – Hydrocarbon fluorescence in pre-spawning exposed tanks. E – Hydrocarbon fluorescence in spawning exposed tanks. F – Hydrocarbon in post-spawning exposed tanks.


A
B
C
D
E
F
Naphthalene
Phenanthrene
Pyrene
General Oil
Benzo (α) Pyrene
TPH
Fluorescence Intensity
Experimental Duration (h)
0
2
4
6
8
10
12
0
5
10
15
20
25
30
35
40
45
50
55
0
20
40
60
80
100

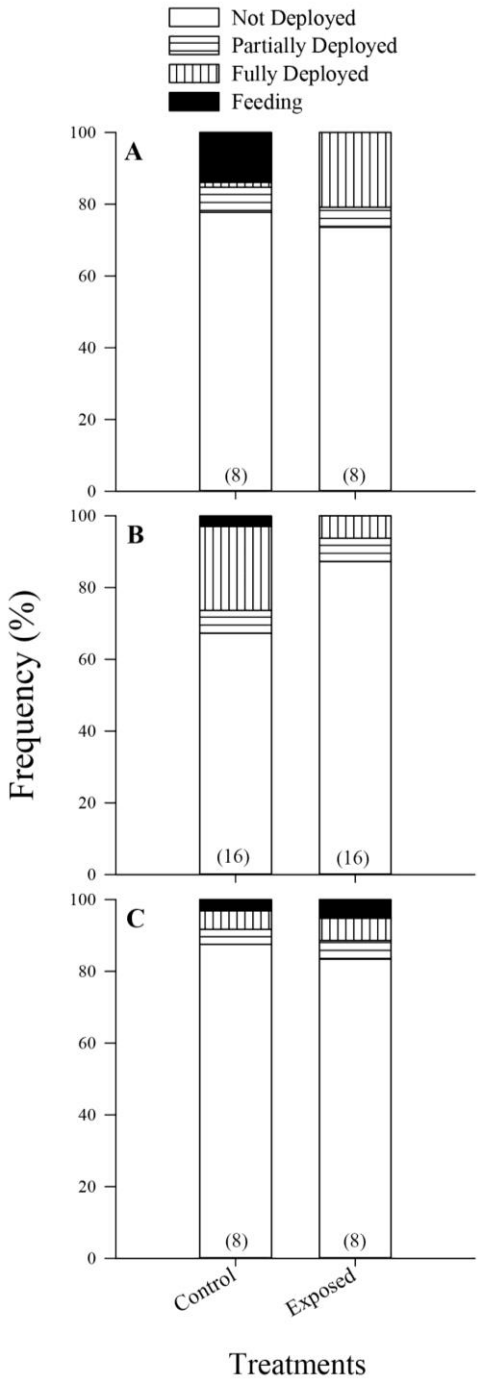


Figure 2.2: Sea cucumber (*C. frondosa*) tentacle deployment under high concentration of ULO WAF. A: Spawning season. B: Post-spawning season. C: Post-spawning depuration. Sample sizes are provided on the bars.

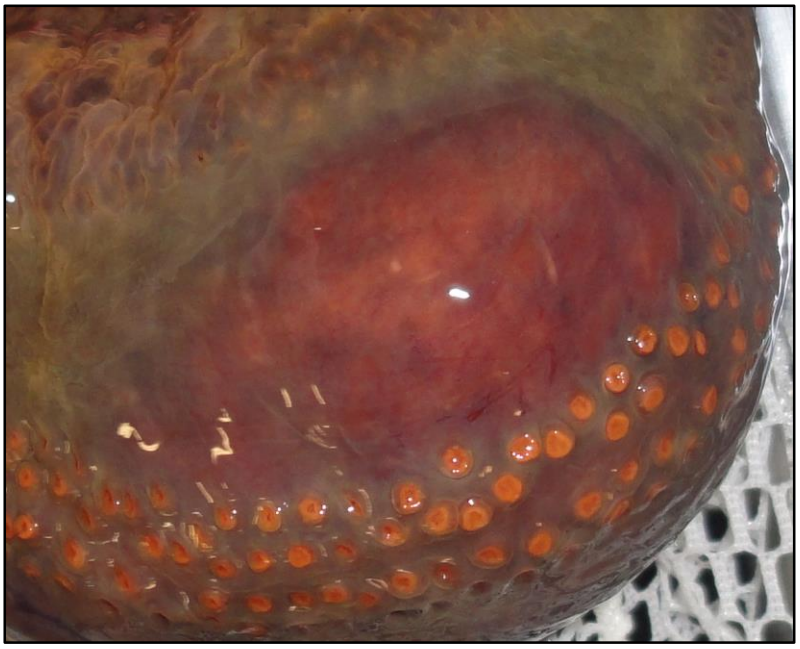


Figure 2.3: Blister along the left lateral side, directly above the tube feet of a sea cucumber (*C. frondosa*). (Credit: B. Gianasi)

2.4.3 Fluctuations in Baseline Enzyme Activity

In this segment, control groups were used to determine baseline enzymatic activity across trials and assess any intrinsic influence of season and/or sex prior to examining the effect of ULO WAF exposure on the same enzymes (section 2.4.4).

During trial 3 in the post-spawning season, 50 % of control individuals (n=16 per species) were collected after the initial 96-h exposure, while the remaining 50 % were sampled after an additional 96-h corresponding to the depuration period in a flow-through system. Both control groups were statistically analyzed to determine if their baseline enzyme activity differed. In the absence of a statistical difference, they were pooled into a

single baseline (control) group for the post-spawning season to increase the sample size and statistical power of the baseline data.

2.4.3.1 Glutathione Peroxidase (GPx)

Baseline GPx activity was detected in the gonads of all focal species as well as in the pyloric caeca of sea stars and the intestines of sea cucumbers (Table 2.3). Of the tissues tested, only respiratory trees of sea cucumbers did not exhibit any GPx activity, while sea urchin intestines yielded inconclusive results, i.e. they did not provide linear or logarithmic regressions of change in absorbance over time. These species/tissues were therefore omitted from the GPx analyses and not tested further for GPx activity. Overall, when activity levels were pooled across tissues for comparisons, baseline GPx activity in sea stars was higher than in all other species, which did not differ significantly from one another (Table A3).

Baseline GPx activity was not significantly affected by season nor sex in any of the sea star tissues (Table 2.4). Baseline GPx activity in the pyloric caeca was similar in the spawning (11.03 ± 1.59 pdu, n=8) and post-spawning seasons (8.38 ± 0.98 pdu, n=15). Stomach baseline activity during the spawning season was also similar between females (2.52 ± 0.57 pdu, n=3) and males (2.16 ± 0.77 pdu, n=5). Post-spawning baseline levels were similar to spawning levels, with no disparity between females (3.37 ± 0.39 pdu, n=11) and males (3.17 ± 1.43 pdu, n=4). An apparent (non-significant) sex disparity was observed in the baseline GPx activity of the gonads during the spawning season, with testes (3.05 ± 1.06 pdu, n=5) exhibiting more than double the GPx activity of

ovaries (1.30 ± 0.22 pdu, n=3). Baseline activity appeared higher in ovaries during the post-spawning season (2.46 ± 0.38 pdu, n=12) than during the spawning season.

Neither season nor sex significantly influenced the GPx activity in the intestines and gonads of sea cucumbers (Table 2.4). The GPx activity in the intestines of females varied from pre-spawning levels of 0.60 ± 0.30 pdu (n=3) to 1.15 ± 0.30 pdu (n=8) in the post-spawning season, whereas male baseline intestine activity varied from 0.73 ± 0.22 pdu (n=5) in the pre-spawning season to 1.49 ± 0.22 pdu (n=8) in the post-spawning season. Again, a slight seasonal trend was observed in female gonads, whereby baseline activity in the ovaries remained ≤0.09 (n=3) in the pre-spawning season and was 1.01 ± 0.30 pdu (n=8) in the post-spawning season. During the same period, activity in the testes remained relatively constant from 0.47 ± 0.43 pdu (n=5) to 0.50 ± 0.15 pdu (n=8).

GPx activity in brittle star gonads was not affected by season nor sex at a statistically significant level (Table 2.4). During the spawning season both ovaries (1.08 ± 0.07 pdu, n=3) and testes (1.24 ± 0.30 pdu, n=3) displayed similar levels. Baseline activity levels in the post-spawning season were also quite similar between ovaries (0.88 ± 0.10 pdu, n=8) and testes (0.87 ± 0.15 pdu, n=8), and only slightly lower than in the spawning season.

Table 2.3: General overview of results for GPx and EROD assays in all the tissues.

Species	**Tissue**	**GPx Activity**	**EROD Activity**
Ophiopholis aculeata	Gonad	Low	Moderate
Cucumaria frondosa	Gonad	Low	None
	Intestine	Low	None
	Respiratory tree	None	None
Asterias rubens	Gonad	Moderate	None
	Stomach	Moderate	None
	Pyloric caeca	High	Low
Strongylocentrotus droebachiensis	Gonad	Moderate	High
	Intestine	N/A	N/A

GPx: None – No activity detected; Low – < 2 pdu; Moderate – 2 - 6 pdu; High – > 6 pdu; N/A – Inconclusive
EROD: None – No activity detected; Low – > 0 , <0.2 pdu; Moderate – >0.2 – 0.4 pdu; High – > 0.4 pdu; N/A – Inconclusive

Table 2.4: Statistical analyses of baseline GPx activity. Independent variable/s indicated along with interactions (×) analyzed. Results of ANOVAs are given.

Species	Tissue	GPx activity		F	df	p
Sea Star	Pyloric Caeca	Season × Sex				
			Spawning = Post-Spawning	3.05	1, 20	0.096
			Females = Males	0.99	1, 20	0.331
	Stomachs	Season x Sex				
			Spawning = Post-Spawning	2.17	1, 20	0.157
			Females = Males	0.17	1, 20	0.686
	Gonads	Season × Sex				
			Spawning = Post-Spawning	1.32	1, 17	0.267
			Females = Males	2.35	1, 17	0.144
Sea Cucumber	Intestines	Season × Sex				
			Pre-Spawning = Spawning = Post-Spawning	2.72	2, 27	0.084
			Females = Males	1.85	1, 27	0.185
	Gonads	Season × Sex				
			Pre-Spawning = Spawning = Post-Spawning	0.93	2, 28	0.405
			Females = Males	1.45	1, 28	0.239
Sea Urchin	Gonads	Season × Sex				
			Pre-Spawning = Spawning = Post-Spawning	0.91	2, 28	0.414
			Females = Males	3.53	1, 28	0.071
Brittle Star	Gonads	Season × Sex				
			Spawning = Post-Spawning	2.38	1, 19	0.139
			Females = Males	0.02	1, 19	0.887

2.4.3.2 Ethoxyresorufin-*O*-Deethylase (EROD)

Prior to performing the EROD assay on experimental samples, tests were conducted to determine optimal assay conditions that would provide consistent measurements, using a minimum of reagents, while not limiting the enzyme activity. All of the species sampled during the trials were tested for EROD activity in various tissues (Table 2.3), and assessed for optimal supernatant (S9) volume and incubation period. It was determined that 50 µL of S9 and 15 min of incubation provided optimal conditions for brittle stars gonads and sea stars pyloric caeca. The gonads of sea urchins required 25 µL of supernatant and an incubation period of 15 min. Of the focal species, only sea cucumbers did not exhibit any EROD activity in any of their tissues (Table 2.3).

While a marginally significant ($p=0.047$) tank effect was detected among post-spawning control depurated sea urchins, the post-hoc analysis using the Games-Howell test did not reveal any significant pairwise differences (Table A2). Additionally, depending on the software used to perform the Welch's ANOVA, the results alternated between nonsignificant and significant. Finally, the post-spawning exposed depurated sea urchins did not display tank effects even though they were paired with the control depurated tanks within the lab environment, and no other tank effects were detected anywhere in the study. Therefore, it was decided to combine controls. A comparison among the other species determined that sea urchins displayed higher levels of EROD activity in their pooled tissues than sea stars and brittle stars, while the latter two did not differ significantly (Table A3).

A significant interaction between season and sex on the baseline activity of EROD was detected in the gonads of sea urchins (Table 2.5). In sex-specific analyses, activity in the ovaries of sea urchins was significantly affected by season (Fig. 2.4A). It was higher and less variable during the spawning season (0.69 ± 0.04 pdu, n=6) than in earlier (0.12 ± 0.12 pdu, n=4) and later seasons (0.28 ± 0.08 pdu, n=9). EROD activity in testes was not significantly affected by season (pre-spawning: 0.09 ± 0.04 pdu, n=3; spawning: 0.10 ± 0.03 pdu, n=2; post-spawning: 0.04 ± 0.02 pdu, n=8). In season-specific analyses, EROD activity in ovaries was significantly higher than in testes during the spawning and the post-spawning seasons (Fig. 2.5). However, sex did not significantly affect EROD activity in the pre-spawning season (Table 2.5).

In the gonads of brittle stars, baseline EROD activity significantly varied across seasons (Table 2.5). Activity was higher in the spawning season (0.20 ± 0.10 pdu, n=7) than the post-spawning season (0 pdu, n=16; Fig. 2.4B). Baseline EROD activity was not sex-dependent in gonads of brittle stars (Table 2.5).

Baseline EROD activity in the pyloric caeca of sea stars was not significantly affected by season or sex, but a significant interaction between the two factors was detected (Table 2.5). However, independent tests at each level of each factor did not reveal any significant differences. Nevertheless, EROD activity was visibly maximal in the pyloric caeca of males during the spawning season (0.05 ± 0.03 pdu, n=5) relative to females in the same season (0.003 ± 0.003 pdu, n=3) and to both sexes in the post-spawning season (Female: 0.002 ± 0.002 pdu, n=12; Male: 0.003 ± 0.003 pdu, n=4).

No EROD activity was detected in the stomachs of sea stars during the spawning or post-spawning seasons.

Figure 2.4 (next page): Significant seasonal variation in baseline EROD activity of various tissues/species combinations. A – EROD activity in the ovaries of sea urchins was higher in the spawning than in the pre-spawning and post-spawning seasons. B – EROD activity in the gonads of brittle stars was higher in the spawning season than in the post-spawning season (no data for the pre-spawning season). Mean activity and standard error are shown. Dissimilar letters indicate statistical significance (ANOVA, $p < 0.05$, sample sizes provided on the bars).

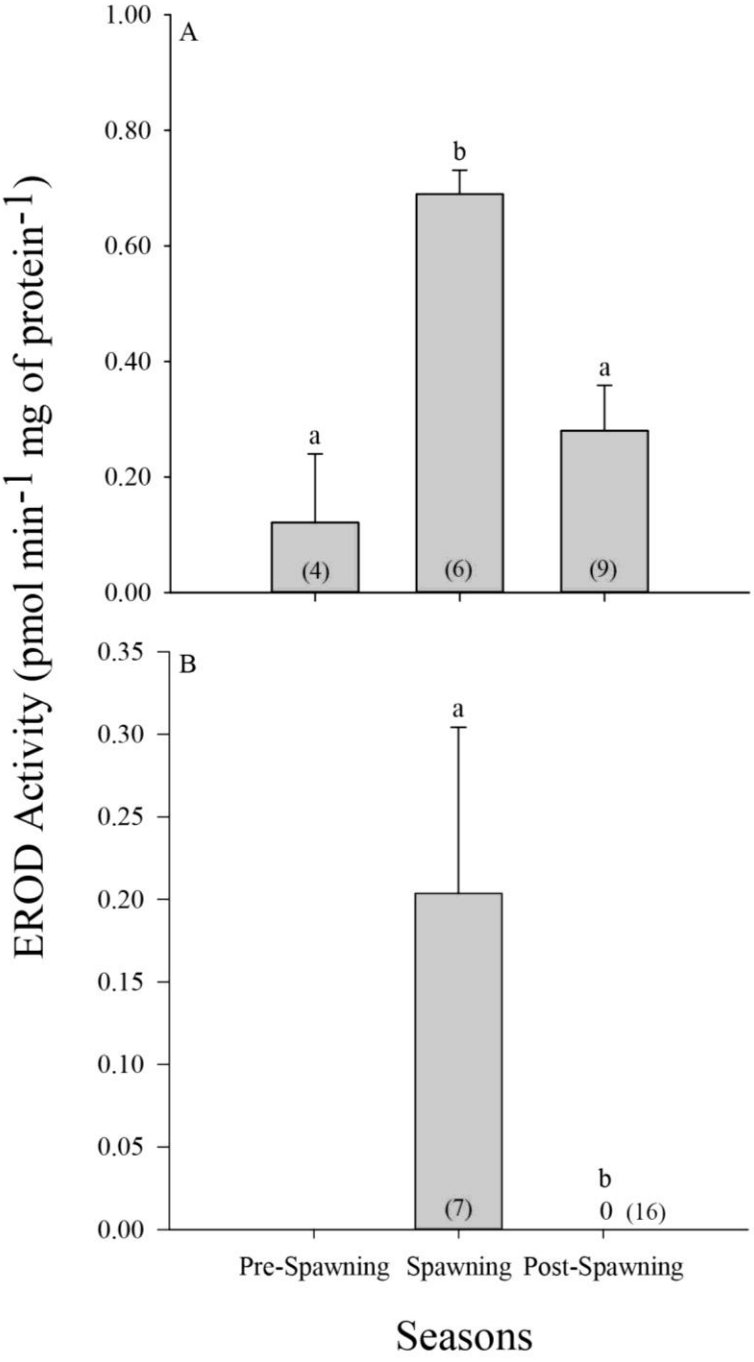

1.00
0.80
0.60
0.40
0.20
0.00
A
a
b
a
(4)
(6)
(9)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
B
a
b
0
(7)
(16)
Pre-Spawning
Spawning
Post-Spawning
Seasons
EROD Activity (pmol min-1 mg of protein-1)

Table 2.5: Statistical analyses of EROD activity. Independent variable/s indicated along with interactions (×) analyzed. Results of ANOVAs are given. Significant ($p \leq 0.05$) results are shown in bold.

Species	**Tissue**	**EROD activity**	**F**	**df**	**p**
Sea Urchin	Gonads	**Season × Sex**	**4.58**	**2, 25**	**0.020**
		Female: Pre-Spawning < Spawning			**0.027**
		Female: Pre-Spawning = Post-Spawning			0.539
		Female: Spawning > Post-Spawning			**0.002**
		Male: Pre-Spawning = Spawning = Post-Spawning	1.02	2, 9	0.399
		Pre-Spawning: Females = Males	0.05	1, 5	0.829
		Spawning: Females > Males	**106.0**	**1, 6**	**<0.0001***
		Post-Spawning: Females > Males	**8.20**	**1, 14**	**0.013***
Brittle Star	Gonads	Season × Sex			
		Spawning > Post-Spawning	**6.68**	**1, 20**	**0.018**
		Females = Males	0.26	1, 20	0.616^
Sea Star	Pyloric Caeca	**Season × Sex**	**4.62**	**1, 20**	**0.044***
		Female: Spawning = Post-Spawning	0.006	1, 13	0.938
		Male: Spawning = Post-Spawning	2.07	1, 7	0.170
		Spawning: Female = Male	1.75	1, 6	0.234
		Post-Spawning: Female = Male	0.06	1, 14	0.809

Symbols indicate that data were either (*) square root or (^) power transformed to obtain homogeneity of variance.

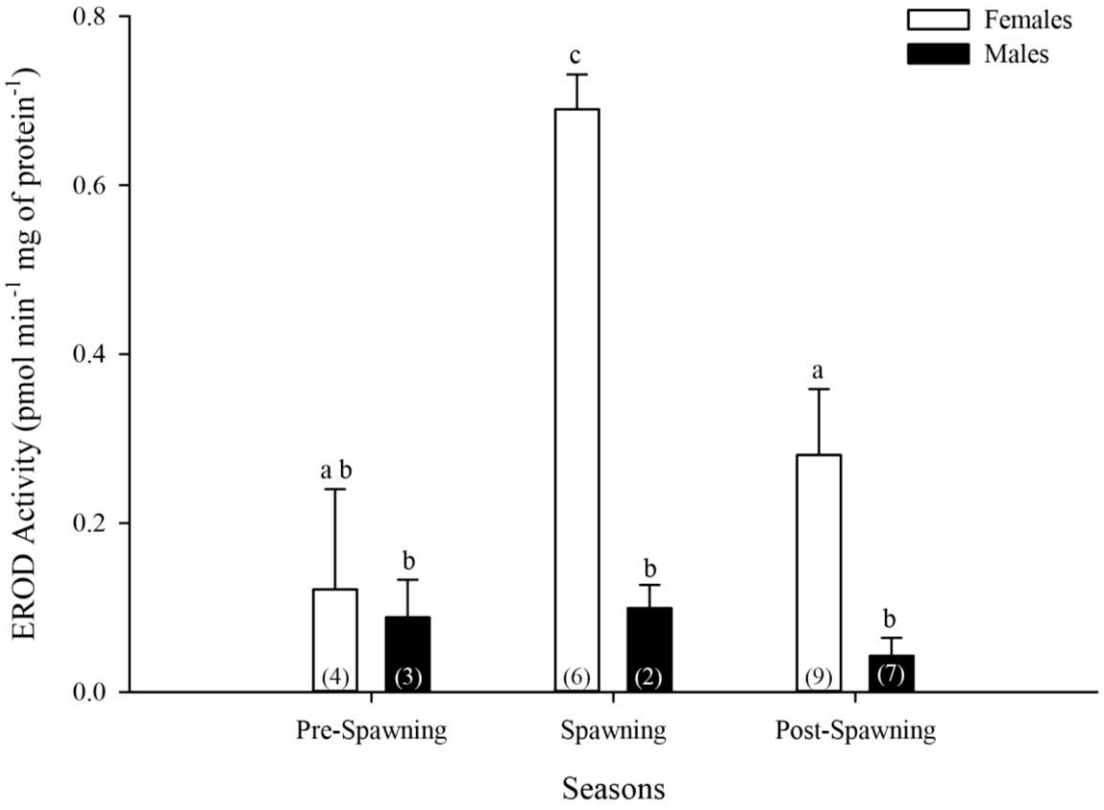


Figure 2.5: Significant season-specific influence of sex on baseline EROD activity in the gonads of sea urchins. EROD activity in male and female gonads was only similar in the pre-spawning season. Female gonads in the spawning season exhibited significantly greater activity than all other female or male gonads at any other time. Female gonads in the post-spawning season exhibited significantly greater activity than any male gonads. EROD activity in male gonads remained similar across seasons. Mean activity with standard error are shown. Dissimilar letters between sexes inside a season or across seasons within a sex indicate statistical differences (ANOVA, $p < 0.05$, sample sizes provided on the bars).

2.4.4 Effect of Oil Exposure on Baseline Enzyme Activity

2.4.4.1 Glutathione Peroxidase (GPx) Activity

To determine the effect that the water-accommodated fraction (WAF) of used lubricating oil (ULO) had on GPx activity, the activity in the various tissues of animals exposed to ULO WAF was compared to baseline levels during the same period. The significance of treatment (control, exposed), depuration after exposure, ULO WAF concentration (0, 0.22, 0.33 mL L^{-1}), sex, and season was assessed.

The GPx activity in the pyloric caeca of sea stars did not differ significantly based on experimental treatment, season, sex, nor depuration (Table 2.6). No significant difference between spawning and post-spawning activity were measured.

A significant interaction between treatment and sex on the GPx activity in stomachs of sea stars was detected (Table 2.6). Within treatment-specific analyses, exposed males (5.68 ± 1.77 pdu, n=3) had significantly more GPx activity in their stomachs than exposed females (2.49 ± 0.30 pdu, n=13; Fig. 2.6A). In sex-specific analyses, GPx activity in stomachs was not significantly affected by season, nor did it differ significantly from baseline after depuration.

Table 2.6: Statistical analyses of the response to the ULO WAF exposure and depuration on GPx activity in the focal species. Independent variable/s indicated along with interactions analyzed. Results of ANOVAs are given. Significant ($p \leq 0.05$) results are shown in bold.

Species	Tissue	GPx activity		F	df	p
Sea star	Pyloric caeca	Treatment × Sex				
			Control = Exposed	0.48	1, 36	0.495
			Females = Males	2.33	1, 36	0.136
		Treatment × Season				
			Control = Exposed	0.38	1, 36	0.540
			Spawning = Post-Spawning	2.78	1, 36	0.104
		Depuration × Sex				
			Control = Depurated	0.19	1, 20	0.665
			Females = Males	0.18	1, 20	0.679
	Stomachs	**Treatment × Sex**		**9.20**	**1, 35**	**0.005**
			Female: Control = Exposed	2.41	1, 25	0.133
			Male: Control = Exposed	3.71	1, 10	0.083
			Control: Female = Male	0.65	1, 21	0.428
			Exposed: Females < Males	**10.67**	**1, 14**	**0.006**
		Treatment × Season				
			Control = Exposed	0.20	1, 36	0.661
			Spawning = Post-Spawning	2.15	1, 36	0.151
		Depuration × Sex				
			Control = Depurated	0.18	1, 20	0.680
			Females = Males	1.53	1, 20	0.231
	Gonads	Treatment × Sex				
			Control = Exposed	0.17	1, 33	0.685
			Females < Males	**7.31**	**1, 33**	**0.011**
		Treatment × Season				
			Control = Exposed	0.08	1, 33	0.786

			Spawning = Post-Spawning	0.70	1, 33	0.408
		Depuration × Sex				
			Control = Depurated	0.83	1, 17	0.377
			Females = Males	2.98	1, 17	0.103
Sea Cucumber	Intestines	Treatment × Sex				
			Control = Exposed	0.27	1, 36	0.606
			Females > Males	2.74	1, 36	0.107
		Sex × ULO Concentration				
			Females = Males	2.60	1, 51	0.113
			0 = 0.22 = 0.33 mL L^{-1}	0.65	2, 51	0.527
		Treatment × Season				
			Control = Exposed	0.02	1, 51	0.899
			Pre-Spawning = Spawning			0.069
			Pre-Spawning < Post-Spawning			**0.019**
			Spawning = Post-Spawning			0.899
		Depuration × Sex				
			Control = Depurated	0.004	1, 21	0.953
			Females = Males	0.48	1, 21	0.496
	Gonads	Treatment × Sex				
			Control = Exposed	0.09	1, 37	0.762
			Females > Males	**6.09**	**1,37**	**0.018**
		Sex × ULO Concentration				
			Females > Males	**5.17**	**1, 52**	**0.027**
			0 = 0.22 = 0.33 mL L^{-1}	0.57	2, 52	0.569
		Treatment × Season				
			Control = Exposed	0.009	1, 52	0.927
			Pre-Spawning = Spawning = Post-Spawning	2.23	2, 52	0.118
		Depuration × Sex				
			Control = Depurated	0.04	1, 21	0.852
			Females = Males	3.00	1, 21	0.098
Sea Urchin	Gonads	Treatment × Sex				
			Control = Exposed	0.05	1, 37	0.833

			Females < Males	**5.24**	**1, 37**	**0.028**
		Sex × ULO Concentration				
			Females < Males	**4.31**	**1, 52**	**0.043**
			0 = 0.22 = 0.33 mL L^{-1}	0.87	2, 52	0.424
		Sex within Season				
			Pre-Spawning: Females = Males	0.28	1, 14	0.604
			Spawning: Females = Males	1.13	1, 14	0.306
			Post-Spawning: Females = Males	3.58	1, 22	0.072^
		Treatment × Season				
			Control = Exposed	0.06	1, 52	0.814
			Pre-Spawning = Spawning = Post-Spawning	1.23	2, 52	0.300
		Depuration × Sex				
			Control = Depurated	0.03	1, 21	0.855
			Females < Males	**5.01**	**1, 21**	**0.036**
Brittle Star	Gonads	Treatment × Sex				
			Control = Exposed	0.04	1, 34	0.845#
			Females = Males	1.23	1, 34	0.275#
		Treatment × Season				
			Control = Exposed	0.10	1, 35	0.758
			Spawning = Post-Spawning	0.45	1, 35	0.509
		Depuration × Sex				
			Control = Depurated	0.67	1, 21	0.422
			Females = Males	0.59	1, 21	0.451

Symbols indicate that data were either (^) power or (#) $\log_{10}$ transformed to obtain homogeneity of variance.

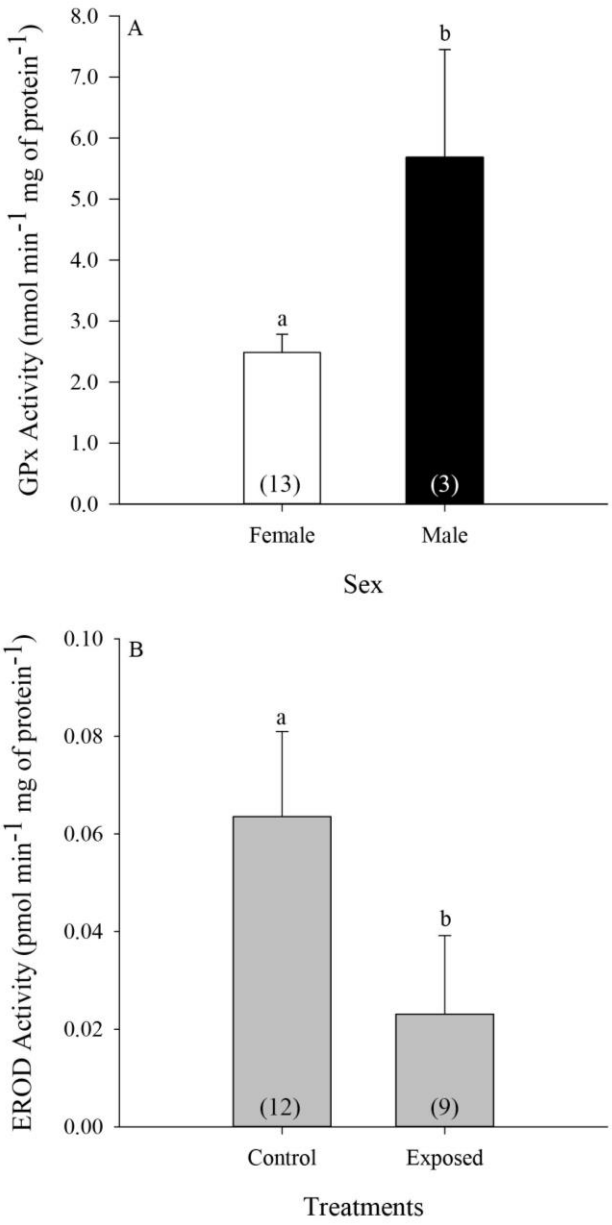


Figure 2.6: Significant treatment-dependent and sex-dependent enzyme activity in the exposed groups. A – GPx activity in the stomachs of exposed sea stars was higher in males than females. B – EROD activity in the testes of sea urchins was higher in control than exposed individuals. Mean activity with standard error are shown. Dissimilar letters indicate statistical significance (ANOVA, $p < 0.05$, sample sizes provided on the bars).

A significant effect of sex on the GPx activity in the gonads of sea stars was measured (Table 2.6). Overall, when combining control and experimental data, activity was significantly higher in testes (3.57 ± 0.92 pdu, n=9) than in ovaries (2.10 ± 0.21 pdu, n=28; Fig. 2.8A). In contrast, no significant sex-based differences were observed within seasons. No significant effect on GPx activity in sea star gonads was detected based on season or treatment, nor did levels differ significantly from baseline after depuration (Table 2.6).

The GPx activity in the intestines of sea cucumbers was significantly affected by season. Overall activity was significantly higher in the post-spawning (1.32 ± 0.18 pdu, n=16) than in the pre-spawning (0.73 ± 0.11 pdu, n=16) season (Fig. 2.7A). No difference between the pre-spawning and spawning season was measured. Activity was not significantly affected by treatment, sex, or concentration of WAF, and did not significantly differ from baseline after depuration (Table 2.6). Nonetheless, some trends were evidenced. During the pre-spawning and spawning seasons, intestine activity levels did not appear to differ between sexes. However, during the post-spawning season a disparity in the exposed treatment was observed, with females (0.80 ± 0.80 pdu, n=2) seeming to have nearly half the activity of males (1.28 ± 0.32 pdu, n=6). In contrast, the GPx activity in the intestines of depurated females (1.35 ± 0.40 pdu, n=5) and males (1.26 ± 0.18 pdu, n=3) were very similar.

Figure 2.7 (next page): Significant overall season-dependent activity [in combined control (baseline) and experimental (exposed) groups]. A – GPx activity in the intestines of sea cucumbers was higher in the post-spawning season than the pre-spawning season. B – EROD activity in the ovaries of sea urchins was higher in the spawning season than the pre-spawning and post-spawning seasons. Mean activity with standard error are shown. Dissimilar letters indicate statistical significance (ANOVA, $p < 0.05$, sample sizes provided on the bars).

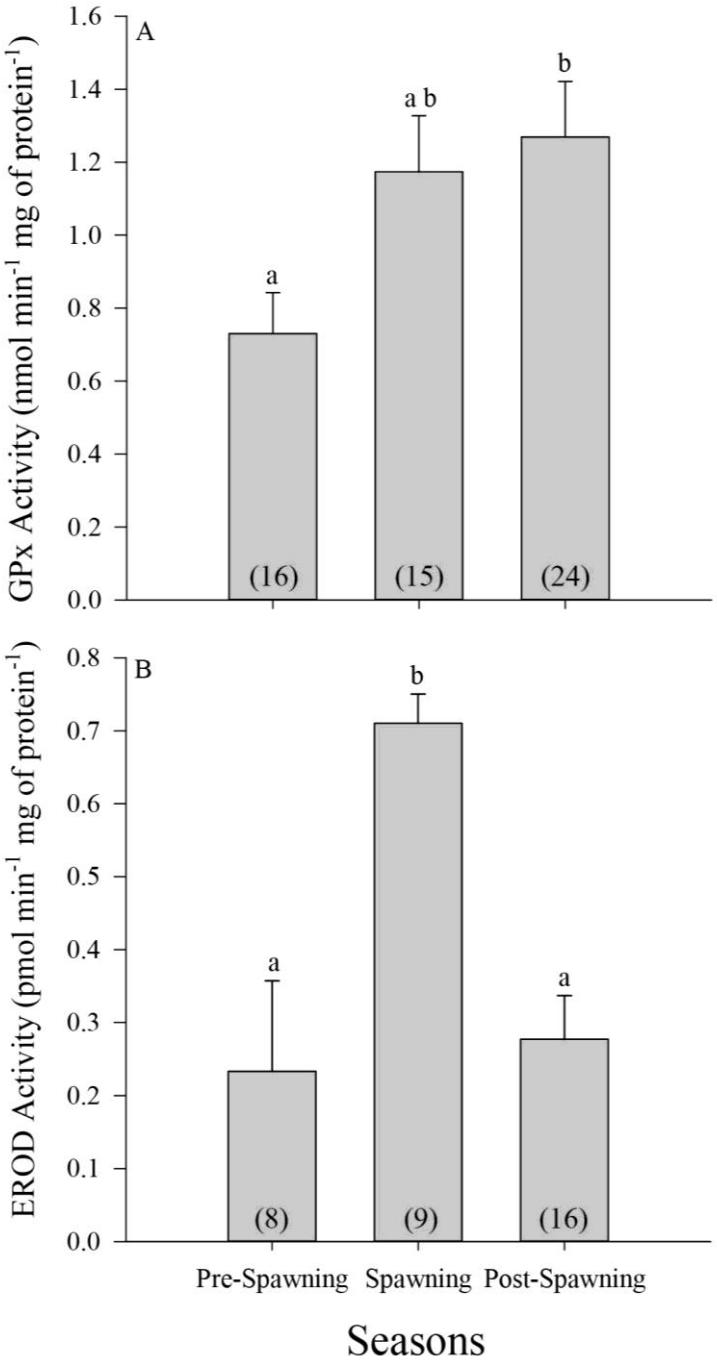
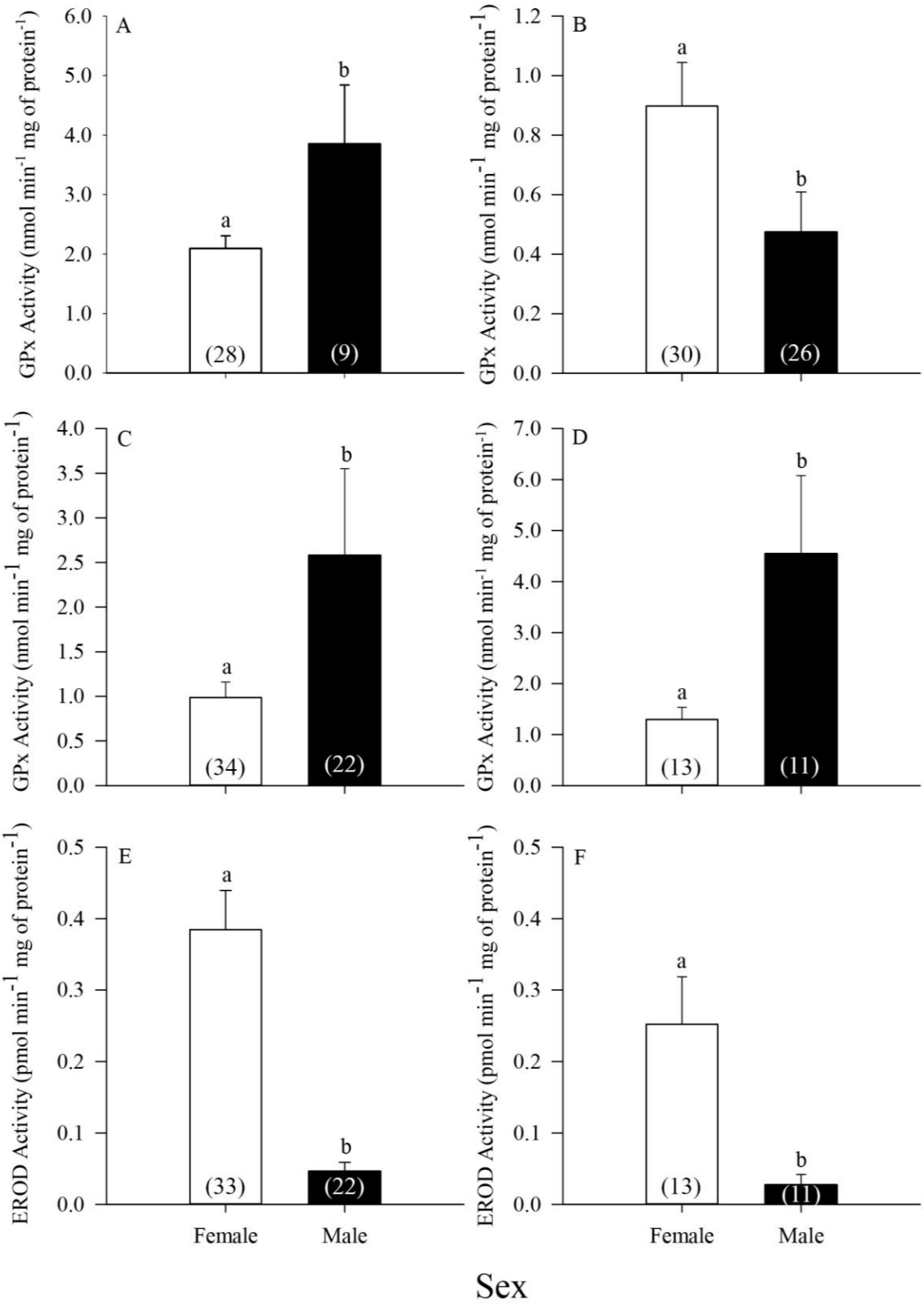

A
GPx Activity (nmol min-1 mg of protein-1)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a b
b
(16)
(15)
(24)
B
EROD Activity (pmol min-1 mg of protein-1)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
b
a
(8)
(9)
(16)
Pre-Spawning
Spawning
Post-Spawning
Seasons

Figure 2.8 (next page): Significant overall sex-dependent activity [in combined control (baseline) and experimental (exposed) groups]. A – GPx activity in the gonads of sea stars was higher in males than females. B – GPx activity in the gonads of sea cucumbers was higher in females than males. C – GPx activity in the gonads of sea urchins was higher in males than females. D – GPx activity in the depurated gonads of sea urchins was higher in males than females. E – EROD activity in the gonads of sea urchins was higher in females than males. F – EROD activity in the depurated gonads of sea urchins was still higher in females than males. Mean activity with standard error are shown. Dissimilar letters indicate statistical significance (ANOVA, $p < 0.05$, sample sizes provided on the bars).


A
B
C
D
E
F
GPx Activity (nmol min-1 mg of protein-1)
EROD Activity (pmol min-1 mg of protein-1)
a
b
(28)
(9)
(30)
(26)
(34)
(22)
(13)
(11)
(33)
(22)
(13)
(11)
Female
Male
Sex

The overall GPx activity in the gonads of sea cucumbers was significantly affected by sex; with the ovaries (0.90 ± 0.15 pdu, n=30) of sea cucumbers exhibiting more activity than testes (0.47 ± 0.14 pdu, n=26; Fig. 2.8B). The activity in gonads was not significantly affected by treatment, sex, concentration of WAF, season, nor differ significantly from baseline after depuration (Table 2.6). The activity in ovaries exposed to the low WAF concentration was 0.69 ± 0.37 pdu (n=6) in the pre-spawning season, while in the high concentration it ranged from 0.98 ± 0.26 pdu (n=4) in the spawning season to 1.32 ± 1.01 pdu (n=2) in the post-spawning season. GPx activity in the testes after exposure to the low of concentration of WAF was 0.23 ± 0.23 pdu (n=2) in the pre-spawning season. At the higher WAF concentration it ranged from 1.11 ± 0.58 pdu (n=4) in the spawning season to 0.19 ± 0.11 pdu (n=6) in the post-spawning season. Testes of male sea cucumbers exposed in the post-spawning season exhibited GPx activity that was seven times lower than ovaries of concurrently exposed females, mirroring the disparity observed between sexes in the post-spawning baseline (section 2.4.3.1).

The overall GPx activity in the gonads of sea urchins was significantly affected by sex; with the testes (2.58 ± 0.97 pdu, n=22) having significantly more activity than ovaries (0.99 ± 0.17 pdu, n=34; Fig. 2.8C). Similarly, levels in post-spawning baseline and depurated testes (4.55 ± 1.53 pdu, n=11) were significantly higher than in ovaries (1.30 ± 0.24 pdu, n=13; Fig. 2.8D). GPx activity was not significantly affected, at a statistical level, by exposure to WAF, concentration of WAF, the season in which they were exposed, nor differ significantly from baseline after depuration (Table 2.6). The most striking trends were those of the testes of sea urchins exposed during the spawning season, when GPx activity appeared highly up-regulated after exposure (3.45 ± 2.92 pdu,

n=5) relative to baseline levels (0.28 ± 0.28 pdu, n=2). With the low sample sizes for testes in the post-spawning season it was not possible to determine whether exposure to WAF had an effect in males. Activity in the ovaries of sea urchins exposed to low WAF concentration was 0.56 ± 0.34 pdu (n=4) in the pre-spawning season, while in the high concentration trials, activity in ovaries ranged from 0.53 ± 0.40 pdu (n=3) in the spawning season to 1.48 ± 0.45 pdu (n=7) in the post-spawning season.

The GPx activity in the gonads of brittle stars was not significantly affected by treatment, sex, season, nor differ significantly from baseline after depuration (Table 2.6). However, there did appear to be some effect of WAF on GPx activity in testes. During the post-spawning season, testes of exposed (1.35 ± 0.43 pdu, n=4) and depurated brittle stars (1.30 ± 0.53 pdu, n=4) appeared to have increased activity in comparison to concurrent baseline levels (0.87 ± 0.15 pdu, n=8). Meanwhile, testes of brittle stars exposed to WAF in the spawning season appeared to have activity (1.30 ± 0.87 pdu, n=4) very similar to spawning season baseline levels (1.24 ± 0.30 pdu, n=3). GPx activity in ovaries did not appear to be influenced by exposure to the high WAF concentration; it ranged from 0.82 ± 0.15 pdu (n=4) in the spawning season to 0.75 ± 0.16 pdu (n=4) in the post-spawning season.

2.4.4.2 Ethoxyresorufin-*O*-Deethylase (EROD) Activity

Overall EROD activity in the gonads of sea urchins was significantly affected by sex, being significantly higher in ovaries than in testes (Fig. 2.8E). In sex-specific analysis, exposed testes (0.02± 0.02 pdu, n=9) exhibited significantly lower activity than the baseline level (0.06 ± 0.02 pdu, n=12; Fig. 2.6B). The EROD activity in ovaries was

significantly influenced by season; activity was higher in the spawning than in either the pre-spawning or post-spawning seasons (Fig. 2.7C). Activity in the ovaries of exposed sea urchin was 0.35 ± 0.22 pdu (n=4) in the pre-spawning season (low WAF concentration), while results of the high WAF concentration trials varied from 0.75 ± 0.10 pdu (n=3) in the spawning season, to 0.27 ± 0.10 pdu (n=7) in the post-spawning season. In sex-specific analyses, the concentration of WAF did not significantly influence EROD activity in the ovaries or testes (Table 2.7). Within the gonads of depurated animals, sex significantly affected the overall activity (Fig. 2.8F), with females exhibiting more activity than males, based on the combined control and exposed groups. However, EROD activity was not significantly affected by treatment, or concentration of WAF for pooled sexes, nor did it differ significantly from baseline after depuration.

EROD activity in the gonads of brittle stars was not significantly influenced by season, treatment, or sex, nor did it differ significantly from baseline after depuration (Table 2.7). EROD activity within the gonads did not differ significantly during the spawning (0.15 ± 0.06 pdu, n=15) and post-spawning (0 pdu, n=24) seasons. Ovaries of brittle stars exposed in the spawning season (0.22 ± 0.10 pdu, n=4) displayed levels similar to the baseline (0.21 ± 0.12 pdu, n=4), whereas activity in testes (0.00 pdu, n=4) appeared to be suppressed relative to baseline (0.20 ± 0.20 pdu, n=3). No activity was detected in the gonads of any exposed brittle stars in the post-spawning season; although depurated ovaries did express faint activity (0.03 ± 0.03 pdu, n=4).

EROD activity in the pyloric caeca of sea stars was not significantly affected by season, treatment, sex, concentration of WAF, and did not differ significantly from baseline after depuration (Table 2.7).

Table 2.7: Statistical analyses of the response to the ULO WAF exposure and depuration on EROD activity in the focal species. Independent variable/s indicated along with interactions analyzed. Results of ANOVAs are given. Significant ($p \leq 0.05$) results are shown in bold.

Species	Tissue	EROD activity		F	df	p
Sea Urchin	Gonads	Treatment × Sex				
			Control = Exposed	1.55	1, 36	0.221*
			Females > Males	**27.67**	**1, 36**	**<0.0001***
		Sex × ULO Concentration				
			Females > Males	**19.35**	**1, 50**	**<0.0001***
			0 = 0.22 = 0.33 mL L^{-1}	0.27	2, 50	0.767*
		Female: Treatment × Season				
			Control = Exposed	0.59	1, 29	0.448
			Pre-Spawning < Spawning			**0.014**
			Spawning > Post-Spawning			**<0.0001**
			Pre-Spawning = Post-Spawning			0.95
		Male: Treatment × Season				
			Control > Exposed	**4.66**	**1, 17**	**0.045**
			Pre-Spawning = Spawning = Post-Spawning	1.81	2, 17	0.193
		Depuration × Sex				
			Control = Depurated	0.77	1, 21	0.392
			Females > Males	**8.85**	**1, 21**	**0.007**
Brittle Star	Gonads	Treatment × Sex				
			Control = Exposed	0.02	1, 36	0.894
			Females = Males	1.14	1, 36	0.294
		Treatment × Season				
			Control = Exposed	1.60	1, 36	0.215^
			Spawning = Post-Spawning	3.06	1, 36	0.089^
		Depuration × Sex				
			Control = Depurated	2.10	1, 21	0.162

			Females = Males	1.05	1, 21	0.317
Sea star	Pyloric caeca	Treatment × Sex				
			Control = Exposed	0.26	1, 37	0.614
			Females = Males	1.02	1, 37	0.320
		Treatment × Season				
			Control = Exposed	0.04	1, 37	0.844
			Spawning = Post-Spawning	0.41	1, 37	0.524
		Depuration × Sex				
			Control = Depurated	0.89	1, 21	0.357
			Females = Males	0.06	1, 21	0.808

Symbols indicate that data were either (*) square root or (^) power transformed to obtain homogeneity of variance. na = not performed

2.5 Discussion

2.5.1 Natural Variations in Enzyme Baseline Activity

The prevalence of GPx activity in the majority of the species/tissues studied here suggests that it is universally present in echinoderms. In comparison, the presence of EROD activity, indicative of CYP450 1A, was found to vary markedly among species/tissues examined. This pattern followed initial predictions, based on the low specificity of GPx to operate on hydrogen peroxide/organic hydroperoxides (Doyotte et al., 1997) and the greater specificity of CYP450 1A to operate on exogenous compounds such as dioxins, furans, PCBs, and PAHs (Goksøyr, 1995; Sarkar et al., 2006). Previous research on CYP450 1A activity within different phyla has shown activity to be highest in tissue responsible for digestion and detoxification, e.g. liver, kidney, hepatopancreas, digestive gland (Goksøyr, 1991; Kirby et al., 1999; Livingstone, 1991; Martín-Díaz et al., 2007; Pohl and Fouts, 1980). The detection of EROD activity in the pyloric caeca of sea stars therefore supports previous research, whereas the presence of EROD activity in the gonads of brittle stars and sea urchins is intriguing. Unlike digestive tissues, gonads are not involved in processing ingested material, which begs the question of its purpose. It can be proposed that due to the lack of any complex respiratory or circulatory system within echinoderms, gonad tissue, which is known to obtain oxygen from the coelomic fluid (Shick, 1983), may require detoxification from xenobiotics present in the coelomic fluid. Alternatively, some CYP450 enzymes are known to metabolize steroids that may vary from cholesterols to estradiols; this process can include the hydroxylation of steroids, which leads to their clearance from the organism (Spies and Rice Jr, 1988). The

metabolism of steroids may explain the EROD activity in the gonads of brittle stars and sea urchins. However, it remains very hypothetical given the lack of EROD activity in the gonadal tissues of sea cucumbers and sea stars.

In the present study, season and/or sex influenced the baseline activities of the focal biomarkers. Specifically, an interaction between season and sex was detected in the EROD activity of sea urchin gonads, whereby seasonal fluctuations (significantly higher activity during the spawning than either pre-spawning or post-spawning periods) were restricted to females. Additionally, ovaries had significantly higher EROD activity than testes during some seasons (spawning and post-spawning). Season was also found to affect EROD activity in the gonads of brittle stars. When seasonal variation occurred, the highest activity was consistently measured during the spawning season, suggesting that reproductive activity could confound responses to a pollutant, and highlighting the need to identify, understand, and account for sources of seasonal variation, as previously recommended by Nahrgang et al. (2013).

Natural variations in biomarker expression may be explained by a combination of biotic and abiotic factors. The former include sex, reproductive stage, trophic activity, and growth of the organism (Sheehan and Power, 1999), whereas the latter includes water temperature, photoperiod, and food availability (Viarengo et al., 1991). Abiotic factors have been shown to influence the activity and induction of CYP450 enzymes in fish. For instance, Sleiderink et al. (1995) observed that EROD activity was inversely proportional to water temperature in North Sea dab (*Limanda limanda*). In the current study, water temperature was kept constant during holding and experimental trials since previous studies have shown that it can affect EROD activity (Lange et al., 1998; Shaw et al.,

2004; Sleiderink et al., 1995). Ambient photoperiod was used throughout since it is an important driver of reproductive activity. All individuals also had access to baseline food supplies over the study period; feeding of the grazers and carnivores was only withheld just before the trials to minimize interference with the measured biomarkers (Hodson et al., 1996; Vigano et al., 1993). Therefore, abiotic factors are less likely effectors than biotic processes, which are also known to elicit seasonal fluctuations in CYP450.

Seasonal differences between sexes in the expression of CYP450 have been documented in striped mullet *Mullus barbatus*, whereby males displayed the highest levels of CYP450 and mixed function oxidase (MFO) activities in the liver, particularly during the reproductive season (Mathieu et al., 1991). Previous research on the pyloric caeca of sea star (*A. rubens*) have also shown CYP450 activity to be highest near the reproductive period, and greater in females than males (den Besten, 1998). Here, reproduction emerged as a source of seasonal variation in biomarker activity, with the peculiar exception of sea stars. Seasonal fluctuations in EROD activity occurred in the gonads of brittle stars, without sex-based differences. In contrast, sex-based differences were most apparent during the spawning season of sea urchins, when ovaries displayed the highest EROD activity. Finally, no sex-based differences nor seasonal fluctuations in EROD activity occurred here in sea star pyloric caeca. The CO-difference spectrum method described by den Besten (1998) differed from the methodology used in the present study, which could account for some of the disparities. Additionally, CYP450 1A (indicated by EROD activity) belongs to a broad CYP450 family, therefore it may express fluctuations that deviate from those observed in the broader CYP450 family. Raymond et al. (2007) had shown that differences existed between sexes in resource allocation pre-

and post-spawning in the sea star *A. vulgaris* (= *rubens*). The energetic content in the pyloric caeca decreased for both sexes after spawning occurred, with lipids accounting for the majority of decreased energy (Raymond et al., 2007). However, variances in methodology and species/tissues make it hard to tease out universal trends, stressing the need for species-specific and tissue-specific measurements across multiple seasons.

The present study showed that, unlike sea stars, sex-based seasonal variation in EROD activity occurred in the gonads of green sea urchins (*S. droebachiensis*). Previous research on resource allocation in pre- and post-spawning purple sea urchin (*Paracentrotus lividus*) by Fernandez (1998) found the gonads to contain high levels of protein, relatively high lipid, and low carbohydrate content, with the primary difference between sexes being a higher lipid content in ovaries than testes. Protein, lipid, and carbohydrate levels initially increased in the gonads during early gametogenesis. As the gonads matured, lipid and carbohydrate levels decreased (Fernandez, 1998). The higher lipid content in ovaries relative to testes supports the greater EROD activity measured here in female sea urchins. However, there is a possibility that the increased levels of EROD activity near spawning may merely reflect its role during the maturation of gonads (Lange et al., 1998). Alternatively, a study by Cooreman et al. (1993) on flatfish (*Limanda limanda*) suggests that increased activity near spawning may be a coincidental response to the increased mobilization of lipids. A similar response may have occurred in the ovaries of sea urchins in the present study, while the lack of response in testes may be explained by their lower lipid content.

In the current study, neither seasonal nor sex-based variation in GPx activity were observed in sea cucumber gonads and intestines. A study on biochemical storage in the

orange-footed sea cucumber (*C. frondosa*) by David and MacDonald (2002) found lipids to constitute the largest portion of gonads, followed by proteins and carbohydrates. They found higher levels of lipids in ovaries than testes with no seasonal variation, and no sex-based differences in protein or carbohydrate content of gonads. Protein stores in gonads were found to decrease after spawning (David and MacDonald, 2002). Carbohydrate levels were highest in gonads during the feeding (March-September) period and decreased during the non-feeding (January-February and October-December) periods (David and MacDonald, 2002). The lack of seasonal variation in lipid content supports the lack of variation in GPx activity found here in sea cucumber gonads. Unfortunately, David and MacDonald (2002) did not study seasonal variation in resource allocation of sea cucumber intestine, making it impossible to compare it to the lack of seasonal variation in GPx observed in the present study.

With respect to the brittle stars used here, seasonal variation in EROD activity was noted. Unfortunately, to the best of our knowledge no previous research on seasonal variation in resource allocation exists for this taxonomic group. However, it is highly probable that gametogenesis in brittle stars requires a net input of resources with variation in resource allocation occurring in a similar manner as that observed in sea star and sea urchin gonads by Raymond et al. (2007) and Fernandez (1998), respectively.

Since lipid, protein, and carbohydrate resources of sea stars, sea urchins, and sea cucumbers are known to vary within sexes and seasons, their availability may play an integral part in the ability of these tissues to respond to environmental stressors. Enzymes such as CYP450 1A and GPx both require an input of resources in order to maintain activity levels, through both enzyme action (i.e. NADPH) (Michiels et al., 1994; Omura,

2010) and synthesis (ATP and protein) (Koehn, 1991). In the case of enzyme induction (i.e. increased synthesis), the energy and protein input may greatly exceed typical maintenance requirements (Koehn, 1991). Furthermore, enzymes may significantly impact the overall cost of an organism's baseline metabolism when the host tissues constitute a large portion of the organism (Koehn, 1991). In the present study, all tissues analyzed represented large portions of the organisms, suggesting that the detected enzyme levels, especially those elevated in the spawning season, required a significant input of energy and resources in order to maintain activity.

The increase in the synthesis of enzymes such as CYP450 1A (shown by enhanced EROD activity), resulting in an inflation of resource expenditure near the spawning season, may provide increased protection from exogenous compounds (pollutants), as well as an increased metabolism of endogenous compounds (steroids). Intriguingly, the increased EROD activity measured here in sea urchin ovaries and brittle star gonads appears to be the inverse of results obtained previously with boreal vertebrates. In polar cod (*Boreogadus saida*) EROD activity was negatively correlated with the reproductive period (Nahrgang et al., 2010), likely due to the production of estradiol-17β in female fish, which is known to inhibit CYP450 activity (Stegeman et al., 1982). The suppression of EROD activity in brook trout (*Salvelinus fontinalis*), demonstrated by Stegeman et al. (1982), occurred after injecting estradiol-17β intramuscularly at concentrations 1000 times greater than those measured in echinoderms. While sea urchins and sea stars are known to produce estradiol-17β (Botticelli et al., 1961; Schoenmakers and Voogt, 1981; Voogt and Dieleman, 1984), this compound did not appear to inhibit EROD activity. However, it is not certain that estradiol-17β did not

influence EROD activity at some level in the current study. It is important to note that the influence of biotic and abiotic factors may not necessarily be consistent within biomarkers or within species. Case in point, polar cod exhibited seasonal variation in EROD activity while Atlantic cod (*Gadus morhua*) did not (Nahrgang et al., 2013). Since even similar species (e.g. in Gadidae family) can have divergent activity induced by external factors, the use of multiple species and tissues when measuring biomarker activity may prove beneficial.

It can be hypothesized that GPx activity near the spawning period may convey protection against oxyradicals to prevent damage to gametes. The orange-footed sea cucumber (*C. frondosa*) is a broadcast-spawner that releases yolky oocytes, which develop into lecithotrophic (non-feeding) larvae (Hamel and Mercier, 1996). Newly released gametes of some broadcast-spawners, like *C. frondosa*, float to the surface where they are exposed to ultra-violet radiation (UVR) (Lesser, 2006). Since exposure to UVR leads to the production of reactive oxygen species (ROS) within the mitochondria of cells (Lesser, 2006), protection by antioxidants such as GPx may be necessary. However, no relation between gamete maturity (season) and GPx activity was detected in the gonad of sea cucumbers; suggesting that either the basal GPx activity is sufficient or that GPx does not play a role in the protection of gametes from UVR.

While year-round (monthly) spawning and protracted annual spawning (over several months) occurs in several tropical species of echinoderms around the equator (Hendler, 1991; Mercier and Hamel, 2009; Montero-Torreiro and Garcia-Martinez, 2003; Pearse, 1968; Pearse and Cameron, 1991; Smiley et al., 1991), cold-water species typically reproduce annually and may thus undergo more pronounced seasonal variations

in biomarkers. To compound this, the seasonal availability of food for adults and juveniles may be critically important in determining and maintaining seasonal reproductive periods (Hendler, 1991; Pearse and Cameron, 1991). Since, the present study used cold-water species that breed annually, it would be expected that they would place high priority on protecting their gametes so as to increase survival rates. Therefore, increased activity of enzymes would be expected near the spawning period, which is precisely what was measured in sea stars and sea urchins.

Sea stars, sea urchins, sea cucumbers, and brittle stars exhibit different diets and occupy different trophic positions, which suggests they may express different levels of GPx and EROD activity based on energy availability and the need for enzyme activity. Sea stars, such as *A. rubens*, have a carnivorous diet that is sustained by active predations on mollusks, such as whelks and mussels. This strategy results in a greater assimilation efficiency, but requires a higher respiratory metabolism and causes a lower growth efficiency (Welch, 1968). A greater respiratory metabolism leads to the increased production of ROS (Nahrgang et al., 2013) leading to a requirement for antioxidants such as GPx. It has also been well documented that sea stars can draw energy stored in the pyloric caeca for use in gametogenesis (Barker and Nichols, 1983; Boivin et al., 1986). These energy stores could potentially be used, instead of or in addition to dietary energy, to help regulate detoxification enzyme synthesis during exposure to stressors. In comparison, sea urchins such as *S. droebachiensis* are omnivores known to actively forage for seaweeds and seagrasses, and to scavenge opportunistically on animal tissue (Briscoe and Sebens, 1988). Compared to carnivory, herbivory and occasional scavenging would provide sea urchins with lower resources for use in enzyme synthesis, since plants

provide a much lower quantity of energy per gram of tissue than meat (Boyd and Goodyear, 1971). Both sea cucumbers and brittle stars are suspension feeders that occupy similarly low trophic positions, mainly consuming phytoplankton and particulate organic matter (Hamel and Mercier, 1998; Roushdy and Hansen, 1960). Due to its size and posture, *C. frondosa* can extend its feeding tentacles much higher in the water column than *O. aculeata*, and potentially has access to fresher organic material. In the present study, sea stars and sea urchins were found to express significantly more GPx and EROD activity, respectively, than all other species, possibly because of their carnivorous/scavenging habits. However, the fact that neither species exhibited consistently higher levels of the two biomarkers shows that dietary lifestyle alone is not a good predictor of biomarker activity.

Growth, which would result in a net re-allocation of resources away from enzyme production and gametogenesis, is unlikely to have influenced the baseline variation in GPx or EROD activity over the course of the present study, due to the use of fully grown adult specimens, which are known to exhibit little to no growth (Lawrence, 1987a; Lawrence and Lane, 1982). Echinoderms in the Northwest Atlantic typically experience slow sporadic growth. Moreover, growth is generally limited during winter when low food availability and water temperatures occur, and enhanced when warmer waters and higher food supplies prevail (Chia and Walker, 1991; Franz, 1986; Hamel and Mercier, 1996; Lawrence and Lane, 1982).

2.5.2 Response to Used Lubricating Oil (ULO)

Previous studies of the effect of ULO have found a linear increase in EROD activity within the liver and kidneys of rainbow trout (*Oncorhynchus mykiss*) three days after injection of ULO into the fish (Upshall et al., 1993). Additionally, exposure to ULO WAF was shown to decrease the rate of wound healing in polychaetes (Nusetti et al., 2005) and induce lesions in the vascular systems of Atlantic silversides (*Menidia menidia*) and eastern oysters (*Crassostrea virginica*) (Gardner et al., 1975).

The occurrence of PAHs in the present study of ULO WAF is of particular interest since they are known to persist in the environment and accumulate in marine organisms (Bechmann et al., 2010; Galt et al., 1991). The low solubility in water and hydrophobicity makes these ring-based hydrocarbons difficult to breakdown in the environment (Martínez-Gómez et al., 2010b). A relation between ring number to turnover rate was found to exist, whereby the number of rings increased persistence in the environment (Meador et al., 1995). PAHs (high ring numbers) typically remain in the water column and attach to particles, slowly settling to the bottom (Lee and Page, 1997; Meador et al., 1995). Particle-bound PAHs can take anywhere from days to years to degrade in aquatic environments (Meador et al., 1995). The benthic sedentary life style of echinoderms makes them particularly suitable to the detection of oil contamination long after the toxicant has been dispersed due to their direct contact with sediment that can contain particle-bound PAHs.

Elevated concentrations of the heavy metal zinc were found in the WAF of ULO studied here, due to its use as an engine oil additive (Vazquez-Duhalt, 1989). The effect

of zinc on echinoderms has typically been tested on gametes and embryos since adults are known to have greater tolerance and are less suitable for short-term experiments and low test-volumes (Bay et al., 1993). Previous studies on the impact of zinc on larval echinoderms have yielded EC_{50} at zinc concentrations orders of magnitude greater than the ones measured here, as well as at concentrations similar (23 $\mu g\ L^{-1}$) (King and Riddle, 2001) to the WAF of ULO (prepared at the high concentration) of this study (60 $\mu g\ L^{-1}$). Due to the greater tolerance of adults and EC_{50} generally being orders of magnitude greater than those in the current study, it is unlikely that zinc was the primary driver of the responses recorded here.

Evidence of physical and behavioural pathology was documented in the high ULO WAF treatment groups, i.e. blistering on sea cucumbers that was particularly prevalent during the depuration period. Furthermore, differences in tentacle deployment behaviour were observed between control and exposed sea cucumbers. The feeding response was strictly observed in control individuals, suggesting its absence can be an indication of stress induced by exposure to oil. While full tentacle deployment was observed to differ between treatment groups, the dominance of this trait switched between trials 2 and 3, indicating it may not be a reliable response. Induction of the mixed function oxidase system (MFO), of which CYP450 1A is a part, is considered a primary response to a pollutant and therefore is expected to occur prior to more serious pathologies (Payne et al., 1987). Additionally, induction of the MFO system at colder temperatures may require a longer period of time due to decreased uptake and distribution of the pollutant (Payne et al., 1987). Since, physical pathologies were documented, it is believed that both the exposure period and experimental temperature were adequate to allow for induction of the

MFO system, if it were to occur. However, evidence of induction was not expressed in the EROD activity measured in the present study.

2.5.3 Drivers of Enzyme Responses to ULO Exposure

Intra-specifically, responses to ULO WAF exposure measured here were affected by the sex of the individual, season of measurement, and varied across tissue types. For instance, in sea stars stomachs, exposure to the high concentration ULO WAF elicited sex-differences in GPx activity, whereby females displayed significantly less activity than males. The fact that such a difference did not occur in the baseline study suggests a complex response to ULO exposure related to energy metabolism and gonad development. Similarly, the gonads of male sea urchins exposed to ULO WAF displayed seasonal variations in EROD activity that were not present in baseline (natural) levels. In particular, EROD activity was suppressed in testes following exposure to ULO WAF in comparison to baseline levels. This response is the opposite of what was expected since induction has previously been observed in fish in response to petroleum pollution (Aas et al., 2000; Gagnon and Holdway, 2000; Upshall et al., 1993). These responses suggest that reproductive status may mediate how enzymes respond to stressors. Again, these variations may reflect a disparity in energy reserves among tissues and between sexes.

2.5.4 Applicability of Examined Biomarkers

When determining the effect of a pollutant it is preferential to use ideal biomarkers, which can provide both high sensitivity and specificity (Livingstone, 1993). Biomarkers with high sensitivity will respond at very low thresholds while those with high specificity will only respond when the correct inducer is present. In the present

study, sensitivity to ULO WAF varied markedly across tissues and species. GPx activity measured in sea star gonads and stomachs emerges as a particularly promising tool for detecting low levels of water soluble hydrocarbons in cold-water environments.

Over the course of the present study, GPx activity was found to occur in all tissues tested, which is a very positive attribute, since widespread activity increases applicability. Secondly, the lack of seasonal variation is very promising, indicating that the sampling period is not likely to affect the detection of the factor of interest (e.g. oil pollution). Notably, the response of GPx to the ULO WAF in the gonads and stomachs of sea stars was sex-specific; i.e. levels in exposed females were significantly lower than in males, which was not the case for baseline activity. While the sex-differences in a response are not necessarily optimal, they can easily be accounted for during field sampling or laboratory experiments. These attributes indicate that GPx activity in sea stars could be used in the measurement of oil exposure. However, further research is needed to determine induction rate and persistence of activity.

The suitability of EROD as a biomarker of hydrocarbon pollution in cold-water organisms was much less conclusive. Seasonal variation in the baseline of EROD activity in sea urchin gonads and brittle star gonads was evidenced, which is not a desirable feature in a biomarker, since it introduces variation that may obscure the detection of the focal effect, i.e. pollutant exposure. This drawback can only be overcome if the sources of variations have been identified, are understood, and can be accounted for (Nahrgang et al., 2013). Lastly, the suppression of EROD activity in exposed testes is the opposite of what would be expected. While the sex-differences in a response are not necessarily optimal, they can easily be accounted for during field sampling or laboratory experiments.

2.6 Conclusion

While it would ideally be possible to choose the conditions under which organisms are exposed to petroleum products, so as to elucidate its effects, the reality is that conditions under which contact occurs can be highly variable and unpredictable. Therefore, prior to an incident (e.g. oil spill, ship sinking, marine accident) for which biomarkers could be used, it is vital to understand all of the factors that govern the activity of focal biomarkers.

The current study outlined the importance of different sources of variation affecting baseline measurements of GPx and EROD biomarker activity in cold-water benthic organisms. In particular, interspecific differences combined with the influence of sex, tissue, and season of measurement were highlighted. Importantly, it confirmed that variability can exist within a single phylum (Echinodermata). It also determined that of the two biomarkers tested, GPx appeared to be the best suited based on its lack of seasonal variability and response to the presence of ULO WAF, particularly for the sea stars tested here.

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Chapter 3: General Conclusions

Petroleum pollution has led to unprecedented levels of hydrocarbons being released into marine environments. While legislation exists to minimize the extent of petroleum release (Farrington, 2013), it still occurs in massive volumes worldwide (Chukwu and Odunzeh, 2006; Ssempebwa and Carpenter, 2009). Visually dramatic (point source) oil spills are often highly publicized; for example, the 2010 Deepwater Horizon oil spill in the Gulf of Mexico and the 1987 spill of the Exxon Valdez off the coast of Alaska. Long-term low-volume releases are comparatively understudied and their effects still remain poorly known (Martínez-Gómez et al., 2010b). Marine pollution through extraction, transportation, and consumption annually releases an estimated 739 million litres of petroleum products into the world's oceans (NRC, 2003), highlighting a need for the identification of biomonitors to detect its effects. As shown in the present study, echinoderms might serve a role in detection of petroleum exposure, due to their global distribution, occupation of ecological niches that are essential for marine ecosystems, and their benthic and sedentary lifestyle (Lawrence, 2001; Mah and Blake, 2012; Ruppert et al., 2004).

Chapter 2 investigated the response of two biomarkers, glutathione peroxidase (GPx) and ethoxyresorufin-*O*-deethylase (EROD), in the reproductive and digestive tissues of four species of echinoderms (*A. rubens, C. frondosa, S. droebachiensis,* and *O. aculeata*). Activity of the biomarkers were recorded over a series of seasons in control and exposed individuals. The exposure treatment involved the water accommodated fraction (WAF) of used lubricating oil (ULO). The GPx assay was used to measure antioxidant activity within the tissues (Ceballos-Picot et al., 1992; Forstrom et al., 1978;

Paglia and Valentine, 1967), while cytochrome P450 1A levels were measured within tissues using the EROD assay (Pohl and Fouts, 1980).

GPx activity was found to be prevalent in the majority of species and tissues, while EROD activity was only expressed in a limited number of tissues and/or species. No natural variations in the activity of GPx were recorded, suggesting a lesser contribution of seasonal or biotic factors on its activity. On the other hand, natural variations in the baseline activity of EROD due to season and sex were measured. With respect to the group exposed to high concentrations of ULO WAF, responses were found to be dependent not only on sex, but season as well. Male sea stars displayed higher GPx activity than females after exposure, which was not found in the baseline study. Additionally, suppression of EROD activity was recorded within the exposed testes of sea urchins. In reference to the two primary sources of variations, the influence of sex can easily be accounted for at the time of sampling, whereas the seasonal variation highlights the need to measure biomarker activity during multiple times of the year.

Of the two biomarkers tested, GPx was found to have the most promising characteristics. (1) It was widely expressed in all species and the majority of tissues. (2) It did not exhibit seasonal variation in the baseline and the sex-based response to the pollutant could easily be controlled for at the time of sampling. Of the focal species, carnivorous sea stars emerged as the best candidates for monitoring cold-water environments, based on high baseline levels of enzyme markers (particularly GPx) and response to oil exposure.

3.1 Future Directions

- The findings presented here indicate that future studies of biomarker activity should ideally consider biotic (age, sex, reproductive status) and abiotic (temperature and trophic activity) factors that have previously been observed to influence biomarker activity.
- Based on the variability shown here around the reproductive activity between January and May, year-round monitoring of natural variations in biomarker activity could be extremely useful. Biotic and abiotic factors may interact in novel ways that may fluctuate over the year based on processes other than reproduction (e.g. cycles of feeding, growth, and prey-predator interactions). Since oil pollution can occur at any time without warning it is essential that we understand how baseline biomarker activity can vary over biologically relevant cycles.
- Additional CYP450 assays, such as ethoxycoumarin-*O*-deethylase (ECOD) and benzyloxyresorufin-*O*-dealkylase (BROD) assays could be tested. These assays use different substrates than the EROD assay used in the present study while still requiring the action of CYP450 enzymes. The level of detected CYP450 activity within the different species could differ based on the substrate.
- Now that candidate species and tissues have been identified as potential cold-water bioindicators, the choice of biomarkers may be refined. Functional genomics tools and techniques (e.g. RNAseq, qPCR) could be used to identify and validate ULO-responsive transcripts (mRNAs) that could serve as molecular biomarkers and shed light on the mode of action. This would allow for the

detection of effects at the earliest stages of induction, instead of relying on the enzymes activity after it has been present in the cell for an unknown period of time. Similarly, testing for the formation of DNA adducts caused by the metabolites of some exogenous compounds would be useful.

- Based on the results of this study, additional species to be tested should concentrate on asteroids (sea stars) and echinoids (sea urchins). Particularly species with wide global distributions, including the Arctic.
- Other pathways of contamination, apart from water soluble oil fraction, could be explored. PAHs with higher ring numbers can attach to particles in the water column and eventually settle to the bottom (Lee and Page, 1997; Meador et al., 1995). Therefore, they can take a very long time to degrade; anywhere from days to years (Meador et al., 1995). The presence of these contaminated particles in the sediment could have a longer lasting influence on the environment than transient soluble pollution. The benthic and sedentary nature of echinoderms makes them well suited to studying this type of sediment-based pollution. Therefore, the use of both field-collected and laboratory-prepared contaminated sediment is proposed. Ideally, sediment and pollutant should reproduce past and present sources of sediment-based pollution.

3.2 References

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Appendix

Table A1: Analysis of tank effects within treatment groups with respect to GPx activity. Symbols indicate which statistical test was performed depending on meeting test assumptions. (#) One-way ANOVA, (*) Welch's ANOVA, (^) Kruskal-Wallis ANOVA on Ranks.

Species	Tissue	Season and Treatment	F or H	df	p
Sea Star	Pyloric Caeca	Spawning Control	* 0.26	3, 2.16	0.851
		Spawning Exposed	* 1.78	3, 2.07	0.374
		Post-Spawning Control	* 3.62	3, 2.14	0.212
		Post-Spawning Depurated Control	^ 0.32	3	0.956
		Post-Spawning Exposed	* 0.49	3, 2.16	0.722
		Post-Spawning Depurated	* 0.43	3, 1.94	0.754
Sea Star	Stomachs	Spawning Control	* 17.83	3, 1.98	0.055
		Spawning Exposed	* 2.90	3, 1.95	0.271
		Post-Spawning Control	^ 0.21	3	0.975
		Post-Spawning Depurated Control	* 0.75	3, 2.01	0.615
		Post-Spawning Exposed	* 0.65	3, 2.10	0.651
		Post-Spawning Depurated	# 0.05	3, 4	0.985
Sea Star	Gonads	Spawning Control	* 0.20	3, 2.11	0.891
		Spawning Exposed	* 3.95	3, 1.67	0.242
		Post-Spawning Control	^ 0.86	3	0.836
		Post-Spawning Depurated Control	^ 2.89	3	0.408
		Post-Spawning Exposed	* 1.03	3, 1.99	0.528
		Post-Spawning Depurated	* 2.08	3, 1.69	0.369
Sea Cucumber	Intestines	Pre-spawning Control	* 0.88	3, 1.69	0.586
		Pre-spawning Exposed	* 3.82	3, 1.74	0.240
		Spawning Control	^ 5.04	3	0.169
		Spawning Exposed	* 1.41	3, 2.14	0.432
		Post-Spawning Control	* 2.51	3, 1.89	0.307
		Post-Spawning Depurated Control	* 0.37	3, 1.67	0.788
		Post-Spawning Exposed	* 0.77	3, 2.00	0.608

		Post-Spawning Depurated	* 3.76	3, 1.74	0.243
Sea Cucumber	Gonads	Pre-spawning Control	^ 1.33	3	0.721
		Pre-spawning Exposed	* 0.20	3, 2.04	0.886
		Spawning Control	* 0.51	3, 1.76	0.720
		Spawning Exposed	* 1.92	3, 2.07	0.355
		Post-Spawning Control	* 5.87	3, 2.01	0.148
		Post-Spawning Depurated Control	* 2.65	3, 1.67	0.318
		Post-Spawning Exposed	* 0.35	3, 1.89	0.799
		Post-Spawning Depurated	* 0.56	3, 2.02	0.691
Sea Urchin	Gonads	Pre-spawning Control	* 0.69	3, 2.06	0.637
		Pre-spawning Exposed	^ 5.16	3	0.161
		Spawning Control	* 0.25	3, 1.68	0.856
		Spawning Exposed	* 0.45	3, 1.73	0.750
		Post-Spawning Control	* 0.69	3, 1.70	0.648
		Post-Spawning Depurated Control	* 0.35	3, 2.04	0.799
		Post-Spawning Exposed	* 0.60	3, 1.97	0.673
		Post-Spawning Depurated	* 0.297	3, 2.02	0.829
Brittle Star	Gonads	Spawning Control	* 1.15	2, 1.14	0.504
		Spawning Exposed	* 0.18	3, 1.97	0.901
		Post-Spawning Control	* 0.32	3, 1.92	0.818
		Post-Spawning Depurated Control	* 0.90	3, 2.13	0.558
		Post-Spawning Exposed	* 2.93	3, 1.67	0.297
		Post-Spawning Depurated	* 0.63	3, 1.68	0.672

Table A2: Analysis of tank effects within treatment groups on EROD activity. Symbols indicate which statistical test was performed depending on meeting test assumptions. (#) One-way ANOVA, (*) Welch's ANOVA, (^) Kruskal-Wallis ANOVA on Ranks. Post-hoc analysis was performed using a Games-Howell test.

Species	Tissue	Season and Treatment		F or H	df	p
Sea Urchin	Gonads	Pre-spawning Control		^ 3.12	3	0.374
		Pre-spawning Exposed		^ 5.08	3	0.166
		Spawning Control		* 0.42	3, 2.04	0.762
		Spawning Exposed		^ 5.91	3	0.418
		Post-Spawning Control		^ 4.22	3	0.239
		Post-Spawning Depurated Control		* 22.65	3, 1.92	0.047
			Tank 9 =11			0.995
			Tank 9 = 13			0.130
			Tank 9 = 15			0.876
			Tank 11 = 13			0.067
			Tank 11 = 15			0.850
			Tank 13 = 15			0.816
		Post-Spawning Exposed		# 0.28	2, 4	0.839
		Post-Spawning Depurated		^ 2.36	3	0.502
Brittle Star	Gonads	Spawning Control		^ 1.76	3	0.623
		Spawning Exposed		^ 1.53	3	0.675
		Post-Spawning Control		^ 0.00	3	1.000
		Post-Spawning Depurated Control		^ 0.00	3	1.000
		Post-Spawning Exposed		^ 0.00	3	1.000
		Post-Spawning Depurated		^ 3.00	3	0.392

Sea Star	Pyloric Caeca	Spawning Control	* 0.67	3, 1.73	0.653
		Spawning Exposed	^ 3.07	3	0.380
		Post-Spawning Control	^ 0.00	3	1.000
		Post-Spawning Depurated Control	^ 2.36	3	0.502
		Post-Spawning Exposed	^ 5.91	3	0.116
		Post-Spawning Depurated	^ 0.00	3	1.000

Table A3: Statistical analyses of GPx and EROD activity across species (combined tissues). Results of a Tukey's test after an ANOVA are given with ordering based on significance. Significant results are shown in bold.

Assay	Species Compared	p
GPx		
	Sea Star > Sea Cucumber	**<0.001**
	Sea Star > Brittle Star	**<0.001**
	Sea Star > Sea Urchin	**<0.001**
	Sea Urchin = Sea Cucumber	0.49
	Sea Urchin = Brittle Star	0.72
	Brittle Star = Sea Cucumber	1.00
EROD		
	Sea Urchin > Sea Star	**<0.001**
	Sea Urchin > Brittle Star	**<0.001**
	Brittle Star = Sea Star	0.46
	Sea Cucumber	N.A.

N.A. – Not applicable