Toxicological effects of MV Rena pollutants to New Zealand fish and lobster

By

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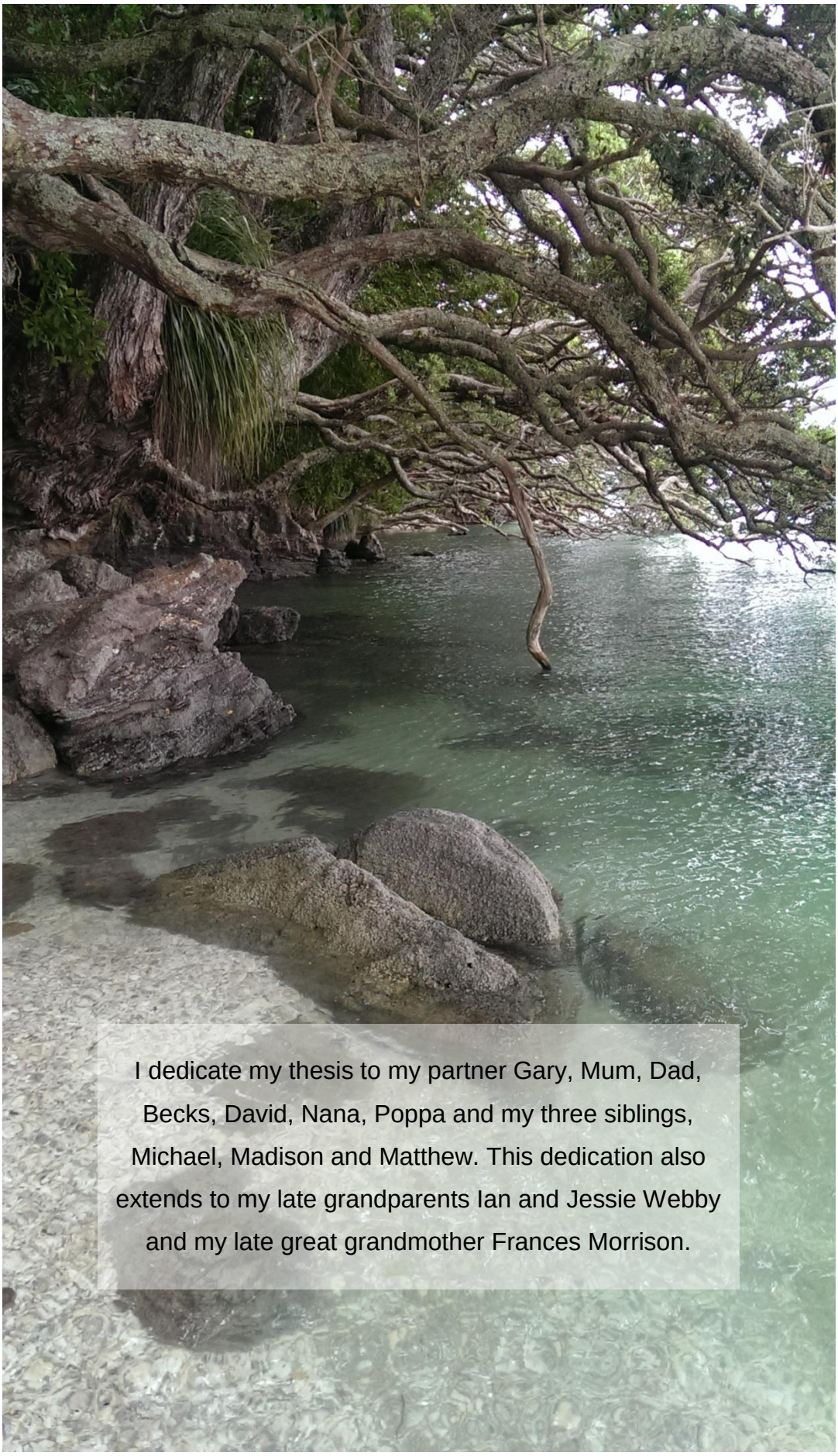
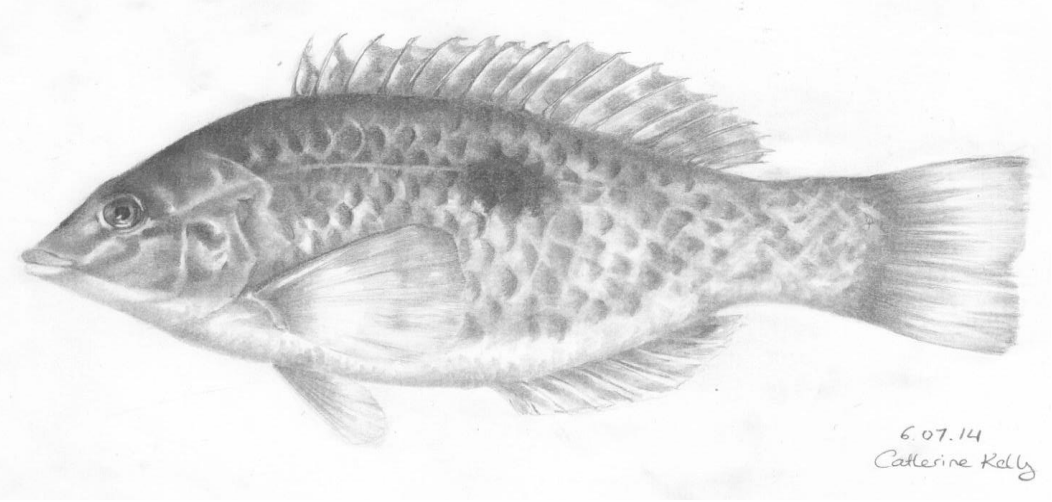


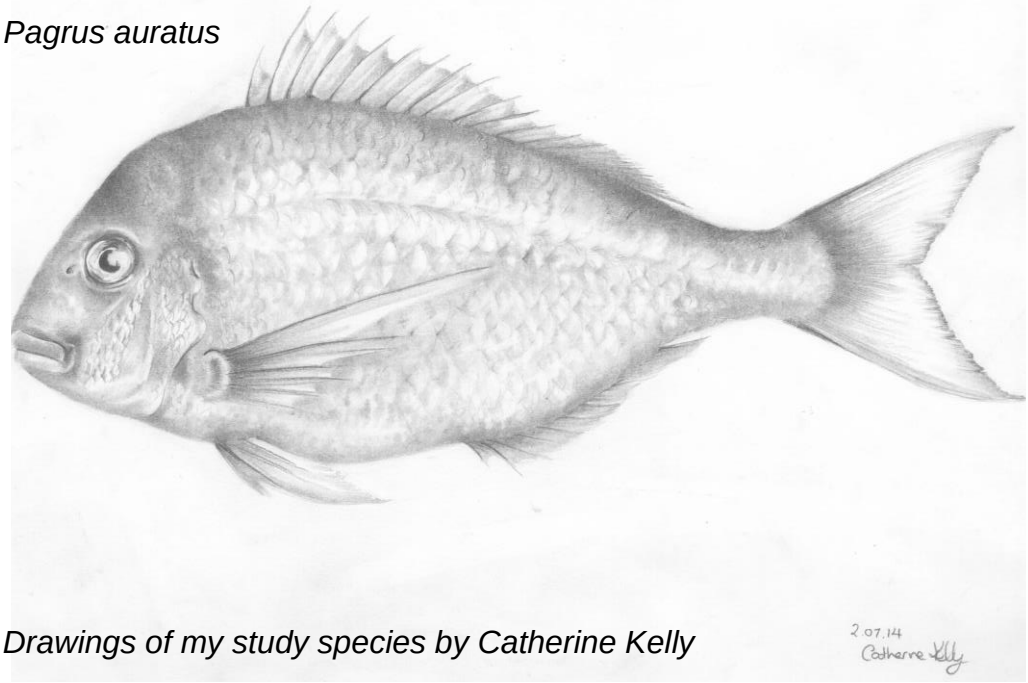
I dedicate my thesis to my partner Gary, Mum, Dad, Becks, David, Nana, Poppa and my three siblings, Michael, Madison and Matthew. This dedication also extends to my late grandparents Ian and Jessie Webby and my late great grandmother Frances Morrison.

Jasus edwardsii

Notolabrus celidotus



Pagrus auratus



Drawings of my study species by Catherine Kelly

Abstract

As part of the Rena Long Term Environmental Recovery Programme commissioned by the Ministry for the Environment in response to the grounding of the MV Rena on Astrolabe Reef (Otaiti), an experimental study of ecotoxicological effects was initiated to examine potential effects of major pollutants discharged from or associated with the Rena shipwreck. This project is one of the first examinations of ship wreck and oil spill pollution in New Zealand. Research will aid responses to future oil pollution events such as ship groundings and oil industry pollution.

The container ship *MV Rena* ran aground on Astrolabe Reef in the Bay of Plenty in October 2011 and discharged approximately 350 tonnes of heavy fuel oil (HFO). During the response HFO was treated with approximately 3 m^3 of the oil dispersant Corexit 9500 at sea. Other pollutants associated with the Rena grounding included general cargo and other goods classified as environmentally hazardous, in particular, 560 tonnes of sodium hexafluoroaluminate or cryolite. Given the almost total absence of toxicity data relevant to HFOs, Corexit 9500 and other contaminant mixtures on New Zealand marine species, this project sought to assess the acute sublethal toxicity of these contaminants to a range of culturally, ecologically and commercially important species.

Sub-adult snapper (*Pagrus auratus*), spotted wrasse (*Notolabrus celidotus*) and red rock lobster (*Jasus edwardsii*) were exposed to 1:1000 cryolite, 1:10000 HFO (HFO WAF), 1:400000 Corexit 9500 and 1:40 HFO/Corexit 9500 combination (HFO CEWAF: 1:10000 HFO, 1:400000 Corexit 9500) for up to 96 h followed by recovery for up to 10 d. These concentrations and exposure durations were viewed as being environmentally realistic. Fish were necropsied and examined for haematology and accumulation of polycyclic aromatic hydrocarbons (PAHs).

Exposure to toxicants caused no lethality but did indicate sub-lethal effects. Measurements of blood parameters indicated two main effects in

fish of erythrocyte swelling and haemoconcentration. The overall pattern of response in fish appeared to be a greater degree of erythrocyte swelling in response to the HFO WAF treatment. HFO WAF and HFO CEWAF treatments caused changes in leukocyte differential counts indicating negative responses of immunosuppression in fish. Corexit 9500 and cryolite exposure caused negligible/minimal changes in haematology in all species. Haematological assessment of *Jasus edwardsii* indicated immune effects of HFO WAF, HFO CEWAF and cryolite exposure as evident by changes in differential haemocyte counts.

Bile PAHs in fish and red rock lobster reached levels several orders of magnitude higher than controls. Corexit 9500 increased PAH body burden when combined with oil, however in fish it also appeared to accelerate depuration of PAHs during post-exposure recovery. Depuration was not as evident in red rock lobster. Exposure to HFO appeared to stimulate an increase in melanomacrophages in the spleen of *Pagrus auratus*. Overall HFO exposure with or without the addition of Corexit 9500 caused apparent sublethal changes in haematology that mostly recovered by 10 d post-exposure.

Acknowledgments

I am indebted to a number of people that have helped me to produce my thesis to its final state. My upmost gratitude goes to my supervisor Associate Professor Nicholas Ling. A master's thesis is never complete without a supervisor's high level of patience, assistance, guidance and advice. I thank you for your teaching efforts and always answering my questions no matter how ignorant, stupid or repetitive they may have seemed. Thanks for all the help in the lab and with my data analysis. Secondly, I'd like to acknowledge by my secondary supervisor Professor Chris Battershill for all of his teaching, editing and guidance, as well as being there whenever I needed help. Thanks for the messages of support and encouragement. I particularly acknowledge the easy-going nature of both of you and the ample supply of helpful information you supplied always.

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helped me with my research; helped in the lab and provided valued friendship. I am truly blessed to have you all as friends. Each of you have helped me in many ways; whether it was in the lab, in the field, proof reading or just being a great friend. You guys always provide great laughs and are great support. In particular I'd like to thank Andrew Daly, Sarah Cross, Nikki Webb, Alicia Catlin, Jordan Cooper, Kate Pepper, Ryan Mallet, Jeremy Garrett-Walker and mum, for their help with my practical work and editing my thesis. I would also like to thank Caleb Valler for his expert fishing skills and his lab assistance. I thank my friends and fellow R block students for many great memories and crazy adventures we had – these were a great distraction and allowed me to take a break from my thesis.

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expertise, and your help. Whether it was helping me to collect animals, helping to look after them, keeping me safe in the lab or offering assistance, guidance and friendship; I am grateful for all that you have done. Thank you to Gloria, Vicki, and Gillian in the school of science office for all of their help and guidance.

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Last but certainly not least, I would like to acknowledge the Rena Recovery Program for awarding me a scholarship to complete my research and the Bay of Plenty regional council for the funding I received in 2014.

Table of contents

List of Figures

List of Tables

Chapter One: General Introduction

1.1 The Rena Oil Spill

On the 5th of October, 2011, the 37,209 t container ship the MV Rena ran aground on Astrolabe Reef (Otaiti) in the Bay of Plenty (WGS84: 37°33′37″S 176°23′47″E) (Figure 1.1). The disaster occurred at 0214 as Rena was travelling at 17 knots carrying 1,368 containers and approximately 1,733 t of heavy fuel oil (HFO) used to fuel the ship. Rena struck the reef as the ship's course had been allegedly altered trying to reach the window for pilotage into Tauranga harbour which closed at 0300 (Transport Accident Commission 2011; Maritime New Zealand 2014).

Figure 1.1: Rena aground on Astrolabe reef. Image credit: Allan Gibson.

During the initial grounding the hull was damaged and an oil leak was confirmed from Rena on the 6th of October. Maritime New Zealand declared the disaster as a tier three emergency, the highest level of response to an oil spill (Maritime New Zealand 2014). Salvors began removing oil from the ship on the 9th of October however, bad weather, equipment breakdown and hazardous changeable conditions halted salvage efforts. As a consequence, an overnight storm on the 11th of

October resulted in the loss of around 350 t of HFO from the wreck, with a further estimated 5 – 10 t lost from the vessel overnight on 22nd – 23rd October (Maritime New Zealand 2014).

1.1.1 Oil spill response and salvage

In aid of the Rena oil spill around 3 m^3 of the oil spill dispersant Corexit 9500 was applied offshore in an attempt to disperse oil from the surface waters in order to reduce negative impacts to seabirds and other surface associated species such as marine mammals. At the height of the response, around 9,500 people were involved, which consisted mostly members of the public but also included a team of about 90 salvors from Svitzer and Smit International (The Rena Project 2014), who were later replaced by the Resolve Salvage Company. Around 1,000 t of oily waste were recovered from the Bay of Plenty open coastal beaches, and over 1,300 t of HFO was eventually recovered from Rena along with more than 500 tonnes of debris (The Rena Project 2014).

Of the 1,368 containers on board, 814 containers were stored below deck, 121 contained perishable food stuffs and 32 contained dangerous goods (Maritime New Zealand 2014). Of these, of particular concern was the estimated 560 t of potentially toxic cryolite on board. Salvors have since recovered 1,039 containers leaving 329 unrecovered. A total of 58 containers were located on the seabed, with the other 271 either trapped in inaccessible parts of the wreck, lost at sea or lying unidentified in the debris field where the sea has largely reduced them to scrap (The Rena Project 2014).

Much of the shipwreck still lies on the ocean floor on Astrolabe Reef, with the deepest part of the ship lying at a depth of 65 m. The bow section has been reduced to 1 m below the surface of the lowest astronomical tide and the 11,000 t stern section is fully submerged resting at a 55-60 degree list on the reef in 23 m of water (Rena Project 2014). Around the shipwreck there is a debris field that covers approximately 10,000 m^2 with the majority lying on parts of the reef above 30 m. The field contains two main types of debris; scrap metal from the ships structure, containers and

cargo; and material such as packaging and timber (Rena Project 2014). As the stern section sank it was estimated that there was approximately 0.4 m^3 of hydraulic oil, 5 m^3 of diesel oil and 2 m^3 of HFO adhered to the sides of tanks and pipelines which was predicted to slowly release overtime (Rena Project 2014).

1.1.2 Rena response and the legacy of the Rena pollutants

There are a number of pollutants connected with the Rena oil spill that have the potential to cause deleterious effects on marine organisms and the environment. Toxicologically, of most concern are effects from HFO, Corexit 9500, and cryolite. These pollutants were of primary concern as they were considered to be the major contaminants associated with the spill in terms of volume and reported 'potential' toxicity.

The Rena grounding was arguably one of New Zealand's worst maritime environmental disasters. There was considerable Moana a Toi Iwi and public concern about the potential effects from the contaminants from Rena, and in response to the oil spill, it became clear that New Zealand was not well prepared for such an incident, particularly in terms of being able to provide predictions of likely ecotoxicological effects. Literature within the field was absent or sparse and relative field data was fundamentally absent (Te Mauri Moana 2013). Addressing the lack of information on the possible environmental effects of HFO and contaminants mixtures was deemed to be of upmost importance.

Due to the large amount of uncertainty, it was necessary to carry out appropriate research during the environmental response to the Rena disaster, using the situation as a rigorous case study (Te Mauri Moana 2013). The Rena Recovery Programme was set up to further our understanding of the effects of contaminant mixtures in the marine environment and to investigate the lethal and sub-lethal responses of important New Zealand marine organisms to pollution. The aim was to provide answers to the numerous questions raised by the public. The first few phases of the investigation are now published in the form of a report that reviews the environmental effects and recovery.

1.2 Heavy fuel oil

1.2.1 Heavy fuel oil

Heavy fuel oil (HFO) is the most commonly used marine fuel oil used to power large marine vessels. It is a general term used to describe a range of products including residual fuel oil, bunker fuel, bunker C, fuel oil No 6, industrial fuel oil, marine fuel oil and black oil (CONCAWE 1998). Heavy fuel oils are produced from residuals and distillates derived from crude oil refining (CONCAWE 1998; American Petroleum Institute Petroleum HPV Group 2012).

The main constituents of fuel oil can be grouped into four categories: saturates, aromatics, resins and asphaltenes, which are combined with smaller amounts of heterocyclic compounds containing sulphur, nitrogen and oxygen (Uhler et al. 2007). Heavy fuel oils are a complex mixture of high-molecular weight compounds consisting of aromatic, aliphatic and naphthenic hydrocarbons, typically having carbon numbers from C_{20} to C_{50} (Figure 1.2) (boiling range from 350 to 650°C) depending on the manufacturing process (Uhler et al. 2007). They also contain trace amounts of metals such as vanadium, nickel, iron and copper. Lower molecular weight material may also be added to reduce viscosity and improve flow characteristics (McKee et al. 2014).

Terms such as heavy fuel oil, medium fuel oil and light fuel oil are used to describe commercial fuel products and give a general indication of the viscosity and density of the product (CONCAWE 1998). The viscosity value is the controlling product specification which is noted after the oil type. Higher numbered oils contain higher boiling point components and are more viscous than lower numbered oils. The number indicates the maximum viscosity of that oil at 50°C; i.e. HFO380 is heavy fuel oil with a maximum viscosity of 380 $mm^2/_s$ cSt (Uhler et al. 2007). The viscosity of any petroleum oil increases when cooled and decreases when heated.

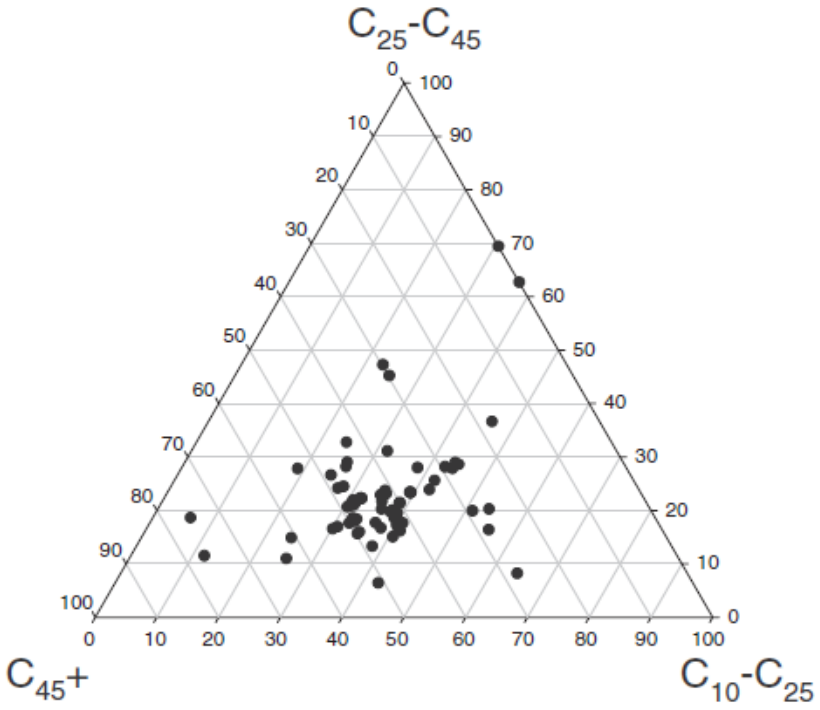


Figure 1.2: A ternary diagram taken from Uhler et al. (2007), illustrating the variability among 71 heavy fuel oil 380 samples showing the large variation in the bulk hydrocarbon composition.

1.2.2 The toxicity of oil

Heavy fuel oil may be up to 50 times more toxic than lighter crude oils (Bornstein et al. 2014; Martin et al. 2014). There are many physical, chemical and biological factors that affect oil toxicity, including state of weathering, water salinity, wave energy, light, shore type, pH, topography, sulphides, phenols, ammonium compounds, suspended material, dispersants, temperature, species, sex, age, and resilience of the ecosystem (Baker 1978; Hylland 2006). These factors have the potential to increase or decrease the toxicity of oil and they demonstrate the wide range of factors that govern oil toxicity along with oil type.

There are a number of ways in which oil exposure can occur, whether it is through physical contact, ingestion, absorption, smothering, toxic contamination or via a trophic cascade (Fingas 2000). Oil can have deleterious effects on a number of organisms which are susceptible to toxicity in different ways. The broad range of impacts can include (but are

not limited to); physical or chemical alteration of natural habitats, toxicological lethal or sub-lethal effects, changes in biological communities, altered metabolic and/or feeding rates, physiological disruption, immune suppression, changes in behaviour and reproduction, asphyxiation via oil slick coating and bioaccumulation (Dicks 1999; Fingas 2000; Kazlauskienė et al. 2004).

Neustonic organisms (organisms living at the water-air interface) are typically most at risk of contamination (Hylland 2006). They are subject to surface contamination from floating oil and oil entrained into the wave mixed layer by breaking waves (French-McCay 2011). Toxic components from oil may be absorbed by organisms in the neuston which often carries a large number of eggs and larvae which are generally more susceptible to toxic substances than adults due to a more permeable integument and lack of development (Fingas 2000; Couillard et al. 2005). Birds and marine mammals are at particular risk from either smothering, ingestion or other toxic effects when they breach or land on the sea surface (Hylland 2006).

When oil is dispersed, both physically and chemically, it may become more available to pelagic organisms. This dispersion can also enhance uptake of contaminants via the gills (Liang et al. 2007). Similarly, when dispersed oil mixes with sand and other debris it can sink increasing availability to benthic organisms (United States Environmental Protection Agency 1999; Peterson et al. 2003). Sessile animals are at particular risk as they are unable to avoid contamination, making them highly vulnerable to smothering. As oil washes ashore it can become highly concentrated and absorb into sediments affecting a range of invertebrate species living in inter-tidal areas (Fingas 2000).

There is no shortage of literature examining the potential toxicity of oils to marine organisms, (Knap et al. 1983; Carls et al. 1999; Kazlauskienė et al. 2004; Fodrie & Heck 2011; Martin et al. 2014; McKee et al. 2014) although much of the research tends to focus on the more vulnerable earlier life stages such as larval and embryonic development. Much of the literature evaluates the effects of crude oil spills, however, peer reviewed literature

on HFO is harder to come by. Many papers also discuss the various effects of oil with the addition of oil spill dispersants which have been known to increase the toxicity of oil (discussed further in section 1.3), as well as the effects of oil weathering on oil.

1.2.2.1 Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a diverse group of hydrocarbons. The simplest aromatic hydrocarbon is benzene which is an organic compound consisting of six carbon atoms connected to six hydrogen atoms in a circular arrangement known as a benzene ring. The configuration of two or more benzene rings is called a polycyclic aromatic hydrocarbon.

PAHs range from two-ring naphthalenes to complex ring structures containing up to ten rings (Figure 1.3) (Hylland 2006; Pampanin & Sydnes 2013). PAHs are found in fossil fuels and include a diverse range of hydrogen and carbon chemical structures which vary significantly in molecular weight (Hattori et al. 1998). Considerable concentrations of PAHs can be present in heavy fuel oil, depending on the nature and amount of low viscosity diluent used and whether the residue component is cracked or un-cracked (CONCAWE 1998). PAHs are recognized as the most acutely toxic hydrocarbons for aquatic organisms and many of them are known to have toxic, mutagenic and/or carcinogenic properties (Hylland 2006). Oil toxic effects are generally attributed to elevated levels of PAHs and alkyl PAHs (Bornstein et al. 2014).

Although found naturally in the environment, anthropogenic activity has largely increased environmental levels of PAHs, which are increased further by oil spill pollution (Hylland 2006). There are four categories of PAHs: biogenic - those produced by organisms; pyrogenic – those produced from incineration products; petrogenic – those produced from fossil fuels; and diagenic – those derived from transformation in the soils and sediments (Hylland 2006). These four processes produce different PAHs and it is generally possible to identify the relative contribution from each PAH source in an environmental sample (Hylland 2006).

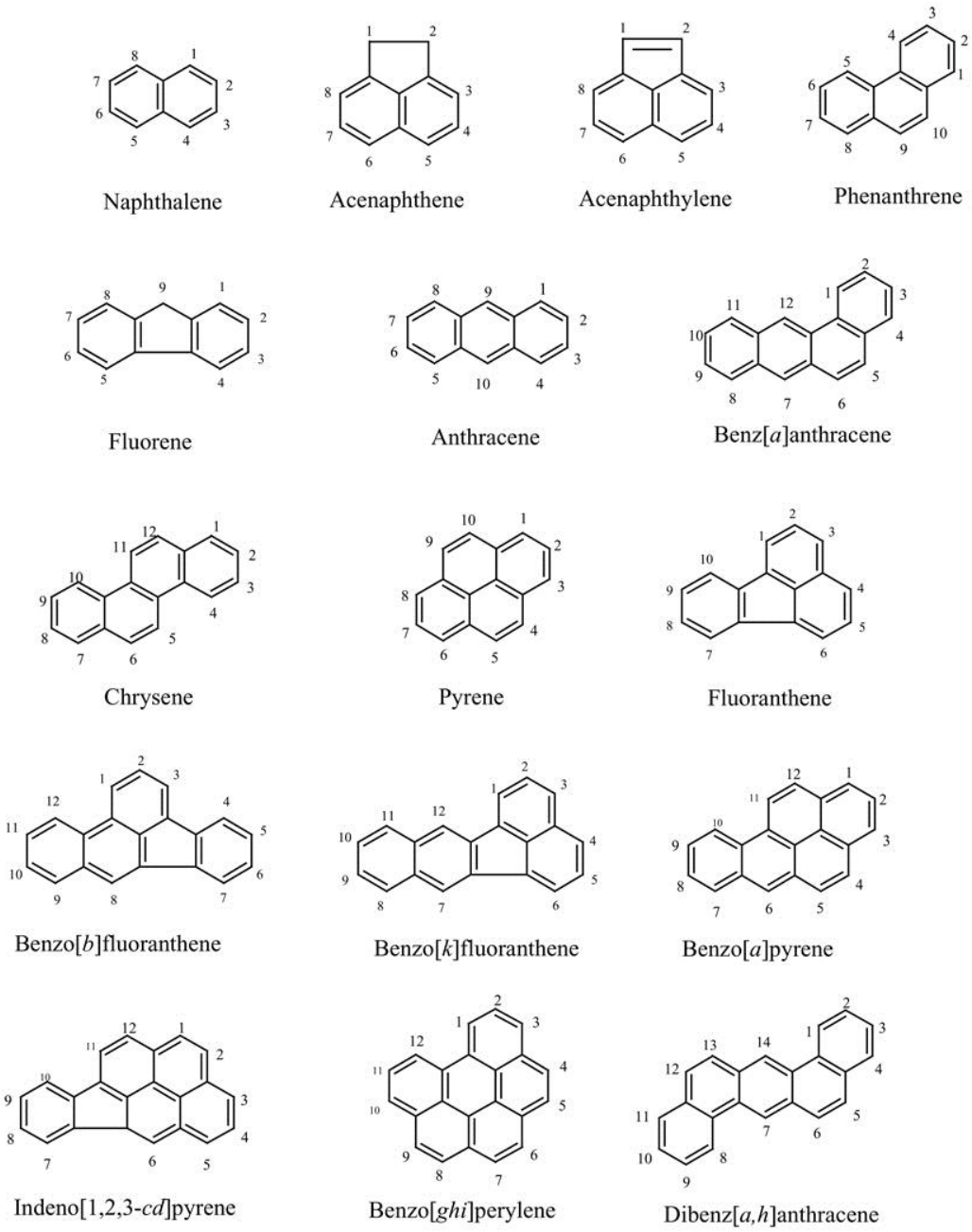


Figure 1.3 The chemical structure of 16 commonly occurring PAHs on the priority pollutant list produced by the USEPA. Image obtained from Yan et al. 2004.

The potential for exposure and accumulation of PAHs is governed by the bioavailability and solubility. The uptake route for more water soluble PAHs is thought to occur through the gills in aquatic organisms; however the uptake of other individual PAHs by respiration will depend on the K_{ow} and degree of alkylation. A PAHs hydrophobic character is represented by the octonal water co-efficient (K_{ow}) (K_{ow} - A coefficient representing the ratio of the solubility of a compound in octanol (a non-polar solvent) to its

solubility in water (a polar solvent)). Bioavailability will vary with each PAH compound as generally solubility decreases with increasing K_{ow} and molecular weight. Furthermore, PAHs have a high affinity for particles and organic material (Baumard et al. 1999; Hylland 2006) meaning that the presence of organic matter in the water column or sediments may influence bioavailability (Logan 2007).

Low-molecular weight hydrocarbons, with one or two benzene rings, are relatively water soluble and quickly reach high concentrations in the water column a few hours after an oil spill. They are not considered to be persistent in the environment because they are susceptible to physical processes such as dilution and evaporation (Couillard et al. 2005). However, low-molecular weight PAHs remain an environmental concern as they are capable of penetrating cell membranes and causing toxicity through a nonspecific mode of action, such as narcosis (Couillard et al. 2005).

High-molecular weight PAHs are less water soluble, more persistent, and 10 to 1,000-fold more toxic than low-molecular weight PAHs (Couillard et al. 2005). The low solubility of high-molecular weight compounds makes them relatively unavailable for uptake by organisms. Intermediate molecular-weight PAHs on the other are easily absorbed by organisms due to the high K_{ow} that favours the rapid transfer from the aqueous phase to lipid-rich tissues (Sette et al. 2013). Hence, medium-molecular weight aromatic compounds are arguably of most concern (Hylland 2006; Uhler et al. 2007; Martin et al. 2014).

Polycyclic aromatic hydrocarbons are common micro-pollutants and their hydrophobic character and low biodegradability and solubility can cause accumulation of these compounds in organisms beyond their concentration levels within the environment; this process is known as bioaccumulation and may include direct uptake from the environment (bioconcentration) and/or uptake by ingestion of contaminated food (biomagnification) (D'Adamo et al. 1997). Bioaccumulation is dependent upon uptake and depuration rates. If the accumulated substances are

stored within the body and not broken down by cellular process then a high level of toxicant will be transferred to organisms higher in the food chain (biomagnification) (Hylland 2006). PAH accumulation within the body will typically correlate with tissue lipid content with consequently high levels in fat, liver, brain, etc (Logan 2007).

1.2.3 Oil degradation

Annual estimates of petroleum discharge or spills into the sea by commercial vessels worldwide is estimated at 1 million t per year (Uhler et al. 2007). In comparison it is estimated that natural crude oil seepage accounts for 47% all of oil entering into the marine environment worldwide (Kvenvolden & Cooper 2003). Previous oil spill events have demonstrated large diversity in behaviours and fate of oil when spilt at sea (Centre of Documentation Research and Experimentation on Accidental Water Pollution 2006). The effects of oil vary depending on the character of the spillage, dispersion rate, the type of oil, the location of the spill, the organisms involved, breeding cycles, seasonal migrations and adhesion to surfaces that may affect the partitioning rates of the toxic components into water (Hylland 2006; Martin et al. 2014).

Immediately after oil is spilt, it undergoes a number of chemical and physical changes, known as weathering, which alter or remove many of the chemical constituents (Abdallah et al. 2005; Hylland 2006; Uhler et al. 2007). Among these processes are: evaporation, dissolution, dispersion, tar lump formation, photo-oxidation, polymerization, microbial degradation, emulsification, sedimentation and biodegradation (Figure 1.4) (Abdallah et al. 2005; Hylland 2006; Gong et al. (In Press)). Lighter aromatic compounds immediately begin to evaporate or dissolve and are diluted or lost to the atmosphere within minutes or hours, while the remaining constituents may float, sink or become incorporated into the sediments (CONCAWE 1998). Heavier aromatic compounds are more resistant to weathering as they are very insoluble.

Biodegradation, the process by which microorganisms break down oil, is a major mechanism for removal of spilled petroleum hydrocarbons from

aquatic environments (Lindstrom & Braddock 2002). Low-molecular weight compounds are preferentially degraded by microorganisms and when these are depleted they begin to degrade more complex molecules (Pampanin & Sydnes 2013). High-molecular weight aromatics exhibit extremely low rates of biodegradation (Abdallah et al. 2005).

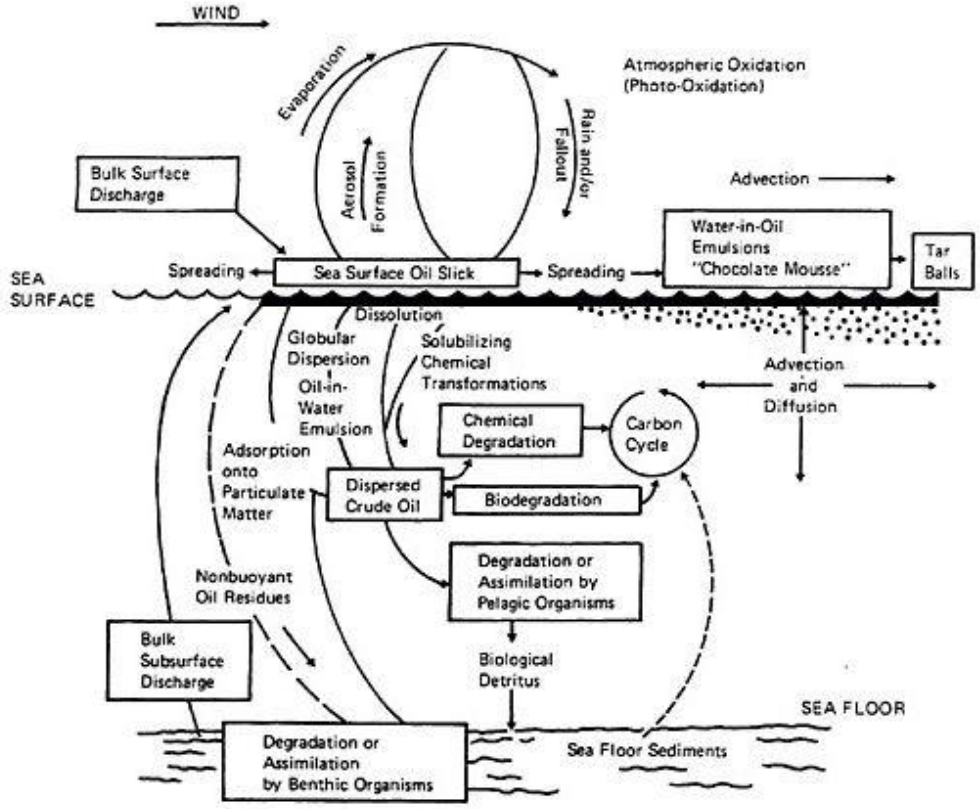


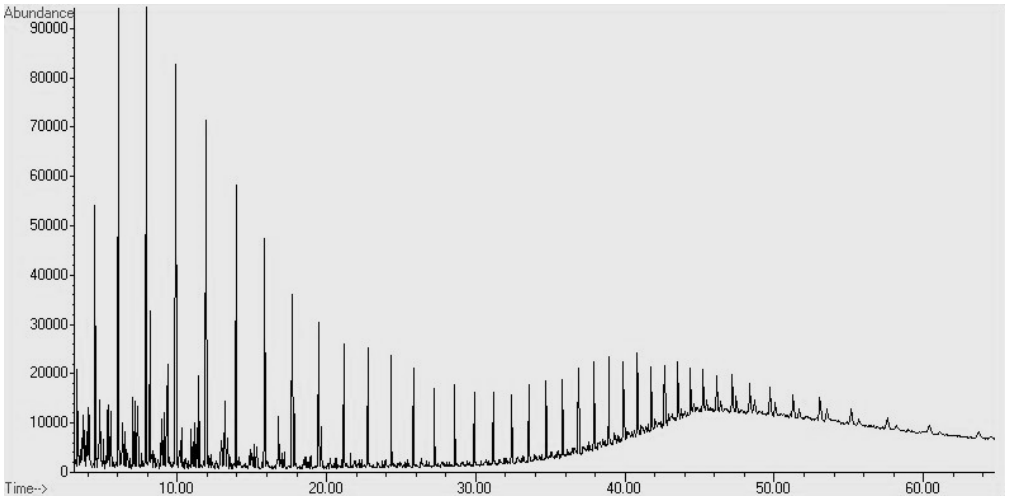
Figure 1.4: A diagram of the interactions of oil in the aquatic environment. Diagram obtained from The National Research Council 2005.

When released into the environment, HFO will break into small masses and does not spread as rapidly as less viscous oil. The density of some HFOs means that they have a higher tendency to sink resulting in the potential for oil to mix with the sediments and become available to benthic marine animals (CONCAWE 1998). Crude oil behaves differently in aquatic environments compared to HFO due to the greater amounts of low density components. Upon weathering, crude oil may lose up to 40% of its weight compared to HFO which may lose only 2.5%, demonstrating that weathering is often not an effective measure in the dissipation of HFO (Martin et al. 2014).

1.2.3.1 Rena polycyclic aromatic hydrocarbons and weathering

Rena HFO fingerprinting analysis was carried out by Professor Alistair Wilkins at the University of Waikato. Initial results from the Rena tank oil (Figure 1.5A) showed a greater abundance of low-molecular weight compounds (Wilkins 2013). Over time the substantial loss of low to medium-molecular weight hydrocarbons is apparent while higher molecular hydrocarbons were more persistent (Figure 1.5B & C). Results indicated that depleted hydrocarbons in a sample of oily sediment from Papamoa Beach (26/10/2012) were greater than could be attributed to loss by evaporation to the atmosphere alone indicating that the oil had undergone a number of weathering processes (Wilkins 2013).

A)



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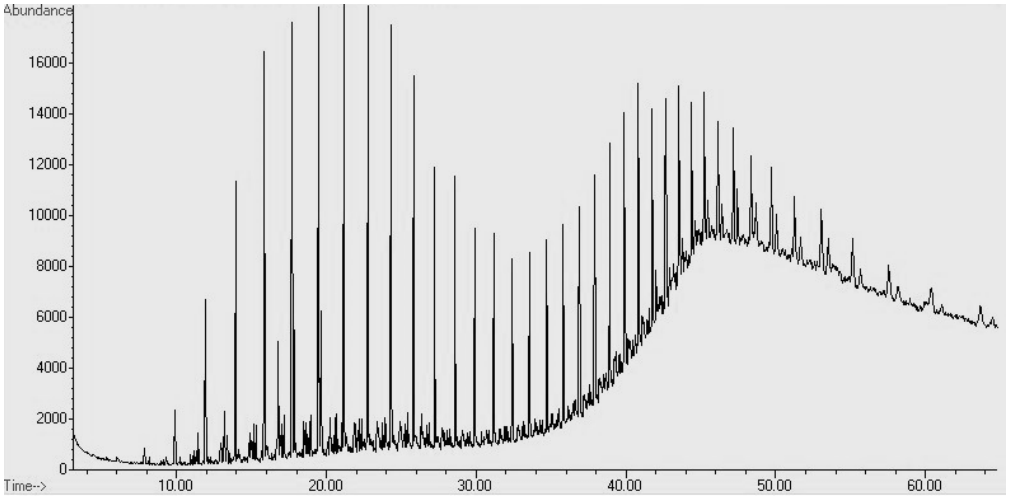

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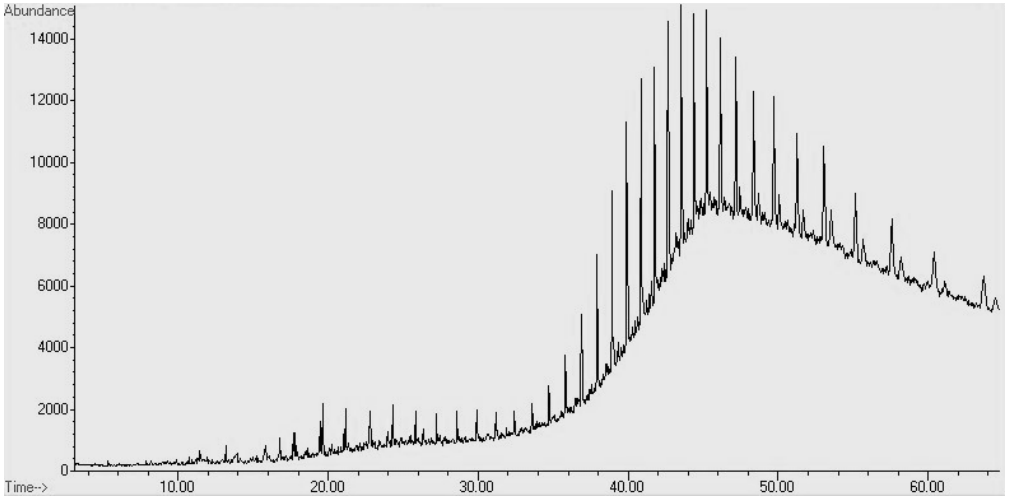

Figure 1.5: Gas chromatography-mass spectrometry (GC-MS) ion m/z 57 ion profiles determined for hydrocarbons (alkanes). A) Rena tank oil B) Papamoa Beach, Taylors Reserve, tarball 27/10/2011. C) Papamoa Beach, Concord Ave, oily sediment (100 mm), 26/10/2012. Analysis by Professor Alistair Wilkins.

1.3 Oil spill dispersants

The use of dispersants is a widely employed and immediately effective method of combating oil pollution on water surfaces (Abdallah et al. 2005). Dispersants are a combination of solvents, surfactants and additives designed to accelerate the dispersion of oil slicks into fine droplets (Fiocco & Lewis 1999; National Research Council 2005). The essential components are surfactants, which contain both an oil-compatible (lipophilic) and water-compatible (hydrophilic) group (Chandrasekar et al. 2006).

Each small oil droplet is covered by a layer of surfactant molecules which prevents the droplets from recombining and keeps them dispersed in the water (Figure 1.6) (Fiocco & Lewis 1999; Schmidt-Etkin 2011). Most dispersant formulations consist of mixtures of one or more surfactants, which may be either nonionic or anionic. Solvents are added primarily to promote the suspension of surfactants and additives into a homogeneous dispersant mixture. In addition to keeping the surfactants in solution, these solvents reduce the product's viscosity and affect the dispersant's solubility in oil (National Research Council 2005). Additives are present for a number of purposes, such as improving the dissolution of the surfactants into an oil slick and increasing the long-term stability of the dispersant formulation (National Research Council 2005).

Over time, oil will break up and disperse naturally via wave action and weathering, however this process is often time consuming and inadequate. As a dispersant is applied to an oil spill, it diffuses through the oil and reduces the interfacial tension between the oil by orienting the hydrophilic groups to interact with the water and the hydrophobic groups to interact with the oil, lowering the energy needed to mix the oil into the water (Fiocco & Lewis 1999). The formation of tar balls and droplets generated by dispersants are typically much smaller than would otherwise form by the natural energy of the sea (Fiocco & Lewis 1999). Dispersion also reduces surface drift and oil exposure to marine birds and mammals along with reducing adhesion to surfaces (Brandvik & Daling 1998).

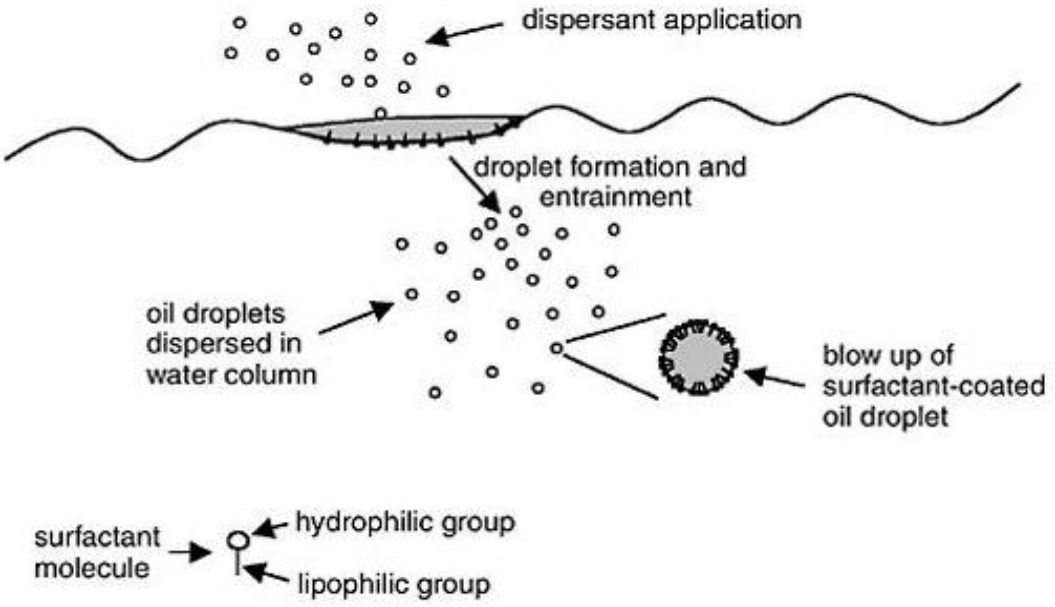

Figure 1.6: The mechanism of chemical dispersion: dispersants disperse oil into small droplets and surfactants bind to oil molecules. The enlarged oil droplet indicates the orientation of the hydrophilic group projecting into the water phase and the lipophilic group projecting into the oil phase. Diagram obtained from the National Research Council 2005.

Factors influencing dispersant effectiveness include oil composition, sea energy, state of oil weathering, type of dispersant used, the amount applied, temperature and the salinity of the water (Abdallah et al. 2005; Chandrasekar et al. 2006; Chapman et al. 2007; Schmidt-Etkin 2011; Campo et al. 2013). Several laboratory studies have demonstrated that there is a large variation in effectiveness and performance of dispersants (Brandvik & Daling 1998; Abdallah et al. 2005; Chapman et al. 2007; Belore et al. 2009). Dispersants are not universally effective on all types of oil as oils with higher viscosity mixtures are not as readily dispersed as lower viscosity oils(Fiocco & Lewis 1999).

The recommended dispersant application in New Zealand is a ratio of 1:20 (oil: dispersant) (Stevens 2006). This concentration is dependent on oil type, thickness of the slick, sea temperature and the degree of oiling and weathering. Only dispersants approved by Maritime New Zealand and the marine protection rules (Part 132, 1998) may be considered for use in New Zealand waters (Stevens 2006). Currently there are 23 approved dispersants, six of which are held in stock: Corexit 9500, Corexit 9527,

Gamlen Oil Dispersant LT, Tergo 2-005, Slickgone LTSW, and Shell Dispersant VDC. Each of these has been tested for effectiveness against different types of fuel oils in both marine and freshwater environments (Stevens 2006).

The decision to use any dispersant during a spill response is only made after full consideration of the factors which affect dispersant properties and an evaluation of the environment. This allows the selection of a dispersant that is formulated to be effective with regard to oil type and environmental conditions. Decisions should be made regarding the impact on ecosystem functioning after consideration of trade-offs including ecological, social and economic values that may be associated with the potentially affected resources. The comparison of possible ecological and socio-economic consequences versus the toxicological impacts can be difficult due to conflicting interests (Brandvik & Daling 1998; National Research Council 2005).

Net Environmental Benefit Analysis (NEBA) principles are applied before dispersant use. These principles focus on the toxicity of dispersed oil in the water column compared to potential impacts that might occur if the oil persists on the water or adheres to the shoreline. The primary aim of this analysis, and the use of dispersants, is to minimise the environmental harm caused by the oil spill (Stevens 2006).

1.3.1 Toxicity of dispersants and dispersed oil

Dispersants have been used in oil spill clean-up since the 1950s to help minimise the impact of oil spills, although there continues to be some reluctance surrounding their use (Belore et al. 2009). They have been labelled as controversial due to the potential for increased exposure to dispersed oil and toxic effects from the dispersant itself (Ramachandran et al. 2004)

There are a number of factors which will influence the toxicity of dispersants such as temperature, life stage, exposure duration, species and salinity (George-Ares & Clark 2000). The risk of ecological effects will

depend on whether oil dispersion increases or decreases the exposure of aquatic species to the oils toxic components (Ramachandran et al. 2004; Chapman et al. 2007). The effects of oil spills on shorelines and megafauna have been well documented. The potential effects on benthic fauna, fish populations and trophic relationships are much less studied (National Research Council 2005).

Many of the early dispersant formulations (prior to 1970) were solvent based degreasing agents. These dispersants proved to be highly toxic (examples in: Swedmark et al. 1973) to aquatic organisms as seen following treatment of the 1967 Torrey Canyon spill (Portmann & Connor 1968; Swedmark et al. 1973; Southward & Southward 1978; Lessard & DeMarco 2000). This incident resulted in a highly unfavourable view of dispersants by the public which still exists today despite a number of studies demonstrating that modern dispersants are relatively benign compared to their predecessors (Mitchell & Holdway 2000; National Research Council 2005).

Although exposure of oil to aquatic organisms in the water column is temporarily increased following dispersant application, dispersed oil is subject to greater advection and dilution by currents and wave action, which accelerates biodegradation (George-Ares & Clark 2000). Studies have shown that dispersion can increase the rate of microbial degradation and the number of degenerated oil droplets (Swannell & Daniel 1999; Lindstrom & Braddock 2002).

1.3.2 Corexit 9500

In aid of the MV Rena HFO spill, approximately 3 m^3 of Corexit® 9500 was applied to the oil slick. Corexit is a product line of oil spill dispersants produced by NALCO Environmental Solutions LLC (NALCO Envionmental Solutions 2011). NALCO produce two variants of Corexit used to disperse oil spilt at sea: Corexit 9527 and Corexit 9500 (formally known as EC9500A) both of which are listed on the Environmental Protection Agency National Contingency Plan Product Schedule (NALCO Envionmental Solutions 2011). The difference between the two Corexit

variants is the 2-butoxyethanol solvent present only in Corexit 9527 (NALCO Envionmental Solutions 2011).

Both products contain a mixture of nonionic (48%) and anionic (35%) surfactants (Table 1.1) Corexit 9527 was developed in the 1970s whereas Corexit 9500 was reformulated in the 1990s using a different solvent because the prolonged exposure of Corexit 9527 caused adverse health effects which were attributed to its glycol ether solvent (2-butoxyethanol) content (George-Ares & Clark 2000). Moreover, the change in formulation extended the window of opportunity for dispersant use and is able to be applied to more varieties of oil. Corexit 9500 has been shown to be slightly more effective against high-viscosity oils compared to Corexit 9527 (George-Ares & Clark 2000; National Research Council 2005).

Table 1.1: Corexit ingredient list obtained from NALCO Environmental Solutions 2011.

Cas #	Name
1338-43-8	Sorbitan, mono-(9Z)-9-octadecenoate
9005-65-6	Sorbitan, mono-(9Z)-9-octadecenoate, poly(oxy-1,2-ethanediyl) derivs.
9005-70-3	Sorbitan, tri-(9Z)-9-octadecenoate, poly(oxy-1,2-ethanediyl) derivs
577-11-7	* Butanedioic acid, 2-sulfo-, 1,4-bis(2-ethylhexyl) ester, sodium salt (1:1)
29911-28-2	Propanol, 1-(2-butoxy-1-methylethoxy)
64742-47-8	Distillates (petroleum), hydrotreated light
111-76-2	** Ethanol, 2-butoxy
** Contains 2-Propanediol ** This chemical component (Ethanol, 2-butoxy-) is not included in the composition of COREXIT® 9500*	

Corexit 9500 has been shown to be an effective tool in oil spill clean-up (Lessard et al. 1999; Lindstrom & Braddock 2002). However, there have also been a number of studies that indicate the potential toxic effects of Corexit 9500 to a number of organisms (Burridge & Shir 1995; George-Ares & Clark 2000; Gulec & Holdway 2000; Mitchell & Holdway 2000; Lindstrom & Braddock 2002; Long & Holdway 2002; Liu et al. 2006; Belore et al. 2009; Wang et al. 2012; Lee et al. 2013; Rico-Martínez et al. 2013).

1.3.3 Toxicity of Corexit 9500

Notably, there are no articles in the mainstream scientific literature regarding information on the toxicity of Corexit 9500 to New Zealand organisms. Moreover, there information is scarce regarding the toxicity of heavy fuel oil/Corexit 9500 combinations. Literature is strongly based around crude oils along with combinations of various dispersant products. A number of studies have indicated that oil and dispersant mixtures can impart a greater negative response in aquatic organisms than the oil alone.

Researchers have investigated the toxicity of oil and Corexit 9500 mixtures on a wide range of marine organisms with examples including fish (Couillard et al. 2005; Jung et al. 2009), algae (Burridge & Shir 1995; Duarte et al. 2010), copepods (Gardiner et al. 2013) cephalopods ((Long & Holdway 2002), rotifers (Rico-Martínez et al. 2013), coral (Lewis 1971) hydra (Mitchell & Holdway 2000), bivalves (Singer et al. 1996), and bacteria (Bruheim et al. 1999; Hamdan & Fulmer 2011). Research also suggests that some dispersants may be toxic to the bacteria that degrade oil (Tsomides et al. 1995). Studies have also focused on the factors that may influence the effectiveness of dispersants such as temperature (Belore et al. 2009; Campo et al. 2013) and salinity (Chandrasekar et al. 2006), and how these factors may influence toxicity.

The Corexit series of dispersants have been said to have a low (LC_{50} or EC50 >100 ppm) to moderate toxicity (LC_{50} or EC50 of 1-100 ppm) to most aquatic organisms (George-Ares & Clark 2000); however the experimental species, life stage, temperature, and exposure duration can significantly affect measurements of toxicity (George-Ares & Clark 1997). For example, George-Ares and Clark (1997) found that toxicity of Corexit 9527 significantly decreases in colder temperatures. The scallop (*Argopecten irradians*) had a 96 h LC_{50} value ranging from 200 ppm (20°C) – 2500 ppm (2°C); similarly, the glass shrimp (*Palaemonetes pugio*) had a 96 h LC_{50} range of 640 ppm (27°C) - 840 ppm (17°C). It is

suggested that lower temperatures may result in decreased uptake of dispersant (George-Ares & Clark 1997).

In a review paper by George-Ares & Clark (2000), the range for 24 - 95 h LC_{50} values ranged from 0.7 ppm to >400 ppm in studies that included zygote, embryo, larval, juvenile and adult life stages of a variety of marine organisms. Data indicated that fish were generally less sensitive to Corexit 9500 compared to crustaceans. They found that juvenile and adult fish had 48 - 96 h LC_{50} values of 50 - 354 ppm. By comparison, a higher sensitivity was seen in crustaceans at the same life stages with 48-96 h LC50 values estimated at 3.5 - 36 ppm. Larval fish sensitivity was found to be intermediate between the two with 48 - 96 h LC_{50} values of 25 – 75 ppm (George-Ares & Clark 2000).

George-Ares & Clark (2000) reported that increased exposure duration generally resulted in increased dispersant toxicity and found that environmental factors such as salinity and temperature may affect toxicity. They highlight that sensitivity to dispersants, oil or dispersed oil, varies significantly among species and life stage and that embryo-larval and early juvenile life stages are generally more sensitive than adults of the same species to both dispersants and dispersed oil. Reasons for this could be related to smaller size (greater relative surface area for uptake) and less developed metabolic capability (George-Ares & Clark 2000; National Research Council 2005).

1.3.4 Summary

There is a significant amount of literature that investigates Corexit 9500. Much of the literature focuses on the potential effects of environmental factors that will determine the effectiveness of the dispersants whilst fewer examine the toxicity of Corexit 9500. When investigating the toxicity of dispersants, studies often investigate its toxicity with and without the addition of oil because Corexit has the potential to increase oil toxicity. Because of oils complex nature and varying properties it is hard to generalise from one dispersant/oil mixture to another because types of oil widely vary.

1.4 Cryolite (Sodium hexafluoroaluminate)

Cryolite (CAS No.: 15096-52-3) or sodium hexafluoroaluminate (Na_3AlF_6) is an uncommon naturally occurring mineral belonging to the fluorine inorganic chemical family. Also made synthetically, cryolite is registered on the United States Environmental Protection Agency (USEPA; 2009) database as having two main uses; in the aluminium industry and as a pesticide. Minor uses include the production of abrasives, a colouring agent in fireworks and use in soldering and welding appliances (Solvay 2011).

In New Zealand, cryolite is used in aluminium smelting processes. Rena was carrying approximately 560 t of used cryolite or cryolite recovery sludge from New Zealand Aluminium Smelters Limited at Tiwai Point, which was lost at sea during the Rena disaster. The composition of the cryolite recovery sludge is somewhat unknown and may vary from that of pure cryolite with the possibility that it may contain a number of impurities.

1.4.1 Cryolite toxicity

Literature in relation to the potential toxic effects of cryolite or cryolite recovery sludge is scarce. The USEPA explains that acute risk is not expected to birds, mammals or aquatic organisms as, in the presence of sufficient water, cryolite is quickly converted to near natural background levels of simple inorganic compounds containing its constituent elements (sodium, aluminium and fluorine). Furthermore, chemical equilibriums in soil and aquatic environments are said to buffer concentrations of cryolite residue (United States Environmental Protection Agency 2011b).

The USEPA recognises that despite more than 50 years of use as a pesticide the chronic effects of cryolite are relatively unknown. They released a document registration review on cryolite in March 2011 and comments were accepted through May 31, 2011. Questions were addressed to the USEPA on the information within the document and a review and response document was released in September 2011. Although the USEPA does not believe that cryolite has long lasting toxic

effects (apart from particular pests that it is used to kill), in the final document a summary of data gaps for the toxicity of cryolite was presented (Table 1.2). Data submission for research data to fill these gaps was accepted up until June 2014 and a draft risk assessment is expected to be released in October 2015 (United States Environmental Protection Agency 2011a).

Table 1.2 A table of data gaps presented in the USEPA Cryolite Final Work Plan Registration Review (United States Environmental Protection Agency 2011a). TGAI = Technical Grade Active Ingredient. Estimated time frame for completion = months. Tier II =A Tier II study is expected to be required.

Data requirement	Test material	Estimated Time frame (Months)
Environmental Fate and Effects		
Freshwater Fish Toxicity (Estuarine/Marine)	TGAI	12
Avian Acute Oral Toxicity (Passerine Species)	TGAI	12
Avian Reproduction	TGAI	24
Toxicity to Sediment Dwelling Organisms		
Acute Toxicity Estuarine/Marine Organisms (Oyster)	TGAI	12
Toxicity to Non-target Plants		
Non-target Area Phototoxicity. Tier II, Seedling Emergence2 TGAI 12	TGAI	12
Non-target Area Phytotoxicity: Tier II, Vegetative Vigor	TGAI	12
Non-target Area Phytotoxicity:Tier II, Aquatic Plant Growth (Aquatic Vascular Plants)	TGAI	12
Non-target Area Phytotoxicity: Tier II, Aquatic Plant Growth (Algal Plants)	TGAI	12
Human Health effects		
90-Day Inhalation Toxicity	TGAI	24
Immunotoxicity Study	TGAI	12

According to material safety data sheets (MSDS), the solubility of cryolite is approximately 0.4 g/L – 0.6 g/L (Glassworks Services Limited 1998; Solvay Flourides Inc 1998; Rio Tinto Alcan 2006; Dupre Minerals 2008; Alcoa 2011; Solvay 2013). A range of environmental factors may affect the solubility such as salinity, temperature (Los Angeles Chemical Company 1993) and pH (Solvay 2013). Results from Cawthron Institute suggest that

the solubility of cryolite in seawater is between 44 and 95 mg/l (L. Tremblay, Cawthron Institute, *pers. comm.*, 2014).

The majority of toxic effects from cryolite occur after ingestion rather than uptake/exposure from a solution. In terrestrial environments, cryolite's insecticidal mode of action requires ingestion and non-target terrestrial invertebrates are not expected to be sensitive to this pesticide via a contact route of exposure (United States Environmental Protection Agency 2011b). Additionally, insects with piercing/sucking mouthparts (including bees) are not expected to be affected by cryolite via an oral route as they cannot ingest crystals (United States Environmental Protection Agency 2011b). Aquatic, avian and mammalian risks are also said to be negligible due to the uptake route of exposure (United States Environmental Protection Agency 2011b).

The toxic effects of cryolite's constituents (in particular aluminium and fluoride) have been documented in relation to aquatic organisms and human health (Wright & Davison 1975; Gensemer & Playle 1999; Connet et al. 2012). Of the components in cryolite, fluoride is of most concern as the toxicity of cryolite is generally attributed to its dissociation of fluoride ions (United States Environmental Protection Agency 2011b). In freshwater environments aluminium is known to have toxic effects upon a number of organisms (Gensemer & Playle 1999). However low pH (pH 2-5) has been proven to have a large influence on the toxicity of aluminium and due to the high pH in seawater (pH 8) it is considered less toxic than in freshwater environments. Furthermore aluminium is less toxic to fish in the presence of fluoride (Gensemer & Playle 1999).

Aquatic organisms living in soft waters may be more susceptible to fluoride than those living in hard or seawaters as the bioavailability of fluoride ions (and consequently, their toxic action) is reduced with water hardness (Camargo 2003). Moreover, Wright and Davidson (1975) suggested that dilution by seawater renders contamination of fluoride negligible and it is suggested that tissue fluoride concentrations often represent background levels (Wright & Davison 1975). Fluoride concentration in seawater when

saturated with cryolite ranges from 25 to 52 mg/L (L. Tremblay, Cawthron Institute, *pers. comm.*, 2014).

Fluoride is known to cause a range of toxic effects in aquatic organisms (Camargo 2003; Cao et al. 2013). Fluoride toxicity has most often been studied in the freshwater environment as opposed to the marine environment probably due to reduced risk resulting from greater dilution. Aquatic organisms uptake fluoride either directly from the water or to a lesser extent by ingestion (Camargo 2003). Uptake is directly correlated with environmental concentrations and exposure time (Pankhurst et al. 1980) and in some marine organisms, prolonged exposure to moderate fluoride levels appears to be tolerable (Wright & Davison 1975).

Some studies indicate little or no toxicity of fluoride in the marine environment, for example; Wright and Davidson (1975) investigated the potential toxicity of fluoride to a range of marine organisms (including fish and crustaceans). The work was commissioned due to the construction of an aluminium smelter which discharged large amounts of fluoride-loaded effluent into the sea. Animals were caught and collected from the Lynemouth in Northumberland and frozen immediately. Fluoride concentration in the area was measured at 3.4 µg/ml. After tissue analysis they concluded that there were no adverse effects seen even at these unnaturally high ambient fluoride levels (Wright & Davison 1975).

Fluoride is readily accumulated in the shell and tissues of marine bivalves (Nell & Livanos 1988). Nell and Livanos (1988) found limited fluoride toxicity to either Sydney rock (*Saccostrea commercialis*) or flat oyster (*Ostrea angasi*) spat; however there were significant reductions in growth at very high seawater fluoride concentrations of 30000 and 50000 µg F/L^{-1}. Fluoride concentrations in Sydney rock oyster spat increased linearly from 45 to 204 μg^{-1} dry spat with increasing seawater fluoride additions from 0 to 30000 µg F/L^{-1} (Nell & Livanos 1988).

Hemens and Warwick (1972) investigated the potential toxicity of fluoride on a number of marine animals. No adverse effects were seen in two species of fish and one species of prawn for 96 h, at concentrations up to

100000 µg F/L^{-1} (Hemens & Warwick 1972). However, long term exposure (72 d; salinity 20ppm; 52000 µg F/L^{-1}) resulted in physical deterioration and increased mortality in grey mullet (*Mugil cephalus*) and the crab (*Tylodiplax blephariskios*) and the reproductive processes of the shrimp (*Palaemon pacificus*) appeared to be adversely effected. In marine algae, toxicity varies between 50000 - 200000 µg F^-/L. Results in a review by Comargo (2003) indicated that some species were unaffected while others displayed signs of growth inhibition or growth enhancement (Camargo 2003). Similarly, Pankhurst et al. (1980) investigated the acute toxicity using both seawater dilutions of effluent and solutions of NaF in seawater examining the sub-lethal impact of fluoride to brine shrimp (*Artemia salina*). They found that effluent solutions of 50 ppm F^- and 100 ppm F^- (pH of < 5) produced high mortality.

In studies using laboratory animals, cryolite has been shown to be 'slightly' to 'practically non-toxic' on an acute basis (United States Environmental Protection Agency 1996). A search of the USEPA ECOTOX database aided in research and produced data from a range of tests which indicated relative toxicity estimates. The search produced results for eight aquatic organisms, three of which were marine species: *Crassostrea gigas* (mollusc), *Uca pugilator* and *Penaeus duorarum* (crustaceans) (Figure 1.7). Endpoint values varied from no observed effect level (NOEL), median concentration that causes 50% of maximal response (EC50) and a lethal concentration that causes death in 50% of the test population (LC_{50}). Concentrations ranged from 5,000 µg/L to > 400,000 µg/L (United States Environmental Protection Agency 2009).

Results from the USEPA database (2009; Figure 1.7) showed results for three different marine species and five freshwater species. The LC_{50} values ranged from 14, 000 µg/L, to >400,000 µg/L, whilst EC_{50} values range from < 10,000 µg/L - > 100,000 µg/L and NOEL values range from just over 10,000 µg/L - 100,000 µg/L. Crustaceans appear to be more sensitive to cryolite than fish and marine crustaceans also appear to be more sensitive than marine molluscs and freshwater fish. Of the freshwater crustaceans, *Daphnia pulex* and *Simocephalus serrulatus*

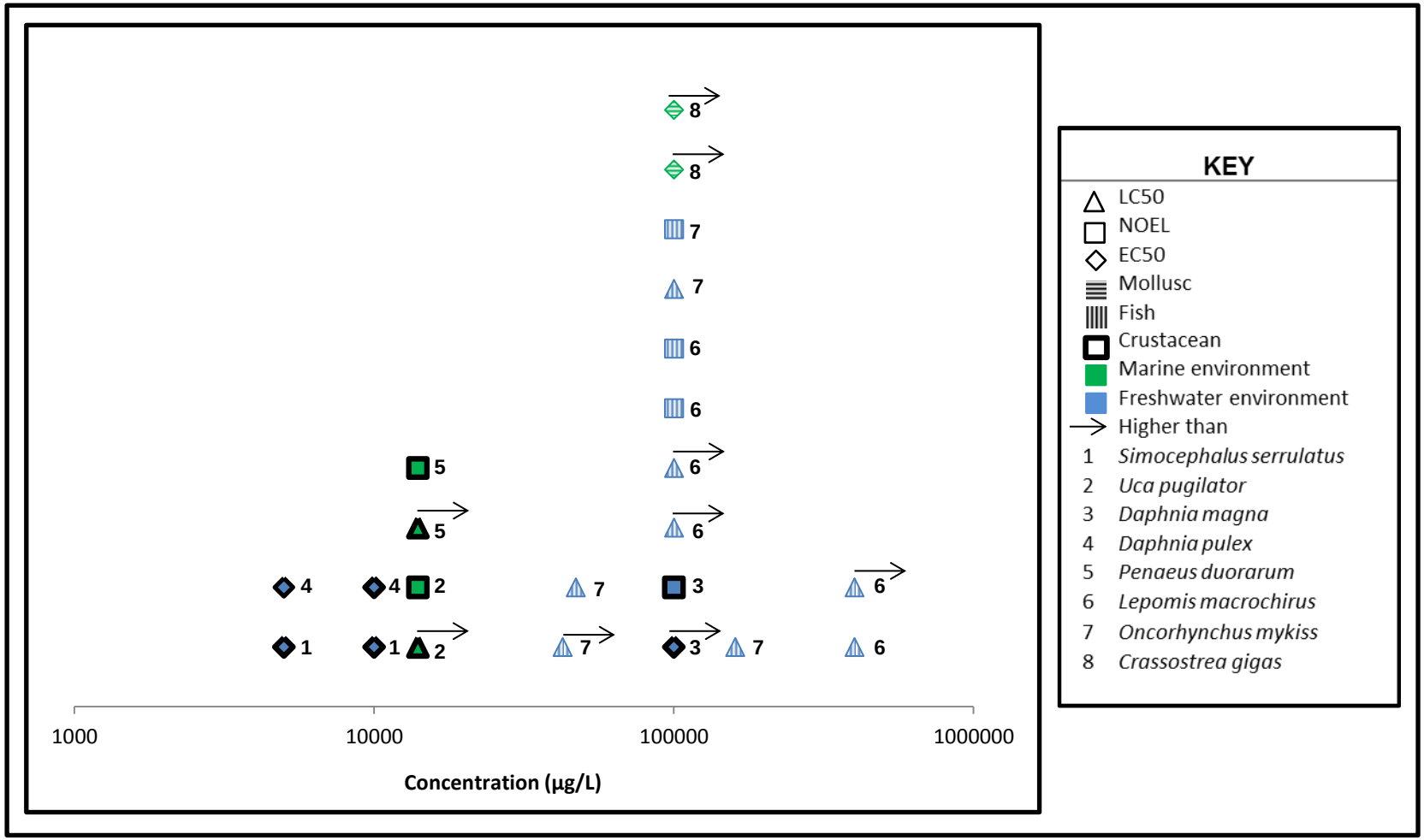

Figure 1.7: Cryolite toxicity values obtained from the USEPA database (United States Environmental Protection Agency 2009).

(EC50 – 5000 µg/L and 10,000 µg/L) are more sensitive to cryolite than *Daphnia magna* (EC_{50} and LC_{50} – 100,000 µg/L).

In studies that determined NOEL, LC_{50} concentrations were estimated above the tested concentration; consequently both the method and result for each are presented in Figure 1.7. For example: effects on *Oncorhynchus mykiss* and *Lepomis macrochirus* at 100,000 µg/L exhibited NOEL, therefore LC_{50} values are estimated to be above this concentration. Similarly, NOEL was also seen for *Penaeus duorarum* and *Uca pugilator* at 14,000 µg/L and consequently LC_{50} values were estimated above this.

Of the limited literature available on the potential toxicity of cryolite or cryolite recovery sludge, only one study was found to be relevant indicating a large gap in the literature. The Department of Agriculture and Fisheries for Scotland (1982) investigated the impact of cryolite recovery sludge to a range of aquatic animals. The study was undertaken in Moray Firth where cryolite recovery sludge (CRS) had been actively dumped from the aluminium smelter from 1972. They found that cryolite recovery sludge (CRS) had no adverse measurable effects on behaviour of salmon (*Salmo salar*). However they did find that salmon exposed to CRS (concentrations above 1%) and CRS filtrate (concentrations above 0.6%) for up to 1 h resulted in clear changes in heart rate, gill ventilation rate and oxygen consumption increases. Results of this study indicated that although the fish were able to detect the CRS they would not actively avoid the solution.

In the Department of Agriculture and Fisheries for Scotland (1982) investigation of *Nephrops* sp. they found that CRS is unlikely to affect the survival rate due to a survivability test that included caged animals inside and outside the dump area. No concentrations of CRS where given and the number of replicates were unreported. After evaluation of catch rates of a variety of benthic organisms inside and outside of the CRS disposal zone they suggested that catch rates of a variety of marine organisms did not vary between areas affected by CRS and those that were not (Department of Agriculture and Fisheries for Scotland 1982). Catch rate

however is highly variable and dependent upon a range of influencing factors such as habitat suitability.

Toxicity values obtained from the USEPA database (United States Environmental Protection Agency 2009) on terrestrial animals indicated 25 records. Most results indicated the toxicity value per acre; however two results indicated LD50 values in g/kg. The LD50 value for the bob white quail (*Colinus virginianus*) was estimated at 2.2 g/kg; similarly, the value for the honey bee (*Apis mellifera*) was estimated at 2 g/kg. For quail the exposure was tested orally via capsule whilst exposure to the honey bee was considered to be topical (United States Environmental Protection Agency 2009). To gain some sort of estimation into toxicity, from these values we could infer that if ingested the LD50 for a 430 g red rock lobster would be around 903 mg or for a 100 g fish it would be approximately 210 mg.

1.4.2 Conclusions

Data is severely lacking when it comes to cryolite toxicity studies and relatively little is known about its potential toxicity. The one relevant marine study on cryolite recovery sludge suggests that toxicity is low as it did not affect survivability or benthic ecology. Uncertainty remains around the potential toxicity of fluoride in aquatic environments; however literature suggests that organisms living in soft waters may be more affected by fluoride pollution than those living in hard or seawaters.

1.5 Study animals

Three study species were chosen; snapper (*Pagrus auratus*), spotted wrasse (*Notolabrus celidotus*) and the New Zealand red rock lobster (*Jasus edwardsii*), which have importance ecologically or for recreational or commercial fisheries and which also hold high cultural values to Māori as valuable kai moana species.

1.5.1 Species biology

1.5.1.1 Snapper (*Pagrus auratus*)

Snapper (*Pagrus auratus*) (Figure 1.8) commonly reside in shallow waters (20 - 50 m) and are found in the warm coastal waters of the North Island and the northern part of the South Island (Parsons et al. 2014). Snapper are one of the most abundant continental shelf species, found in a range of habitats including estuaries, reefs, harbours and the open ocean. Most snapper range between 30 - 80 cm, weigh 2 - 5 kg and are long lived, living up to 60 years of age (Francis 2001). Snapper feed on invertebrates, echinoderms, polychaete worms, crustaceans, shellfish and other small fish (Ayling & Cox 1982; Francis 2001).

Figure 1.8: A snapper (*Pagrus auratus*). Image credit: Richard Ling.

Snapper are extensively studied and are considered to be one of New Zealand's most valuable inshore commercial finfish species having had high commercial importance since the 1900's (The South Australian

Centre for Economic Studies 1999; Parsons et al. 2014). Snapper are most often targeted by recreational fishers and are considered to have high cultural significance to Māori. Snapper also have a high ecological value as they are an important predator within the inshore waters of New Zealand as well as being considered a conspicuous and important part of the ecosystem by having the ability to influence and modify the environment they live in (NIWA 2013; Parsons et al. 2014).

Snapper are protogynous hermaphrodites, beginning life as females before they mature at around 23 - 30 cm or 3 - 4 years old, where approximately half of them undergo a sex change and become males (Parsons et al. 2014). Gonads become ripe in spring and spawning occurs in summer when the sea surface temperatures reach 18°C. Spawning occurs in the warmer surface waters as the pair will rise and fall in unison releasing large amounts of gametes. Larvae drift in the plankton for 18 - 32 days before juveniles settle at the bottom at a length of 20 - 30 mm (Parsons et al. 2009).

1.5.1.2 Spotted wrasse (*Notolabrus celidontus*) (spotty)

Spotted wrasse (*Notolabrus celidontus*) (Figure 1.9) are endemic to New Zealand and are one of the most common reef fish in New Zealand. They are abundant on coasts, reefs, harbours and sometimes estuaries. Recreational fishing targeting spotted wrasse is not common and usually restricted to children catching them from wharfs (Ayling & Cox 1982). They are often located in water less than 30 m deep and are most abundant in the upper 10 m particularly in sheltered areas. Their diet consists of a wide variety of invertebrates including crabs, hermit crabs, amphipods, barnacles, brittle stars, gastropods, various shellfish and worms (Ayling & Cox 1982; Francis 2001). As with the snapper, spotted wrasses are a protogynous hermaphrodite; young reach maturity at 12 cm long and spend one or two years as mature females before changing colour and sex and becoming a male when around 18-22 cm (Francis 2001).

Males protect and control a territory that is attractive to females, courting and spawning with any female that enters from July – December. Ideal

territories contain patches of broken rock and kelp that are ideal for young to mature. Larvae drift in the plankton for about two months, before settling in the fronds of kelp as juveniles at around 15-20 mm in length. Spotted wrasse reach a maximum length of around 26-30 cm and weigh less than a kilogram and may live at least 7 years. Spotted wrasse were chosen as their abundance indicates that they are a very successful and fundamental part of our shallow reef ecosystem. All study animals were female as they were more prevalent within the environment.

Figure 1.9: A female spotted wrasse (*Notolabrus celidotus*). Image retrieved from Te Ara Encyclopedia of New Zealand.

1.5.1.3 Red rock lobster (*Jasus edwardsii*) (Crayfish)

Red rock lobster (*Jasus edwardsii*) (Figure 1.10) are widespread around the entire coastline of New Zealand and its offshore islands, especially in areas where there is plenty of cover. Red rock lobsters are considered to be a shallow water species which mainly inhabit rocky reefs and light foul ground that extends from the intertidal zone down to a depth of 200 m (Jeffs et al. 2013). Adults can reach up to 8 kg and can have a ocular carapace length up to 23 cm. Rock lobster are considered to be nocturnal opportunistic feeders with studies indicating that their diet consists of a wide range of prey including molluscs, crustaceans, annelid worms, macroalgae, echinoderms, sponges, bryozoans, fish, foraminifera and brachiopods (Jeffs et al. 2013; MacDiarmid et al. 2013).

Red rock lobster are considered to be a valuable resource as food, revenue and recreation. They are arguably the most abundant dominant benthic predator on coast reefs and play an important role in ecosystem functioning (MacDiarmid et al. 1991; MacDiarmid et al. 2013; Eddy et al. 2014). In New Zealand the recreational fishery is based largely around diving, however red rock lobster pots are also used. Commercial fishing of lobster through the use of pots represents the main source of lobster fishery revenue in New Zealand (Eddy et al. 2014). Red rock lobster are also an important kai moana species highly valued by Māori who have been fishing for them for centuries (MacDiarmid et al. 2013).

Figure 1.10: A red rock lobster (*Jasus edwardsii*). Image retrieved from: South Australian Research and Development Institute 2014.

Red rock lobsters are known to make great migratory movements in large groups where they may travel up to 460 km. On a day to day basis lobster will tend to have an average foraging range of 24 km, movement will be influenced by local topography (MacDiarmid et al. 2013).

Female maturity is estimated to range from 3 - 7 years or 72 – 122 mm ocular carapace length (MacDiarmid 1989). Peak mating season occurs between May and June and females carry eggs on their pleopods under their tails and can bear up to 550,000 eggs. Eggs are carried for up to 6 months before hatching in September to late October. The initial

naupliosoma larvae swim vigorously towards the surface within 30 minutes of hatching and subsequently moult into stage one phyllosoma larvae which remain positively phototaxic and form surface swarms (MacDiarmid et al. 2013).The phyllosoma spend around 1-2 years in the planktonic phase during which ocean currents may carry them a significant distance offshore. They then return to inshore areas where they settle on the bottom as transparent puerulii (MacDiarmid et al. 1991; MacDiarmid et al. 2013).

1.6 Immunotoxicology

Toxicants are those which are created through human activity (Bols et al. 2001). Ecotoxicology deals with the risk of toxic chemicals that disrupt the functioning of ecological systems (Segner et al. 2012). Ecotoxicants have two general properties – they are discharged into the environment and they have the potential to impact ecosystems at relatively low concentrations (Bols et al. 2001). Two main study designs often carried out to investigate effects: chronic studies which investigate the prolonged exposure of a toxicant, and acute studies which investigate the adverse effects from a single short-term exposure.

The immune system is an important objective when studying the toxicity of substances as it functions in resistance to diseases and aids in protection. The immune system protects organism homeostasis and integrity by monitoring any alteration of cells and tissues caused either by internal (age, neoplastic proliferation etc.) or external (pathogens, disease, chemicals etc.) stressors. Primarily, the immune system is the recognition and the discrimination of the self and non-self (Segner et al. 2012).

1.6.1 Fish immune systems

The immune systems of fish are physiologically similar to that of mammalian vertebrates with some minor differences (Anderson & Zeeman 1995; Uribe et al. 2011). The immune system of teleosts can be broadly subdivided into the categories represented in Figure 1.11.

In teleosts the first line of defence is represented by external barriers separating the fish from its environment (i.e. skin, gills, and the alimentary canal) which act as physical barriers. The innate immune system is the second category of the immune system which responds to invading pathogens. This system contains humoral (anti-bacterial peptides, complement factors, antibodies etc.) and cellular immune (immune cells) factors (Logan 2007). Cellular elements of the immune system include monocytes, granulocytes, macrophages and natural killer cells. The adaptive immune system involves a number of humoral and cellular

responses. T and b lymphocytes mediate humoral and cellular responses respectively and they possess antigen-specific receptors.

Lymphoid organs are known as the tissues of the immune system. Unlike vertebrates, the advanced teleosts lack bone marrow and lymph nodes, and instead possess hematopoetic tissues primarily in the spleen, head kidney, thymus, circulating lymphocytes and gut associated lymphoid tissue (Zelikoff 1994).

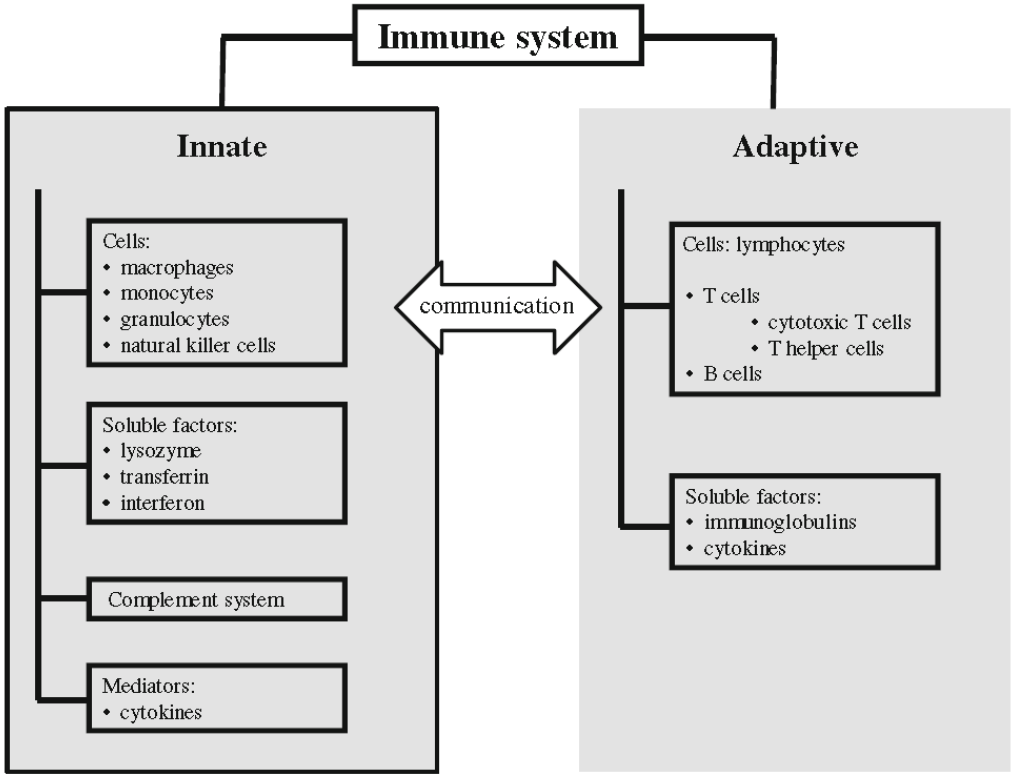

Figure 1.11: A simplified schematic of the fish immune system in teleost fish. Image obtained from Segner et al. 2012.

1.6.2 Crustacean immune systems

Like fish, in crustaceans the first line of defence includes external barriers such as an exoskeleton and gills. Invertebrates lack an adaptive immune system and instead they have developed defence systems which respond against antigens (Vazquez et al. 2009). Crustaceans possess the innate immune system and hematopoietic tissues (Lin & Söderhäll 2011).

1.6.3 Haematology and immune suppression

Haematological assessment can be useful to detect diseases that affect the cellular component of blood as they are important parameters for the evaluation of fish physiological status (V´azquez & Guerrero 2007). Indications of potential immune effects may include changes in lymphoid organ weights; changes in leukocyte differential counts and increased susceptibility to infections or the development of cancer or tumours (Anderson & Zeeman 1995). Toxicants also affect the erythrocyte parameters of fish and may persist for several days after removal of the stressor (Campbell & Ellis 2007).

1.6.3.1 Fish haematology

The introduction of xenobiotics alters functions and balances in the immune system leading to undesirable effects such immunosuppression, uncontrolled cell proliferation, neoplasia and alteration of defence mechanisms (Rowley et al. 1988).

Erythrocytes

Erythrocytes are the most common cell type in the blood of the vast majority of teleosts. The main function of these cells is the transport of oxygen (Rowley et al. 1988).

Leukocytes

Leukocytes are derived from stem cells within the spleen, kidneys and various other immunopoietic sites. The majority of leukocytes are divided into lymphocytes, granulocytes (three types: basophils, neutrophils, eosinophils), monocytes, macrophages and thrombocytes (Campbell & Ellis 2007). Monocytes and macrophages are involved in phagocytosis. Lymphocytes aid in defence against pathogens and toxins while the role of granulocytes is phagocytosis and inflammation. Thrombocytes are responsible for blood clotting and control of fluid loss from a surface injury in fish (Campbell & Ellis 2007).

1.6.3.2 Crustacean haematology

Like fish, in crustaceans the first line of defence includes external barriers such as the exoskeleton and gills. However, the circulatory system of invertebrates is physiologically different to vertebrates. Crustaceans have an open circulatory system which transports oxygen, hormones and cells in the haemolymph (Vazquez et al. 2009). The circulating cells (haemocytes) are considered to be functionally analogous to vertebrate leukocytes due to their role in the recognition of the non-self, phagocytosis and coagulation (Vazquez et al. 2009). Crustaceans possess several defence mechanisms that initiate in response to different pathogen characteristics (Vazquez et al. 2009).

Effector defence mechanisms in crustaceans include semi granulocytes, granulocytes and hyaline cells (Le Moullac & Haffner 2000; Vazquez et al. 2009). Hyaline cells function in phagocytosis, semi-granulocytes are involved with encapsulation, melanisation and coagulation and granulocytes function in cytotoxicity, melanisation and function as antimicrobial peptides (Lin & Söderhäll 2011).

1.6.4 The stress response

Stress is a physiological response to disruption of homeostasis and is an adaptive mechanism that allows organisms to cope with real or perceived stressors in order to maintain homeostasis (Barton 2002). Stress responses occur in all animals when physiological systems are extended beyond their normal range by external stressors (Chang 2005).

1.6.4.1 The stress response in fish

The primary stress response involves the release of catecholamines and release of corticosteroids. Secondary responses include changes in metabolite levels, plasma and tissue ions, haematological features, and heat shock or stress proteins, which among other things, relate to immune function and cellular responses (Barton 2002). Tertiary responses involve whole animal performance which involves changes in growth, condition, resistance to disease, metabolic scope, behaviour and ultimately survival (Figure 1.12) (Barton 2002).

Cortisol is the principal steroid hormone in fish and its plasma concentration rises in response to stress. It is an essential component in the stress response in fish and also plays a significant role in osmoregulation, growth and reproduction (Mommsen et al. 1999). In a stressed state, elevated cortisol causes hyperglycaemia and elevates blood pressure which are important mechanisms for coping with stress. However, cortisol is also supresses the inflammatory and immune responses as well as growth and reproduction (Bonga 1997; Mommsen et al. 1999; Falahatkar et al. 2009).

Reponses in the blood occur as a consequence of released hormones into the blood stream. Increases in packed cell volume and haemoglobin in fish subjected to stressors may be due to changes in erythrocyte number, erythrocyte swelling, haemodilution, or haemoconcentration (Falahatkar et al. 2009). A decrease in white blood cells is also a typical response to acute stress in fishes and may be meditated by cortisol (Falahatkar et al. 2009). Erythrocyte counts, leukocyte cell counts, haemoglobin and packed cell volume are commonly used to assess acute stress and health in fishes (Mommsen et al. 1999).

The leukocyte composition of fish is affected by stress, inflammatory and infectious diseases and nutritional disorders (Campbell & Ellis 2007). Lymphocytes are the most common leukocyte found in fish and the effect of stress in fish is usually measured by a decrease in lymphocytes in differential counts and a relative increase in granulocytes. This is because a decrease in lymphocytes concentration typically results from conditions that reduce the number of circulating lymphocytes such as stress (Campbell & Ellis 2007). Increases in cortisol during stress are well known to cause lymphocyte apoptosis (Ellsaesser & Clem 1987; Poursaeid et al. 2012).

1.6.4.2 The stress response in crustaceans

Crustacean immune systems and response to xenobiotics are not well studied. The concept of stress as outlined in Figure 1.12 is similar in crustaceans, however stress responses in invertebrates have received far

less study than those of vertebrates. One of the main stress responses examined has been hyperglycaemia which is controlled by the crustacean Hyperglycemic Hormone (cHH). For example: Chang (2005) investigated stress in lobsters examining crustacean hyperglycemic hormone and stress proteins (Chang 2005).

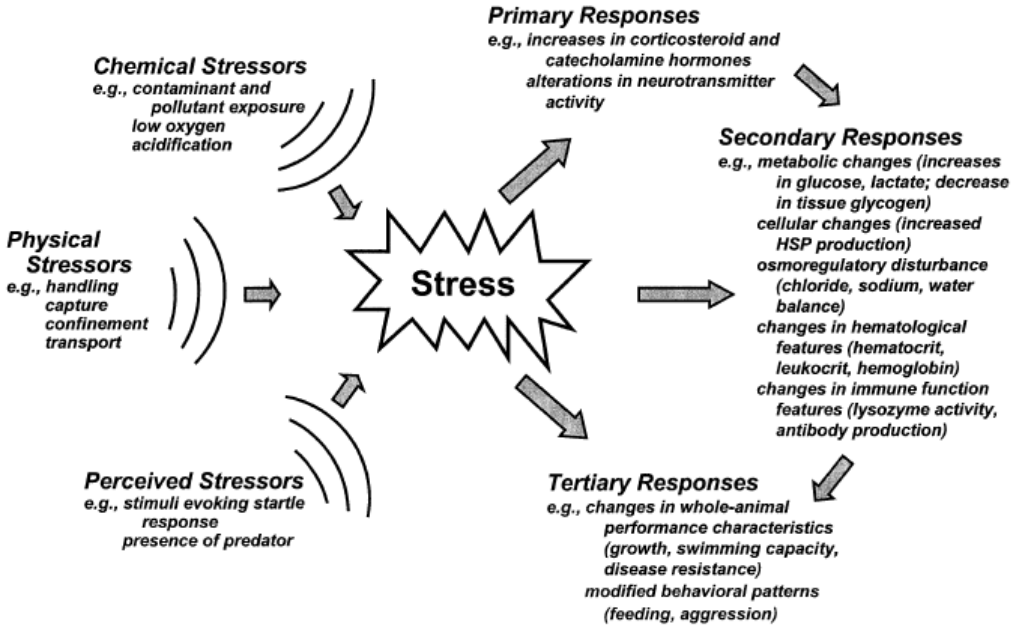

Figure 1.12: Physical, chemical and other perceived stressors that act on fish and induce physiological change and related effects. As indicated by the arrows primary and secondary responses may consequently affect secondary and tertiary responses. Image taken from Barton 2002.

1.6.5 Polycyclic aromatic hydrocarbon uptake

Once polycyclic aromatic hydrocarbons (PAHs) are absorbed they may be transported via the bloodstream to other parts of the body. There are a number of studies which have demonstrated their toxic effects on the immune system (Logan 2007).

1.6.6 Polycyclic aromatic hydrocarbons biomarkers

Polycyclic aromatic hydrocarbons act to supress cell meditated immune responses and may also alter both specific and non-specific immunity in

fish (Reynaud & Deschaux 2006). The liver is essential for the metabolic disposal of essentially all xenobiotics which often occurs without damage. Because of this exposure in the liver will often be higher than in other tissues. Due to their lipophilic nature, PAHs tend to accumulate in the most lipid rich tissues such as the liver (Logan 2007). The bile is a major excretion route for PAHs in fish. After biotransformation in the liver, PAH metabolites are excreted into the bile and concentrated making biliary concentration a good indicator of PAH exposure (Aas et al. 1998; Logan 2007). Bile analysis has also proved very suitable to study the uptake of other xenobiotics, like saturated hydrocarbons (Ariese et al. 1993). The risk to fish from oil and oil/dispersant treatments can be assessed in terms of exposure to PAH, and changes in exposure after the use of dispersants (Ramachandran et al. 2004).

Knowledge of human health risks related to environmental exposure to hazardous chemicals is a current concern. Because some PAHs pose a serious threat to human health (Franco et al. 2008), it is important to monitor and assess PAH build up and depuration in marine organisms that are commonly consumed by humans. One way that PAH uptake may occur in humans is in absorption of contaminated food (Jongeneelen 1994). Marine animals represent an important food source and following an oil spill both the general public and members of the public health board want to be assured that these animals are safe to eat. Therefore, it is important to assess the uptake rates in organisms as well as assessing how long it takes these animals to depurate, a process of which generally results from the biotransformation of xenobiotics.

1.7 Project funding and wider research projection

Funding for this project was part of the New Zealand Government's 2.4 million dollar Rena Long Term Environmental Recovery Plan administered by the Ministry for the Environment, and was obtained from the Rena Recovery Project to be used to study ecotoxicology of Rena pollutants.

The recovery plan involved a number of stakeholders and research partners including The University of Waikato, The University of Bremen, The University of Canterbury, Bay of Plenty Polytechnic, Maketu Taiapure, Intercoast, Ngati Makino, Manaaki Taha Moana, Manaaki Te Awanuni, Te Whare Wananga o Awanuiarangi, Hill Laboratories, the Cawthron Institute, Moana a Toi Iwi groups, Bay of Plenty Regional Council and the District Health Board (Figure 1.13).

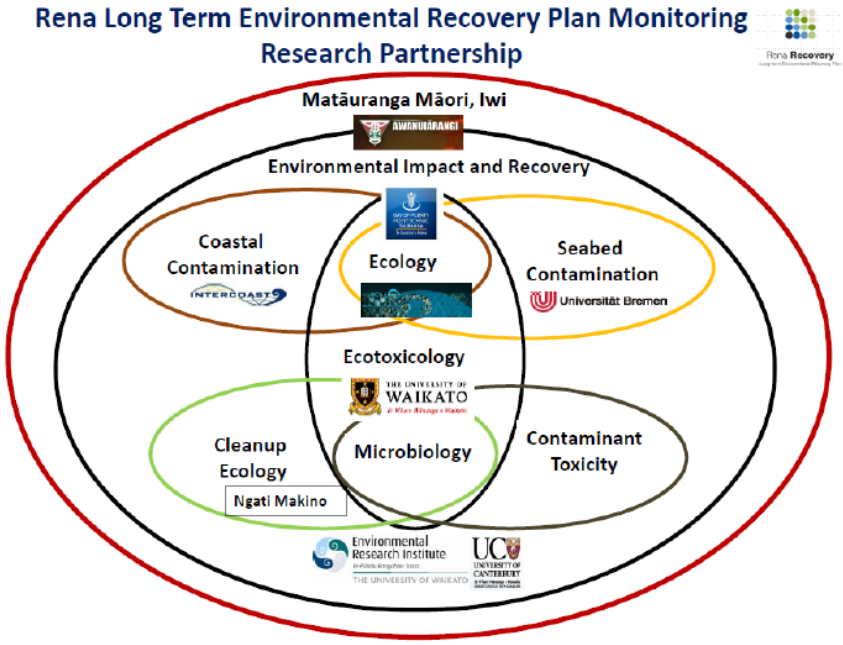

Figure 1.13: Rena long term recovery plan partnerships. Image obtained from Te Mauri Moana 2013.

The Rena project aims to investigate immediate and long-term impact through assessments including science, water, seafood and sediment chemistry; ecohydrology; physiological and sub-lethal impact assessment, oil and dispersant toxicology, microbiology and microbial degradation rates. The scientific sampling and monitoring programme commenced following the grounding on the 5th of October 2011 and is ongoing.

1.8 Objectives

The primary objective of this study was to assess the toxicological impacts of pollutants associated with the Rena oil spill. Specific aims were to:

- Assess the acute toxicity of Rena pollutants including heavy fuel oil (HFO), Corexit 9500 (oil dispersant), cryolite, and a combination of HFO and Corexit 9500.
- To gain basic PAH toxicological data on the chosen species including magnitudes of uptake and rates of depuration
- Investigate whether or not the addition of Corexit 9500 enhanced the toxicity of HFO or accumulation of PAHs
- As HFO is highly variable in component organics, the focus was to collect data on the specific effects of the Rena HFO.

Although there were a large number of contaminants associated with the Rena disaster, this study assessed the three contaminants which were present in the largest amounts and of most concern, particularly to the public. Study animals were chosen based upon their commercial, recreational, cultural and ecological value. Selection was also based upon their ecological niche; Red rock lobster represented the benthic habitat, snapper resented the pelagic habitat and spotted wrasse represented the coastal habitat. Furthermore, as much of the larvae and juvenile life stages are subject to high predation and mortality, sub-adult organisms were chosen because they are more likely to survive and live on after the disaster even though they may be acutely affected. They are also of most concern due to potential human consumption.

To assess this, concentrations and exposure durations that were considered environmentally relevant were used. The concentration selection also took into consideration the findings of previous work. This study was set up to investigate a grey area in the literature which addressed the lack of information on ecotoxicological data relevant to New Zealand marine animals as well as addressing uncertainty of the potential effects from the contaminants. Toxic effects from the Rena pollutants were

understandably of great concern to the public, media and scientific community. Of significance, the uncertainty about the potential effects of the associated contaminants that arose from the disaster caused the most frustration in the public domain.

Overall this project aimed to provide information to cultural communities, the public health system and advisories for management of future environmental oil spill disasters.

Chapter Two: Methods

2.1 Animal collection and maintenance

All study animals were collected from the Waikato and Bay of Plenty coastal regions in the North Island of New Zealand outside of areas affected by the Rena oil spill.

Juvenile red rock lobsters (*Jasus edwardsii*) (ocular carapace length 86 ± (±SEM) 7.9 mm, weight 428 ± 7.9 g) were collected by SCUBA diving using snares from Bowentown, Raglan and Tairua, as well as from a commercial fishing vessel in Tairua using red rock lobster pots. Juvenile spotted wrasse (*Notolabrus celidotus*) (length 170 ± 3.7 mm fork length, weight 85 ± 6.7 g), were caught from Bowentown and juvenile snapper (*Pagrus auratus*) (length 181 ± 2.7 mm fork length, weight 140 ± 5.8 g), from Tairua and Raglan by baited angling using fishing rods, equipped with sabiki lures (size 10 -15) and baited with squid. Animals were transported in aerated bins filled with seawater. Water changes were carried out regularly.

Study organisms were held at the Waikato University Aquatic Research Centre in 5000 L fibreglass tanks (Figure 2.1). Tanks were supplied with artificial seawater via a recirculating seawater system and a compressed air system provided oxygenation. Seawater was made up using Crystal Sea Marinemix salt (Marine Enterprises International, Baltimore, Maryland, U.S.A). The salinity of the water varied from 32 ppt - 34 ppt and water temperature varied between 15°C - 18°C. To provide a natural photoperiod the lighting system (fluorescent tubes) was set up on a 12L:12D photoperiod. The holding tanks had sheltered areas such as cinder blocks, half pots and large plastic tubes where the organisms could take refuge (Figure 2.2).

Study animals were fed twice a week on chopped green-lipped mussels with excess food waste removed up to 2 h after feeding. Animals were monitored daily for signs of injury, changes in behaviour or disease symptoms. Unwell animals were removed from the tanks and euthanized.

Levels of ammonia, nitrate, nitrite and phosphate were measured every three days using API® water test kits, and water changes were made accordingly to maintain levels below toxic thresholds.

Figure 2.1: Animal holding tank in the Aquatic Research Centre.

Figure 2.2: Underwater photo inside one of the holding tanks.

2.2 Toxicant treatments

The HFO used in this study was obtained from Rena fuel tanks during salvage, Corexit 9500 was supplied by Maritime New Zealand and the cryolite was sourced from Tiwai Point aluminium smelter. Toxicant concentrations were based upon environmentally relevant concentrations and findings in Singer et al. (2000). The concentration also needed to be below the LC_{50} value in order to examine acute sub-lethal effects rather than mortality.

Toxicant mixtures included Rena heavy fuel oil (HFO) water accommodated fraction (WAF), Corexit 9500, Rena HFO combined with Corexit 9500 also referred to as chemically enhanced water accommodated fraction (CEWAF) and cryolite. All toxicants were prepared using a standard procedure of mixing over a 24 h period on a magnetic stirrer in the laboratory using 10 cm stirrer bars with sufficient energy to produce a vortex 25% the depth of the volume following the methods presented in Singer et al. (2000). Toxicant mixtures were most often used immediately after the 24 h mixing period, however if not used, they were stored at 6°C and used within a 24 h period or otherwise discarded. All toxicant mixtures were prepared in 2 L borosilicate glass bottles using filtered (0.45 µ) artificial seawater (Figure 2.3).

2.2.1 Rena heavy fuel oil water accommodated fraction (HFO WAF)

Rena HFO water accommodated fractions (WAF) were prepared following methods presented in Singer et al. (2000) at a concentration of 1 mL/L HFO in seawater. Two mL of Rena HFO was added to 2 L of seawater and mixed for 24 h as noted above. The WAF was diluted 10-fold in seawater to give a final exposure concentration of 1:10,000 (HFO WAF:seawater). The WAF was aspirated from the mixing vessel through a glass tube with a 7 mm thick wall using a 100 mL glass syringe to prevent including oil in the solution.

Figure 2.3: Toxicant solutions being mixed. Left: Rena HFO WAF, Middle: Rena HFO and Corexit 9500 (HFO CEWAF) and Right: Corexit 9500.

2.2.2 Corexit 9500 mixture

Corexit 9500 toxicant treatments were prepared using 25 µL/L (Corexit 9500:seawater) and mixed in 2 L bottles following the standard mixing procedure (section 2.2). The final solution was then diluted 1:10 in seawater.

2.2.3 Corexit 9500 combined with Rena heavy fuel oil water accommodated fraction (Chemically enhanced water accommodated fraction (CEWAF)) (HFO CEWAF)

A chemically enhanced water accommodative fraction (CEWAF) mixture of Corexit 9500 and Rena HFO was prepared as above with the addition of 1 g/L of Rena HFO (1:40, Corexit 9500:HFO). After 24 h mixing, the HFO CEWAF was diluted 1:10 in seawater. The HFO CEWAF was extracted from the mixing vessel as outlined in section 2.2.1.

2.2.4 Cryolite

Powdered cryolite was mechanically sieved through a 25 µm sieve to remove large particles. Cryolite mixtures were prepared with 1 g/L cryolite: seawater. Two grams of cryolite was added to 2 L of seawater and mixed following the standard mixing procedure. This suspension was further diluted to produce a final exposure concentration of 0.1 g/L.

2.3 Experimental conditions

Study organisms were randomly chosen from the holding tanks and were housed in 25 L glass aquaria (30 cm x 30 cm x 30 cm) throughout the experiment. The aquaria were specially made and constructed without silicon glue as this can adsorb hydrophobic compounds in water. The experiment took place in a temperature controlled room set at 16°C. Each tank was covered with a glass lid and was aerated using glass tubing and an air stone. Tanks were surrounded by black plastic to prevent further stress to the study organisms.

Figure 2.4: Red rock lobster (*Jasus edwardsii*) experiments in progress.

All test organisms were tested in 10 L of water; during toxicity tests, tanks were filled with 9 L of artificially made seawater and 1 L of toxicant treatment stock solution. One animal was placed into each test chamber and left for the prescribed time limit of 24 h, 48 h or 96 h (Figure 2.5 and

Figure 2.4). Toxicant-free control organisms were tested for 96 h (96 h control) in experimental tanks containing 10 L of artificially made seawater to test for effects of confinement stress. Half of the water (50%) in each test chamber was changed every 24 hours and replaced with the same concentration of toxicant (4.5 L of seawater, 0.5 L of toxicant treatment). Tanks were cleaned thoroughly with detergent and hot freshwater after each experiment. Control organisms were taken directly from the holding tank and were immediately euthanized and sampled.

To test the time course of depuration, study organisms were exposed to toxicants for 96 h following the 96 h method. After 96 h the animals were then placed into recovery tanks for either 4 d or 10 d. The recirculating recovery system (Figure 2.5) consisting of four 200 L tanks, was filled with seawater and set up with a protein skimmer, a bio filter, a UV light and activated carbon to remove organics such as PAHs.

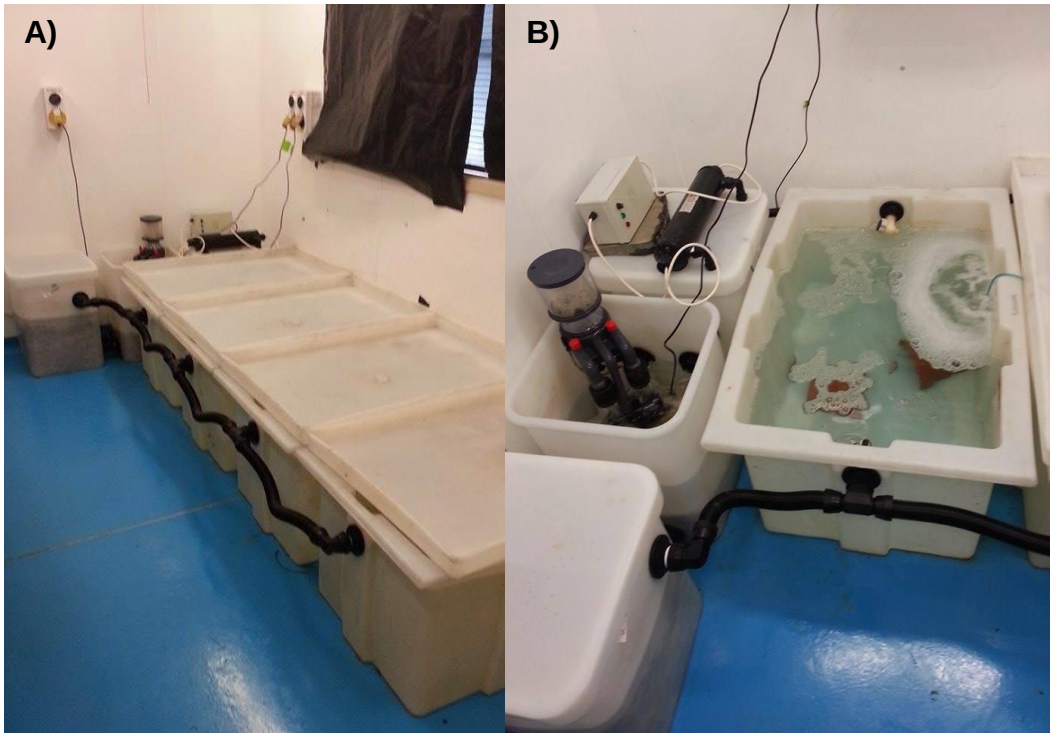

Figure 2.5: A) the recirculating recovery system, B) one of the recovery tanks, the bio filter, protein skimmer and UV light.

Snapper reach maturity at around 23 - 30 cm or 3 - 4 years old, and although two of the study snapper were over 23 cm, none were over 30 cm, therefore we can reasonably assume that all of the snapper tested were female. A total of 42 male 46 female red rock lobster were tested amongst the four toxicant treatments and two control treatments. All spotted wrasse were female and were assumed to be maintained in this state because of the addition of a large male wrasse to each holding tank.

Three or six replicates were established per treatment for each study organism. Sample size was limited due to a range of factors listed in 2.10: Research limitations.

2.4 Necropsy

At sampling, fish were euthanized with an overdose of anaesthetic (0.1 g/L benzocaine); red rock lobster were euthanized by hypothermia followed by brain ablation. Organism weights (± 1 g) and lengths (total length for fish and ocular carapace length for red rock lobster, ± 1 mm) were recorded and a visual inspection of the organism was carried out, checking for any obvious signs of external damage. The sex of the specimen was noted by the gonads or by the urogenital pore and pleopods in red rock lobster.

2.4.1 Blood sampling

Using a 1 mL heparinised (heparin ammonium salt: 5000 i.u./mL; Sigma U.S.A) syringe (23 G needle) a blood sample (400 µL) was taken from the caudal vein of fish (caudal venepuncture) (Figure 2.6). The needle and syringe dead space were filled with heparin solution and dilution due to heparin was accounted for in subsequent analyses. Blood samples (500 µL) from rock lobster were collected using a 1 mL syringe (23 G needle) from the pericardinal sinus under the thoracic carapace (cardiac puncture) and mixed with 10% neutral buffered formalin fixative (1:1). Blood samples were stored in Eppendorf tubes on ice until analysis within 2 h.

Figure 2.6: Blood being drawn from a spotted wrasse (*Notolabrus celidotus*).

2.4.2 Organ sampling

To expose internal organs of fish, an incision was made ventrally down the mid-line of the fish and the wall of the body cavity was cut away on one side (Figure 2.7 A and B). Red rock lobster were cut open on the ventral side to expose internal organs. For each organism, tissue samples (fish; gill, liver, spleen, gonad where present: lobster; gill, hepatopancreas, gonad where present) were collected. Each of the liver and hepatopancreas samples where halved, creating one sample for analysis of PAHs, which were stored in a -20°C freezer in Whirlpak bags for later analysis and the other for histological analysis. Histological samples (gills, liver, haepatopancreas, gonads, spleen) were placed into histocasettes and stored in 10% neutral buffered formalin. Gill samples were always taken from the second gill arch on the left hand side of the animal.

2.4.3 Bile sampling

Using a 0.5 mL syringe (29 G fixed needle), a sample of bile was extracted from the gall bladders of fish (Figure 2.7 C) and stored in a glass vial in a -20°C freezer for later analysis.

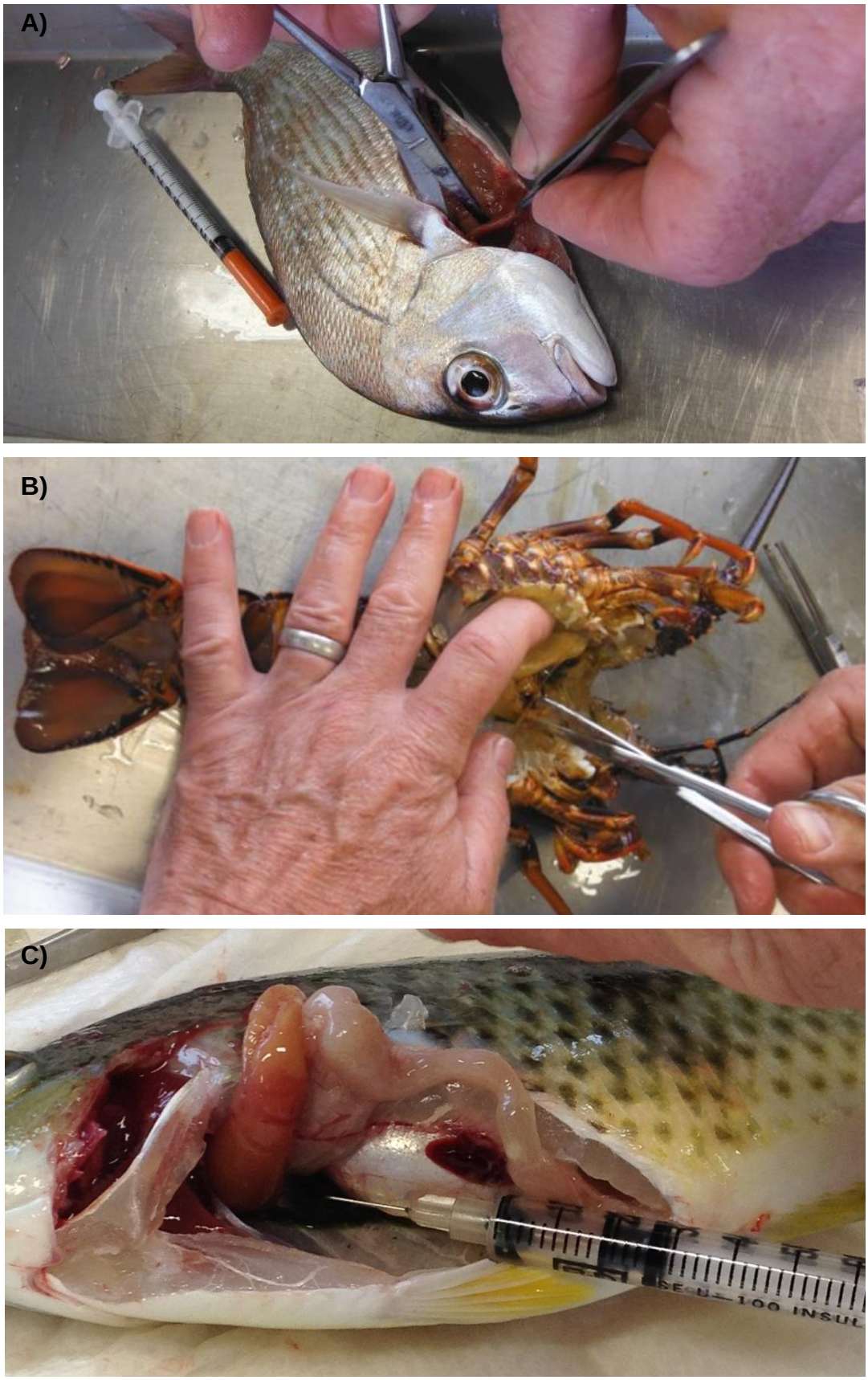

Figure 2.7: A) liver being extracted from a snapper (*Pagrus auratus*), B) haepatopancreas being extracted from a red rock lobster (*Jasus edwardsii*), C) bile being drawn from a spotted wrasse (*Notolabrus celidotus*).

2.5 Haematology

Within 1 hour of collection, fish blood was analysed or prepared for total red blood cell counts (RBCC), haemoglobin concentration (Hb), packed cell volume (PCV), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), mean cell volume (MCV) and differential leukocyte cell counts according to standard methods presented in Dacie and Lewis 1991. Lobster haematological analysis included haemocyte counts (HC) and differential haemocyte counts.

2.5.1 Packed cell volume

Packed cell volume was determined using the micro-capillary method and capillary centrifugation. Blood samples were drawn into 1-5 µL micro-haematocrit tubes (Drummond Scientific Company, USA), sealed with Critoseal and centrifuged for 5 min at 12,000 rpm. The packed cell volume value was determined using a packed cell volume reader and was calculated as a percentage in relation to total blood volume.

2.5.2 Cell counts

Fish blood was diluted 1:200 in red blood cell counting fluid (1% formalin with 32 g/L trisodium citrate) and lobster blood was counted without further dilution. Haemocyte counts were completed under a microscope using an Improved Neubauer haemocytometer (magnification 40×).

2.5.3 Differential leukocyte/ haemocyte analysis

2.5.3.1 Blood smears

A drop of blood (2 µL) was pipetted onto one end of a microscope slide and blood smears were made using the standard procedure presented in Dacie and Lewis 1991.

2.5.3.2 Blood slide staining

Stock solutions of Giemsa stain and May-Grunwald stain were made with 1 g of Giemsa (Sigma Alrich) added to 100 mL of methanol and warmed for 15 min at 50°C and then through 150 mm filter paper. May-Grunwald stain was prepared with 0.3 g of May Grunwald stain (Sigma Alrich)

added to 100 mL of methanol and warmed for 15 min at 50°C and then filtered through 150 mm filter paper. Dried blood smears were placed into a slide holder (10 per holder) and were fixed by placing them into a staining jar of methanol (300 mL) for 20 min. Slides were transferred to a staining jar containing May-Grunwald stain (150 mL) and buffered water (150 mL) (1:1) and left for 15 min before being transferred to a jar containing Giemsa stain (30 mL) (1:9) and buffered water (270 mL) for 15 min. After time, slides were then transferred and washed rapidly through four jars of buffered water (300 mL). Slides were left in the final jar for 3 min to differentiate. Slides were removed from the jar and placed vertically in slide-holders and left to dry.

Dried slides were then cover-slipped by streaking a small amount of DPX mountant down the slide surface with a glass rod, and lowering the coverslip onto the slide with a pin. Slides were then left on a flat surface overnight to allow the mountant to set.

2.5.3.3 Differential leukocyte/ haemocyte counts

Using a 100× magnification immersion oil objective lens a differential cell count was carried out for each slide by counting the first 100 cells seen (Figure 2.8 and Figure 2.9). Relative proportions of the cell types were recorded on a tally sheet. Each slide was scanned from side to side, moving one field of view down each time at the end of the slide. Two blood smears were made and counted for each study animal and averaged to obtain a final differential count. However, occasionally bad blood smears and staining sometimes prevented a second count.

2.5.4 Haemoglobin concentration

Blood samples were diluted 1:200 by placing 5 µL blood in 1 mL of Modified Drabkins solution (1 L of water, 50 mg/L of potassium cyanide, 200 mg/L of potassium ferricyanide). Samples were then analysed using a Shimadzu UV spectrophotometer UV1601 and a 1 cm path length cuvette. Modified Drabkins solution was used to zero the machine and samples were analysed at a wavelength of 540 nm. Three replicates were analysed for each blood sample and an average was used in the final data set.

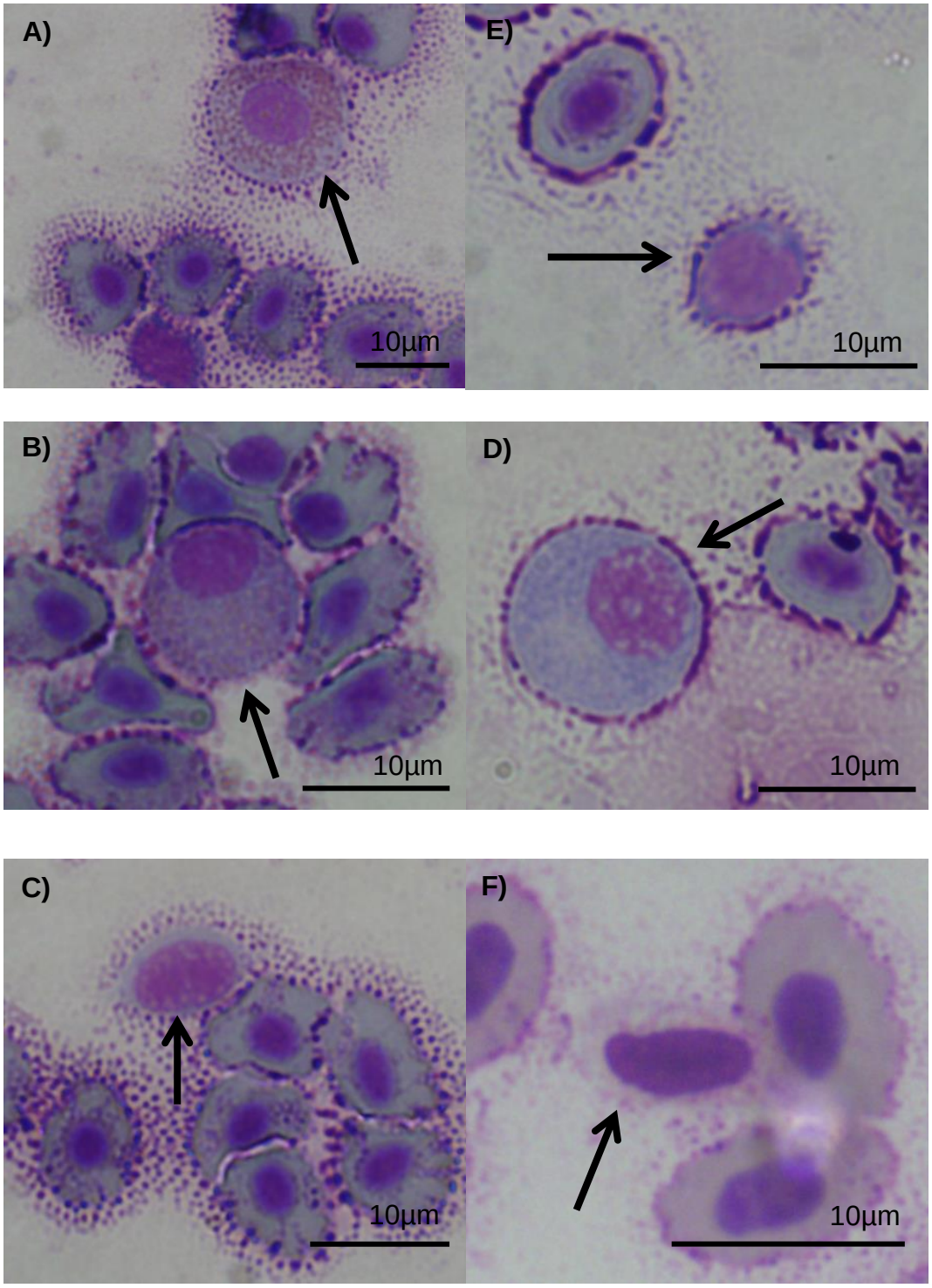

Figure 2.8: Leukocytes of *Pagrus auratus* (left) and *Notolabrus celidotus* (right). A) *Pagrus auratus* lymphocyte, B) *Pagrus auratus* granulocyte, C) *Pagrus auratus* thrombocyte D) *Notolabrus celidotus* lymphocyte, E) *Notolabrus celidotus* granulocyte, F) *Notolabrus celidotus* thrombocyte. Arrows indicate the leukocyte named.

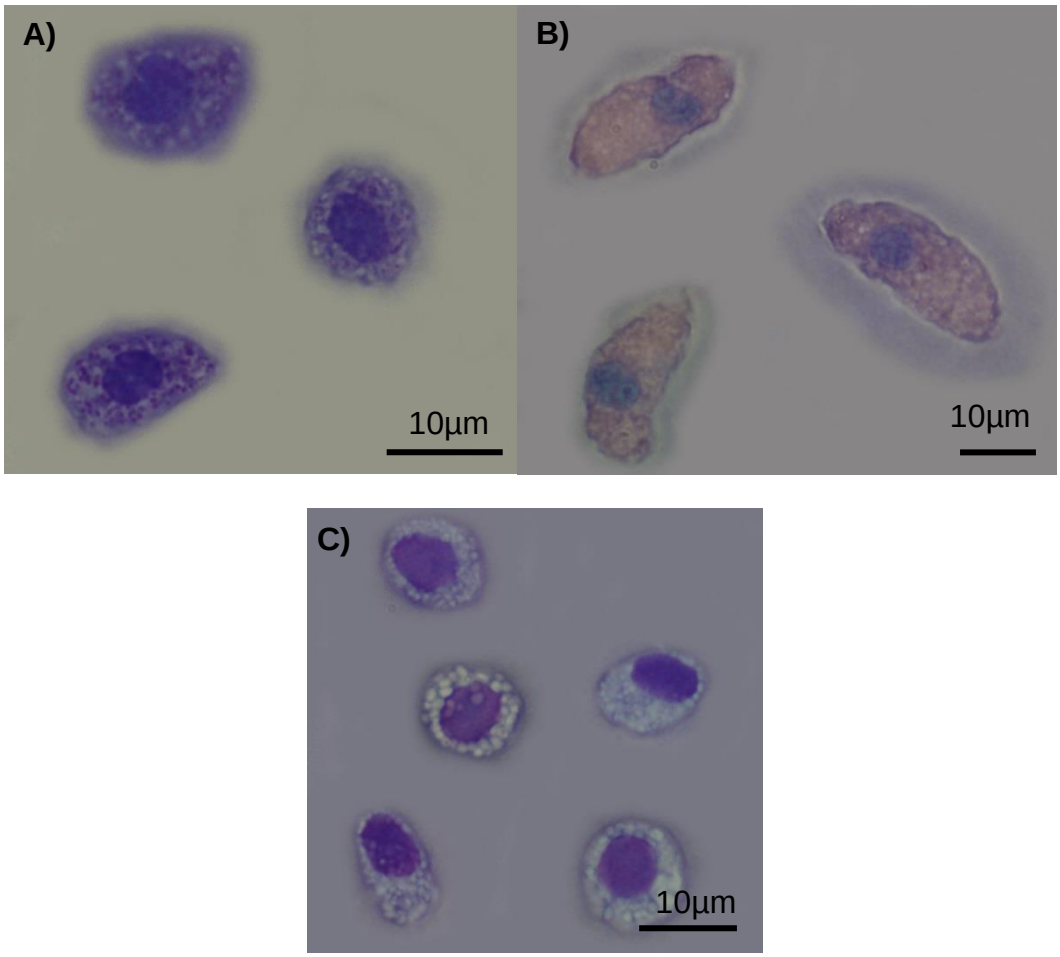

Figure 2.9: White blood cells of *Jasus edwardsii*. A) granulocytes, B) semi-granulocytes, C) hyalines.

2.6 Polycyclic aromatic hydrocarbon analysis

2.6.1 Bile and haepatopancreas preparation

2.6.1.1 Bile

Frozen bile samples were thawed and diluted between 1:500 (controls) to 1:25000 (treatments) in ethanol: water (1:1) and analysed by synchronous fluorescence spectrometry.

2.6.1.2 Haepatopancreas

Samples of frozen haepatopancreas tissue were thawed and 1 g was weighed and placed into a 15 mL falcon tube with 5 mL of ethanol: water (1:1). Samples were then homogenized using an Ultra Turrax T8 homogeniser for 1 min. Samples were then ultrasonicated using a ultrasonic processor Misonix sonicator for a total of 1 min 20 s on power setting 3, consisting of 20 s of sonication, followed by 20 s of cooling. Homogenates were centrifuged at 3500 RCF for 10 min and the supernatant aspirated from between the surface fat layer and the tissue pellet using a 1 mL syringe with 19 G needle. 50 µL of supernatant was diluted in 4950 µL ethanol:water (1:1) and analysed by synchronous fluorescence spectrometry.

2.6.1.3 Synchronous fluorescence spectrometry

Bile and haepatopancreas samples were analysed for pyrene-1-glucuronide by synchronous fluorescence spectrometry (SFS) using a Shimadzu RF5301 scanning spectrofluorometer and a 1 cm path length quartz cuvette. SFS spectra were scanned from 263 to 413 nm (excitation wavelength), scanning both monochromators simultaneously at a fixed wavelength difference (Δλ) of 37 nm and bandwidth of 5 nm. Quantification of pyrene-1-glucuronide concentration was determined by measuring the net peak area of the SFS spectrum from 372 to 392 nm (emission wavelength). Earlier studies have identified that the highest concentrations of PAHs are detected in the haepatobiliary system (Aas et al. 1998; Hosnedl et al. 2003). The selection of pyrene-1-glucuronide as a biomarker was based upon findings in Hosnedl et al. 2003. They reported

that pyrene-1-glucuronide represents the predominant product of biotransformation of pyrene; that the bioavailability of pyrene to aquatic organisms is relatively high and that it exhibits a strong fluorescence which enables sensitive detection (Hosnedl et al. 2003).

Because pyrene-1-glucuronide standards are not available, peak areas were calibrated against a series of unconjugated 1-hydroxypyrene (Toronto Research Chemicals Inc.) standards and corrected for the greater fluorescence intensity and blue shifted emission spectrum (by 5 nm) of pyrene-1-glucuronide using a calibration factor of 2.2. Figure 2.12 includes representative synchronous fluorescence spectra of control and oil exposed hepatopancreas extracts along with a hydroxypyrene standard.

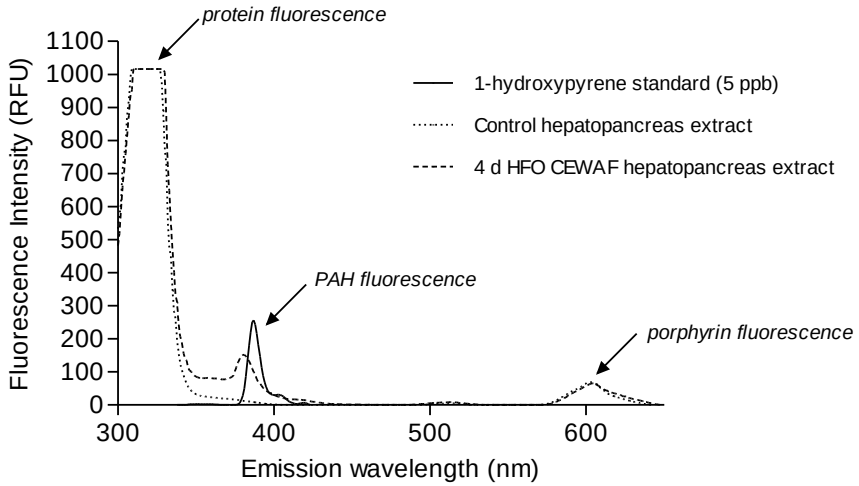

Figure 2.10: Representative synchronous fluorescence spectra (SFS) (Δλ = 37 nm) of control and PAH exposed and a 1-hydroxypyrene standard. RFU - relative fluorescence units.

2.7 Histology

Snapper spleens were fixed in 10% buffered formalin and paraffin embedded. Thin sections were mounted on standard microscope slides and stained with haematoxylin and eosin using standard methods. Each stained section was examined at 100× magnification on a Leica micro systems DMRE microscope and ten random fields of view were captured for image analysis. Images were analysed using ImageProPlus

(ImageProPlus, 2009, version 7.0.0.591, Media Cybermetrics) and the relative area of melanomacrophage centers (MMC) was calculated by drawing regions of interest around each MMC and comparing to the total area of the image field. Analysis was carried out for controls, 96 h and 10 d for HFO WAF, HFO CEWAF and Corexit 9500.

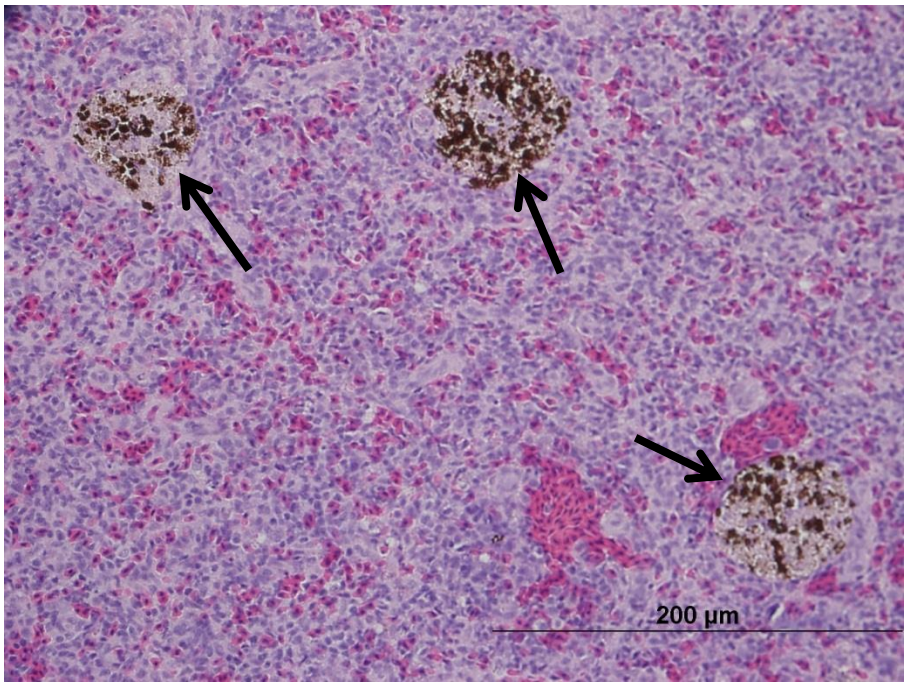

Figure 2.11: Histological image of a *Pagrus auratus* spleen. Arrows indicate melanomacrophage centers.

2.8 Statistical analysis

All statistical analysis were carried out in Statistica (version 11, Statsoft, USA). Statistical differences between blood parameters and treatments were tested using one way analysis of variance (ANOVA) combined with Tukey's honest significant difference post-hoc tests. Results for four snapper were removed as they were found to be anaemic when sampled. Statistics showed that their blood parameters fell below the 5th percentile and that any causation was not obvious from treatment.

Melanomacrophage centre area significant differences were tested using ANOVA, combined with T-tests.

2.9 Animal Ethics

All tests carried out within this study had been approved by the University of Waikato Animal Ethics Committee.

2.10 Research limitations

This study was somewhat limited due to time, resources and ethics approval. Replicates were limited by ethics approval which allowed up to 6 animals at each time point per treatment. Secondly, a limited supply of Rena HFO was supplied, and where possible six replicates were completed for the more crucial time points (control, 96 h and 10 d). Spotted wrasse replicates in particular were limited as the numbers of study organisms needed were unable to be obtained.

Chapter Three: Results

Sub-adult snapper *(Pagrus auratus*), spotted wrasse (*Notolabrus celidotus*), and red rock lobster (*Jasus edwardsii*) were exposed to environmentally relevant concentrations of 1:10000 cryolite, 1:400000 Corexit , 1:10000 Rena HFO WAF or 1:10000 HFO and 1:400000 Corexit 9500 (CEWAF: 1:40 HFO dispersant ratio) for up to 96 h. Exposure for up to 96 h caused no mortality of any species.

3.1 Fish haematology

Although no significant differences were found in any single haematological parameter between control and treatment values, noticeable changes in blood composition were observed for all treatments in *Pagrus auratus* and *Notolabrus celidotus*. Blood data analysis indicated two main signs of acute stress that were occurring during toxicant exposure: erythrocyte swelling and/or haemoconcentration arising as a result of either erythrocyte release from the spleen or haemoconcentration due to reduced plasma volume. Analysis of blood parameters allows assessment of physiological condition in the animal but generally requires evaluation of more than one parameter to conclude what effects may be occurring.

For example, an increase in PCV may occur in response to swelling of erythrocytes, release of erythrocytes from the spleen or a decrease in blood plasma volume due to the loss of water as a result of diminished osmoregulatory function or greater water retention in muscle cells or the extracellular compartment. However, erythrocytic swelling can be confirmed if the increase in PCV is accompanied by a simultaneous decrease in MCHC and increase in MCV. If, on the other hand, no changes in the MCHC and MCV were observed but increases instead in whole blood haemoglobin concentration (Hb) and RBCC then one could conclude that haemoconcentration was the principal cause of the rise in PCV. However, discriminating between splenic erythrocyte release and

plasma volume loss is impossible without further information such as relative plasma and spleen volumes.

3.1.1 Haematology effects

The overall pattern of response in fish appears to be a greater degree of erythrocyte swelling in response to the HFO WAF treatment. Lesser cell swelling occurred in response to HFO CEWAF and virtually no response to Corexit 9500 alone. This pattern was apparent in both fish species with a shorter time course in *Pagrus auratus* compared with *Notolabrus celidotus*, such that the response returned to control levels within 96 h in *Pagrus auratus* but lasted longer in *Notolabrus celidotus*. However, it is also apparent that some degree of response is caused simply by confinement of fish in the small volume treatment aquaria which can be discerned by comparing the haematology of the 96 h confined control fish (96 h control) with the controls.

The tables within this section only note significant differences between the controls and 96 h controls. All other differences are stated in text.

3.1.1.1 Snapper (*Pagrus auratus*)

ANOVA indicated no significant differences in RBCC or MCH for *Pagrus auratus*. No significant differences were found between the control and treatment values. Some significant differences were seen between the 96 h control and treatments for Hb, MCHC and PCV (Table 3.1 & Table 3.3) (p = <0.05).

HFO WAF, Corexit 9500 and HFO CEWAF treatments

Significant differences in PCV were found between HFO 24 h and HFO 96 h, HFO 4 d, and HFO 10 d. Significant differences in MCV were found between CEWAF 48 h and 4 d, and 10 d (p = <0.05).

Differences between controls and treatments usually occur within the treatment periods and begin to return to normal levels in post-exposure recovery. In relation to the HFO WAF and HFO CEWAF exposure, results for *Pagrus auratus* indicated blood cell swelling and haemoconcentration

in HFO WAF (Figure 3.1 - Figure 3.3); however blood cell swelling was more prominent in the HFO WAF treatment compared to the HFO CEWAF treatment. Slight blood swelling was observed in response to Corexit 9500 exposure (Figure 3.1 - Figure 3.3).

Cryolite treatment

Cryolite results indicated effects of blood cell swelling with an increase in PCV and MCV accompanied by decrease in MCHC, however, statistical comparisons within any one parameter were non-significant (Figure 3.4 Table 3.4).

3.1.1.2 Spotted wrasse (*Notolabrus celidotus*)

ANOVA indicated no significant differences in PCV, MCV, or MCHC for *Notolabrus celidotus*. Some significant differences were seen between the 96 h control and treatments for MCH, and Hb (Table 3.5 - Table 3.8) (p = <0.05). Only one significant difference was found between the controls: this was between the Corexit 96 h control and control MCH. However, no significant differences were seen between controls and treatments (Table 3.5 - Table 3.8).

HFO WAF, Corexit 9500 and HFO CEWAF treatments

In relation to HFO WAF results indicated effects of blood cell swelling and haemoconcentration. In response to HFO CEWAF exposure, results indicate slight blood cell swelling while exposure to Corexit 9500 alone indicated no significant effects on blood parameters (Figure 3.5 - Figure 3.7).

Cryolite treatment

RBCC results indicated one significant difference between cryolite 4 d and 10d and comparatively, significant differences in MCH were found between cryolite 4 d and 10 d (Table 3.8), however, over all exposure to cryolite indicated no significant effects on blood parameters (Figure 3.8).

Table 3.1: Means (± SEM) of blood parameter responses in *Pagrus auratus* in relation to heavy fuel oil (HFO WAF) exposure. Total haemoglobin (Hb), packed cell volume (PCV), red blood cell count (RBCC), mean erythrocyte haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), and mean cell volume (MCV). * Indicates a significant difference between the 96 h control and treatment (*p <0.05).

Treatment & time	Replicates n	Hb (g L^{-1})	PCV (%)	RBCC (x10^{12} cells L^{-1})	MCHC (g L^{-1})	MCH (pg cell $^{-1}$)	MCV (fl)
Control	6	72.6 ± 3.02	33.1 ± 1.20	3.18 ± 0.17	220 ± 11	23.0 ± 1.22	106 ± 7.92
HFO WAF 24 h	3	73.3 ± 4.36	41.1 ± 3.88	3.31 ± 0.22	182 ± 21 *	22.6 ± 3.03	125 ± 11.71
HFO WAF 48 h	3	75.4 ± 2.70	41.1 ± 0.81	3.23 ± 0.03	184 ± 10	23.4 ± 0.63	127 ± 3.69
HFO WAF 96 h	6	66.3 ± 4.59	30.1 ± 2.39	2.82 ± 0.23	223 ± 11	23.7 ± 0.84	107 ± 5.04
Control 96 h	6	83.2 ± 5.25	35.0 ± 1.77	3.27 ± 0.12	238 ± 11	25.8 ± 2.37	108 ± 7.97
HFO WAF 4 d	3	58.1 ± 7.38 *	24.4 ± 2.11 *	2.76 ± 0.35	236 ± 12	21.0 ± 0.39	89 ± 3.87
HFO WAF 10 d	5	70.2 ± 1.85	30.7 ± 0.60	3.14 ± 0.80	228 ± 4	22.4 ± 1.37	98 ± 6.68

Table 3.2: Means (± SEM) of blood parameter responses of *Pagrus auratus* in relation to heavy fuel oil and Corexit 9500 (Chemically enhanced water accommodated fraction (CEWAF)) exposure. Total haemoglobin (Hb), packed cell volume (PCV), red blood cell count (RBCC), mean erythrocyte haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), and mean cell volume (MCV). N.D – No data.

Treatment & time	Replicates n	Hb (g L^{-1})	PCV (%)	RBCC (x10^{12} cells L^{-1})	MCHC (g L^{-1})	MCH (pg cell $^{-1}$)	MCV (fl)
Control	6	72.6 ± 3.02	33.1 ± 1.20	3.18 ± 0.17	220 ± 11	23.0 ± 1.22	106 ± 7.92
HFO CEWAF 24 h	3	N.D	37.8 ± 3.66	3.08 ± 0.12	N.D	N.D	122 ± 7.41
HFO CEWAF 48 h	3	80.8 ± 2.70	37.8 ± 1.41	2.68 ± 0.35	214 ± 10	30.9 ± 2.72	146 ± 18.83
HFO CEWAF 96 h	5	69.8 ± 5.00	30.3 ± 2.04	2.91 ± 0.32	231 ± 9	23.9 ± 1.23	104 ± 5.45
Control 96 h	6	83.2 ± 5.25	35.0 ± 1.77	3.27 ± 0.12	238 ± 11	25.8 ± 2.37	108 ± 7.97
HFO CEWAF 4 d	3	76.5 ± 5.05	32.5 ± 3.88	3.58 ± 0.51	239 ± 19	21.9 ± 1.74	92 ± 3.45
HFO CEWAF 10 d	5	67.8 ± 4.17	29.8 ± 1.62	3.03 ± 0.72	229 ± 18	22.5 ± 1.60	99 ± 7.08

Table 3.3: Means (± SEM) of blood parameter responses of *Pagrus auratus* in relation to Corexit 9500 exposure. Total haemoglobin (Hb), packed cell volume (PCV), red blood cell count (RBCC), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), and mean cell volume (MCV). * Indicates a significant difference between the 96 h control and treatment (*p <0.05).

Treatment & time	Replicates *n*	Hb (g L^{-1})	PCV (%)	RBCC (x10^{12} cells L^{-1})	MCHC (g L^{-1})	MCH (pg cell $^{-1}$)	MCV (fl)
Control	6	72.6 ± 3.02	33.1 ± 1.20	3.18 ± 0.17	220 ± 11	23.0 ± 1.22	106 ± 7.92
Corexit 9500 24 h	3	73.5 ± 5.01	34.6 ± 3.61	3.16 ± 0.11	216 ± 18	23.3 ± 1.11	110 ± 12.93
Corexit 9500 48 h	3	75.7 ± 2.49	31.3 ± 1.47	3.29 ± 0.36	242 ± 4	23.4 ± 1.72	97 ± 6.09
Corexit 9500 96 h	5	67.5 ± 3.16	30.5 ± 1.68	3.15 ± 0.34	224 ± 14	21.4 ± 0.69	97 ± 5.76
Control 96 h	6	83.2 ± 5.25	35.0 ± 1.77	3.27 ± 0.12	238 ± 11	25.8 ± 2.37	108 ± 7.97
Corexit 9500 4 d	3	64.4 ± 6.35	25.6 ± 0.70	3.20 ± 0.50	251 ± 20	21.2 ± 3.72	84 ± 13.13
Corexit 9500 10 d	5	66.5 ± 5.69	26.8 ± 2.53 *	2.92 ± 0.36	249 ± 9	22.9 ± 0.73	92 ± 4.16

Table 3.4: Means (± SEM) of blood parameter responses of *Pagrus auratus* in relation to cryolite exposure. Total haemoglobin (Hb), packed cell volume (PCV), red blood cell count (RBCC), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), and mean cell volume (MCV).

Treatment & time	Replicates *n*	Hb (g L^{-1})	PCV (%)	RBCC (x10^{12} cells L^{-1})	MCHC (g L^{-1})	MCH (pg cell $^{-1}$)	MCV (fl)
Control	6	72.6 ± 3.02	33.1 ± 1.20	3.18 ± 0.17	220 ± 11	23.0 ± 1.22	106 ± 7.92
Cryolite 24 h	3	70.6 ± 8.21	36.2 ± 3.48	2.92 ± 0.27	194 ± 5	24.1 ± 1.37	124 ± 6.87
Cryolite 48h	3	72.1 ± 1.38	38.2 ± 2.15	3.24 ± 0.25	189 ± 7	22.5 ± 1.38	119 ± 7.27
Cryolite 96 h	3	74.2 ± 2.91	38.2 ± 2.15	2.78 ± 0.25	195 ± 3	27.3 ± 3.16	140 ± 17.23
Control 96 h	6	83.2 ± 5.25	35.0 ± 1.77	3.27 ± 0.12	238 ± 11	25.8 ± 2.37	108 ± 7.97
Cryolite 4 d	3	73.2 ± 1.18	35.8 ± 0.41	3.19 ± 0.02	205 ± 4	23.0 ± 0.50	112 ± 1.10
Cryolite 10 d	3	75.7 ± 4.69	32.5 ± 0.41	3.30 ± 0.25	233 ± 15	23.3 ± 2.55	100 ± 6.97

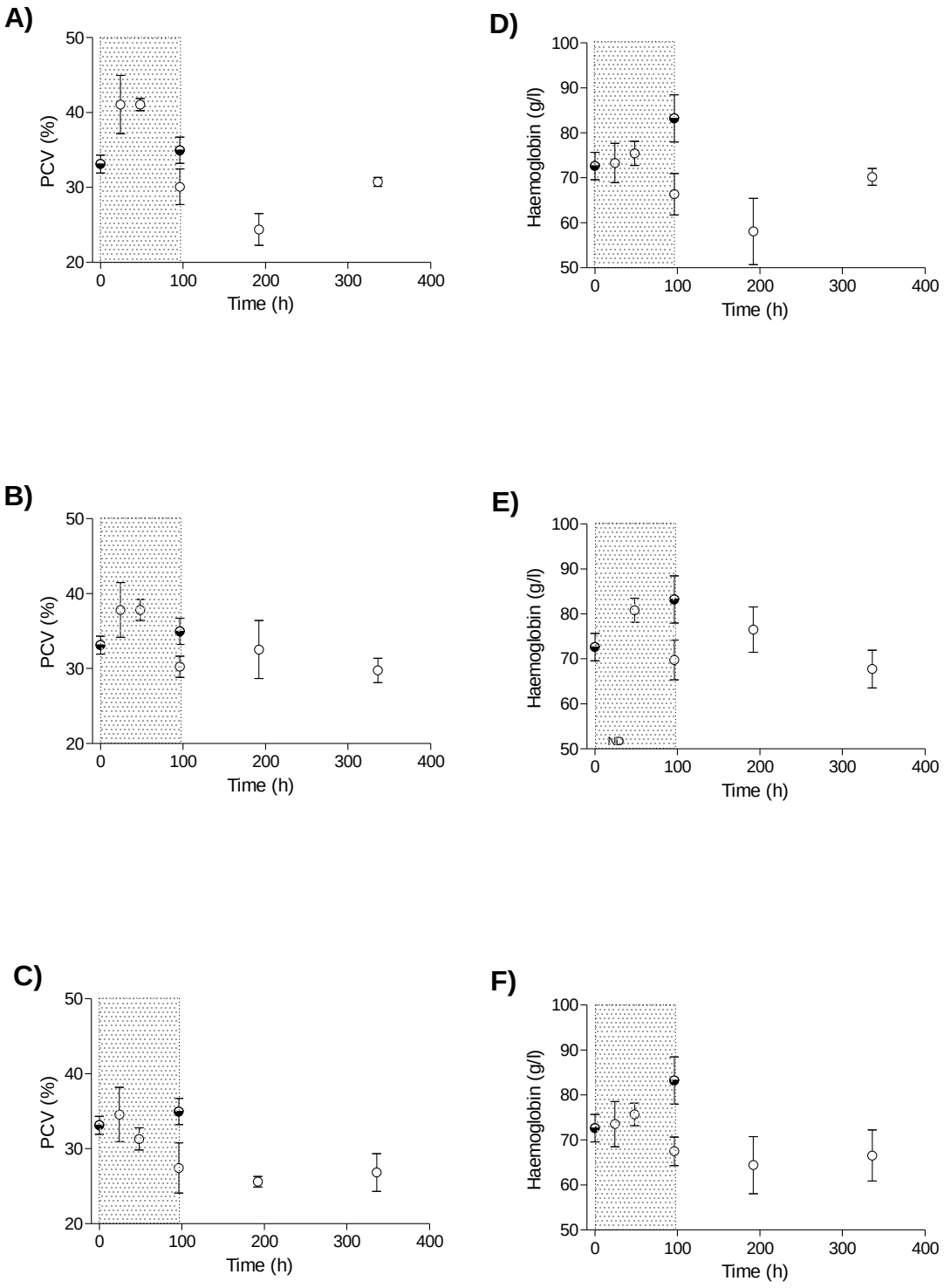

Figure 3.1: Mean (±SEM) haemoglobin responses of *Pagrus auratus*. Packed cell volume (PCV) changes in response to: A) heavy fuel oil (HFO WAF), B) HFO and Corexit 9500 (HFO CEWAF), C) Corexit 9500; and haemoglobin concentration changes to: D) HFO WAF, E) HFO CEWAF, F) Corexit 9500. Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

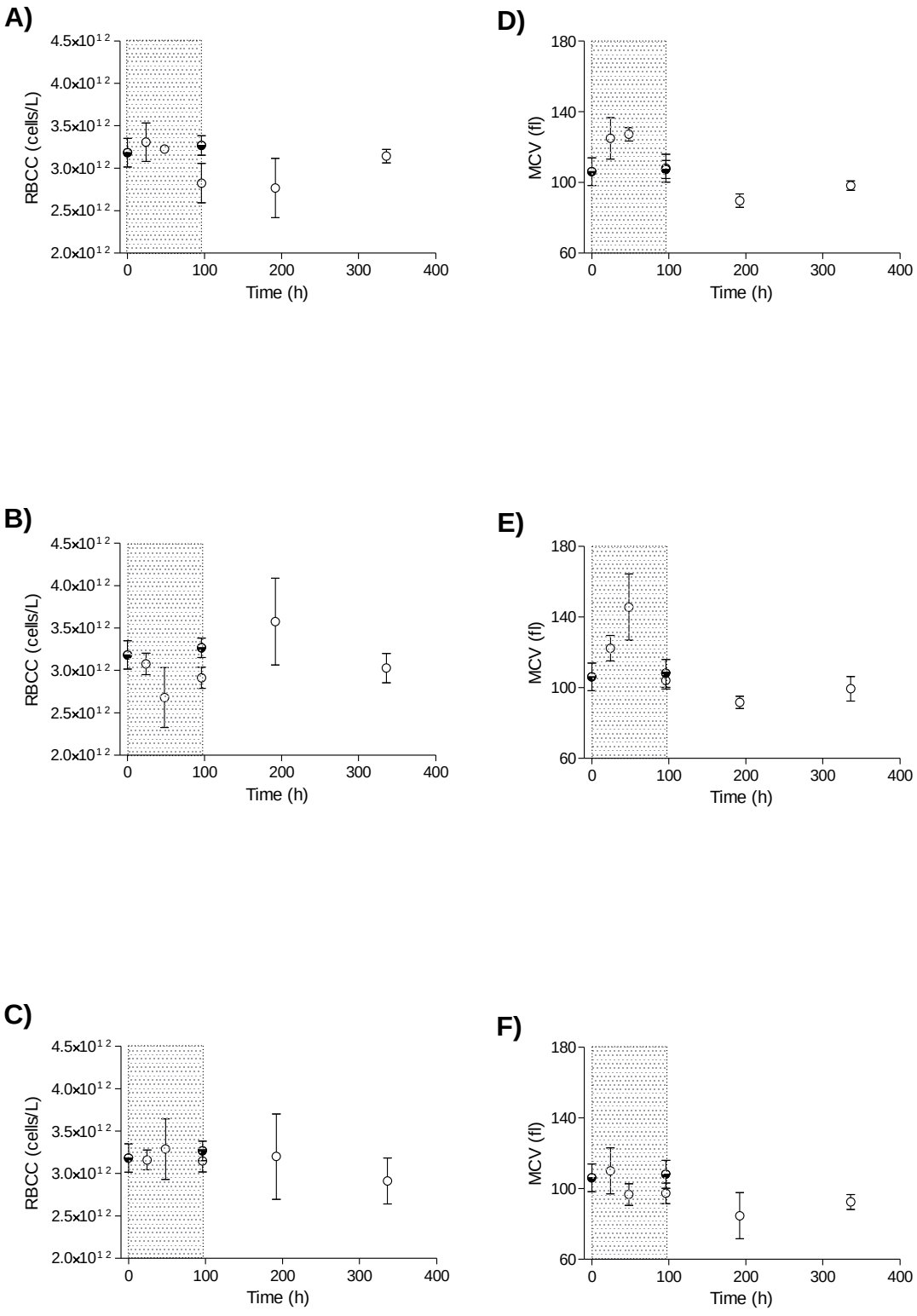

Figure 3.2: Mean (±SEM) haemoglobin responses of *Pagrus auratus.* Red blood cell count (RBCC) changes in response to: A) heavy fuel oil (HFO WAF), B) HFO and Corexit 9500 (HFO CEWAF), C) Corexit 9500; and mean cell volume (MCV) responses to: D) HFO WAF, E) HFO CEWAF, F) Corexit 9500. Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

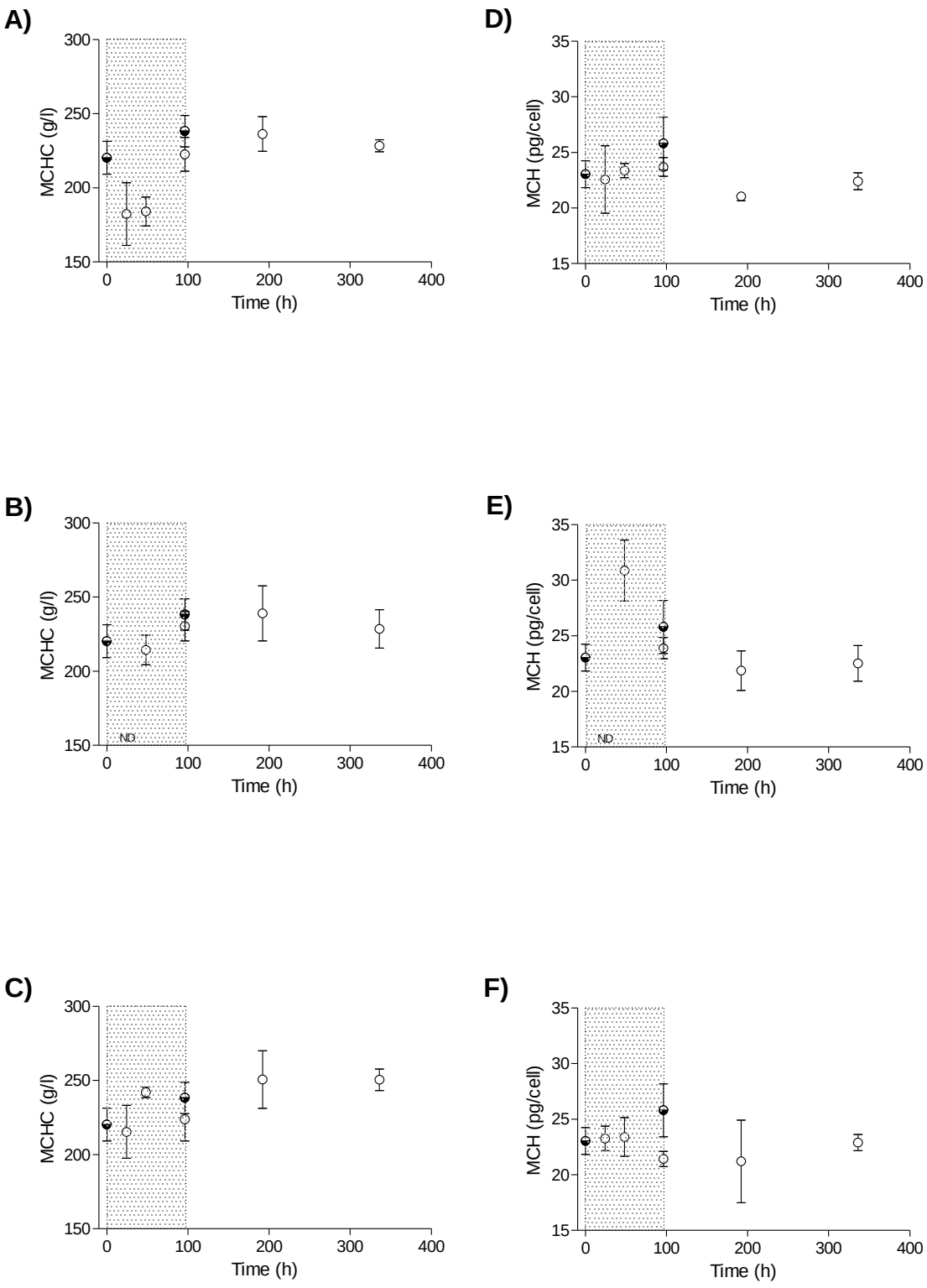

Figure 3.3 Mean (±SEM) haemoglobin responses of *Pagrus auratus*. Mean cell haemoglobin concentration (MCHC) in response to: A) heavy fuel oil (HFO WAF), B) HFO and Corexit 9500 (HFO CEWAF) C) Corexit 9500; and responses of Mean cell haemoglobin (MCH) in response to: D) HFO WAF, E) HFO CEWAF, F) Corexit 9500. Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

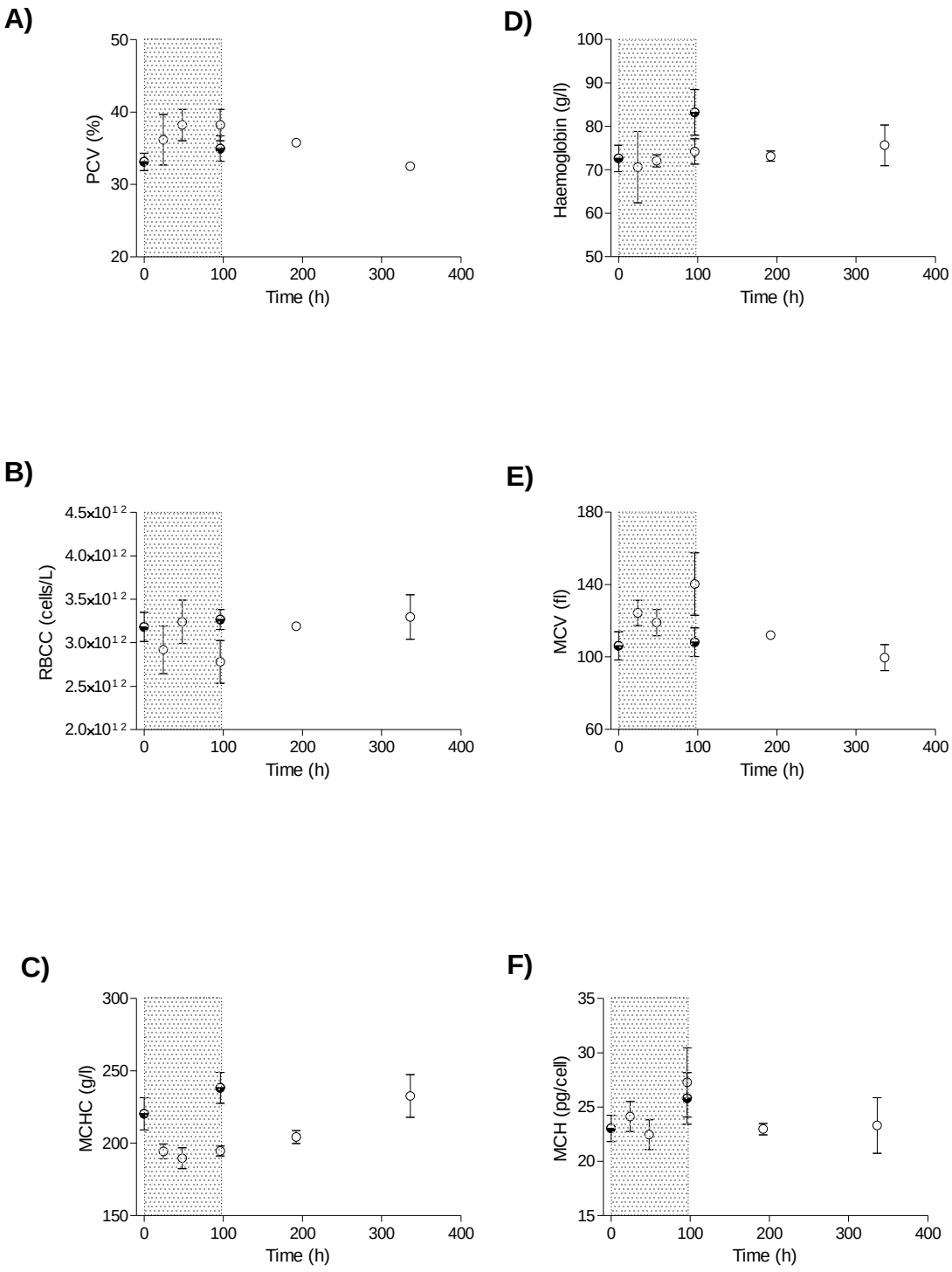

Figure 3.4: Mean (±SEM) haemoglobin responses of *Pagrus auratus* in relation to cryolite exposure. A) packed cell volume (PCV), B) erythrocyte count, C) mean cell haemoglobin concentration (MCHC), D) haemoglobin concentration (Hb), E) mean cell volume (MCV), F) mean cell haemoglobin (MCH). Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

Table 3.5: Means (± SEM) of blood parameter responses of *Notolabrus celidotus* in relation to heavy fuel oil (HFO WAF) exposure. Total haemoglobin (Hb), packed cell volume (PCV), red blood cell count (RBCC), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), and mean cell volume (MCV).

Treatment & time	Replicates *n*	Hb ($g\ L^{-1}$)	PCV (%)	RBCC ($x10^{12}$ cells L^{-1})	MCHC ($g\ L^{-1}$)	MCH (pg $cell^{-1}$)	MCV (fl)
Control	6	62.4 ± 2.99	30.5 ± 1.57	3.42 ± 0.18	206 ± 13.02	18.3 ± 0.78	90 ± 7.68
HFO WAF 24 h	3	77.8 ± 7.65	46.1 ± 6.55	3.97 ± 0.66	173 ± 22	20.4 ± 2.42	117 ± 4.10
HFO WAF 48 h	3	90.6 ± 17.22	46.5 ± 5.99	4.28 ± 0.74	195 ± 26	21.4 ± 2.20	111 ± 7.37
HFO WAF 96 h	3	71.6 ± 5.89	55.8 ± 9.66	4.06 ± 1.07	134 ± 15	19.6 ± 4.06	150 ± 37.24
Control 96 h	6	81.5 ± 5.85	39.1 ± 5.32	3.43 ± 0.23	224 ± 29.91	23.8 ± 1.04	114 ± 14.70
HFO WAF 4 d	3	64.2 ± 6.07	40.3 ± 2.11	4.05 ± 0.19	159 ± 10	15.8 ± 1.00	99 ± 0.51
HFO WAF 10 d	3	57.6 ± 7.69	33.8 ± 5.88	2.83 ± 0.19	177 ± 26	20.3 ± 1.87	118 ± 13.72

Table 3.6: Means (± SEM) of blood parameter responses of *Notolabrus celidotus* in relation to (Chemically enhanced water accommodated fraction (CEWAF)) exposure. Total haemoglobin (Hb), packed cell volume (PCV), red blood cell count (RBCC), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), and mean cell volume (MCV). * Indicates a significant difference between the 96 h control and treatment (*p <0.05).

Treatment & time	Replicates *n*	Hb ($g\ L^{-1}$)	PCV (%)	RBCC ($x10^{12}$ cells L^{-1})	MCHC ($g\ L^{-1}$)	MCH (pg $cell^{-1}$)	MCV (fl)
Control	6	62.4 ± 2.99	30.5 ± 1.57	3.42 ± 0.18	206 ± 13	18.3 ± 0.78	90 ± 7.68
HFO CEWAF 24 h	3	54.0 ± 2.33 *	39.4 ± 3.57	3.30 ± 0.11	139 ± 9	16.3 ± 0.41 *	119 ± 7.25
HFO CEWAF 48 h	3	60.3 ± 2.13	30.8 ± 3.20	2.58 ± 0.26	202 ± 29	23.7 ± 1.77	123 ± 22.67
HFO CEWAF 96 h	3	72.2 ± 1.62	35.8 ± 0.81	3.35 ±0.03	202 ± 4	21.6 ± 0.67	107 ± 3.09
Control 96 h	6	81.5 ± 5.85	39.1 ± 5.32	3.43 ± 0.23	224 ± 30	23.8 ± 1.04	114 ± 14.70
HFO CEWAF 4 d	3	72.1 ± 2.47	40.7 ± 2.15	3.52 ± 0.40	178 ± 8	20.9 ± 2.19	119 ± 18.80
HFO CEWAF 10 d	3	74.5 ± 10.73	37.7 ± 4.44	3.23 ± 0.36	198 ± 21	23.2 ± 3.10	117 ± 3.76

Table 3.7: Means (± SEM) of blood parameter responses of *Notolabrus celidotus* in relation to Corexit 9500 exposure. Total haemoglobin (Hb), packed cell volume (PCV), red blood cell count (RBCC), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), and mean cell volume (MCV). * Indicates a significant difference between the 96 control and treatment (*$p < 0.05$).

Treatment & time	Replicates *n*	Hb ($g\ L^{-1}$)	PCV (%)	RBCC ($x10^{12}$ cells L^{-1})	MCHC ($g\ L^{-1}$)	MCH (pg cell $^{-1}$)	MCV (fl)
Control	6	62.4 ± 2.99	30.5 ± 1.57	3.42 ± 0.18	206 ± 13.02	18.3 ± 0.78	90 ± 7.68
Corexit 9500 24 h	3	56.5 ± 7.47 *	29.3 ± 2.11	3.46 ± 0.55	193 ± 22	16.5 ± 0.49 *	89 ± 14.00
Corexit 9500 48 h	3	63.9 ± 3.07	32.5 ± 0.81	2.83 ± 0.45	196 ± 4	23.4 ± 2.57	120 ± 13.60
Corexit 9500 96 h	3	54.9 ± 6.97	34.1 ± 3.36	2.86 ± 0.18	160 ± 7	19.2 ± 1.93	119 ± 7.13
Control 9500 96 h	6	81.5 ± 5.85	39.1 ± 5.32	3.43 ± 0.23	224 ± 29.91	23.8 ± 1.04	114 ± 14.70
Corexit 9500 4 d	3	62.3 ± 4.67	37.0 ± 4.01	3.29 ± 0.27	174 ± 28	19.0 ± 0.28	116 ± 21.14
Corexit 950010 d	3	65.0 ± 1.57	33.7 ± 2.59	3.35 ± 0.33	195 ± 15	19.7 ± 1.78	102 ± 7.96

Table 3.8: Means (±SEM) of blood parameter responses of *Notolabrus celidotus* in relation to Cryolite exposure. Total haemoglobin (Hb), packed cell volume (PCV), red blood cell count (RBCC), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), and mean cell volume (MCV). * Indicates a significant difference between the 96 control and treatment (*$p < 0.05$).

Treatment & time	Replicates *n*	Hb ($g\ L^{-1}$)	PCV (%)	RBCC ($x10^{12}$ cells L^{-1})	MCHC ($g\ L^{-1}$)	MCH (pg cell $^{-1}$)	MCV (fl)
Control	6	62.4 ± 2.99	30.5 ± 1.57	3.42 ± 0.18	206 ± 13.02	18.3 ± 0.78	90 ± 7.68
Cryolite 24 h	3	62.5 ± 5.48	38.6 ± 2.05	3.44 ± 0.58	163 ± 17	18.9 ± 2.30	119 ± 20.40
Cryolite 48 h	3	74.6 ± 13.0	39.0 ± 1.22	3.36 ± 0.42	192 ± 37	22.3 ± 2.64	120 ± 13.30
Cryolite 96 h	3	65.0 ± 3.6	37.8 ± 1.22	3.27 ± 0.11	172 ± 12	19.9 ± 0.64	116 ± 6.90
Control 96 h	6	81.5 ± 5.85	39.1 ± 5.32	3.43 ± 0.23	224 ± 29.91	23.8 ± 1.04	114 ± 14.70
Cryolite 4 d	3	62.1 ± 7.21	42.8 ± 4.82	4.12 ± 0.23	148 ± 23	15.0 ± 1.45 *	104 ± 6.02
Cryolite 10 d	3	56.0 ± 1.81	29.7 ± 2.85	2.37 ± 0.20	194 ± 26	24.0 ± 2.31	126 ± 15.22

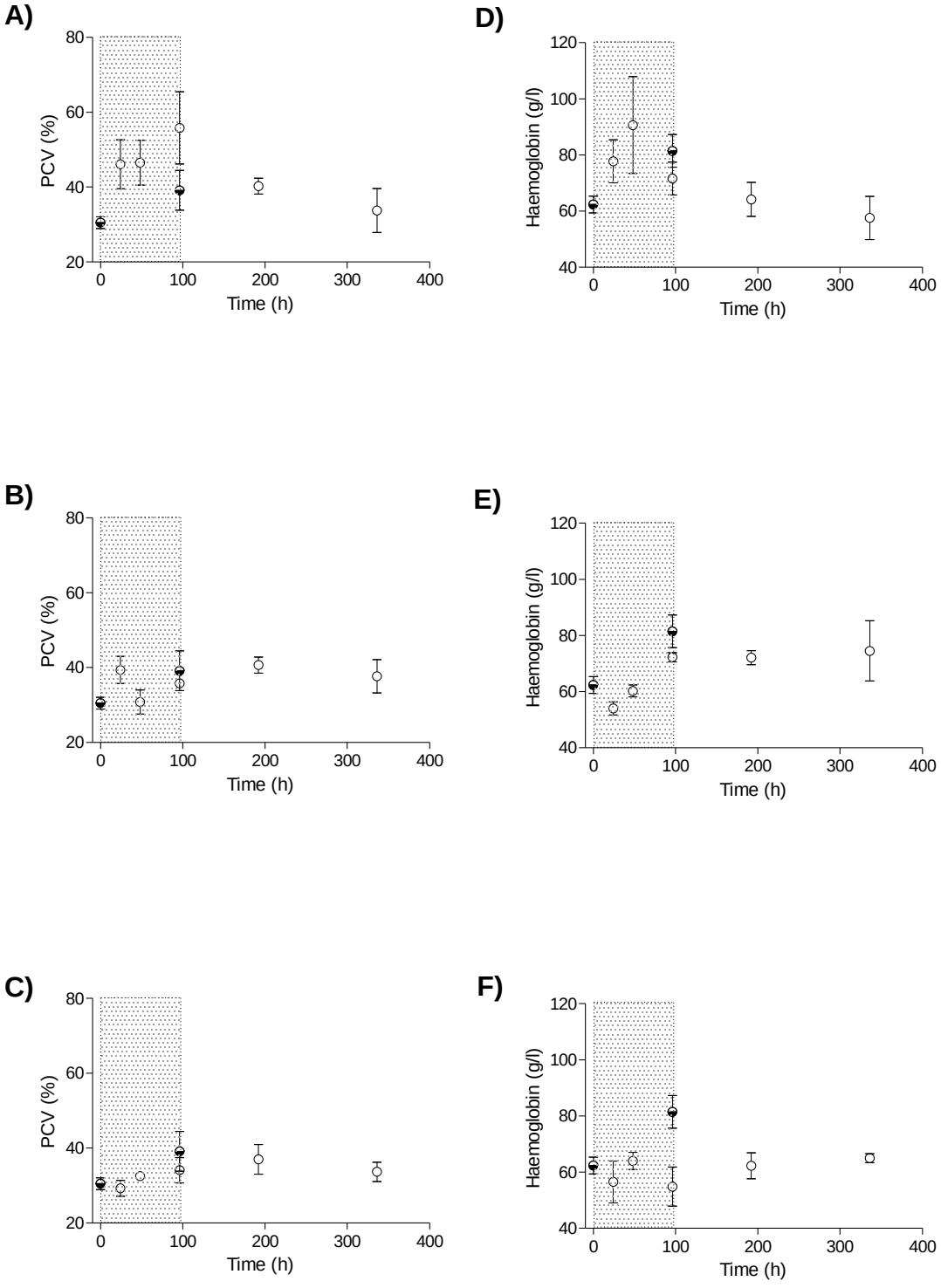

Figure 3.5: Mean (±SEM) haemoglobin responses of *Notolabrus celidotus.* Packed cell volume (PCV) values in response to: A) heavy fuel oil (HFO WAF), B) HFO and Corexit 9500 (HFO CEWAF), C) Corexit 9500; and responses of haemoglobin concentration (Hb) in response to: D) HFO WAF, E) HFO CEWAF, F) Corexit 9500. Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

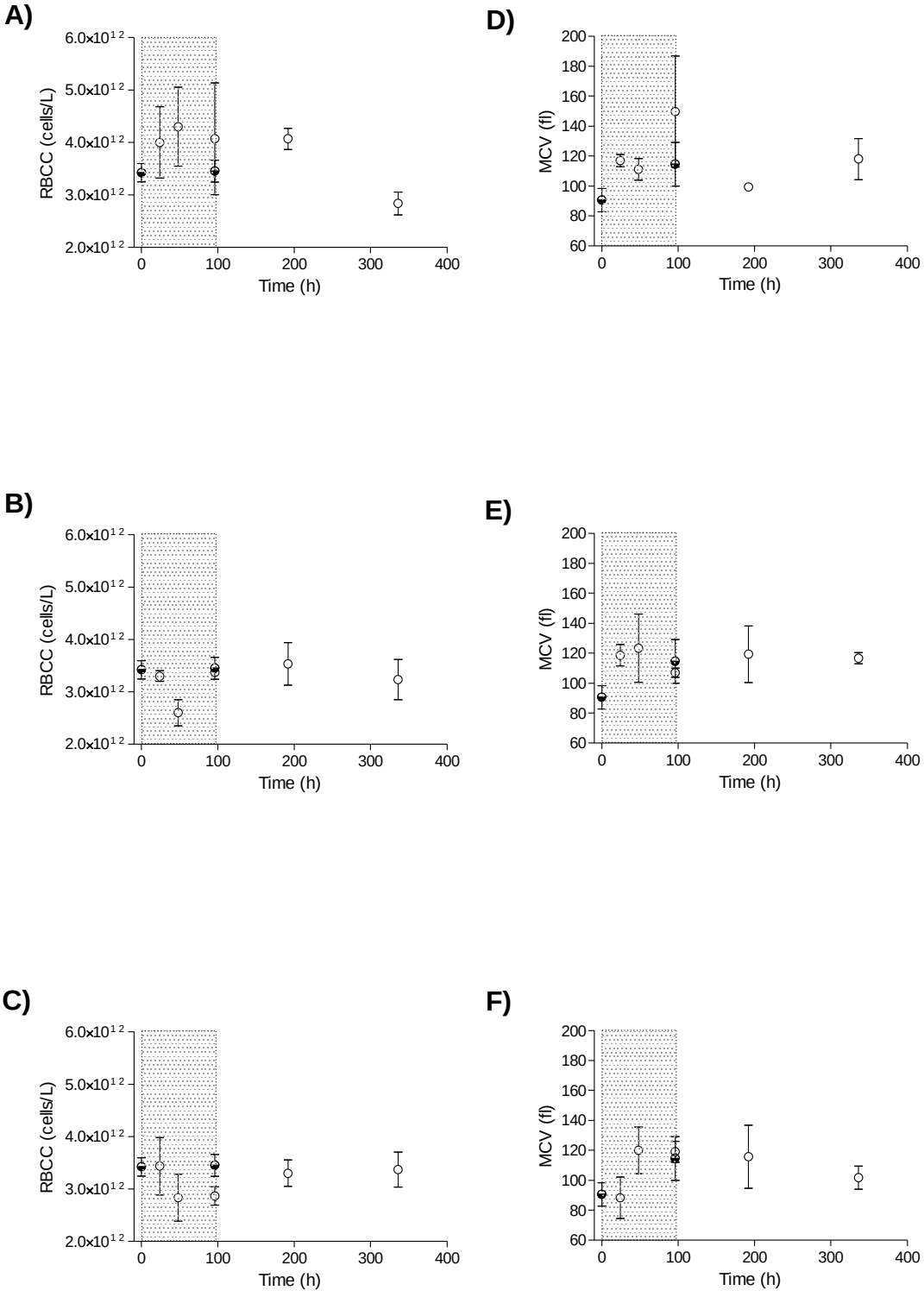

Figure 3.6: Mean (±SEM) haemoglobin responses of *Notolabrus celidotus.* Red blood cell count (RBCC) changes in response to: A) heavy fuel oil (HFO WAF), B) HFO and Corexit 9500 (HFO CEWAF), C) Corexit 9500; and mean cell volume (MCV) responses to: D) HFO WAF, E) HFO CEWAF, F) Corexit 9500. Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

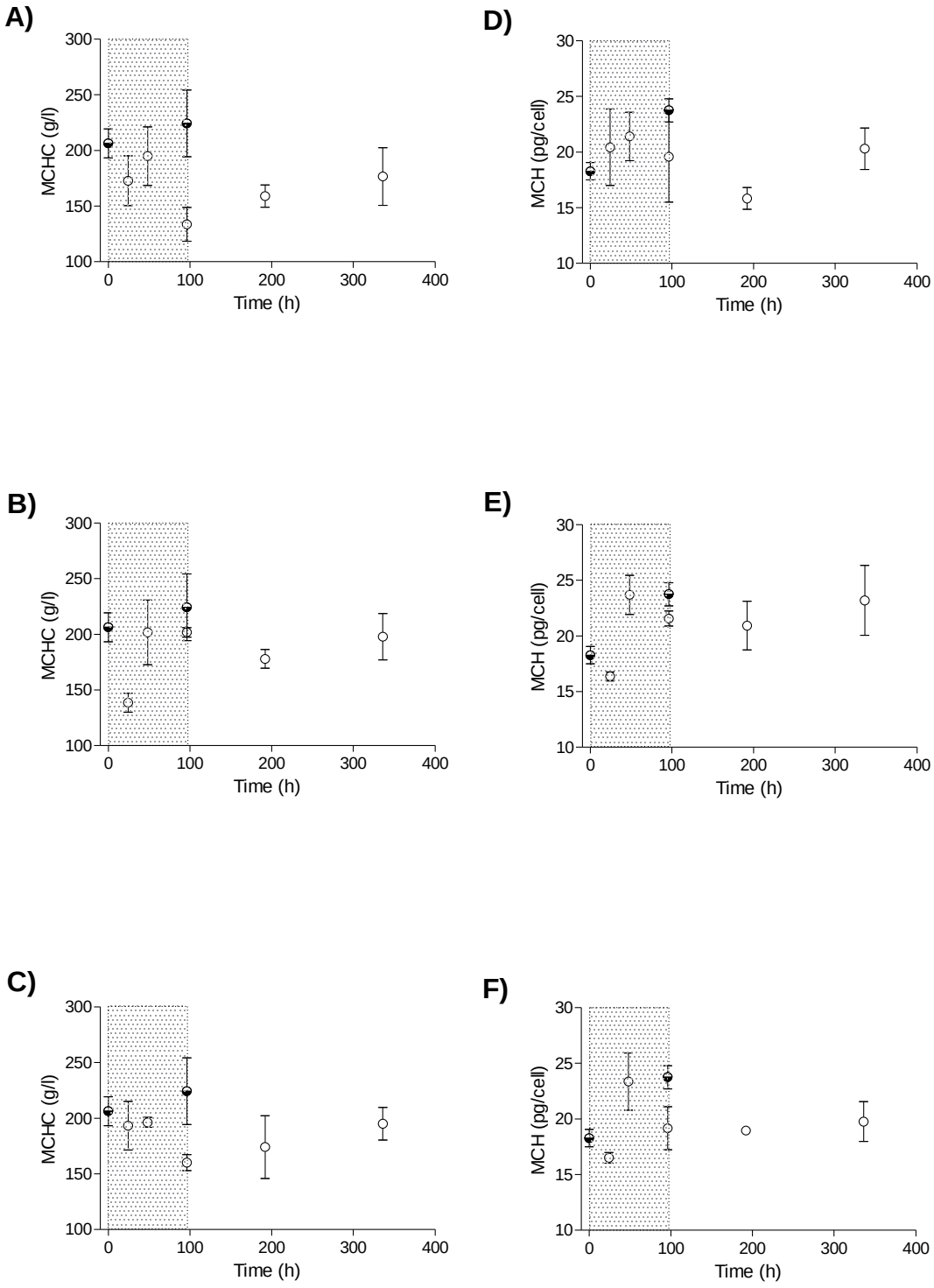

Figure 3.7: Mean (±SEM) haemoglobin responses of *Notolabrus celidotus*. Mean cell haemoglobin concentration (MCHC) in response to: A) heavy fuel oil (HFO WAF), B) HFO and Corexit 9500 (HFO CEWAF), C) Corexit 9500; and responses of Mean cell haemoglobin (MCH) in response to: D) HFO WAF, E) CEWAF, F) Corexit 9500. Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

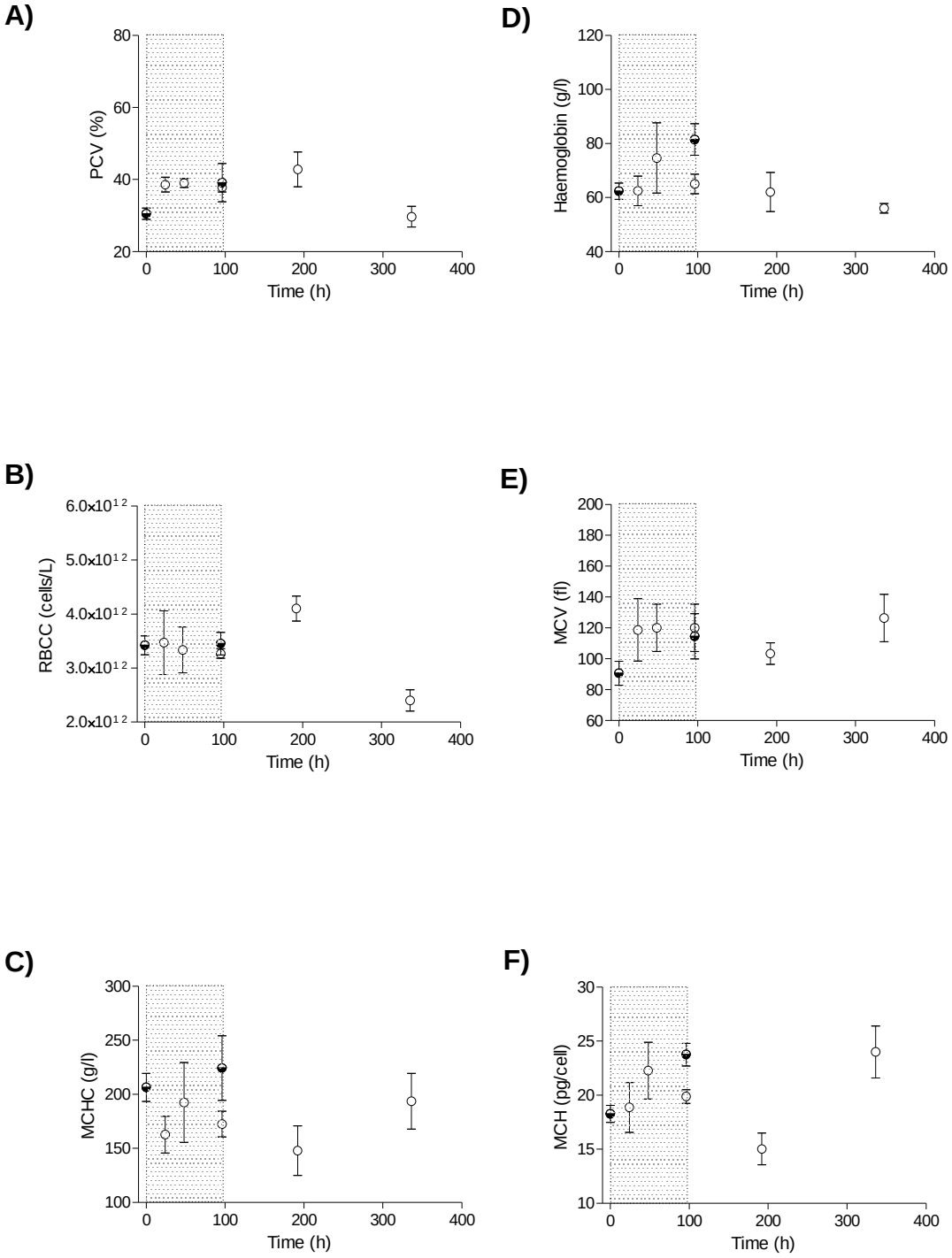

Figure 3.8: Mean (±SEM) haemoglobin responses of *Notolabrus celidotus* in relation to cryolite exposure. A) packed cell volume (PCV), B) red blood cell count (RBCC), C) mean cell haemoglobin concentration (MCHC), D) haemoglobin concentration (Hb), E) mean cell volume (MCV), F) mean cell haemoglobin (MCH). Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

3.1.2 Fish leukocyte analysis

3.1.2.1 Snapper *(Pagrus auratus)*

HFO WAF, Corexit 9500 and HFO CEWAF treatments

ANOVA indicated significant differences between control and 24 h HFO CEWAF between all measured cell types (Table 3.9). Between treatments significant differences were found between HFO WAF 24 h and 96 h; 4 d; 10 d and HFO CEWAF 96 h and 48 h. Significant differences in thrombocytes were found between HFO CEWAF 24 h and 96 h; 4 d and 10d (Table 3.9).

The significant difference seen in all leukocyte cell types between the control and 24 h HFO CEWAF (and between the 96 h control and 24 h HFO CEWAF for the lymphocytes and granulocytes) was due to a significant drop in lymphocytes at 24 h (Figure 3.9 and Table 3.9) which begin to increase again after 48 h but return to control levels by 96 h. This decrease coincides with a proportional increase in granulocytes leading to the significant difference observed in granulocytes for the HFO CEWAF treatment. The HFO WAF and Corexit 9500 treatments showed no major change in lymphocyte numbers (Table 3.9 and Figure 3.9).

Cryolite

ANOVA indicated one significant difference in thrombocytes in relation to 24 h cryolite treatment (Table 3.9). Although no other significant differences were found between leukocyte cell counts in relation to cryolite exposure a noticeable difference in proportion of lymphocytes and granulocytes was observed at the 24 h and 48 h time points (Figure 3.10 and Table 3.10) indicating that some lymphocyte apoptosis may be occurring.

Table 3.9: Mean (±SEM) differential leukocyte counts (%) of *Pagrus auratus* in relation to heavy fuel oil (HFO WAF), HFO and Corexit 9500 (HFO CEWAF), Corexit 9500 and cryolite. * Indicates a significant difference between the 96 h control and treatment (p* <0.05). † Indicates a significant difference between the control and treatment (†*p* <0.05).**

Treatment & time	*n*	Lymphocytes	Granulocytes	Thrombocytes
Control	6	74 ± 2.72	25 ± 2.49	2 ± 0.50
HFO WAF 24 h	3	67 ± 4.51	33 ± 5.01	1 ± 0.50
HFO WAF 48 h	3	71 ± 5.55	29 ± 5.42	1 ± 0.19
HFO WAF 96 h	3	83 ± 5.98	14 ± 6.00	3 ± 0.87
Control 96 h	6	76 ± 8.10	23 ± 8.07	2 ± 0.51
HFO WAF4 d	3	68 ± 3.33	30 ± 2.84	3 ± 0.44
HFO WAF 10 d	6	86 ± 3.16	13 ± 3.54	2 ± 0.44
HFO CEWAF 24 h	3	23 ± 6.33 * †	78 ± 6.01 * †	0 ± 0.00 †
HFO CEWAF 48 h	3	51 ± 3.09	48 ± 2.32	1 ± 0.50
HFO CEWAF 96 h	3	88 ± 1.47	9 ± 0.93	3 ± 0.60
Control 96 h	6	76 ± 8.10	23 ± 8.07	2 ± 0.51
HFO CEWAF 4 d	3	84 ± 2.00	14 ± 2.33	3 ± 1.00
HFO CEWAF 10 d	6	80 ± 4.12	19 ± 4.31	2 ± 0.38
Corexit 9500 24 h	3	79 ± 4.09	20 ± 4.60	1± 0.67
Corexit 9500 48 h	3	79 ± 8.66	20 ± 8.67	3 ± 0.33
Corexit 9500 96 h	3	85 ± 2.34	14 ± 2.52	2 ± 0.25
Control 96 h	6	76 ± 8.10	23 ± 8.07	2 ± 0.51
Corexit 9500 4 d	3	86 ± 3.06	13 ± 3.11	2 ± 0.44
Corexit 9500 10 d	6	83± 5.47	14 ± 5.16	3 ± 0.64
Cryolite 24 h	3	57 ± 7.32	43 ± 7.49	0 ± 0.17 †
Cryolite 48h	3	57 ± 9.57	43 ± 10.23	1 ± 0.67
Cryolite 96 h	3	80 ± 8.04	18 ± 7.49	3 ± 1.17
Control 96 h	6	76 ± 8.10	23 ± 8.07	2 ± 0.51
Cryolite 4 d	3	82 ± 1.67	17 ± 1.42	2 ± 0.33
Cryolite 10 d	3	85 ± 5.08	13 ± 6.27	2 ± 0.44

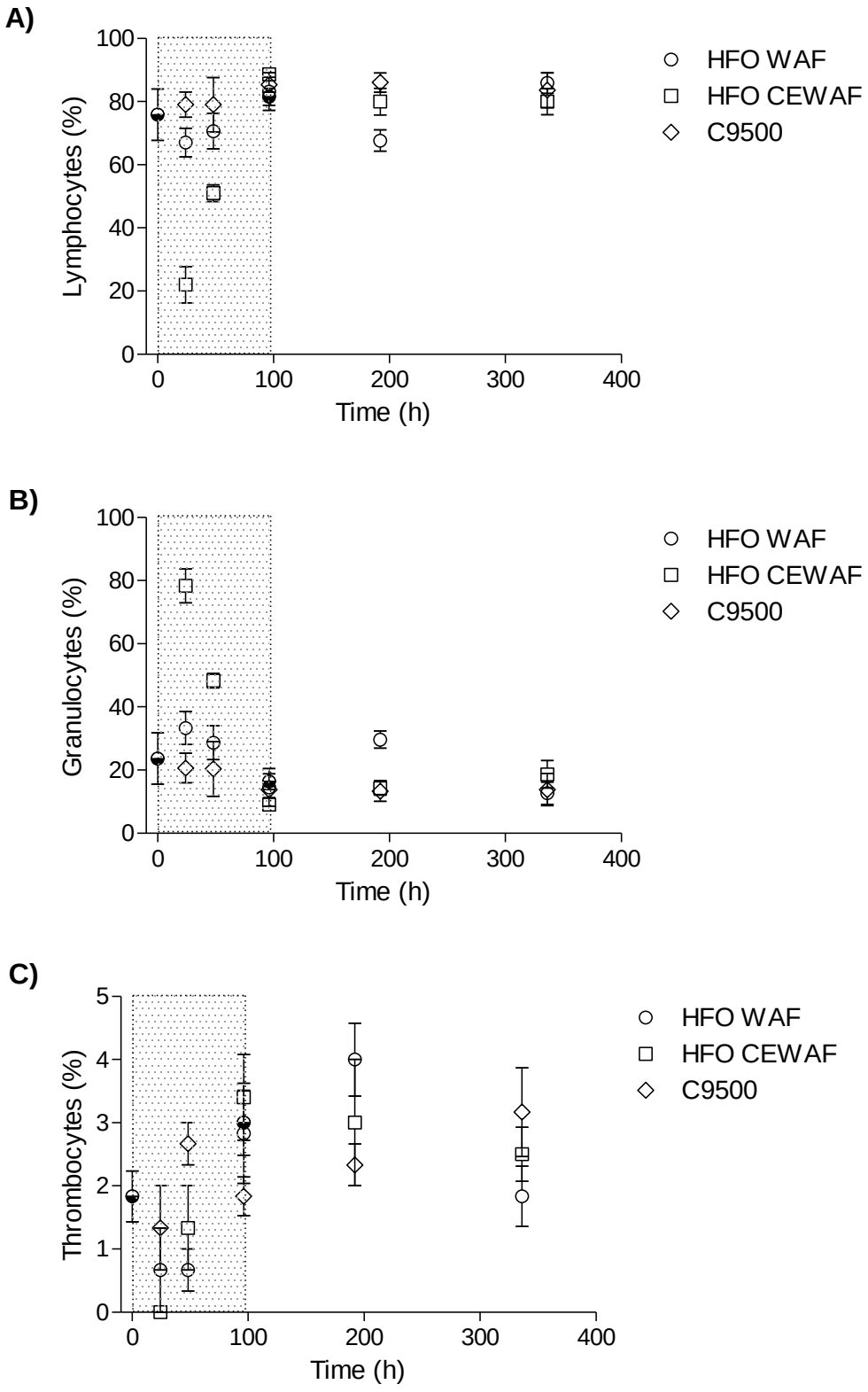

Figure 3.9: Mean (±SEM) differential leukocyte counts of *Pagrus auratus* in response to: heavy fuel oil (HFO), HFO and Corexit 9500 (HFO CEWAF), and Corexit 9500 (C9500). A) lymphocytes, B) granulocytes, C) thrombocytes. Half-closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

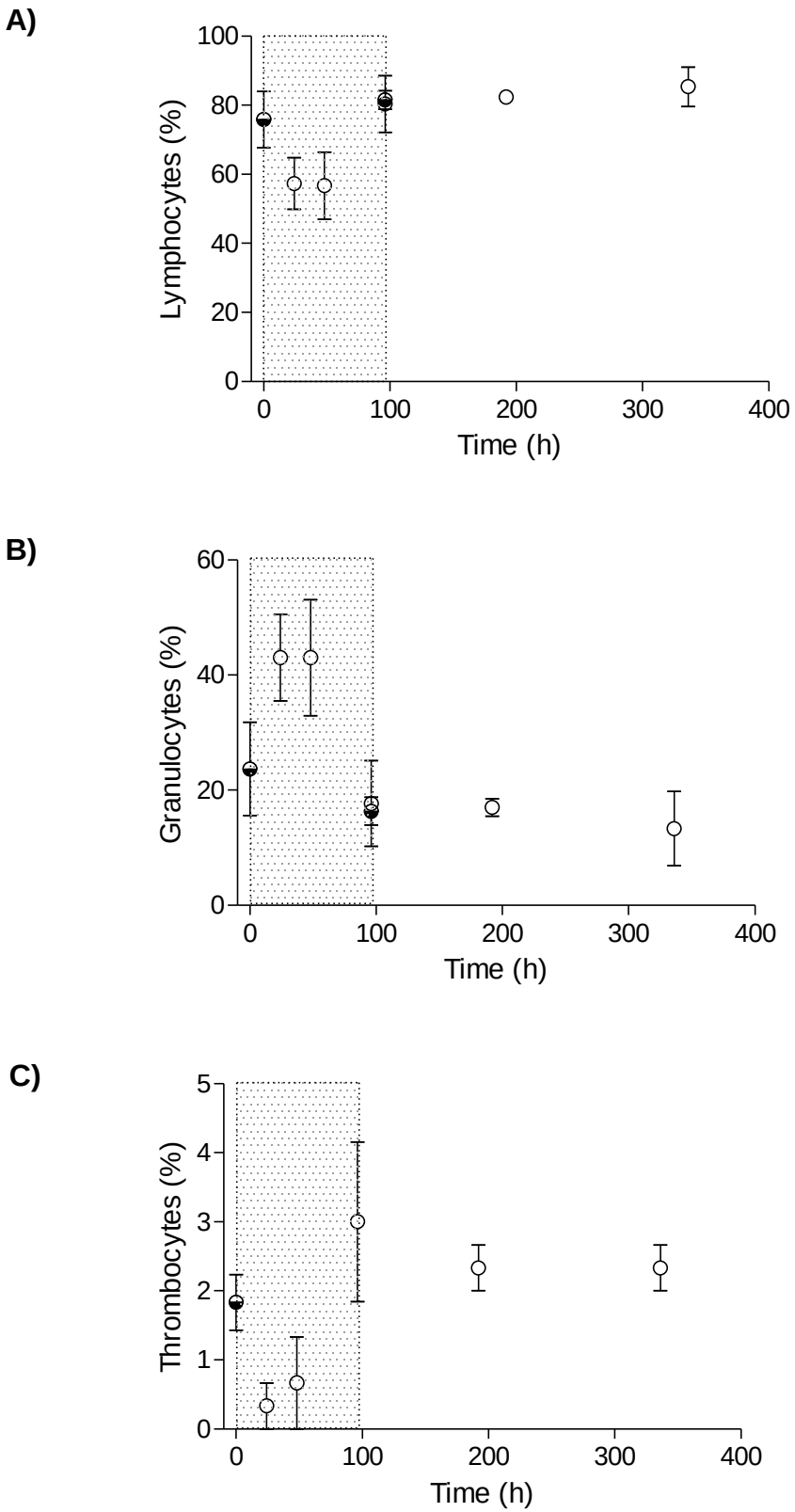

Figure 3.10: Mean (±SEM) differential leukocyte counts of *Pagrus auratus* in in response to cryolite. A) lymphocytes, B) granulocytes, C) thrombocytes. Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

3.1.2.2 Spotted wrasse (*Notolabrus celidotus*)

ANOVA results indicated no significant differences between control and treatments or controls and 96 h controls (Table 3.10). Significant differences between treatments were observed in thrombocytes between HFO CEWAF 24 h and; 48 h and 96 h. In granulocytes a significant difference was observed between HFO WAF 48 h and 10 d.

HFO WAF, Corexit 9500 and HFO CEWAF treatments

Three significant differences were observed between 96 h controls and HFO CEWAF (Table 3.10). A noticeable decrease in lymphocytes is seen during 24, 48, and 96 h in response to HFO WAF and HFO CEWAF treatment (Table 3.11 and Figure 3.11). The significant differences found between 96 h HFO CEWAF and controls are due to the largest decline in lymphocytes from control levels, coinciding with an increase in granulocytes and thrombocytes. A large increase in thrombocytes in HFO CEWAF 48 h was also observed leading to a significant difference between this treatment and controls, these levels return to control levels at 4 d. A smaller decrease in lymphocytes is seen in relation to Corexit 9500 and lymphocyte numbers return to control levels after 96 h (Figure 3.11).

Cryolite treatment

No significant differences between cryolite treatments were observed. However, a decrease in lymphocytes after 24 h is evident and 48 h. These levels returned to control levels at the 96 h time point (Figure 3.12).

Table 3.10: Mean (±SEM) differential leukocyte counts (%) of *Notolabrus celidotus* in relation to heavy fuel oil (HFO WAF), HFO and Corexit 9500 (HFO CEWAF), Corexit 9500 and cryolite. † Indicates a significant difference between the control and treatment (†*p* <0.05).

Treatment & time	*n*	Lymphocytes	Granulocytes	Thrombocytes
Control	6	83 ± 8.75	14 ± 8.50	3 ± 0.46
HFO WAF 24 h	3	55 ± 5.00	28 ± 7.25	17 ± 6.50
HFO WAF 48 h	3	53 ± 9.45	35 ± 3.17	13 ± 6.57
HFO WAF 96 h	3	55 ± 1.92	21 ± 3.78	24 ± 5.33
Control 96 h	3	71 ± 1.88	23 ± 2.00	6 ± 0.96
HFO WAF 4 d	3	87 ± 2.57	9 ± 2.02	5 ± 0.87
HFO WAF 10 d	3	78 ± 14.08	8 ± 3.91	15 ± 11.98
HFO CEWAF 24 h	3	58 ± 12.04	40 ± 11.54	2 ± 11.54
HFO CEWAF 48 h	3	60 ± 7.98	20 ± 6.11	20 ± 6.11 †
HFO CEWAF 96 h	3	52 ± 12.38 †	22 ± 10.13	26 ± 10.13 †
Control 96 h	3	71 ± 1.88	23 ± 2.00	6 ± 0.96
HFO CEWAF 4 d	3	83 ± 2.17	10 ± 1.09	7 ± 1.09
HFO CEWAF 10 d	3	82 ± 1.20	13 ± 1.00	5 ± 1.00
Corexit 9500 24 h	3	69 ± 10.77	27 ± 10.84	5 ± 10.84
Corexit 9500 48 h	3	63 ± 8.85	34 ± 8.32	3 ± 8.32
Corexit 9500 96 h	3	82 ± 1.36	13 ± 0.83	5 ± 0.83
Control 96 h	3	71 ± 1.88	23 ± 2.00	6 ± 0.96
Corexit 9500 4 d	3	85 ± 0.44	10 ± 0.76	5 ± 0.76
Corexit 9500 10 d	3	80 ± 4.42	14 ± 2.35	6 ± 2.35
Cryolite 24 h	3	69 ± 10.54	24 ± 10.97	8 ± 10.97
Cryolite 48h	3	60 ± 2.40	37 ± 4.40	3 ± 4.40
Cryolite 96 h	3	71 ± 4.48	21 ± 2.60	7 ± 2.60
Control 96 h	3	71 ± 1.88	23 ± 2.00	6 ± 0.96
Cryolite 4 d	3	85 ± 2.42	10 ± 1.32	5 ± 1.32
Cryolite 10 d	3	77 ± 7.22	19 ± 7.13	4 ± 7.13

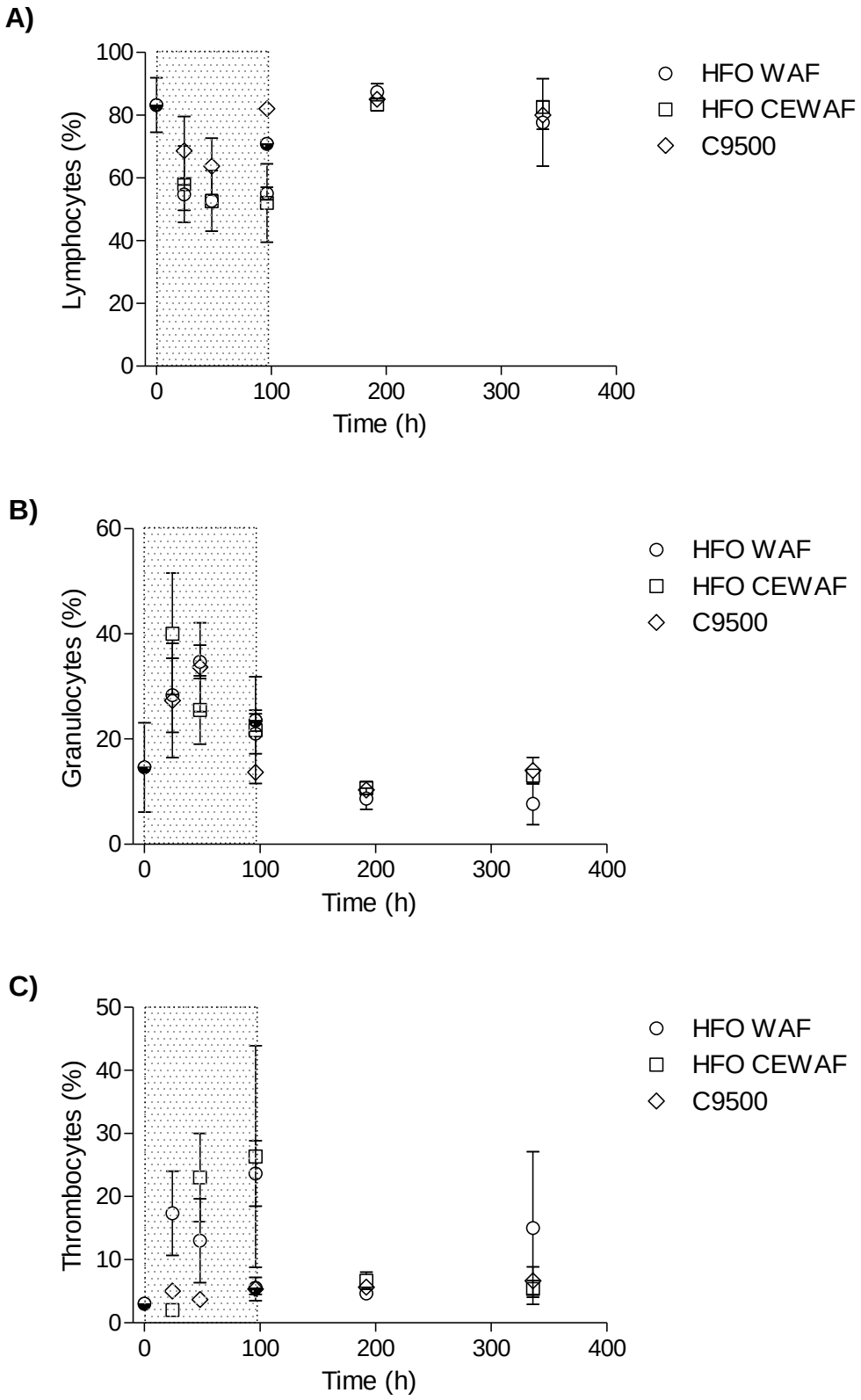

Figure 3.11: Mean (±SEM) differential leukocyte counts of *Notolabrus celidotus* in response to: heavy fuel oil (HFO), HFO and Corexit 9500 (HFO CEWAF), and Corexit 9500 (C9500). A) lymphocytes, B) granulocytes, C) thrombocytes. half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

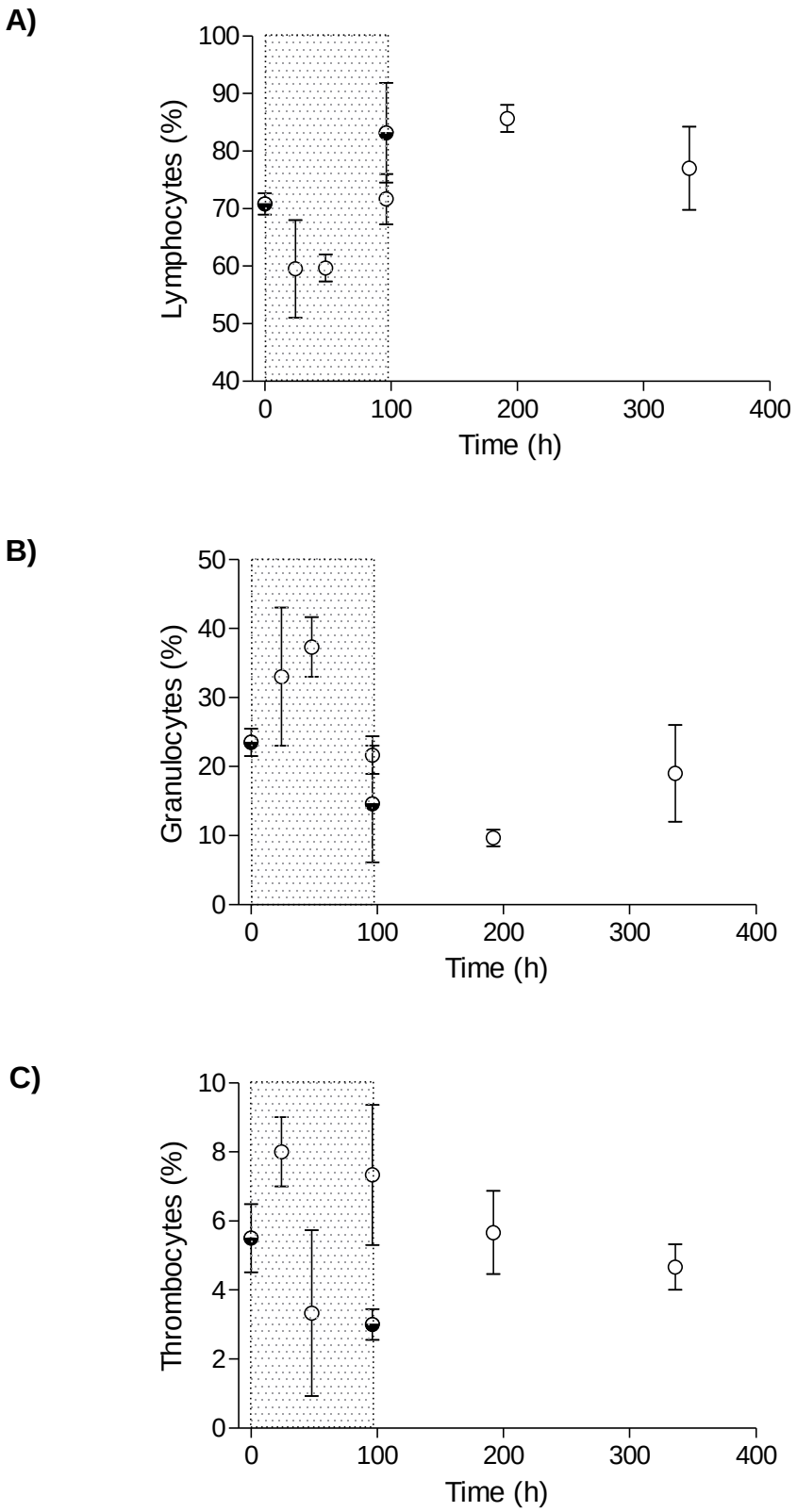

Figure 3.12: Mean (±SEM) differential leukocyte counts of *Notolabrus celidotus* in in response to cryolite. A) lymphocytes, B) granulocytes, C) thrombocytes. Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

3.2 Melanomacrophage centers (MMC) analysis

ANOVA results indicated no significant difference (P <0.05) in MMCs for all treatments. However, there is a noticeable increase in MMCs overtime for all treatments with the largest increase seen in the HFO WAF treatment. Measurements of MMCs in relation to Corexit 9500 and HFO CEWAF were more similar to control values. F tests show that all treatments at 10 days have a significant increase in variance compared to control. T-test results revealed that the Control vs HFO WAF 10 days were significantly different (P = 0.0139).

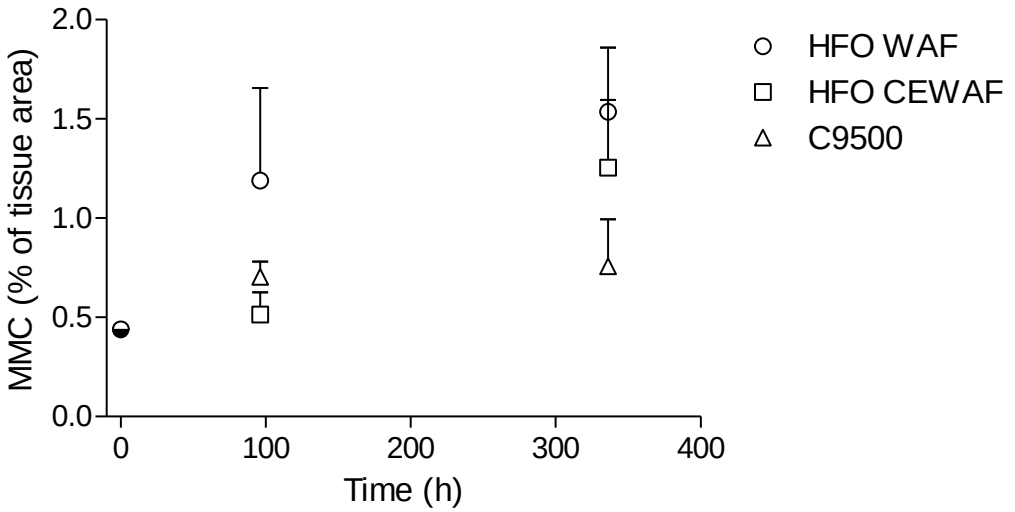

Figure 3.13: Melanomacrophage tissue percentage area (mean ±SEM) in the spleens of *Pagrus auratus* in response to heavy fuel oil (HFO), heavy fuel oil and corexit 9500 (HFO CEWAF) and Corexit 9500 (C9500). NB: Only one direction of error bars (±SEM) are shown for clarity. The half-closed circle represents the controls.

3.3 Polycyclic aromatic hydrocarbon uptake in fish

Pyrene bile metabolite (pyrene-1-glucuronide) levels reached several orders of magnitude higher than in controls for HFO WAF and HFO CEWAF treatments for both *Notolabrus celidotus* and *Pagrus auratus* (Figure 3.14). General trends across species included: an increase in pyrene-1-glucuronide over time and then a decrease during recovery. A noticeably higher decrease in pyrene-1-glucuronide was seen in the HFO CEWAF treatment recovery period compared to the HFO WAF treatments,

which indicates a quicker depuration rate of pyrene-1-glucuronide. Depuration levels however did not return to control levels.

A significant increase in pyrene-1-glucuronide for the HFO CEWAF treatment was seen between the 24 h and 48 h time periods for *Pagrus auratus*, whereas a significant increase in uptake was seen between the 48 h and 96 h time periods for *Notolabrus celidotus*. Pyrene-1-glucuronide concentrations in response to HFO WAF and HFO CEWAF in *Notolabrus celidotus* were generally lower than in *Pagrus auratus* (Figure 3.14). A very high concentration of pyrene-1-glucuronide was seen at one time point for *Notolabrus celidotus* at 96 h but this was considered to be rather anomalous (and unexplained) result as it was more than twice the concentration expected from the observed rate of increase and depuration and significantly greater than that seen in the CEWAF treatment at the same point (Figure 3.14 C).

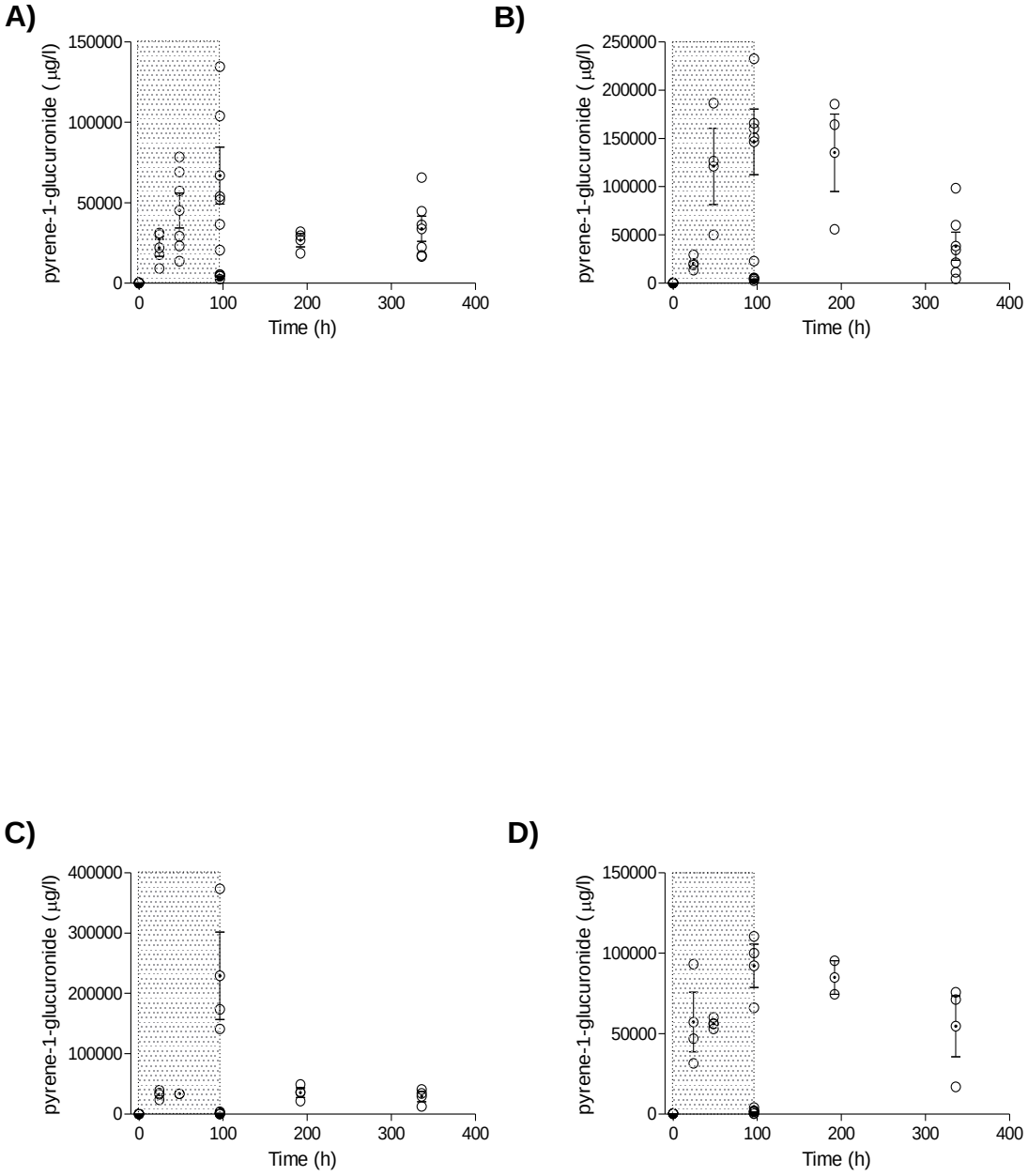

Figure 3.14: Mean (± SEM) bile concentrations of pyrene-1-glucuronide in: *Pagrus auratus* in response to: A) heavy fuel oil (HFO WAF) B) heavy fuel oil and Corexit 9500 (HFO CEWAF); and *Notolabrus celidotus* in response to: C) HFO WAF D) HFO CEWAF. Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

3.4 Red rock lobster (*Jasus edwardsii*) haematology

3.4.1 Haemocyte counts

No significant differences were found between treatment and control variables for red rock lobster haemocyte counts (Table 3.11).

HFO WAF, Corexit 9500 and HFO CEWAF treatments

Results indicate no change in haemocyte numbers observed overtime. However, lower haemocyte numbers in 96 h controls may indicate confinement stress (Figure 3.15).

Table 3.11: Means (± SEM) of haemocyte responses in *Jasus edwardsii* in relation to heavy fuel oil (HFO WAF), heavy fuel oil and Corexit 9500 (HFO CEWAF), Corexit 9500 or cryolite exposure in relation to control and 96 h control values

Treatment & time	Replicates (*n*)	Haemocytes (x10^{12} cells L^{-1})
Control	6	2.04 ± 0.0020
HFO WAF 24 h	3	1.42 ± 0.0037
HFO WAF 48 h	3	2.21 ± 0.0028
HFO WAF 96 h	6	2.41 ± 0.0035
Control 96 h	6	1.39 ± 0.0020
HFO WAF 4 d	3	1.83 ± 0.025
HFO WAF 10 d	6	2.01 ± 0.0022
HFO CEWAF 24 h	3	2.31 ± 0.0025
HFO CEWAF 48 h	3	1.89 ± 0.0046
HFO CEWAF 96 h	5	2.63 ± 0.0040
Control 96 h	6	1.39 ± 0.0020
HFO CEWAF 4 d	3	1.65 ± 0.0009
HFO CEWAF 10 d	5	2.17 ± 0.0014
Corexit 9500 24 h	3	2.08 ± 0.0022
Corexit 9500 48 h	3	2.05 ± 0.0010
Corexit 9500 96 h	6	2.15 ± 0.0014
Control 96 h	3	1.39 ± 0.0020
Corexit 9500 4 d	3	1.86 ± 0.0008
Corexit 950010 d	6	2.05 ± 0.0030
Cryolite 24 h	3	2.07 ± 0.0016
Cryolite 48h	3	9.02 ± 0.0046
Cryolite 96 h	3	1.92 ± 0.0040
Control 96 h	3	1.39 ± 0.0020
Cryolite 4 d	3	9.47 ± 0.0018
Cryolite 10 d	3	1.47 ± 0.0012

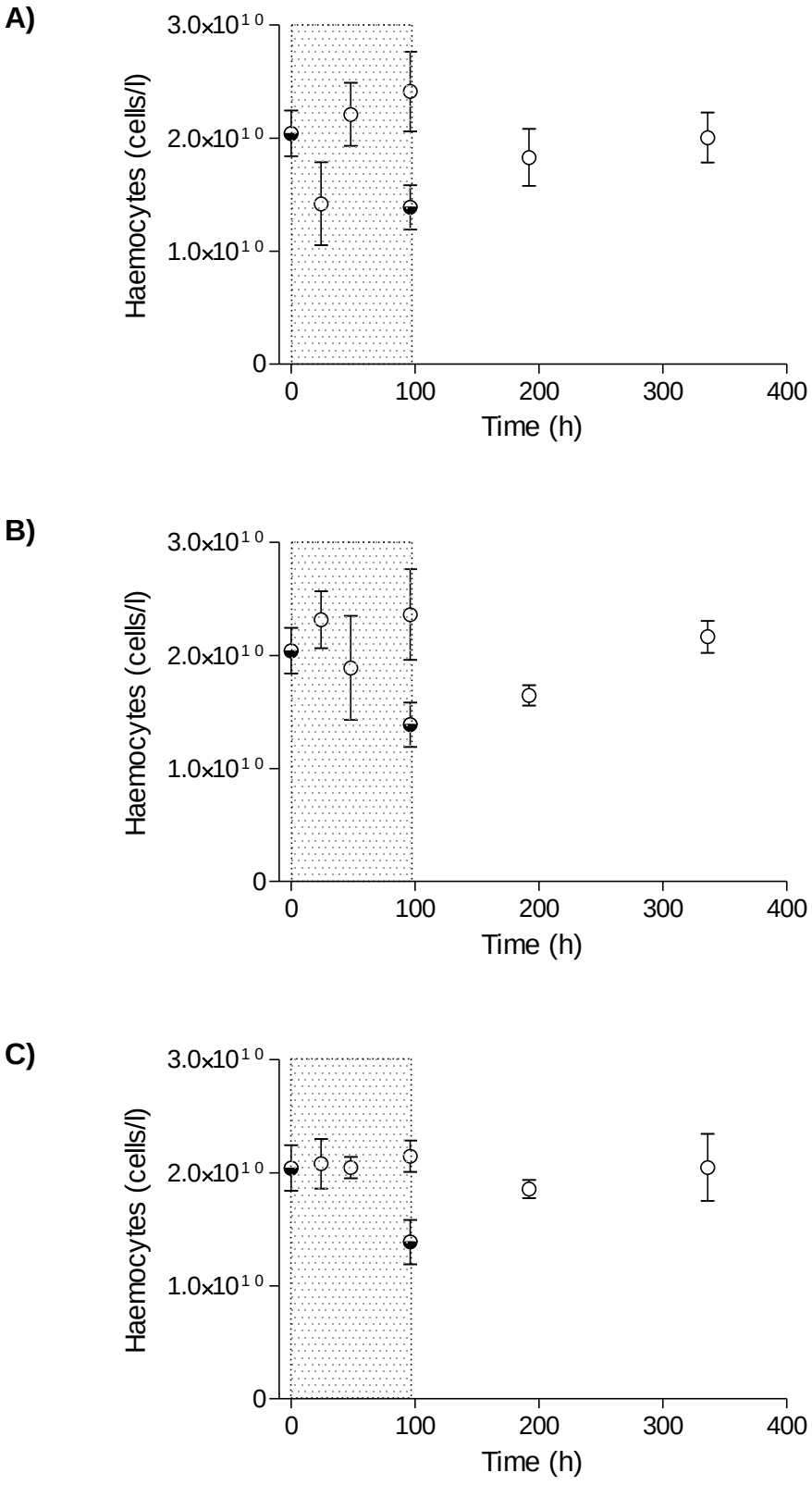

Figure 3.15: Means (± SEM) of haemocyte counts of *Jasus edwardsii* in response to: A) heavy fuel oil (HFO WAF), B) heavy fuel oil and Corexit 9500 (HFO CEWAF), C) Corexit 9500. Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

Cryolite treatments

A decrease in haemocytes is seen at the 48 h time point followed by a return to control levels at 96 h (Figure 3.16). Haemocyte numbers decrease during post-exposure recovery.

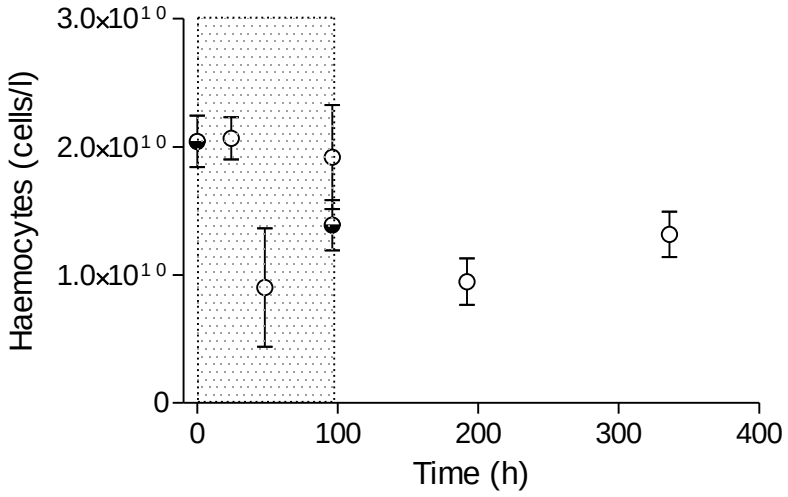

Figure 3.16: Means (± SEM) of haemocyte counts of *Jasus edwardsii* in response to cryolite. Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

3.4.2 Differential haemocyte analysis in *Jasus edwardsii*

ANOVA results for *Jasus edwardsii* indicated a number of significant differences between control and treatments and 96 h control and treatments (Table 3.12). Although cell numbers were different, there was a similar and proportional cell type change in all treatments. As lymphocytes decreased both granulocytes and semi-granulocytes increased however granulocytes increased proportionally more compared to semi-granulocytes.

HFO WAF, Corexit 9500 and HFO CEWAF treatments

Significant differences between treatments were observed in granulocytes of *Jasus edwardsii* in response to HFO WAF 24 and 4 d, HFO WAF 48 h: 4 d and 10 d and HFO CEWAF 24 h: 96 h, 4 d and 10 d and in hyaline cells in response to HFO WAF 4 d: 24 h and 48 h. HFO CEWAF 24 h: 96 h, 4 d and 10d (Figure 3.12).

Significant differences between controls and 96 h controls coincide proportionally with each other, i.e., the significant difference seen in hyaline cells at HFO WAF 24 h is due to a large decrease in cell numbers and an increase in granulocytes at HFO WAF 24 h. Leukocyte counts in relation to Corexit 9500 did not vary far from the control levels.

Cryolite treatment

Significant differences in cryolite treatments in relation to hyaline cells of *Jasus edwardsii* included cryolite 48 h and 10 d and cryolite 96 h: 4 d and 10 d. In semi-granulocytes significant differences were seen between cryolite 10 d: 24 h, 48 h and 96 h and in hyaline cells between cryolite 10 d; 24 h, 48 h and 96 h (Table 3.12). In relation to cryolite exposure results indicated a decrease in hyaline cells and increases in both granulocytes and semi granulocytes during toxicant treatment. These levels returned to normal during post-exposure recovery (Figure 3.18).

Table 3.12: Means (±SEM) differential haemocyte counts of *Jasus edwardsii* in relation to heavy fuel oil (HFO WAF), HFO and Corexit 9500 (HFO CEWAF), Corexit 9500 and cryolite. * Indicates a significant difference between the 96 h control and treatment (p* <0.05). † Indicates a significant difference between the control and treatment (†*p* <0.05).**

Treatment & time	*n*	Granulocytes	Semi-granulocytes	Hyaline
Control		11 ± 1.86	12 ± 1.31	77 ± 3.06
HFO WAF 24 h	3	47 ± 1.14 †	26 ± 8.19	28 ± 7.05
HFO WAF 48 h	3	52 ± 3.26 †	30 ± 3.85	18 ± 1.66 †
HFO WAF 96 h	3	31 ± 8.84	15 ± 4.28	54 ± 12.43
Control 96 h	6	34 ± 5.85	22 ± 4.24	44 ± 9.02
HFO WAF 4 d	3	6 ± 2.35 *	7 ± 1.12	87 ± 3.44
HFO WAF 10 d	6	18 ± 6.12	20 ± 5.74	62 ± 11.28
HFO CEWAF 24 h	3	27 ± 1.26 †	40 ± 0.31	33 ± 0.96 †
HFO CEWAF 48 h	3	43 ± 3.61 †	30 ± 5.13	27 ± 8.72
HFO CEWAF 96 h	3	46 ± 2.73	23 ± 2.05	31 ± 4.29 *
Control 96 h	6	34 ± 5.85 †	22 ± 4.24	44 ± 9.02 †
HFO CEWAF 4 d	3	13 ± 0.42	14 ± 1.61	73 ± 1.58 *
HFO CEWAF 10 d	6	9 ± 4.34 *	11 ± 2.20	81 ± 5.28 *
Corexit 9500 24 h	3	43 ± 4.86 *	20 ± 1.50 *	37 ± 5.68 *
Corexit 9500 48 h	3	32 ± 1.69	20 ± 5.53	48 ± 4.09
Corexit 9500 96 h	3	14 ± 1.08 *	12 ± 2.13	74 ± 1.96 *
Control 96 h	6	34 ± 5.85 †	22 ± 4.24	44 ± 9.02 †
Corexit 9500 4 d	3	12 ± 1.74	8 ± 0.87	80 ± 2.35
Corexit 9500 10 d	6	13 ± 1.79 *	12 ± 1.13 *	75 ± 2.68 *
Cryolite 24 h	3	11 ± 5.06	7 ± 11.91 †	82 ± 6.86 †
Cryolite 48h	3	16 ± 5.86 †	16 ± 7.76	68 ± 10.36 †
Cryolite 96 h	3	9 ± 1.64 †	12 ± 7.97	79 ± 7.16 †
Control 96 h	6	34 ± 5.85 †	22 ± 4.24	44 ± 9.02 †
Cryolite 4 d	3	13 ± 1.63	12 ± 0.14	75 ± 1.77
Cryolite 10 d	3	11 ± 4.70 *	9 ± 1.86	80 ± 5.46 *

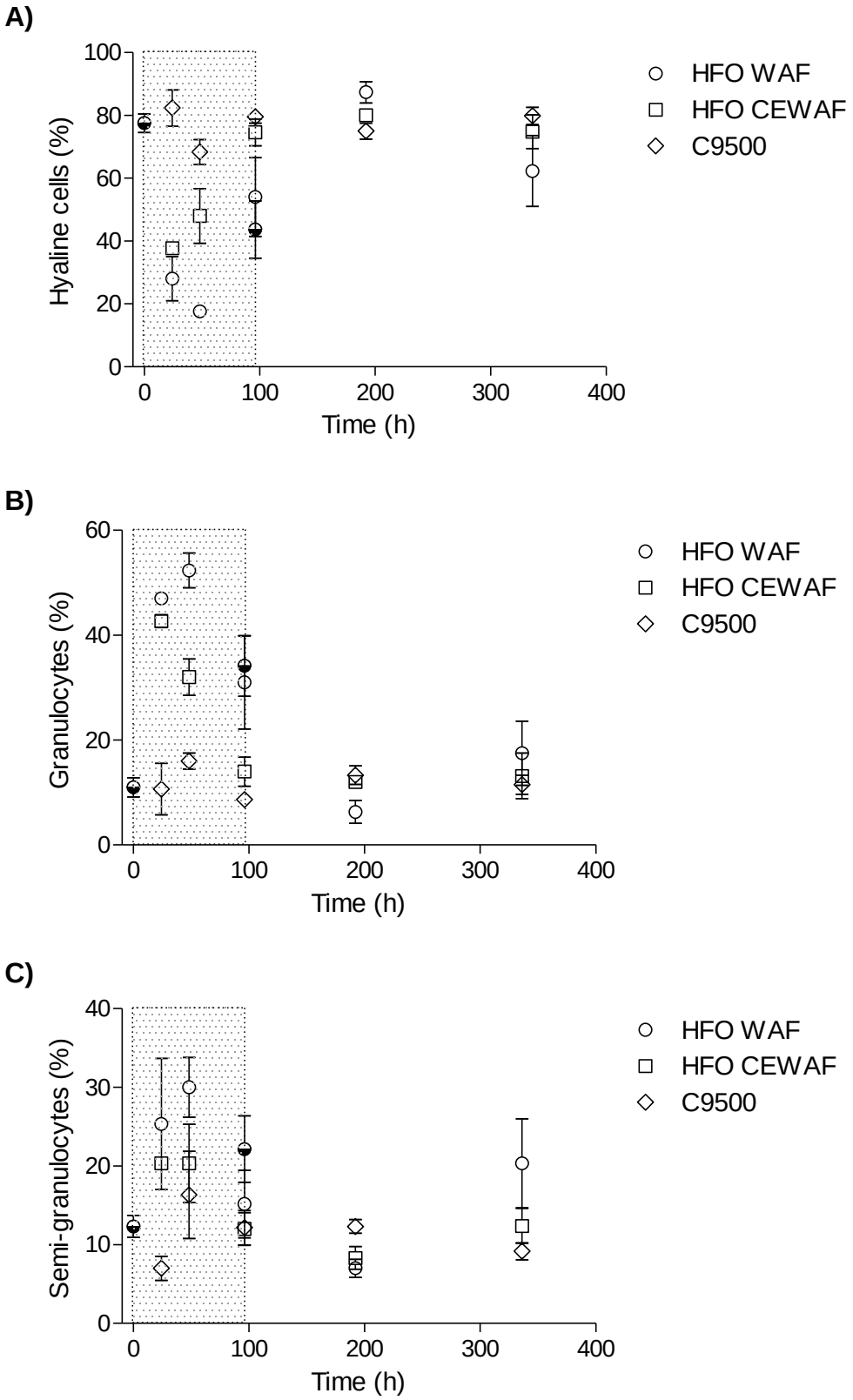

Figure 3.17: Differential haemocyte counts of *Jasus edwardsii* in response to heavy fuel oil (HFO), heavy fuel oil and Corexit (HFO CEWAF) and Corexit 9500 (C9500). A) hyaline cells, B) granulocytes, C) semi-granulocytes. Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

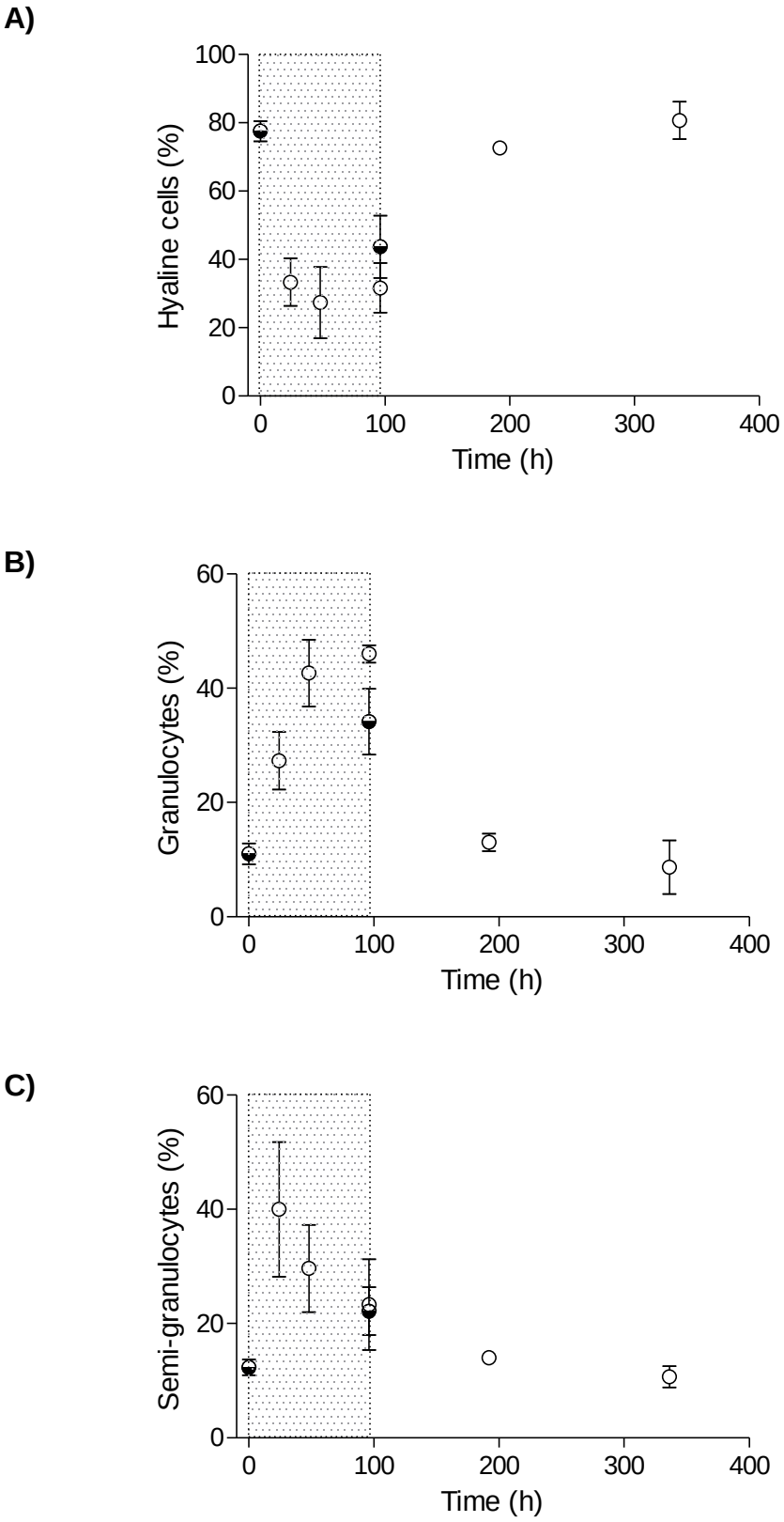

Figure 3.18: Means (±SEM) differential haemocyte counts of *Jasus edwardsii* in response to cryolite. A) hyaline cells, B) granulocytes, C) semi-granulocytes. Circles represent treatments; half closed circles represent the controls. The shaded area represents the treatment period; the area outside of this represents the depuration period.

3.5 Red rock lobster (*Jasus edwardsii*) polycyclic aromatic hydrocarbon analysis

Pyrene-1-glucuronide concentrations reached higher levels in treatment exposure compared to the controls in haepatopancreas tissue. Pyrene-1-glucuronide increased over time for both HFO WAF and HFO CEWAF treatments (Figure 3.19). During recovery a slight decrease in pyrene-1-glucuronide was seen for HFO WAF but not for HFO CEWAF. A significantly higher concentration in pyrene-1-glucuronide was observed for the HFO CEWAF treatment compared to the HFO WAF treatment.

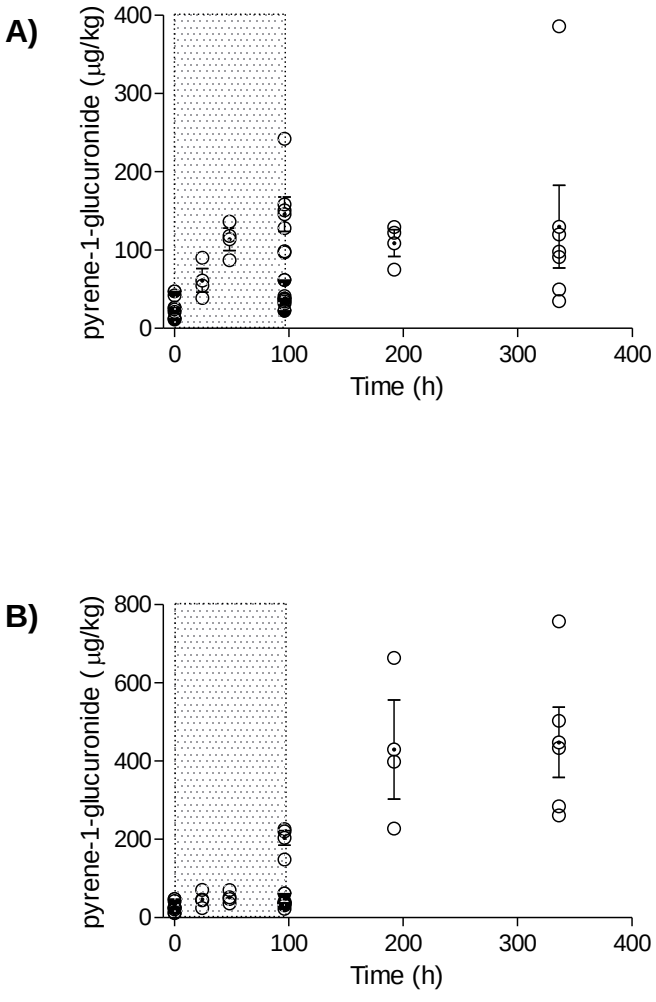

Figure 3.19: Bile metabolite measurements for pyrene-1-glucuronide in *Jasus edwardsii* A) heavy fuel oil (HFO WAF), B) heavy fuel oil and Corexit 9500 (HFO CEWAF). Circles represent treatments; half closed circles represent the controls. Means (± SEM) and individual data values are shown. The shaded area represents the treatment period; the area outside of this represents the depuration period.

Chapter Four: Discussion

This study aimed to assess the ecotoxicity of the MV Rena pollutants to New Zealand fish (*Pagrus auratus* and *Notolabrus celidotus*) and red rock lobster (*Jasus edwardsii*). To achieve this, the study assessed the acute toxicity of three main contaminants that were associated with the Rena oil spill and container debris loss: heavy fuel oil (HFO WAF), Corexit 9500, cryolite, and a combination of HFO and Corexit 9500 (HFO CEWAF). These were selected as they were of most public concern and were present in the largest amounts. Ecologically, culturally, recreationally and commercially important study animals were exposed to environmentally relevant concentrations of toxicants and immunotoxicological parameters of blood and bile metabolites were assessed.

The main objective of this study was to provide some basic polycyclic aromatic hydrocarbon (PAH) toxicity data on New Zealand marine animals in response to the paucity of relevant peer reviewed information in the literature. Secondly, it aimed to assess the magnitude of uptake and subsequent depuration rates of PAHs. As HFO is a highly variable fuel oil in terms of its composition, this study investigated the specific toxicity and effects of Rena HFO 380. The study was therefore limited to some degree by the small quantity of Rena HFO available (a total of ~300 mL). In addition, the project sought to assess whether or not the addition of the oil dispersant Corexit 9500 enhanced the toxicity of HFO or accumulation of PAHs thereby directly examining consequences of the actual deployment of Corexit during the Rena oil spill event.

Exposure to all toxicants for up to 96 h caused no mortality in any animals in any treatments, although evidence of acute sub-lethal effects were found via measurements of blood parameters, induction of melanomacrophage centers and bile metabolites. Effects were most prominent in response to HFO WAF and HFO CEWAF exposure, but were minimal in relation to cryolite exposure and negligible in relation to Corexit 9500 exposure. It was also evident that HFO CEWAF enhanced acute

effects in some aspects of measurement when compared to HFO WAF treatments.

4.1 Rena heavy fuel oil with and without the addition of Corexit 9500

4.1.1 Rena heavy fuel oil

Previous studies have demonstrated the high variability of petroleum products along with demonstrating their varying toxicity (Tatem et al. 1978; Khan & Kiceniuk 1984; Ramachandran et al. 2004). Using Rena HFO 380 sourced from the Rena fuel tanks during salvage, this study was able to investigate effects from the environmental disaster that had the potential to affect organisms and communities. The results obtained from this oil spill event are therefore relevant to future oil and gas industry spill events as well as shipping related spill responses given the common use of HFO 380 in most international shipping activities. However, the exact chemical composition of HFO in any ship and subsequent spill will likely vary from that discharged by the Rena. The complex chemical nature of any oil formulation and the subsequent effects of weathering once discharged into the environment will mean that the exact chemical composition of any oil will differ not only between ships, but also at any time point after discharge. The work represented here represents a realistic scenario reflecting likely in situ oil (and other toxicant) dilution.

4.1.2 Polycyclic aromatic hydrocarbon uptake

To gain basic PAH ecotoxicity data on New Zealand fish and red rock lobster, the assessment of a common PAH component was investigated through analysis of the pyrene metabolite, pyrene-1-glucuronide, in the bile of *Pagrus auratus* and *Notolabrus celidotus* and haepatopancreas of *Jasus edwardsii*.

Exposure to HFO WAF resulted in rapid uptake and partitioning of PAH metabolite compounds into bile and haepatopancreas tissue. Concentrations of PAH inferred from pyrene-1-glucuoronide levels were several orders of magnitude higher than in controls in response to HFO

WAF for *Pagrus auratus*, *Notolabrus celidotus* and *Jasus edwardsii*. *Pagrus auratus* accumulated more PAH in the bile compared to *Notolabrus celidotus* which could indicate that *Pagrus auratus* uptake PAHs more readily than *Notolabrus celidotus*, but the reason for this was not apparent.

In this study, the addition of Corexit 9500 was found to significantly increase PAH uptake in all species. The uptake of PAHs is governed by bioavailability, and a number of studies have shown that dispersants can increase the bioavailability of PAHs (Ramachandran et al. 2004; Sun et al. 2006; Van Scoy et al. 2010).

In examination of Corexit 9500 toxicity and three types of crude oil, Ramachandran et al. (2004), found that at the recommended ratio of 1:20 (dispersant: oil), enzymatic detoxification activity increased in the livers of rainbow trout (*Oncorhynchus mykiss*) in the CEWAF treatment compared to the WAF treatment, indicating a higher metabolization of PAHs within the liver (Ramachandran et al. 2004), an effect also observed in rock fish (*Sebastes schlegeli*) exposed to crude oil and crude oil/Corexit 9500 mixtures (Jung et al. 2009).

4.1.3 Polycyclic aromatic hydrocarbon depuration

Depuration as applied to this study is the process by which metabolism or excretion ultimately eliminates the toxicant/s from the organism's system. Depuration generally results from the biotransformation of xenobiotics after uptake, and subsequent metabolization to produce waste products which are then expelled from the organism.

Results indicated that significant depuration of pyrene-1-glucuronide occurs after as little as 4 d post-exposure in *Pagrus auratus* and *Notolabrus celidotus*, which further decreased after 10 d in both HFO WAF and HFO CEWAF treatments for both species. Although depuration was evident, concentration levels of pyrene-1-glucuronide did not return to control levels suggesting that time to depuration of pyrene-1-glucuronide is much longer than 10 d, an observation previously observed by Aas et al. (2000) in Atlantic cod (*Gadus morhua*). In investigating of exposures of

crude oil up to 1 ppm they found that PAH depuration was evident after 7 d, however PAH levels were still higher than for the control group (Aas et al. 2000).

Because bile is "downstream" to the liver where biotransformation occurs it is likely that the PAHs are being metabolised at a faster rate than was observed by accumulation of PAH metabolites in the bile. Direct analysis and comparison of the PAHs in fish livers could be conducted in future studies to gain a more comprehensive understanding of this process and to obtain information on a wider range of PAH compounds. However, this is considerably more complicated, time consuming and expensive than the simple, rapid and inexpensive assessment of pyrene metabolites in bile using fluorescence spectrometry.

Although it was evident that HFO CEWAF increased the concentration of pyrene-1-glucuronide during exposure, HFO CEWAF results also indicated an apparent quicker depuration rate of pyrene-1-glucuronide as opposed to HFO WAF for *Pagrus auratus* and *Notolabrus celidotus*. An explanation for this is the observation explained above that CEWAF increases the expression of hepatic detoxification mechanisms in fish (Ramachandran et al. 2004). A similar result was observed in rock fish (*Sebastes schlegeli*) in which after 72 h of exposure a greater hepatic enzymatic activity was observed in CEWAF treatments as opposed to WAF treatment indicating a more rapid clearance of PAHs (Jung et al. 2009).

During post exposure recovery, pyrene-1-glucuronide decline was not evident after 4 or 10 d in *Jasus edwardsii.* PAH depuration rates have not been studied in crustaceans but the fact that the pyrene metabolite was accumulating indicates that PAH metabolism was occurring in the hepatopancreas but that this process and subsequent elimination of metabolites is presumably slower than seen in fish.

4.1.4 Haematology

Haematological assessment can be useful as they are important parameters for the evaluation of fish physiological status (Campbell & Ellis 2007).

4.1.4.1 Snapper and spotted wrasse

Haemoglobin parameters

Haemoconcentration and blood cell swelling was observed in response to HFO WAF and HFO CEWAF treatments indicating signs of stress in both *Notolabrus celidotus* and *Pagrus auratus*. For both species the effect of blood cell swelling was more prominent in the HFO WAF treatment. Haemoconcentration was also observed in the HFO WAF treatment for both *Notolabrus celidotus* and *Pagrus auratus*. Previous studies have observed both increases (Zbanyszek & Smith 1984) and decreases (Alkindi et al. 1996; Duarte et al. 2010) in PCV and Hb in fish exposed to oil. Zbanyszek & Smith (1984) observed an increase in PCV, Hb and erhtrocyte counts in rainbow trout (*Oncorhynchus mykiss*) presumably caused by haemoconcentration. Duarte et al. (2009) noted a decrease in PCV after 6 and 24 h of exposure of Corexit 9500 and crude oil (1:1200, oil: dispersant) CEWAF in tambaqui (*Colossoma macropomum*).

Leukocytes

A decrease in lymphocytes is a common response to stress in teleost fish (Bonga 1997; Barton 2002). A large decrease in lymphocyte numbers and the consequent significant difference observed in *Pagrus auratus* in the HFO CEWAF 24 h treatment is likely due to increased cortisol levels leading to lympopenia, a response which has been commonly observed in previous studies in relation to stressors, including oil (Barton 2002). For example, Theron et al. (2014) observed that dispersants alone had minimal effects on juvenile turbot (*Scophthalmus maximus*), however when added with oil, effects included a decrease in white blood cells and gill alteration (Theron et al. 2014). Although no significant differences were found, a stress response is also evident in *Notolabrus celidotus* due to noticeable decrease in lymphocytes in relation to HFO WAF and HFO CEWAF exposure.

4.1.4.2 Red rock lobster

Haemocytes

The circulating haemocyte number in crayfish is a stress indicator (Perez & Fontanetti 2011). A decrease in haemocytes was observed over time

the HFO WAF and HFO CEWAF treatments. Studies in relation to the effects of oil on haematological responses in crustaceans are scarce, however previous studies have reported a decrease in haemocytes in response to environmental stressors and toxicants (Sánchez et al. 2001; Pascual et al. 2003; Flores et al. 2007; Hong et al. 2007; Verghese et al. 2007). For example, Lorenzon et al. (2001) found exposure to heavy metals in the shrimp (*Palaemon elegans*) caused a decrease in haemocytes after 8 h of exposure (Lorenzon et al. 2001).

Differential haemocyte count

The decrease in hyaline cells observed in the differential haemocyte counts for red rock lobster exposed to HFO WAF and HFO CEWAF is thought to be a generalised stress response. Previous studies have reported a decrease in hyaline cells in crustaceans in response to a number of stressors such as in the crab (*Paratelphusa hydrodromous*) in response to cadmium chloride (Victor 1993).

4.1.5 Melanomacrophage centers

The general function of melanomacrophage centers is the focalization of destruction, detoxification or recycling of endogenous and exogenous materials (Agius & Roberts 2003). They are an integrated component of the immune system in fish and consist of aggregates of macrophages filled with fragments of cells consisting of mainly degenerated erythrocytes (Payne & Fancey 1989). Overall the analysis of melanomacrophage centers indicated results that were consistent with the overall trend seen within blood parameters. The largest effect was seen during longer exposure and observed effects were greater following exposure to HFO WAF treatment which appeared to stimulate an increase in melanomacrophages in the spleen of *Pagrus auratus.*

Changes in the tissue occur at a much slower rate than in the blood which can occur within minutes. The increase in melanomacrophage centers at 10 d can be explained by the fact that histological changes are slower and more prolonged than haematological changes. Atlantic cod (*Gadus morhua*) exposed to water-soluble fractions of Venezuelan and Hibernia

crude oils at concentrations of 50 – 300 ppb for 12 – 13 weeks, resulted in an increase of melanomacrophage centers in the spleen and kidney (Khan & Kiceniuk 1984).

4.2 Corexit 9500

Results from this study indicate negligible toxicity to all study animals tested from exposure to Corexit 9500 on its own. No significant differences or effects were seen in blood parameters in all three species studied. However it is evident that Corexit 9500 has the potential to cause synergistic effects when mixed with Rena HFO as shown in section 4.1.

Toxicity testing of Corexit 9500 was essentially a control to the HFO CEWAF treatment in order to observe whether or not the effects observed were due to the Corexit or the increased availability of PAHs. The concentration of Corexit used was well below that of observed LC_{50} values that induce toxic effects in other studies. However, it probably reflects a realistic concentration that might occur in the environment following its use during an oiling event.

LC_{50} values are known to vary largely with species and life stage. George-Ares Clark, (2000), estimated the 48-96 h LC_{50} value to be between 7 ppm to >400 ppm in studies that included zygote, embryo, larval, juvenile and adult life stages of a variety of marine organisms. By comparison, a higher sensitivity was seen in crustaceans at the same life stages with 48 - 96 h LC_{50} values estimated at 3.5 - 36 ppm (George-Ares & Clark 2000). Ramachandran et al. (2004) estimated the 96 h LC_{50} for rainbow trout (*Oncorhynchus mykiss*) to be between 100 – 1000 mg/L.

4.3 Cryolite

Cryolite produced only minor effects on all study animals as ANOVA results indicated no significant differences between control and treatment animals for *Pagrus auratus* and *Notolabrus celidotus,* although significant differences were observed in hyaline cells in *Jasus edwardsii* suggesting an immune response in relation to cryolite exposure.

The observed effects suggest that fluoride toxicity may be occurring via dissolved cryolite. However, previous studies have found negligible effects of fluoride toxicity at higher concentrations than applied in this study. For example; Hemens and Warwick, (1972) reported no toxic effects to two species of estuarine prawns (*Penaeus indicus* and *Penaeus monodon*) after exposures of 96 h to a maximum fluoride concentration of 100 mg F^-/l (Hemens & Warwick 1972). Similarly, Hemens et al. (1975) found that exposure to fluoride concentration of 5.5 mg F^-/l for 113 d did not indicate any physical deterioration or mortality in the mud crab (*Tylodiplax blephariskios*) and the penaeid prawn (*Penaeus indicus*) (Hemens et al. 1975). The maximum possible fluoride concentration in the present study is suggested to be 5.5 mg/L (L. Tremblay, Cawthron Institute, *pers. comm.*, 2014).

Little is known about the toxicity of cryolite and literature within the field is severely lacking. However the results coincide with findings which suggested negligible effects of cryolite to marine organisms (Department of Agriculture and Fisheries for Scotland 1982).

4.4 Implications and future research

4.4.1 Research limitations and implications

Toxicity and toxic effects are well known to vary both between and within species as well as with other biological factors such as age and sex. Blood parameters will also vary with water quality, age and population density in both fish (Campbell & Ellis 2007) and crustaceans (Flores et al. 2007).

Laboratory tests may not be representational of the environmental conditions and processes, however environmental tests are often not practical or feasible (Schmidt-Etkin 2011). Hence, impacts such as weathering, biodegradation and dilution could not be fully studied in this research even though the entire study was designed to generate realistic ‘field’ concentrations of toxicants. However, when examining the toxicity of oil, Fuller et al. (2004) concluded that un-weathered oil was found to be more toxic than weathered oil (Fuller et al. 2004), and this assessment of

the toxicity of Rena HFO WAF and HFO CEWAF therefore represents a "worst case toxic scenario".

4.4.1.1 Capture, anaesthesia and stress

Both erythrocyte swelling and splenic erythrocyte release occur in response to capture and anaesthesia and will be present in all treatment groups, including controls, because both these responses are caused by the rapid release of catecholamines and time delays between capture of fish and subsequent blood sampling cannot be avoided (Ryan 1992; Rothwell et al. 2005). Similarly, handling fish for as little as 20 s can result in the release of catecholamines (Campbell & Ellis 2007). However, this effect should be the same in all groups assuming that the duration between capture, blood sampling and analysis is constant for all groups. Therefore, any differences between treatment groups should represent true treatment effects over and above effects caused by sampling alone.

4.4.2 Future research and wider research perspective

This research provides preliminary PAH data to New Zealand marine animals and can lead to a variety of further investigations. The relatively low sample size was due to constrained research limitations and resulted in a somewhat variable data set. Variability was however not as extreme as in many similar studies, but nevertheless improvements with larger replicate numbers would always be desirable. Future study could aim to improve and add to this data set.

Future study could also investigate toxic effects of these contaminants to a wider range of species. Furthermore, in a wider perspective, research could be directed toward trophic interactions, bioaccumulation and bioconcentration. In a field situation, reduced performance of marine organisms may result in reduced viability as they become more vulnerable to predation.

Further study is needed to evaluate the time it takes for animals to completely rid their body of PAHs if indeed this occurs - especially for important recreational or commercial species given the public concerns about potential toxicity of food fish. From results it became evident that all

three study organisms did not depurate PAH within 10 d, therefore longer depuration rates could be assessed.

Further assessment could also examine lethal concentrations and examination of other parameters in relation to ecotoxicological effects that may affect New Zealand marine animals.

4.5 Conclusions

This project has enhanced the knowledge store of relevant information through a unique and complex study based upon a major maritime oil spill event in New Zealand. This information will be used to respond to future oil spill industry events both in New Zealand and overseas, as well as planning risk assessments, while also improving response actions.

Although sub-lethal effects were observed within the present study, because measured parameters returned to control levels during post-exposure recovery; the effects observed are unlikely to have long lasting effects on the study animals apart from the potential prolonged PAH build up in the liver and bile resulting in exposure to HFO.

The magnitude of uptake and depuration was interesting in the respect that the addition of Corexit 9500 increased depuration rates. This research also highlighted the negligible toxicity of Corexit 9500 which was heavily debated in terms of toxicity during the response, and was also of high public concern. The research was highly relevant as it examined New Zealand species that are considered of high importance particularly in terms of food source. Furthermore, this study found no significant effects regarding the release of large amounts of cryolite which was also viewed of large concern due to its hazardous category and unknown toxic effects.

References

Aas E, Baussant T, Balk L, Liewenborg B, Andersen OK 2000. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: A laboratory experiment with Atlantic cod. Aquatic Toxicology 51(2): 241-258.

Aas E, Beyer J, Goksøyr A 1998. PAH in fish bile detected by fixed wavelength fluorescence. Marine Environmental Research 46: 225-228.

Abdallah RI, Mohamed SZ, Ahmed FM 2005. Effect of biological and chemical dispersants on oil spills. Petroleum Science and Technology 23: 463-474.

Agius C, Roberts RJ 2003. Melano-macrophage centres and their role in fish pathology. Journal of Fish Diseases 26(9): 499-509.

Alcoa 2011. Synthetic cryolite: Material safety data sheet. http://www.alcoa.com/global/en/environment/msds_view.asp?LoadMSDS=203839 (accessed 1 June 2014).

Alkindi A, Brown J, Waring C, Collins J 1996. Endocrine, osmoregulatory, respiratory and haematological parameters in flounder exposed to the water soluble fraction of crude oil. Journal of Fish Biology 49: 1291-1305.

American Petroleum Institute Petroleum HPV Group 2012. Robust Summary Information on Heavy Fuel Oil Category. http://www.petroleumhpv.org/petroleum-substances-and-categories/substance-and-category-list (accessed June 2014).

Anderson D, Zeeman M 1995. Immunotoxicology of fish. In: Rand G ed. Fundamentals of Aquatic Toxicology: Effects, Envionmental Fate, and Risk Assessment. 2nd ed. Washington D.C, Taylor & Francis. Pp. 371-404.

Ariese F, Kok SJ, Verkaik M, Gooijer C, Velthorst NH, Hofstraat JW 1993. Synchronous fluorescence spectrometry of fish bile: A rapid screening method for the biomonitoring of PAH exposure. Aquatic Toxicology 26: 273-286.

Ayling T, Cox GJ 1982. Collins Guide to Sea Fishes of New Zealand. Auckland, New Zealand, Williams Collins Publishers. 343 p.

Baker JM 1978. Marine ecology and oil pollution. Water Pollution Control Federation 50: 442-449.

Barton BA 2002. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. Integrative and Comparative Biology 42: 517-525.

Baumard P, Budzinski H, Garrigues P, Narbonne JF, Burgeot T, Michel X, Bellocq J 1999. Polycyclic aromatic hydrocarbon (PAH) burden of mussels (*Mytilus* sp.) in different marine environments in relation with sediment PAH contamination, and bioavailability. Marine Environmental Research 47: 415-439.

Belore RC, Trudel K, Mullin JV, Guarino A 2009. Large-scale cold water dispersant effectiveness experiments with Alaskan crude oils and Corexit 9500 and 9527 dispersants. Marine Pollution Bulletin 58: 118-128.

Bols NC, Brubacher JL, Ganassin RC, Lee LEJ 2001. Ecotoxicology and innate immunity in fish. Developmental & Comparative Immunology 25: 853-873.

Bonga SEW 1997. The stress response in fish. Physiological Reviews 77(3): 591-625.

Bornstein JM, Adams J, Hollebone B, King T, Hodson PV, Brown RS 2014. Effects-driven chemical fractionation of heavy fuel oil to isolate compounds toxic to trout embryos. Environmental Toxicology and Chemistry 33: 814-24.

Brandvik PJ, Daling PS 1998. Optimising oil spill dispersants as a function of oil type and weathering degree: A multivariate approach using partial least squares (PLS). Chemometrics and Intelligent Laboratory Systems 42: 73-91.

Bruheim P, Bredholt H, Eimhjellen K 1999. Effects of surfactant mixtures, including Corexit 9527, on bacterial oxidation of acetate and alkanes in crude oil. Applied and Environmental Microbiology 65: 1658-61.

Burridge TR, Shir M-A 1995. The comparative effects of oil dispersants and oil/dispersant conjugates on germination of the marine macroalga *Phyllospora comosa* (Fucales: Phaeophyta). Marine Pollution Bulletin 31: 446-452.

Camargo JA 2003. Fluoride toxicity to aquatic organisms: A review. Chemosphere 50: 251-264.

Campbell TW, Ellis CK 2007. Avian and Exotic Animal Haematology and Cytology. 3rd ed. Ames, IA, Blackwell Publishing. 268 p.

Campo P, Venosa AD, Suidan MT 2013. Biodegradability of Corexit 9500 and dispersed south Louisiana crude oil at 5 and 25 °c. Environmental Science & Technology 47: 1960-1967.

Cao J, Chen J, Wang J, Wu X, Li Y, Xie L 2013. Tissue distributions of fluoride and its toxicity in the gills of a freshwater teleost, *Cyprinus carpio*. Aquatic Toxicology 130–131: 68-76.

Carls MG, Rice SD, Hose JE 1999. Sensitivity of fish embryos to weathered crude oil: Part I. Low-level exposure during incubation causes malformations, genetic damage, and mortality in larval pacific herring (*Clupea pallasi*). Environmental Toxicology and Chemistry 18: 481-493.

Centre of Documentation Research and Experimentation on Accidental Water Pollution 2006. Heavy Fuel Oils, Questions Raised: Rational. France. 6p.

Chandrasekar S, Sorial GA, Weaver JW 2006. Dispersant effectiveness on oil spills – Impact of salinity. ICES Journal of Marine Science 63(8): 1418-1430.

Chang ES 2005. Stressed-out lobsters: Crustacean hyperglycemic hormone and stress proteins. Integrative and Comparative Biology 45(1): 43-50.

Chapman H, Purnell K, Law RJ, Kirby MF 2007. The use of chemical dispersants to combat oil spills at sea: A review of practice and research needs in Europe. Marine Pollution Bulletin 54: 827-838.

CONCAWE 1998. Heavy Fuel Oils. Product Dossier No. 98/109, Prepared by CONCAWE's Petroleum Products and Health Management Groups. Brussels, Belgium. 48p.

Connet P, Connet E, Connet M, Neurath C, Blank T 2012. Comments on the U.S. EPA's Amended Cryolite Final Work Plan Registration Review. Conton, NY, Fluoride Action Network. 27p.

Couillard CM, Lee K, Légaré B, King TL 2005. Effect of dispersant on the composition of the water-accommodated fraction of crude oil and its toxicity to larval marine fish. Environmental Toxicology and Chemistry 24: 1496-1504.

D'Adamo R, Pelosi S, Trotta P, Sansone G 1997. Bioaccumulation and biomagnification of polycyclic aromatic hydrocarbons in aquatic organisms. Marine Chemistry 56: 45-49.

Dacie J, Lewis SM 1991. Practical Haematology. 7th ed. New York, Churchill Livingston. 556 p.

Department of Agriculture and Fisheries for Scotland 1982. Report on Research into the Effects of Dumping of Cryolite Sludge in the Moray Firth. Working Paper No. 2/82. Aberdeen, U.K. 32p.

Dicks B 1999. The Environmental Impact of Marine Oil Spills - Effects, Recovery and Compensation. Rio de Janeiro, Brazil, International Tanker Owners Pollution Federation. 8p.

Duarte RM, Honda RT, Val AL 2010. Acute effects of chemically dispersed crude oil on gill ion regulation, plasma ion levels and

haematological parameters in tambaqui (*Colossoma macropomum*). Aquatic Toxicology 97: 134-141.

Dupre Minerals 2008. Material safety data sheet: Cryolite http://www.dupreminerals.com/downloads/products/friction/synthetic-cryolite-msds.pdf (accessed 1 June 2014).

Eddy TD, Pitcher TJ, MacDiarmid AB, Byfield TT, Tam JC, Jones TT, Bell JJ, Gardner JPA 2014. Lobsters as keystone: Only in unfished ecosystems? Ecological Modelling 275: 48-72.

Ellsaesser CF, Clem LW 1987. Cortisol-induced hematologic and immunologic changes in channel catfish (*Ictalurus punctatus*). Comparative Biochemistry and Physiology Part A: Physiology 87: 405-408.

Falahatkar B, Poursaeid S, Shakoorian M, Barton B 2009. Responses to handling and confinement stressors in juvenile great sturgeon *Huso huso*. Journal of Fish Biology 75: 784-796.

Fingas M 2000. The Basics of Oil Spill Cleanup. 2nd ed. USA, CRC Press. 256 p.

Fiocco RJ, Lewis A 1999. Oil spill dispersants. Pure and Applied Chemistry 71: 27-42.

Flores M, Díaz F, Medina R, Re AD, Licea A 2007. Physiological, metabolic and haematological responses in white shrimp *Litopenaeus vannamei* (Boone) juveniles fed diets supplemented with astaxanthin acclimated to low-salinity water. Aquaculture Research 38: 740-747.

Fodrie FJ, Heck KL, Jr. 2011. Response of coastal fishes to the gulf of Mexico oil disaster. PLoS ONE 6: e21609.

Francis M 2001. Coastal Fishes Of New Zealand. An Identification Guide. 3rd ed. Auckland, New Zealand, Reed Books. 103 p.

Franco SS, Nardocci AC, Günther WMR 2008. PAH biomarkers for human health risk assessment: A review of the state-of-the-art. Cadernos de Saúde Pública 24: a569-s580.

French-McCay D 2011. Oil spill modeling for ecological risk and natural resource damage assessment. International Oil Spill Conference Proceedings 2011: abs 415.

Fuller C, Bonner J, Page C, Ernest A, McDonald T, McDonald S 2004. Comparative toxicity of oil, dispersant, and oil plus dispersant to several marine species. Environmental Toxicology and Chemistry 23(12): 2941-2949.

Gardiner WW, Word JQ, Word JD, Perkins RA, McFarlin KM, Hester BW, Word LS, Ray CM 2013. The acute toxicity of chemically and physically dispersed crude oil to key arctic species under arctic conditions during the open water season. Environmental Toxicology and Chemistry 32: 2284-2300.

Gensemer RW, Playle RC 1999. The bioavailability and toxicity of aluminum in aquatic environments. Critical Reviews in Environmental Science and Technology 29: 315-450.

George-Ares A, Clark JR 1997. Acute aquatic toxicity of three Corexit products: An overview. International Oil Spill Conference Proceedings 1997: 1007-1008.

George-Ares A, Clark JR 2000. Acute aquatic toxicity of two Corexit® dispersants. Chemosphere 40: 897-906.

Glassworks Services Limited 1998. Safety Data Sheet: Cryolite, Synthetic Powder. http://www.google.co.nz/url?sa=t&rct=j&q=&esrc=s&frm=1&source=web&cd=40&ved=0CE4QFjAJOB4&url=http%3A%2F%2Fwww.glassworksservices.co.uk%2Fmsds%2Fcryolite.doc&ei=gvScU4fnBcq6lQW_-4CoCQ&usg=AFQjCNECjwSL7tR6639_hPum9KNhL5MOjw (accessed 1 June 2014).

Gong Y, Zhao X, Cai Z, O'Reilly SE, Hao X, Zhao D 2014. A review of oil, dispersed oil and sediment interactions in the aquatic environment: Influence on the fate, transport and remediation of oil spills. Marine Pollution Bulletin 79: 16-33.

Gulec I, Holdway DA 2000. Toxicity of crude oil and dispersed crude oil to ghost shrimp *Palaemon serenus* and larvae of Australian bass *Macquaria novemaculeata*. Environmental Toxicology 15: 91-98.

Hamdan L, Fulmer P 2011. Effects of Corexit EC9500A on bacteria from a beach oiled by the deepwater horizon spill. Aquatic Microbial Ecology 63: 101-109.

Hattori Y, Kanno M, Baumard P, Budzinski H, Garrigues P, Sorbe JC, Burgeot T, Bellocq J 1998. Concentrations of PAHs (polycyclic aromatic hydrocarbons) in various marine organisms in relation to those in sediments and to trophic level. Marine Pollution Bulletin 36: 951-960.

Hemens J, Warwick RJ, Oliff WD 1975. Effect of extended exposure to low fluoride concentration on estuarine fish and crustacea. Progress in Water Technology 7: 579–585.

Hemens J, Warwick RJ 1972. The effects of fluoride on estuarine organisms. Water Research 6: 1301-1308.

Hong M, Chen L, Sun X, Gu S, Zhang L, Chen Y 2007. Metabolic and immune responses in Chinese mitten-handed crab (*Eriocheir sinensis*) juveniles exposed to elevated ambient ammonia. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 145: 363-369.

Hosnedl T, Hajšlová J, Kocourek V, Tomaniová M, Volka K 2003. 1-hydroxypyrene as a biomarker for fish exposure to polycyclic aromatic hydrocarbons. Bulletin of Environmental Contamination and Toxicology 71: 0465-0472.

Hylland K 2006. Polycyclic aromatic hydrocarbon (PAH) ecotoxicology in marine ecosystems. Journal of Toxicology and Environmental Health, Part A 69: 109-123.

Jeffs A, Gardner C, Cockcroft A 2013. *Jasus* and *Sagmariasus* species. In: Phillips B ed. Lobsters: Biology, Management, Aquaculture & Fisheries. Chichester, U.K., Wiley. Pp. 259-288.

Jongeneelen FJ 1994. Biological monitoring of environmental exposure to polycyclic aromatic hydrocarbons; 1-hydroxypyrene in urine of people. Toxicology Letters 72: 205-211.

Jung JH, Yim UH, Han GM, Shim WJ 2009. Biochemical changes in rockfish, *Sebastes schlegeli*, exposed to dispersed crude oil. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 150: 218-223.

Kazlauskienė N, Svecevičius G, Vosylienė MZ, Marčiulionienė D, Montvydienė D 2004. Comparative study on sensitivity of higher plants and fish to heavy fuel oil. Environmental Toxicology 19: 449-451.

Khan RA, Kiceniuk J 1984. Histopathological effects of crude oil on Atlantic cod following chronic exposure. Canadian Journal of Zoology 62(10): 2038-2043.

Knap AH, Sleeter TD, Dodge RE, Wyers SC, Frith HR, Smith SR 1983. The effects of oil spills and dispersant use on corals: A review and multidisciplinary experimental approach. Oil and Petrochemical Pollution 1: 157-169.

Kvenvolden KA, Cooper CK 2003. Natural seepage of crude oil into the marine environment. Geo-Marine Letters 23: 140-146.

Le Moullac G, Haffner P 2000. Environmental factors affecting immune responses in Crustacea. Aquaculture 191: 121-131.

Lee K-W, Shim WJ, Yim UH, Kang J-H 2013. Acute and chronic toxicity study of the water accommodated fraction (WAF), chemically enhanced WAF (CEWAF) of crude oil and dispersant in the rock pool copepod *Tigriopus japonicus*. Chemosphere 92: 1161-1168.

Lessard RR, Aurand D, Coelho G, Fuller C, McDonald TJ, Clark J, Bragin G, Jamail R, Steen A 1999. Design and implementation of a mesocosm experiment on the environmental consequences of nearshore dispersant use. International Oil Spill Conference Proceedings 1999: 1027-1030.

Lessard RR, DeMarco G 2000. The significance of oil spill dispersants. Spill Science & Technology Bulletin 6: 59-68.

Lewis JB 1971. Effect of crude oil and an oil spill dispersant on reef corals. Marine Pollution Bulletin 2: 59-62.

Liang Y, Tse MF, Young L, Wong MH 2007. Distribution patterns of polycyclic aromatic hydrocarbons (PAHs) in the sediments and fish at Mai Po Marshes Nature Reserve, Hong Kong. Water Research 41: 1303-1311.

Lin X, Söderhäll I 2011. Crustacean hematopoiesis and the astakine cytokines. Blood 117: 6417-6424.

Lindstrom JE, Braddock JF 2002. Biodegradation of petroleum hydrocarbons at low temperature in the presence of the dispersant Corexit 9500. Marine Pollution Bulletin 44: 739-747.

Liu B, Romaire RP, Delaune RD, Lindau CW 2006. Field investigation on the toxicity of Alaska north slope crude oil (ANSC) and dispersed ANSC crude to gulf killifish, eastern oyster and white shrimp. Chemosphere 62: 520-526.

Logan DT 2007. Perspective on ecotoxicology of PAHs to fish. Human and Ecological Risk Assessment 13: 302-316.

Long SM, Holdway DA 2002. Acute toxicity of crude and dispersed oil to *Octopus pallidus* (Hoyle, 1885) hatchlings. Water Research 36: 2769-2776.

Lorenzon S, Francese M, Smith VJ, Ferrero EA 2001. Heavy metals affect the circulating haemocyte number in the shrimp *Palaemon elegans*. Fish & Shellfish Immunology 11(6): 459-472.

Los Angeles Chemical Company 1993. Material Safety Data Sheet: Cryolite http://aardvarkclay.com/pdf/raw_materials/CRYOLITE.PDF (accessed 1 June 2014).

MacDiarmid AB 1989. Moulting and reproduction of the spiny lobster *Jasus edwardsii* (Decapoda: Palinuridae) in northern New Zealand. Marine Biology 103: 303-310.

MacDiarmid AB, Freeman D, Kelly S 2013. Rock lobster biology and ecology: Contributions to understanding through the Leigh Marine Laboratory 1962-2012. New Zealand Journal of Marine and Freshwater Research 47: 313-333.

MacDiarmid AB, Hickey B, Maller RA 1991. Daily movement patterns of the spiny lobster *Jasus edwardsii* (Hutton) on a shallow reef in northern New Zealand. Journal of Experimental Marine Biology and Ecology 147: 185-205.

Maritime New Zealand 2014. Response to the Rena Grounding. http://www.maritimenz.govt.nz/Environmental/Responding-to-spills-and-pollution/Past-spill-responses/Rena-response.asp (accessed 4 March 2014).

Martin JD, Adams J, Hollebone B, King T, Brown RS, Hodson PV 2014. Chronic toxicity of heavy fuel oils to fish embryos using multiple exposure scenarios. Environmental Toxicology and Chemistry 33: 677-687.

McKee RH, Reitman F, Schreiner C, White R, Charlap JH, O'Neill TP, Goyak KO 2014. The toxicological effects of heavy fuel oil category substances. International Journal of Toxicology 33: 95s-109s.

Mitchell FM, Holdway DA 2000. The acute and chronic toxicity of the dispersants Corexit 9527 and 9500, water accommodated fraction (WAF) of crude oil, and dispersant enhanced WAF (DEWAF) to *Hydra viridissima* (green hydra). Water Research 34: 343-348.

Mommsen TP, Vijayan MM, Moon TW 1999. Cortisol in teleosts: Dynamics, mechanisms of action, and metabolic regulation. Reviews in Fish Biology and Fisheries 9(3): 211-268.

NALCO Envionmental Solutions 2011. Corexit® http://www.nalcoesllc.com/nes/corexit.html (accessed 18 January 2013).

National Research Council 2005. Oil Spill Dispersants: Efficacy and Effects. Washington. D.C, National Academic Press. 400 p.

Nell JA, Livanos G 1988. Effects of fluoride concentration in seawater on growth and fluoride accumulation by Sydney rock oyster (*Saccostrea commercialis*) and flat oyster (*Ostrea angasi*) spat. Water Research 22: 749-753.

NIWA 2013. The Ecosystem Influences on the Life Cycle of Snapper. https://www.niwa.co.nz/fisheries/research-projects/the-ecosystem-influences-on-the-life-cycle-of-snapper (accessed 18 April 2014).

Pampanin DM, Sydnes MO 2013. Polycyclic aromatic hydrocarbons a constituent of petroleum: Presence and influence in the aquatic environment. In: Kutcherov V ed. Hydrocarbon. Pp. 83-118.

Pankhurst NW, Boyden CR, Wilson JB 1980. The effect of a fluoride effluent on marine organisms. Environmental Pollution Series A, Ecological and Biological 23: 299-312.

Parsons DM, Morrison MA, MacDiarmid AB, Stirling B, Cleaver P, Smith IWG, Butcher M 2009. Risks of shifting baselines highlighted by anecdotal accounts of New Zealand's snapper (*Pagrus auratus*) fishery. New Zealand Journal of Marine and Freshwater Research 43: 965-983.

Parsons DM, Sim-Smith CJ, Cryer M, Francis MP, Hartill B, Jones EG, Le Port A, Lowe M, McKenzie J, Morrison M, Paul LJ, Radford C, Ross PM, Spong KT, Trnski T, Usmar N, Walsh C, Zeldis J 2014. Snapper (*Chrysophrys auratus*): A review of life history and key vulnerabilities in New Zealand. New Zealand Journal of Marine and Freshwater Research 48(2): 256-283.

Pascual C, Sánchez A, Sánchez A, Vargas-Albores F, LeMoullac G, Rosas C 2003. Haemolymph metabolic variables and immune response in *Litopenaeus setiferus* adult males: The effect of an extreme temperature. Aquaculture 218(1): 637-650.

Payne JF, Fancey LF 1989. Effect of polycyclic aromatic hydrocarbons on immune responses in fish: Change in melanomacrophage centers in flounder (*Pseudopleuronectes americanus*) exposed to hydrocarbon-contaminated sediments. Marine Environmental Research 28(1–4): 431-435.

Perez DG, Fontanetti CS 2011. Hemocitical responses to environmental stress in invertebrates: A review. Environmental Monitoring and Assessment 177: 437-447.

Peterson CH, Rice SD, Short JW, Esler D, Bodkin JL, Ballachey BE, Irons DB 2003. Long-term ecosystem response to the Exxon Valdez oil spill. Science 302: 2082-2086.

Portmann JE, Connor PM 1968. The toxicity of several oil-spill removers to some species of fish and shellfish. Marine Biology 1: 322-329.

Poursaeid S, Falahatkar B, Mojazi AB, Vander Kraak G 2012. Physiological responses in cultured great sturgeon, *Huso huso*, implanted by cortisol following endoscopic surgery. Journal Of Fisheries (Iranian Journal of Natural Resources) 65(2): 153-167.

Ramachandran SD, Hodson PV, Khan CW, Lee K 2004. Oil dispersant increases PAH uptake by fish exposed to crude oil. Ecotoxicology and Environmental Safety 59: 300-308.

Rena Project 2014. Status of the Wreck. http://www.renaproject.co.nz/status-of-the-wreck/ (accessed 30 April 2014).

Reynaud S, Deschaux P 2006. The effects of polycyclic aromatic hydrocarbons on the immune system of fish: A review. Aquatic Toxicology 77: 229-238.

Rico-Martínez R, Snell TW, Shearer TL 2013. Synergistic toxicity of Macondo crude oil and dispersant Corexit 9500A® to the *Brachionus plicatilis* species complex (Rotifera). Environmental Pollution 173: 5-10.

Rio Tinto Alcan 2006. Cryolite electrolytic bath: Material safety data sheet http://www.msds.riotintoalcan.com/msds/fiches.nsf/0/701d9ea3ffbca2fe85256fda004604eb/$FILE/FS000209EUEN20120730.pdf (accessed 1 June 2014).

Rothwell SE, Black SE, Jerrett AR, Forster ME 2005. Cardiovascular changes and catecholamine release following anaesthesia in Chinook salmon (*Oncorhynchus tshawytscha*) and snapper (*Pagrus auratus*). Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 140(3): 289-98.

Rowley AF, Hunt TC, Page M, Mainwaring G 1988. Fish In: Rowley AF, Ratcliffe NA eds. Vertebrate Blood Cells. United Kingdom Cambridge University Press. Pp. 19-128.

Ryan S 1992. The dynamics of MS-222 anaesthesia in a marine teleost (*Pagrus auratus*: Sparidae). Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 101: 593-600.

Sánchez A, Pascual C, Sánchez A, Vargas-Albores F, Le Moullac G, Rosas C 2001. Hemolymph metabolic variables and immune response in *Litopenaeus setiferus* adult males: The effect of acclimation. Aquaculture 198(1): 13-28.

Schmidt-Etkin D 2011. Spill occurences and the oil spill problem In: Fingas M ed. Oil Spill Science and Technology Burlington, MA, Gulf Professional Publishing Pp. 7-50.

Segner H, Wenger M, Möller A, Köllner B, Casanova-Nakayama A 2012. Immunotoxic effects of environmental toxicants in fish — how to assess them? Environmental Science and Pollution Research 19: 2465-2476.

Sette CB, Pedrete TdA, Felizzola J, Nudi AH, Scofield AdL, Wagener AdLR 2013. Formation and identification of PAHs metabolites in marine organisms. Marine Environmental Research 91: 2-13.

Singer MM, Aurand D, Bragin GE, Clark JR, Coelho GM, Sowby ML, Tjeerdema RS 2000. Standardization of the preparation and quantitation of water-accommodated fractions of petroleum for toxicity testing. Marine Pollution Bulletin 40: 1007-1016.

Singer MM, George S, Jacobson S, Lee I, Weetman LL, Tjeerdema RS, Sowby ML 1996. Comparison of acute aquatic effects of the oil dispersant Corexit 9500 with those of other Corexit series dispersants. Ecotoxicology and Environmental Safety 35: 183-189.

Solvay 2011. Product Safety Summary of Trisodium Hexafluoroaluminate (Cryolite). http://www.icca-chem.org/Portal/SafetySummarySheets/634593802744407699_PSS%20Cryolite_V01.pdf (accessed 1 June 2014).

Solvay 2013. Material Safety Data Sheet. http://www.solvaychemicals.us/SiteCollectionDocuments/sds/P19190-USA.pdf (accessed 1 June 2014).

Solvay Flourides Inc 1998. Cryolite, Synthetic: Material Safety Data Sheet http://fluoridealert.org/wp-content/pesticides/msds/cryolite.syn.powder.pdf (accessed 1 June 2014).

South Australian Research and Development Institute 2014. Offshore Crustaceans. http://www.sardi.sa.gov.au/fisheries/wild_fisheries/offshore_crustaceans (accessed 1 June 2014).

Southward AJ, Southward EC 1978. Recolonization of rocky shores in cornwall after use of toxic dispersants to clean up the torrey canyon spill. Journal of the Fisheries Research Board of Canada 35: 682-706.

Stevens L 2006. Guidelines for the Use of Oil Spill Dispersants. Cawthron Report 915, Prepared for Maritime New Zealand. Nelson, New Zealand, Cawthron Institute. 33p.

Sun Y, Yu H, Zhang J, Yin Y, Shi H, Wang X 2006. Bioaccumulation, depuration and oxidative stress in fish *Carassius auratus* under phenanthrene exposure. Chemosphere 63(8): 1319-1327.

Swannell RPJ, Daniel F 1999. Effect of dispersants on oil biodegradation under simulated marine conditions. International Oil Spill Conference Proceedings 1999: 169-176.

Swedmark M, Granmo Å, Kollberg S 1973. Effects of oil dispersants and oil emulsions on marine animals. Water Research 7: 1649-1672.

Tatem HE, Cox BA, Anderson JW 1978. The toxicity of oils and petroleum hydrocarbons to estuarine crustaceans. Estuarine and Coastal Marine Science 6(4): 365-373.

Te Mauri Moana 2013. Rena Environmental Recovery Monitoring Programme 2011-2013 - Executive Summary. http://sci.waikato.ac.nz/research/projects-and-case-studies/rena-research/Rena-report (accessed 11 May 2014).

The South Australian Centre for Economic Studies 1999. Value of New Zealand Recreational Fishing. REC9801, Undertaken for New Zealand Ministry of Fisheries. South Australia. 117p.

Theron M, Bado-Nilles A, Beuvard C, Danion M, Dussauze M, Ollivier H, Pichavant-Rafini K, Quentel C, Le Floch S 2014. Chemical dispersion of crude oil: Assessment of physiological, immune, and antioxidant systems in juvenile turbot (*Scophthalmus maximus*). Water, Air, & Soil Pollution 225(3): 1-17.

Transport Accident Commission 2011. Containership MV Rena grounding on Astrolabe Reef 5 October 2011. Interim Report. Marine inquiry 11-204. Wellington, New Zealand 28p.

Tsomides HJ, Hughes JB, Thomas JM, Ward CH 1995. Effect of surfactant addition on phenanthrene biodegradation in sediments. Environmental Toxicology and Chemistry 14: 953-959.

Uhler AD, Stout SA, Douglas GS 2007. Chemical heterogeneity in modern marine residual fuel oils. In: Wang Z, Stout S eds. Oil Spill Environmental Forensics: Finger Printing and Source Identification Burlington, MA, Academic Press Pp. 327-348.

United States Environmental Protection Agency 1996. R.E.D Facts: Cryolite. http://www.epa.gov/oppsrrd1/REDs/factsheets/0087fact.pdf (accessed 10 June 2014).

United States Environmental Protection Agency 1999. Understanding Oil Spills and Oil Spill Response: Understanding Oil Spills in Freshwater Environments Washington, DC, Author. 45 p.

United States Environmental Protection Agency 2009. ECOTOX Database. http://cfpub.epa.gov/ecotox/ (accessed 4 June 2014).

United States Environmental Protection Agency 2011a. Cryolite Final Work Plan - Registration Review, September 2011 (Case No. 0087). http://www.epa.gov/oppsrrd1/registration_review/cryolite/ (accessed 11 May 2014).

United States Environmental Protection Agency 2011b. Cryolite Summary Document: Registration Review - Initial Docket, March 2011. http://www.epa.gov/oppsrrd1/registration_review/cryolite/ (accessed 11 May 2014).

Uribe C, Folch H, Enriquez R, Moran G 2011. Innate and adaptive immunity in teleost fish: A review. Veterinarni Medicina 56(10): 486-503.

V´azquez G, Guerrero G 2007. Characterization of blood cells and hematological parameters in *Cichlasoma dimerus* (Teleostei, Perciformes). Tissue and Cell 39: 151–160.

Van Scoy AR, Yu Lin C, Anderson BS, Philips BM, Martin MJ, McCall J, Todd CR, Crane D, Sowby ML, Viant MR, Tjeerdema RS 2010. Metabolic responses produced by crude versus dispersed oil in

Chinook salmon pre-smolts via NMR-based metabolomics. Ecotoxicology and Environmental Safety 73(5): 710-717.

Vazquez L, Alpuche J, Maldonado G, Agundis C, Pereyra-Morales A, Zenteno E 2009. Review: Immunity mechanisms in crustaceans. Innate Immunity 15: 179-188.

Verghese B, Radhakrishnan E, Padhi A 2007. Effect of environmental parameters on immune response of the Indian spiny lobster, *Panulirus homarus* (Linnaeus, 1758). Fish & Shellfish Immunology 23(5): 928-936.

Victor B 1993. Responses of hemocytes and gill tissues to sublethal cadmium chloride poisoning in the crab *Paratelphusa hydrodromous* (Herbst). Archives of Environmental Contamination and Toxicology 24: 432-439.

Wang H, Shi Y, Major D, Yang Z 2012. Lung epithelial cell death induced by oil-dispersant mixtures. Toxicology in Vitro 26: 746-751.

Wilkins A 2013. Rena PAH and Metals Chemistry (Project 1f - Rena Environmental Recovery Monitoring Programme 2011-2013). http://sci.waikato.ac.nz/__data/assets/pdf_file/0004/180364/chapter-6-project-1f.pdf (accessed 9 July 2014).

Wright DA, Davison AW 1975. The accumulation of fluoride by marine and intertidal animals. Environmental Pollution 8: 1-13.

Yan J, Wang L, Fu PP, Yu H 2004. Photomutagenicity of 16 polycyclic aromatic hydrocarbons from the USEPA priority pollutant list. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 557: 99-108.

Zbanyszek R, Smith LS 1984. The effect of water-soluble aromatic hydrocarbons on some haematological parameters of rainbow trout, *Salmo gairdneri* Richardson, during acute exposure. Journal of Fish Biology 24(5): 545-552.

Zelikoff J 1994. Fish immunotoxicology. In: Dean Jack H, Luster M, Munson AE, Kimber I eds. Immunotoxicology and Immunopharmacology. 2nd ed. New York, Raven Press. Pp. 71-96.