Immunotoxicology in Marine Invertebrates:

Effects of Manganese on Immune Response

Carolina Oweson

UNIVERSITY OF GOTHENBURG

Faculty of Science
Department of Marine Ecology

Akademisk avhandling för filosofie doktorsexamen i marin zoologi vid Göteborgs Universitet, som enligt beslut vid naturvetenskapliga fakulteten kommer att försvaras offentligt fredagen den 5e juni 2009, kl. 10.00 i Föreläsningssalen, Sven Lovén Centrum för Marina Vetenskaper - Kristineberg, Fiskebäckskil.

Examinator: Prof. Michael Thorndyke, Institutionen för Marin Ekologi, Göteborgs Universitet

Fakultetsopponent: Dr. Elisabeth Dyrynda, School of Life Sciences, Heriot-Watt University, Riccarton, Edinburgh, UK

Cover by Andreas Ribbung
Printed by Intellecta Infolog AB

ISBN 91-89677-43-9
http://hdl.handle.net/2077/19653

Abstract

Manganese, Mn, is an abundant element in nature, particularly in soft bottom sediments of the oceans and in bedrock. The metal is predominantly bound to the sediment in the colloid state, MnO_2. Eutrophication caused by the high nutrient load in coastal waters together with over-fishing cause cascade effects in the ecosystem increasing the algal blooms and enhancement of hypoxic condition over large bottom areas. During hypoxic events MnO_2 is reduced and released into the bottom water as bioavailable ions, Mn^{2+}. Mn is essential for several metabolic and enzymatic processes and is necessary for both animals and plants. Elevated levels though, are toxic and severe effects on the nervous system have been known for long. In addition, previous studies have shown an impaired immune system of the bottom living lobster, *Nephrops norvegicus*, when exposed to concentrations that are realistic to find in nature. In this study I aimed to investigate if immunotoxic effects of manganese are general also for other marine invertebrates.

It is widely accepted that invertebrates do not have a documented so called *adaptive* immune response. They lack the genes, proteins and cells for the highly specific recognition and the long-term memory as found in vertebrates. Invertebrates primarily rely on the *innate* immune system to effectively combat a wide array of microbial pathogens. The innate immune system comprises of a first line of defence systems such as coagulation and melanization reactions, often followed by cellular reactions such as phagocytosis, encapsulation and production of antimicrobial substances. Many innate immune reactions are highly evolutionary conserved and are found throughout the whole animal kingdom. In aquatic invertebrates the open coelom or semi-open haemal circulatory system continuously expose them to potential pathogens and their immune response has proved to be exceptionally efficient in pathogen elimination as witnesses by the invertebrates' evolutionary success.

In this thesis species from three different phyla within the Bilaterians were investigated; the Norway lobster, *Nephrops norvegicus* (Crustacea), the blue mussel *Mytilus edulis* (Mollusca) and the common sea star, *Asterias rubens* (Echinodermata), differing in preferred habitats, feeding behaviour and somewhat in their strategies of immune defence. Studies were made on molecular, cellular and organism levels. On molecular and cellular levels we investigated the effects of manganese on the renewal of haemocytes (proliferation and differentiation of new cells), manganese effects on viability of haemocytes and the stress responses measured in both haemocytes and haematopoietic tissue. On the whole organism we investigated the effect of manganese on the ability for the animals to clear their cavity form injected bacteria.

The results of this thesis show that Mn in concentrations found in bottom waters affects the immune system of marine invertebrates differently. In *N. norvegicus* the metal severely suppresses the number of circulating haemocytes by inducing apoptosis, programmed cell death. The impaired immunity made them more susceptible to infections, which was also found in *M. edulis*. In *A. rubens* the same Mn concentration seemed to have a stimulating effect (hormesis) on the haematopoiesis which increased the number of circulating haemocytes. Although manganese was shown stressful to the haemocytes and affected their ability to phagocyte, the increased number of haemocytes compensates these impairments. There was seemingly a negative correlation between the accumulation of the metal in the tissues of the animals and their ability to eliminate bacteria. Although Mn does not cause chronic effects on immunity, the expanding areas with bioavailable Mn might have an impact on species composition since some invertebrates become more susceptible to infections.

Keywords: Invertebrates, immune system, haemocytes, manganese (Mn), immunotoxicology, Crustacea, Mollusca, Echinodermata

Till Mamma & Pappa

Allt ordnar sig alltid till det bästa

Immunotoxicology in Marine Invertebrates: Effects of Manganese on Immune Response

Carolina Oweson

This doctoral thesis is produced as a collection of papers. The papers are throughout the thesis referred to by their Roman numerals. The papers are appended at the end of the thesis.

Paper I **Oweson, C., Baden, S. P., Hernroth, B. E.** (2006). Manganese induced apoptosis in haematopoietic cells of *Nephrops norvegicus* (L.). Aquatic Toxicology 77:322-328.

Paper II **Oweson, C., Sköld, H., Pinsino, A., Matranga, V., Hernroth, B.** (2008). Manganese effects on the haematopoietic cells in *Asterias rubens* (L.). Aquatic Toxicology 89:75-81.

Paper III **Oweson, C., Li, C., Söderhäll, I., Hernroth, B.** (2009). Effects of hypoxia and manganese on haematopoiesis in the common sea star, *Asterias rubens* (L.). Manuscript.

Paper IV **Oweson, C. and Hernroth, B.** (2009). A comparative study on the influence of manganese on the bactericidal response of marine invertebrates. Manuscript. Submitted to Fish and Shellfish Immunology; FSIM-S-09-00134[1].

CONTENTS

1. INTRODUCTION

The immune system, within all animals, is based on two fundamental systems: *recognition*, to distinguish between self and non-self, and *effector* systems. Through evolution species have developed sophisticated solutions to manage invading threats like infectious microbes, i.e. pathogens, and other non-self molecules. The character of the immune system of a species reflects its surrounding environment. The immune actions in different animals are dependant on their way of living and how they have evolved together with their threats. Thus, their susceptibility to environmental stressors may differ.

1.1. Invertebrate immune systems

In general invertebrates have an open or semi-open circulatory system and aquatic invertebrates live in continuous contact with potential pathogens (Auffret & Oubella, 1997; Canesi *et al.*, 2002). This makes them dependent on minute reaction of defence and coagulation mechanisms. They have an immune defence based on activities of the blood cells in their body fluid, which entrap foreign particles (Ratcliffe *et al.*; 1984, Chia & Xing, 1996; Johansson & Söderhäll, 1989; Söderhäll & Cerenius, 1998). In the open circulatory systems of e.g. echinoderms, blood is called coelomic fluid and the blood cells are called coelomocytes. In the semi open circulatory systems of e.g. arthropods, the blood is on the other hand called haemolymph and the blood cells haemocytes. To make it easier for the reader the blood and blood cells are, when discussed in general, in this thesis referred to as haemolymph and haemocytes.

It is widely accepted that invertebrates do not have a documented so called *adaptive* immune response. They lack the genes, proteins and cells for the highly specific recognition and the long-term memory as found in vertebrates (Flajnik & Du Pasquier, 2004). To effectively combat a wide array of microbial pathogens, invertebrates primarily rely on the *innate* immune system. The innate immune system is comprised of a first line of defence systems such as coagulation and melanization reactions, often followed by cellular reactions such as phagocytosis, encapsulation and production of antimicrobial substances. Many innate immune reactions are highly evolutionary conserved and are found throughout the whole animal kingdom (Hoffmann & Reichhart, 2002). The immune defence, based on humoral and cellular actions, is proven exceptionally efficient in pathogen elimination as witnessed by the invertebrates' evolutionary success (Haine *et al.* 2008). The innate immune system

employs germline-encoded pattern recognition receptors (PRRs) to identify invading pathogens. The receptors are able to identify non-self by pathogen-associated molecular patterns (PAMPs). These molecules, for example lipopolysaccarides (LPS), peptidoglucans and β-1-3-glucans, stimulate the immune system unspecifically since they are present on the surface of large groups of bacteria and other microorganisms (Medizhitov & Janeway, 2002; Steiner, 2004). Especially peptidoglucans (PGNs) are excellent targets for recognition by the eukaryotic immune system, because PGN is an essential cell wall component of virtually all bacteria and it is not present in eukaryotic cells (Rosenthal & Dziarski, 1994). PGN is especially abundant in Gram-positive bacteria, in which it accounts for almost half the cell wall mass. In Gram-negative bacteria, a relatively thin PGN layer surrounds the cytoplasmic membrane underneath the LPS-containing outer membrane that is also a unique molecule to be recognized (Doyle & Dziarski, 2001).

The innate immunity uses a set of sensors to recognize foreign patterns as told earlier, which are found either intracellular, on cell surfaces or excreted in the haemolymph of the host for an instant reaction (Steiner, 2004). The recognition receptors of the innate immune system induce the effector system of the immunity. The most frequently studied pattern recognition receptors is the peptidoglucans recognition proteins, PGRPs in insects, which can lead to both cellular and humoral responses. The *cellular responses* include phagocytosis or encapsulation and degranulation of haemocytes resulting in release of cytotoxic substances. Examples of *humoral responses* include activation of proteins constitutively present in the haemolymph, such as the prophenoloxidase- and coagulation cascades, as well as activation of intracellular signalling pathways that stimulate production of different defence proteins, for example antimicrobial peptides (AMPs) (these different responses are explained further below) (Hoffmann & Reichhart, 2002; Cerenius & Söderhäll, 2004; Kurata *et al.*, 2006). All species comprise these different responses to a certain extent, but threats in the species environment have evolved changes in strategies.

Phagocytosis refers to engulfment of entities of an individual cell. It is a highly conserved cellular response and occurs in all metazoan and many protozoan phyla. It is the primary reaction of haemocytes to small particles and targets bacteria, yeast and apoptotic cells (Yokoo *et al.*, 1995). Further, *encapsulation* is the immune response against foreign bodies too large for phagocytosis by a single cell. It refers to the

formation of multicellular nodules following a massive bacteria infection or larger invading objects such as nematodes (Lackie, 1988).

The *prophenoloxidase activating system*, ProPO-AS, can distinguish minute amount of lipopolysaccarides (LPS), peptidoglucans or β-1,3 glucans from bacteria or fungi. ProPO-AS is an antimicrobial cascade reaction in invertebrates, generating melanin in the cuticle, haemolymph and tissue (Fig. 1). Melanin physically shields the intruding organism and constrains the infection. Important in the formation of melanin is the production of its cytotoxic intermediates, for example quinone (Cerenius & Söderhäll, 2004). When recognition receptors on the surface of semigranular and granular haemocytes are activated, the cell releases the ProPO-AS from granules through the degranulation process. Once outside the haemocyte complex pattern recognition proteins activate the ProPO-AS and a proteolytic cascade is initiated resulting in the cleavage of ProPO to the active enzyme phenoloxidase, PO, (Kan *et al*., 2008; Kim *et al*., 2008; Cerenius *et al*., 2008). The PO enzyme starts a complex stepwise pathway to melanization (Smith & Söderhäll, 1983; Söderhäll & Cerenius, 1998). The intermediary cytotoxic compounds are also needed for cell communication to initiate further activities in haemocytes, such as phagocytosis and encapsulation, for example peroxinectin (Jiravanichhpaisal *et al*., 2006). Production of melanin and its intermediates prevents growth of microorganisms by inhibiting proteinases and chitinases (Söderhäll & Cerenius, 1992; Söderhäll & Cerenius, 1998; Johansson *et al*., 2000). Recent research has clarified that activation of the proPO-AS in insects is "cross talking" with the activation of AMP synthesis through the Toll-pathway (Kan *et al*., 2008; Kim *et al*., 2008; Cerenius *et al*., 2008).

Wound healing and coagulation are essential processes in invertebrates since many invertebrates have an open circulatory system, and must therefore instantly seal wounds to prevent body fluid imbalance. Many invertebrates also have the ability to regenerate lost parts of their bodies, which is preceded by a rapid closure of the cut, particularly evident in echinoderms (Smith, 1981; Smith, 1991; Gurther *et al*., 2008).

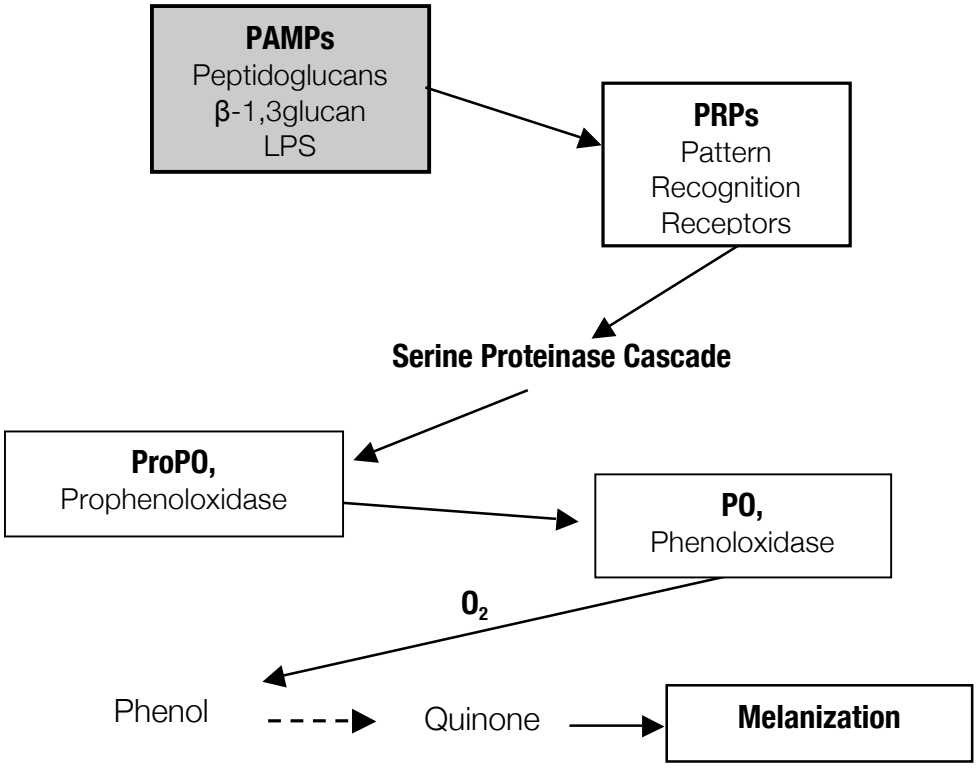

Figure 1. The prophenoloxidase-activating system, ProPO-AS, in crustaceans. The proPO-AS is confined to semigranular- and granular cells in haemolymph and is triggered by minute amount of LPS, peptidoglucans or β-1,3-glucans (Modified after Söderhäll & Cerenius, 1998).

The *secretion of antimicrobial peptides* is generated through different pathways, where two different pathways have been thoroughly described, the Toll pathway and the ImD pathway (Hoffmann & Reichhart, 2002; Dziarski, 2004). The Toll pathway in insects is primarily stimulated by infections of $Gram^+$ bacteria and fungi (Michel *et al.*, 2001). Interaction of PGN on bacteria with host PGRP activates proteases cleaving of an extracellular cytokine-like protein called *Spätzle*, which serves as an endogenous activator of the membrane bound *Toll-receptor*. Activation of the Toll-receptor initiates a signal transduction pathway resulting in translocation of the two transcription factors *Dif* to the nucleus which initiates transcription of *Drosomycin*, a gene encoding an antifungal peptide, and some other AMPs (Hoffmann & Reichhart, 2002; Weber *et al.*, 2003; Steiner, 2004). The second system, the Toll-independent ImD pathway, is mediated through transmembrane host PGRPs reacting on $Gram^-$ bacteria and certain $Gram^+$ bacilli. The PGRPs act as receptors or co-receptors for these bacteria (Hoffmann & Reichhart, 2002; Werner *et al.*, 2003). Activation of this pathway results in a general humoral response, through the transcription factor *Relish*, comprising a number of AMPs predominated by the *Diptericin*, lacking in the Toll

pathway. Both Dif and Relish are members of the Rel family of transcription factors, which are similar to the mammalian NF-κB.

1.2. Animals studied

The species studied in this thesis are from three different phyla within the Bilaterians: Arthropoda, Mollusca and Echinodermata, differing in preferred habitats, feeding behaviour and somewhat in their strategies of immune defence (Fig. 2). There are differences in mobilization and activation of the immune defence between these groups of invertebrates. For example, the filter feeding mussels have developed an immune system based on phagocytosis, probably since they constantly interact with foreign particles and thus also pathogens (Cheng, 1969; Canesi *et al.*, 2002). The immune mechanisms of crustaceans rely mostly on a clotting and melanization systems (Söderhäll & Cerenius, 1992, 1998) since they are more likely to be injured and in need of a fast clotting system. Likewise, the echinoderms often get injured due to predation and need a fast system for preventing blood loss, wound healing and regeneration of tissues. The immune defence of the three invertebrate phyla studied in this thesis is briefly summarized as follows: The circulating haemocytes of various invertebrates are morphologically and functionally diverse. The different types of haemocytes are mainly well characterized in arthropods, for example in *Drosophila melanogaster* (Crozatier *et al.*, 2007) and *Pacifastacus leniusculus* (Johansson *et al.*, 2000; Wu *et al.*, 2008), while for many species characterization is not completed. The major classification of haemocytes in invertebrates is the presence or absence of cytoplasmic granules. The granules contain a range of hydrolytic enzymes including proteinases, glucosidases and sulphatases (Pipe, 1997) and are described as lysosomes.

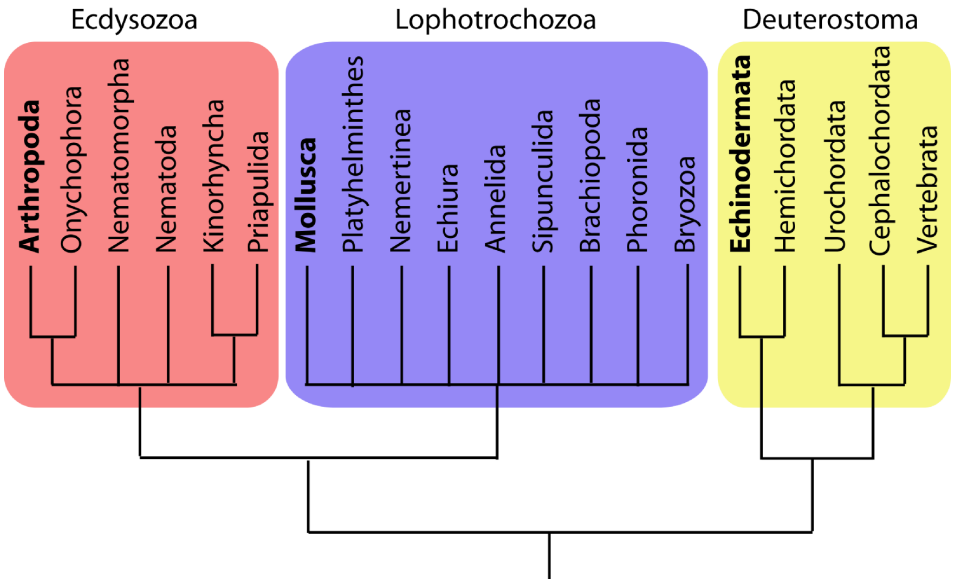

Figure 2. Bilaterian Phylogeny. The three main phyla within the bilaterians; Ecdysozoa, Lophotrochozoa and Deuterostoma. The studied groups within these phyla are marked in bold (by Karolina Larsson, 2008).

1.2.1. Crustacea

The arthropod species used in this thesis is the crustacean Norway lobster, *Nephrops norvegicus* (Linnaeus). The Norway lobster is a stationary inhabitant of borrows in soft bottom sediments at 40 - 800 m depth and is common in waters along the European Atlantic coast. Proliferation and development of haemocytes occur in a specific tissue in crustaceans. It is called the haematopoietic tissue (Hpt), which is a sheet-like tissue found on the dorsal side of the stomach (Chaga *et al.*, 1995). Haematopoietic stem cells, haemoblasts, are densely packed in small lobules of different developmental stages. The haemoblasts are the stem cells for the circulating haemocytes and can be found in the blood cell forming tissue but also in the circulating haemolymph (Wright, 1981). A further differentiation in the haemolymph is shown in crustaceans where specific marker proteins for different cell lineages appear after the release of haemocytes to the circulation (Söderhäll *et al.*, 2003; Wu *et al.*, 2008). Crustaceans have three categories of haemocytes; the *hyalinocytes*, an agranular cell with a phagocytotic function, and two types of cells with granula, *semigranular-* and *granular cells*. The main function of semigranular- and granular cells is the storage of the ProPO-AS (Söderhäll & Cerenius, 1992; Söderhäll & Cerenius, 1998; Johansson *et al.*, 2000). The defence system in crustaceans has evolved to be based on the activity of semigranular- and granular cells. The crustaceans are in some

areas highly infected by the dinoflagellate, *Hematodinium spp.*, which is a parasite invading the haemocoel and connective tissue of most organs and dissolve the muscle tissue (Field & Appleton, 1995; Messick & Shields, 2000). In fisheries, this parasite causes economical losses of great value every year.

1.2.2. Mollusca

The mollusc *Mytilus edulis* (Linnaeus) or the common blue mussel is widespread along the European coastline and lives on hard- and sandy bottoms at 0-10 m. As filter feeders a substantial portion of the diet of molluscs is microorganisms (ZoBell *et al.* 1938). Thus, filter feeding results in concentrations of potential pathogens, but bacteria in large numbers may persist without causing diseases in the animal. Adult molluscs have an efficient defence against pathogens, but stress may comprise the host and outbreaks of different bacterial diseases caused by e.g. the most common *Vibrios* and *Pseudomonas* (Olafsen *et al.* 1993). The site of haematopoiesis in *Mytilus edulis* is currently unknown, but in related organisms such as snails haemocytes are produced in small nodes, primarily in the epithelial cells lining the pericardium (Sminia, 1974). Haemocyte mitosis in molluscs seems also to occur in haemolymph (Mayrand *et al.*, 2005). The immune defence of *M. edulis* has evolved to be specialized on phagocytosis and has very efficient antimicrobial peptides (Mitta *et al.*, 1999; Wootton *et al.*, 2003). The role of granular cells within bivalves is phagocytosis as well as encapsulation of microbes. After engulfment the phagosomes fuse with lysosomes and the microbes are sequestered in the acidic phago-lysosome by the enzymes, reactive metabolites and antimicrobial peptides (Cheng, 1983; Pipe, 1992; Winston *et al.*, 1996). In molluscs, three different categories of haemocytes are found and all of them are able to phagocyte although one of them, the eosinophilic, seems to be more prominent (Pipe *et al.*, 1997; Dyrynda *et al.*, 1997).

1.2.3. Echinodermata

Asterias rubens (Linnaeus) is the common sea star in European waters and lives on hard or soft bottoms at depth between 0 - 200 m. Studies on echinoderm species reveal that their immune system is based on the phagocytotic activity of the immune cells (Coteur *et al.*, 2002). They also have a simplified complement system (Smith *et al.* 2001) and bacteria-inducible transcription factors including a NF-κB homologue (Pancer *et al.* 1999). The coelomic fluid of *A. rubens* possesses large populations of

circulating cells. The circulating cells in *A. rubens* have not been named in a universal way. The same type of cells can be called different names in different literature. Phagocytes constitute the predominated sub-population, comprising approximately 80-95% of the population of coelomocytes (Pinsino *et al.*, 2007). These cells can be transformed to petaloid and filopodial forms. It is found that coelomocytes in *A. rubens* have the ability to form networks and fuse to syncytic formations when non-self organisms are invading the coelomic fluid (Holm *et al.*, 2008). In addition, there are also amoebocytes, so called because of their ability to migrate within tissue and vibratile cells present in the coelomic fluid (Smith, 1981). The coelomocytes are able to efficiently clear bacteria from the coelomic cavity and in case of injury they take part in wound healing by migrating to the injured site, prevent bleeding by clotting and interact with the extracellular matrix during the healing process (Smith, 1981; Dybas & Frankboner, 1986). The recruitment of circulating coelomocytes is not fully understood. The coelomic epithelium has been suggested as one of the most probable potential source of the coelomocytes of echinoderms (Munoz-Chapuli *et al.*, 2005) but also the axial organ (Leclerc *et al.*, 1987) and the Tiedemanns's body have been suggested as well as the possibility of self-replication of the circulating coelomocytes (Ratcliffe & Rowely, 1979). All three of these tissues have shown mitogenic response to LPS, which further indicate their role as haematopoietic tissues (Holm *et al.*, 2008). Pathogen-induced mortalities of echinoderms, in particular of sea urchins, have been reported from several places (Jangoux, 1990). Mass mortalities of the sea star, *Acanthaster planci*, attributed to a sporozoan have been found in the Pacific Ocean (Zann *et al*, 1990).

1.3. Manganese and Hypoxia

Many naturally occurring compounds are increasing in distribution and concentration due to anthropogenic activities. These substances can reach toxic levels and may affect the immune system of living organisms. Manganese, Mn, is an abundant element in nature, particularly in soft bottom sediments of the oceans and in bedrock. The metal is predominantly bound to the sediment in a four-valent colloid state, MnO_2. However, during hypoxic conditions, lower than 16 % O_2 saturation that can occur during periods of days to weeks in the bottom water (Baden *et al.*, 1990; Pihl *et al.*, 1991), MnO_2 is reduced and released into its bioavailable state, Mn^{2+}, and can reach toxic levels in benthic biota (Hall *et al.*, 1996). There have been reports

of measured Mn concentration increased by a factor of 1000 (Trefry *et al.*, 1984). Along the Swedish west coast the Mn^{2+} fraction can increase and reach 19-20 mg L^{-1} in the bottom waters (Magnusson *et al.*, 1996). Mn^{2+} re-oxidizes only on particles and the bioavailable fraction may therefore stay in the water column for quite some time even after hypoxia.

Eutrophication caused of the high input of nutrients in coastal waters together with over-fishing cause cascade effects in the ecosystem increasing the algal blooms and enhances hypoxic condition in large bottom areas (Casini *et al.*, 2008; Diaz & Rosenberg, 2008). The seasonal hypoxia is increasing along the Swedish and European coastline (Diaz & Rosenberg, 1995; Diaz & Rosenberg, 2008) and thus also the level of bioavailable Mn (Fig. 3.).

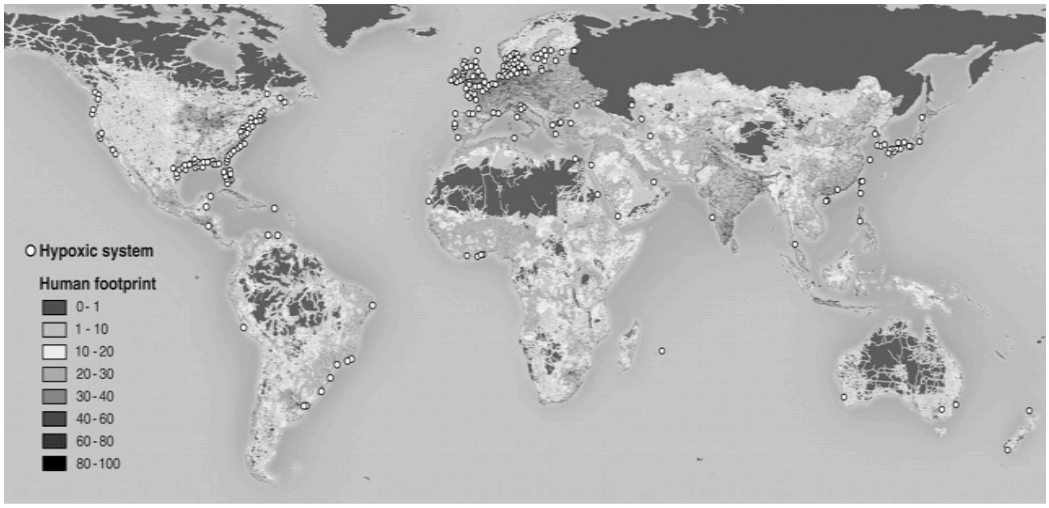

Figure 3. The distribution of documented hypoxic areas in 2008. Diaz & Rosenberg, 2008.

Manganese (Mn) is an essential trace metal accumulating especially in mitochondria in both animals and plants. The metal is involved in metabolic processes as a cofactor or activator of different enzymatic reactions, e.g. electron transfer reactions and phosphorylation (Simkiss & Taylor, 1989). Mn can however act as a toxicant to organisms when the concentrations are elevated and start affecting neuromuscular transmission by interacting with mitochondrial Ca^{2+} and disturbing the ion balance in muscle membranes (Gavin *et al.*, 1999). Ionic Mn can also cross the blood-brain barrier and interfere with chemical synapse functions. The fact that Mn has an effect on the central nervous system has been known for long and a symptom called Manganism, similar to Parkinson's disease can be expressed (Iregren, 1990; Verity, 1999).

Detoxification through metallothioneins known to regulate the sequestration and the metabolism of a variety of metals such as cadmium (Cd) and copper (Cu) might not be the pathway for elimination of Mn (Viarengo, 1985). The intracellular pathways of Mn have been studied in the yeast *Saccharomyces cerevisiae* (Cizewski-Culotta *et al.*, 2005) and include widely conserved transport proteins. When Mn occurs in excess the cell minimizes the uptake by degradation of a transport protein, SMF1. The export is regulated through the Golgi apparatus by a secretory pathway known as PMR1. Both these pathways are also used for Ca transport. Detoxification could also happen through entrapment of the metal by lysosomes (Temara *et al.*, 1998; Sterling *et al.*, 2007).

Earlier studies of *N. norvegicus* reveal that manganese accumulates primarily in the nervous tissue, but also in the haemolymph, where it accumulates three times the exposure concentration. It was shown to reach neurotoxic levels in the bottom living *N. norvegicus* (Baden & Neil, 1998; Holmes *et al.*, 1999; Baden & Eriksson 2006). Recent studies have revealed that a surplus of Mn affected several immunological processes of *N. norvegicus* (Hernroth *et al.*, 2004). Hernroth and co-workers found that proliferation and maturation of haemocytes in *N. norvegicus* are inhibited. One of the observations is a decreased number of circulating haemocytes.

2. AIM OF THE THESIS

The main objective of this study has been to explore the effects of exposure to manganese (Mn) on immunological mechanisms of marine invertebrates and the consequences for the animals' defence against microorganisms. The overall hypothesis is that Mn^{2+} accumulation in haemolymph causes defective mechanisms in haematopoiesis and suppresses the activation of immune response with increased prevalence for microbial infection as a result. The studies were intended to clarify similar/dissimilar influences from manganese exposure in concentrations reported from field conditions on the immune systems of selected invertebrate species from different phyla.

The specific aims were:

Paper I Investigate potential mechanisms behind the lowered number of haemocytes, haemocytopenia, caused by manganese, in *N. norvegicus*. Focus was on whether apoptosis or necrosis contribute to the haemocytepenia.

Paper II Compare *A. rubens*, to earlier studies on *N. norvegicus*. Mechanistic and functional responses were considered, in order to get a broad view of the effects of Mn as a stressor to echinoderms.

Paper III Investigate effects of exposure to Mn in combination with hypoxia on the proliferation and maturation of *A. rubens* coelomocytes.

Paper IV A comparative study of clearance rate of the bacterium, *Vibrio parahaemolyticus*, injected in three different species, *N. norvegicus*, *A. rubens* and *M. edulis* exposed to Mn. In addition, potential acute or chronic effects of elevated concentration of Mn were investigated.

3. METHODOLOGICAL CONSIDERATION

All papers include analysis of the actual level of Mn in haemolymph from Mn exposed and unexposed animals, which makes it possible to draw conclusions that the reason for change is elevated Mn levels. Likewise, the number of circulating coelomocytes or haemocytes in all animals are routinely analysed to check for possible changes when exposed to increased levels of Mn, which is a fundamental hypothesis in the thesis. Analyses of interest from the specific paper are presented below.

3.1. Animal handling

The three studied phyla of invertebrates were collected outside the Sven Lovén Centre of Marine Sciences – Kristineberg, formally known as Kristineberg Marine Research Station in the Gullmar Fjord situated at the Swedish west coast. Animals were maintained in basins supplied with running seawater of ambient temperature and salinity and were fed regularly until acclimatized and used for the experiments. The specimens of *Asterias rubens* and *Mytilus edulis* were collected by scuba divers, *A. rubens* at the depth of 5-15 m and *M. edulis* at 0.5-2 m. *Nephrops norvegicus* were caught in creels by local fishermen at about 60 m depth. All animals used for the study were of similar size within each group; *A. rubens* 10-12 cm across, from arm tip to most distant arm tip, *M. edulis* 5-7 cm across the shell, and *N. norvegicus* 5-8 cm length over carapax and the group was a random mixture of gender.

During time of experiment the lobsters and mussels were kept in containers with seawater allowing mussels 0.5 L per individual and the lobsters about 50 L per individual. The containers used where continuously mixed and aerated through bubbling of the water. To simulate the hiding burrows lobsters naturally use, plastic tubes were available in their tanks. Sea stars, on the other hand, are very delicate to handle in a laboratory environment and we could not use a continuous flow-through system when exposing the animals to manganese. To be able to expose sea stars to controlled Mn concentrations they were placed in 3.5 l glass aquaria on a slowly moving mixing table fulfilling the demand of oxygen without bubbling. The water was exchanged daily and the animals were not fed during the experiment.

During manganese exposure Mn is dissolved in filtered seawater at appropriate nominal concentrations, achieved by using manganese(II)chloride tetrahydrate (GR, Merck, Germany). Animals used as controls were treated in the

same way but in seawater without Mn. When examining the effects of hypoxia on the sea star *A. rubens*, the same type of containers were used as when exposing the animals to manganese, but sealed. Oxygen levels between 14 - 16 % saturation were achieved by aeration with nitrogen gas in the sealed containers and controlled with oxygen meters (Oxi 340, WTW), continuously logged with Achat II Software. Lower saturation levels would be irrelevant in this study, since such oxygen depletion would subordinate the effects of the immune system in the animals.

3.2. Cell viability

Viability of the circulating haemocytes is an important indicator for studying the functionality of the cells. If the cells are less competent than under normal conditions, the whole system is most likely less efficient. To investigate the cytotoxicity of Mn on haemocytes two different methods were used based on; *a*) Metabolic activity, examining calorimetrically the ability of cells to convert tetrazolium to formazan through dehydrogenase activity (Mosmann, 1983) and *b*) Cell membrane integrity, investigating the ability of haemocytes to exclude Trypan Blue. The tetrazolium test gives a good view of how vital the cells are and is used in both Papers I and II. When doing the tetrazolium test *in vitro* in Paper I, we had difficulties with Mn complex binding to the anticoagulation buffer, since it contained EDTA, but since we did the Trypan Blue test in parallel the outcome of the results could be verified. To avoid the complex binding in Paper II, we did not use any anticoagulation buffer and diluted coelomocytes in coelomic fluid after concentrating them.

3.3. Cell proliferation

Increased cell proliferation in the haematopoietic tissue is a way to compensate for loss in number of circulating haemocytes and could as well be a strategy to compensate for loss of viability of the haemocytes. Cell proliferation was not increased in *N. norvegicus*, which would be a normal reaction to the decrease in circulating haemocytes (Hernroth *et al.* 2004). In *A. rubens* the number of circulating haemocytes increased radically when exposed to Mn. In order to investigate the influence of Mn on cell proliferation of the circulating haemocytes and of coelomic epithelium, which is regarded as a source of haemocyte, renewal (Muñoz-Chápuli *et al.*, 2005) two different methods were used. Proliferation was compared between Mn

exposed and un-exposed sea stars by microscopical determination of the ratio of nuclei in mitotic stages found in cells from coelomic epithelium, used in Paper II. However, mitotic nucleus could sometimes be hard to judge. The second method used was to get a less subjective view of mitotic stages of haematopoietic cells, and mitosis was traced and compared by using the substitute nucleotide, 5-Bromo-2´-deoxyuridine, BrdU. BrdU-substitutes for thymidine in S-phase of replicating cells and this was detected with a specific antibody, used in Papers II and III. Both methods, the Mitotic index and the BrdU-incorporation, indicated that Mn induced proliferation of cells in the HPT of *A. rubens*.

3.4. Cell differentiation

Runx-homologous molecules are a family of transcription factors defined by a highly conserved DNA binding Runt-domain (Rennert *et al.*, 2003; Stricker *et al.*, 2003). Runx genes are in generally known to be involved in the transcriptional control of developmental processes (Wheeler *et al.*, 2000; Coffman, 2003), but the Runt gene in invertebrates is also determining the haematopoietic cell fate of granular cells (Tracey & Speck, 2000; Reviewed by Coffman, 2003). Hernroth *et al.* (2004) studied the Runt gene by using c-DNA-probe and *in situ* hybridization technique to examine the effect of manganese on differentiation of haematopoietic cells of *N. norvegicus*. To investigate whether manganese and hypoxia have an effect on differentiation of haemocytes in *A. rubens*, the expression of the Runt gene was quantified with Real-Time Polymerase Chain Reaction (qRT-PCR) technique. Since the Runt gene in *A. rubens* had not been sequenced before, homology cloning and sequencing was done before designing specific Runt primers and the sequence was annotated to BLAST algorithm at the National Centre for Biotechnology Information (http://www.ncbi.nlm.gov/blast). Analysis of the data from the different exposure groups was made with comparative quantification. The qRT-PCR has advantages since the analysis gives a quantitative measurement of the Runt expression compared to the semi-quantitative *in situ* hybridization technique.

3.5. Apoptosis

In *N. norvegicus* the number of haemocytes drastically decreased when the animals were exposed to Mn. Hernroth *et al.* (2004) suggested that Mn inhibited the proliferation, which normally would increase upon such losses. Other possible

reasons for the heamocytopenia could be increased necrosis or apoptosis of both circulating and proliferating haematopoietic cells. By distinguish between apoptosis and necrosis in Paper I we aimed to judge the degree of Mn toxicity to the cells. Agents that can cause apoptosis at low doses could cause necrosis by inhibiting vital metabolic processes at high doses (Raffray & Cohen, 1997). Cell death caused by necrosis involves a catastrophic failure of cellular homeostasis, uncontrolled, degrading enzymatic reactions and cell leakage, which could initiate inflammatory reactions in mammalian systems (Alison & Sarraf, 1995; Raffray & Cohen, 1997). Apoptosis is a gene-derived cell suicide process, found in virtually all metazoan organisms, to eliminate unwanted or damaged cells. During apoptosis the integrity of the cellular organelles and plasma membrane is maintained and the fragments are eliminated through non-traumatic phagocytic clearance (Steller, 1995; Jacobson *et al.*, 1997; Raff, 1998). Apoptosis is in general characterized by generation of DNA fragments that can be recognized through detecting their specific single strand breaks or their typical migration on agarose gel. Both these methods were used in this study to analyze dose and time dependent induction of apoptosis. DNA fragmentation assay, called TUNEL (TdT-mediated dUTP Nick-end Labelling), where a fluorecein-labeled probe is complementary to specific end sequences was used to identify the strand breaks specific to apoptotic fragments. The other test used was a DNA-ladder assay, identifying apoptosis specific DNA fragmentation when separated on agarose gel, forming a so-called DNA-ladder (Wyllie, 1980).

Initially, a pilot study was performed to investigate Mn-induced apoptosis in circulating haemocytes. Due to experimental difficulties recognized as interference between auto-florescence of the haemocytes and the green dye fluorecein-labeled probe, the experiment was instead performed on cells from the Hpt. Both methods, TUNEL and DNA-ladder assays, indicated that Mn induced apoptosis.

3.6. Stress response

When, in Paper II, testing whether a stress response is induced in *A. rubens*, two different methods were performed. One indication of induced stress in animals is increased levels of the so-called heat shock proteins (Hsp). The heat shock proteins are a family of ubiquitous expressed proteins, which help to process misfolded and damaged polypeptide chains and support maturation by functioning as a chaperone protein (Bukau *et al.* 1998). Hsp70, one protein within this family, is an indicator of

stress, since it is upregulated when exposed to a functional or environmental stressor (Matranga *et al.*, 2000; Pinsino *et al.*, 2007; Holm *et al.*, 2008). A specific antibody against Hsp70 was used as a stress marker in haemocytes and coelomic epithelium.

Another attempt of measuring the stress levels induced in animals was done by using the rather new technique, OxyBlot. Previously, protein carbonyls have been used for investigating oxidative damage of proteins due to environmental stress (Almroth *et al.*, 2005). Protein oxidation was analyzed by measuring the levels of dinitrophenylhydrazone derivates of protein carbonyls, by separation with gel electrophoresis and identification through blotting procedure and a specific antibody. This registers the endpoint protein at oxidative damage, which indicates irreversible damage of the proteins. We used Western Blot technique for both analyses. Hsp70 was also detected through immunohistochemistry on tissue sections.

3.7. Functional response

It is of great importance to investigate if increased levels of Mn affect the functional responses in animals since it would be effect their survival in nature. In Paper II effect on phagocytosis was investigated *in vitro* and in Paper IV the bactericidal capacity after *in vivo* injection of bacteria was studied.

3.7.1. Phagocytosis assay

Paper II includes a test of how successful haemocytes from Mn exposed sea stars are to phagocyte dead yeast cells marked with fluorescence, FITC, compared to that of unexposed sea stars. The haemocytes in coelomic fluid were incubated with FITC-marked yeast. The fluorescence of yeast cells that are not engulfed by haemocytes are then quenched with Trypan Blue, which can enter only dead cells through their insufficient cell membrane. The yeast engulfed by active cells is then still fluorescent and detectable with a fluorometer.

First we intended to apply this method on a variety of organisms also including animals from the Baltic Sea. The method was tested on *M. edulis, Macoma baltica* and *Saduria entomon*. Mn effect on total haemocyte number was counted. Some problems occurred when trying to optimize the phagocytosis assay for the different animals. Since the haemocytes for most of the animals were decreasing in number when exposed to Mn it became difficult to get a proper number for the assay. We tried to concentrate the number of haemocytes through centrifugation, but since we

wanted to avoid using EDTA as an anticoagulant because it might bind Mn and change the ion concentration of the metal in the assay, it was impossible to avoid clotting of cells. However, the haemocytes of *A. rubens* were sufficient without concentration and thus this *in vitro* phagocytosis experiment was used to compare the phagocytic index only on haemocytes from Mn exposed and un-exposed sea stars.

3.7.2. Bactericidal capacity

In Paper IV the whole focus of the study was on how effective animals were in defending themselves from a sub-lethal dose of a pathogen injected after Mn exposure when compared to unexposed animals. The study was made on *N. norvegicus, M. edulis* and *A. rubens*, and the pathogen used was a bacterium, *Vibrio parahaemolyticus*. We used *V. parahaemolyticus* as a model organism since the coastal water is their natural habitat and they have the ability to infect fish and shellfish (De Paola *et al.*, 1998, Colwell & Hug, 2001). Studies from Eiler *et al.* (2006) found *V. parahaemolyticus* in Skagerrak and the Baltic Sea to the Gulf of Bothnia. There are reports on increased spreading with increased temperature in water (Ra Londe, 2006). The bacteria were isolated from mussels sampled when water temperature was approximately 20 °C in the area outside the Sven Lovén Centre - Kristineberg.

The appropriate concentration of the bacteria was determined for each species to ensure that the dose was not lethal but still detectable with the viable count method. The animals where first exposed to Mn for 5 days before being injected with *V. parahaemolyticus*. Samples of haemolymph and the digestive gland were then taken from animals in a time course. The fluid was streaked out and incubated on agar plates. When analyzing viable counts during the first hours post injection we could see high variances between individuals. Since the goal was to recognize potential differences between the animals exposed to Mn and the un-exposed rather than the clearance kinetics we decided to avoid such an early investigation. We could see that the clearance from the fluid was quite fast and thus we decided to also include the digestive glands of the animals. Viable counts were determined to compare the bactericidal capacity of the different groups. To investigate if manganese has a prolonged effect after time of exposure a recovery study was performed. The same procedure was repeated after a recovery period of 3 days in water without Mn additive after first being exposed during 5 days to manganese. Samples of haemolymph and the digestive gland were taken after 24h.

Vibrios are known to enter a viable but non-culturable (VBNC) stage (Wang & Gu, 2005) when encountering non-favourable conditions. Since only culturable *V. parahaemolyticus* were investigated in this study those that might be VBNC would be missed. However, it was assumed that the bacteria were equally affected by the environmental conditions and thus viable counts were judged as a satisfactory method.

4. MAIN RESULTS AND DISCUSSION

Measuring the concentration of Mn after exposure reveals an uptake and accumulation in the haemolymph and in the digestive gland of the animals, but varied between the different species. These studies have demonstrated that after 5 days of exposure the levels of Mn in haemolymph of the animals are in steady state with the surrounding water in *A. rubens*, the accumulation is significantly higher in *M. edulis* and increases almost 3 fold in *N. norvegicus*. The accumulation of Mn in the digestive glands of the tested species gives a different picture. Here, the uptake of Mn in *A. rubens* and *N. norvegicus* was slower reaching a lower concentration of Mn than in the blood while in *M. edulis* Mn accumulated to a similar level as found in the haemolymph (Paper IV). The differences in accumulation of Mn observed between the species seem to reflect the different immune response.

When exposed to Mn in concentrations relevant to what is found in nature, 15 mg Mn L^{-1} (Magnusson *et al.*, 1996), the number of haemocytes was affected in all tested animals, although the alteration differs between the animals. Both *N. norvegicus* and *M. edulis* showed reduced numbers of haemocytes after Mn exposure. Opposite to these findings, *A. rubens* significantly increased its circulating haemocytes. The reduction in circulating haemocytes was in agreement with the results from earlier studies on *N. norvegicus* (Hernroth *et al.* 2004) and we could see similar results in pilot studies when testing the effect of Mn on *M. baltica*, *S. entomon* and *Ciona intestinalis* (Table 1.). The contradictory results from *A. rubens* are a very interesting discovery. The numbers of circulating haemocytes of *A. rubens* have previously been shown to be quite stable despite changes in salinity and temperature and as well to Cd exposure (Coteur *et al.* 2004, 2005). It indicates that the relatively low uptake in *A. rubens* initiates a stimulating effect of the immune system. This stimulating effect, hormesis, on the haemocyte numbers of *A. rubens* might have responded differently if the Mn dose was higher than we used. This was not relevant in our study since we wanted to investigate the effects of Mn concentrations occurring in nature. We were not able to see a hormesis effect on *N. norvegicus* when exposed to lower concentrations (Paper I).

Table 1. Total Haemocyte Counts (THC) in different species after 5 days exposure to 15 mg Mn /L. *N. norvegicus* and *C. intestinalis* are exposed for 10 days to 10 resp. 20 mg Mn /L. $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

Group	Species	Number of tested ind. (n)	THC*10^6 ml^{-1} Control (se)	Mn (se)
Arthropoda	*Nephrops norvegicus*	22	14.3 (3.0)	9.3 * (2.0)
Arthropoda	*Saduria entomon*	9	0.7 (0.1)	0.6 (0.2)
Mollusca	*Mytilus edulis* (west coast)	10	2.1 (0.8)	1.0 ** (0.7)
Mollusca	*Mytilus edulis* (east coast)	5	0.8 (0.1)	0.5 (0.1)
Mollusca	*Macoma baltica*	10	0.9 (0.08)	0.5*** (0.04)
Echinodermata	*Asterias rubens*	10	3.0 (2.4)	5.3 * (2.3)
Urochordata	*Ciona intestinalis*	8	57 (20)	32 * (11)

In high concentrations Mn is known to interact with calcium and in that way interrupt the synaptic transmission (Luk *et al.*, 2003). Thus Mn might neurologically affect the ectoderm and the hydrostatic organ, or tube feet, of *A. rubens* and thereby disturb the homeostasis of the coelom. Such a homeostatic change rather than an actual induction of cell proliferation was a theory investigated in Paper II, as a cause for the observed elevated concentrations of haemocytes. However, studies in Paper II revealed that the coelomic fluid density did not change as indicated by its stable protein level and the un-changed body index after Mn-exposure, when length and weight were measured before and after exposure. Instead the proliferation studies in Paper II showed an increase in dividing coelomic epithelial cells pointing out that the manganese induced proliferation and renewal of circulating haemocytes. The proliferation of cells in coelomic epithelium of Mn-treated sea stars was significantly enhanced compared to that of un-exposed sea stars. Mitotic cells were not found in coelomic fluid. The coelomic epithelium, the axial organ and the Tiedemanns's body have been suggested as sources of the haemocytes of echinoderms (Munoz-Chapuli *et al.*, 2005; Holm *et al.*, 2008). In general the proliferation rate in coelomic epithelium was comparatively low to what previously has been described in the Hpt of *N. norvegicus* (Hernroth, *et al.* 2004). The coelomic epithelium though, covers the dorsal

part of the entire coelomic cavity of the animals and given the large size its contribution of renewal of coelomocytes should be significant.

When analyzing hypoxia treated animals, *A. rubens*, in Paper III, there were no changes of proliferation in cells from the coelomic epithelium nor change in amount of circulating haemocytes. Though when exposed to manganese, a 4 fold increase in proliferation was found in both groups, Mn and Mn and hypoxia in combination which showed that Mn rather than hypoxia stimulated the proliferation.. Studies on differentiation of these cells, explored by the expression of the Runt gene, showed a dramatic synergistic effect of Mn in combination with hypoxia. Since Runt is expressed in higher levels when haematopoietic cells differentiate to granular cells, this might be an indication of a change in composition of haemocytes.

Different cell types are most probably different in their resistance to Mn, which might generate toxicant tissue selectively. Hirata (2002) found that the viability of a neuronal cell line (PC2), in terms of its ability to convert tetrazolium to formazan by mitochondrial dehydrogenase, was significantly reduced when kept in culture and exposed to 5 and 55 mg l^{-1} of Mn for 48 h. Such an effect on haemocytes could not be shown in present the studies on Norway lobster. The viability was not reduced when the haemocytes were exposed *in vitro* or in the *in vivo* study in Paper I, although the animals accumulated more than twice the exposure concentration of Mn. The ability of the haemocytes to exclude Trypan blue, which was also tested in Paper I, did confirm the maintenance of their cell membrane integrity.

The results from the viability tests on *N. norvegicus* in Paper I showed that necrosis was most likely not the explanation to haemocyte depletion, but apoptosis was. The apoptotic cells amplified in stem cells with increased Mn concentration when tested with the TUNEL-assay. The degree of apoptosis was related to both time of exposure and concentration. The DNA-ladder assay did also show a tendency to increased fragmentation related to concentration of Mn. However, after five days of exposure using the DNA-ladder assay, only the highest exposure concentration, 20 mg Mn L^{-1}, elicited a pronounced apoptotic fragmentation. Apoptosis is a single cell event and the detection level for a DNA-ladder formation might not be reached at the lower concentrations and the shorter exposure time. Furthermore, typical apoptotic bodies were observed in the microscope when analyzing both kinds of cells. Thus, it was concluded that apoptosis of the circulating haemocytes and their precursor cells obviously contributed to the haemocytopenia of

lobsters that was found after Mn exposure. Contrary to the findings in lobsters, studies in Paper II establish that Mn did decrease the viability of haemocytes in *A. rubens* when tested through the same analysis. These findings point out that even though the cellular number increases significantly in the sea star, the conditions of the cells seemed negatively affected. However, the viability assay does not give enough information concerning possible negative effects on the animal's immunological response in terms of host-parasite interactions. At a cellular level however, there were negative effects of Mn on the phagocytic capacity of coelomocytes in *A. rubens* (Paper II) as the capacity to engulf yeast particles was significantly reduced with approximately 6 %. It has earlier been found that cadmium, Cd, does have a negative effect on the immune system in *A. rubens* (Coteur *et al.*, 2005) since, they found a reduction in phagocytic activity although no differences in haemocyte numbers.

The study on the bactericidal capacity when injected with *V. parahaemolyticus* (Paper IV) showed a rapid clearance of the haemolymph. Thus it was assumed that the bacteria were either killed or translocated to other tissues. The digestive gland in both *M. edulis* and *N. norvegicus* appear to be a sink for the tested bacterium, especially so in *N. norvegicus*. This has been reported before in crustaceans and bivalves (Sahoo *et al.*, 2007; Williams *et al.*, 2009). The bacteria might be translocated to the digestive gland by phagocytotic cells, which have previously been reported (Fontaine & Lightner, 1974; Aldrich *et al.*, 1995) or most probably transported through the haemolymph since they were culturable throughout the experiment. *A. rubens* on the other hand did not show the same translocation as the other two tested species. It seems like the echinoderm can compensate for the negative effect of manganese on phagocytic activity through the induced proliferation of coelomocytes. It was obvious that the Mn-exposed sea stars have a better ability to clear the coelomic fluid and their digestive gland from *V. parahaemolyticus* compared to that of the other species. The phagocytic capacity of the digestive gland in *A. rubens* might be more efficient due to the larger organ compared to the other tested animals.

A. rubens and *M. edulis* might not represent species found in areas frequently exposed to elevated levels of Mn. The study does however demonstrate an accumulation of Mn in different species and effects on the immune system and therefore also the fitness of the animals in nature. It is however remarkable that *N.*

norvegicus, living in an environment with recurrent increase in Mn concentration, seems to be the least prepared to cope with the problem.

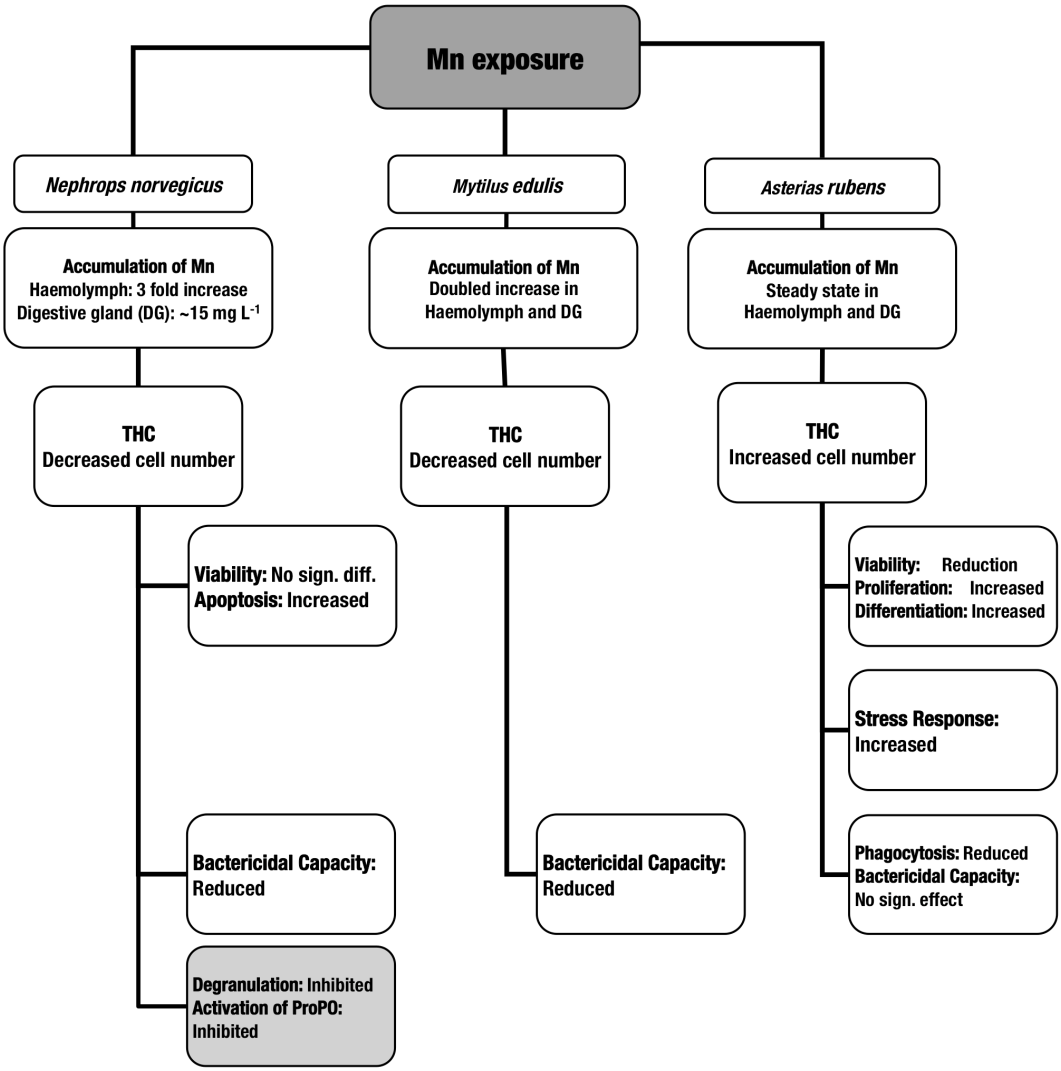

Figure 4. The effects of manganese exposure on the immune systems of the three studied species; *Nephrops norvegicus*, *Mytilus edulis* and *Asterias rubens*. The effect on differentiation in *A. rubens* is in combination with hypoxia. The light grey box at the bottom describes effects from a previous study (Hernroth *et al.*, 2004).

5. CONCLUSIONS

This thesis has shown immune suppressive effects of manganese exposure, in both mechanistic and functional responses, in concentrations realistic to find in bottom waters. The species were not similar in response, however. Taken together; these results showed that Mn exposure significantly affects fundamental immune reactions in species within the studied phyla pointing out the potential harm also for other organisms. In *N. norvegicus* the metal severely suppresses the numbers of haemocytes

by inducing apoptosis. The impaired immunity made them more susceptible to infections. Other invertebrates, such as *M. edulis*, responded in a similar way as the lobsters. *A. rubens* reacted to the same Mn concentration with a stimulating effect on the haematopoiesis which increased the numbers of haemocytes. Although manganese was shown stressful to the haemocytes and affected their ability to phagocyte, the high numbers compensate these impairments. There was seemingly a negative correlation between the accumulation of the metal in the tissues of the animals and their ability to eliminate bacteria. Manganese interferes with proliferation, differentiation and apoptosis, whereby the number of circulating haemocytes is affected. Animals with a lowered cell number are inferior to cope with invasive microbes.

Deficient immune systems increase the prevalence for infections and are of utmost ecological importance. Mobilization and activation of a functional immune system is of great concern for the fitness of all animals and the effects of Mn reported here should be considered in a broader immunotoxicological perspective. Although Mn does not cause chronic effects on immunity the expanding areas with bioavailable Mn might have an impact on species composition since some become more susceptible to infections.

ACKNOWLEDGMENT

I would like to thank the following organizations for financial support to this thesis: The Swedish Research Council for Environment, Agricultural Science and Spatial Planning (FORMAS), the Memory Foundation of Birgit and Birger Wåhlström, the Memory Foundation of Lars Hierta, the Memory Foundation of Carl Tryggers, the Memory Foundation of Wilhelm and Martina Lundgren and the Scientific Foundations of the Royal Swedish Academy of Science.

Jag vill börja med att tacka mina tre handledare; **Bodil Hernroth**, **Irene Söderhäll** & **Susanne Pihl-Baden**, för att jag fick chansen att göra det här projektet. Ni har varit otroligt bra alla tre och kompletterar varandra på ett strålande sätt. **Bodil**, du är värd ett särskilt stort tack! Det har varit fantastiskt att få lära mig inte allt du kan, men en del. Ditt engagemang är inspirerande. Utan dig Bodil, hade jag inte börjat fundera på att doktorera och alldeles säkert inte lyckats få ihop en färdig avhandling. Du är en klippa! Jag vill också tacka **Helen** för att det är kul att jobba och snacka med dig. **Olga**, thank you for all the help with the qPCR. We did it, finally… **Kristina**, det är skönt att vi är två hårdhudingar som snorklar på höst & vinter. Jag tackar självklart alla andra på **JämFys** i Uppsala och fram för allt alla på **Kristineberg** som jag inte jobbat tillsammans med, men som ser till att det är kul att vara på jobbet ändå.

Stor kram till hela gänget i Lyset: **Emma, Ida, Andreas, Sandra, Kikki, Hannah, Linda, Lene, Josefin, Kenta, Maj, Ulrika, Erika, Linus, Pia, Karl, Annelie, Cicci, Marina, Maria, Martin, Olivia, Soffan** och **Tobias** och **den bästa sommargästen, Andreas**. Det är skönt att ni finns, särskilt de dagar vi själva får se till att stan vaknar till.

Big up to **Glen Trash**! Det var väl någon dag där i solgasset på Klubban eller på gamla Belone som min marina karriär och allt annat började. Ni är skönaste gänget!

Mina **Uptown grrls: Anna, Anna, Joel, Zandra** & **Karo**. Var ska jag börja? Ni är helt fantastiska! Tack för att jag alltid får komma och bo hos er när jag dyker upp. Tack för allt ni fixar. Tack för allt snick-snack på dagar & sena kvällar. Kram till **Marie** också, även om du inte bor i Uppsala längre. Ni är mina bästa vänner. Och **Karo**, vi har aldrig tråkigt… jo, en gång ☺. En extra stor kram till dig!

Jag vill ge en jättestor kram till min familj . Tack till **Syrran, Andreas, Freja** & **Tora**. Snart kommer vi äntligen kunna ses mer igen. Jag ser fram emot det. Ett särskilt tack till **Andreas** för hjälpen med att få min bok så fin. Tack **Mamma** & **Pappa** för allt stöd och hjälp på vägen. Det har känts skönt att komma och koppla av hemma hos er ibland.

Den största kramen av alla går till **Pelle**. Det har varit otroligt skönt att du har funnits med och peppat mig. Jag är så glad att det är Du&Jag. Tack för allt du gjort. Nu är det äntligen slutpendlat. Jag kan packa upp väskan och vi får bo ihop – for real. Det kommer bli kalasbra!

REFERENCES

Aldrich, H.C., McDowell, L.M., Tamplin, M.L., Frase, C., Murphree, R., Jackson, J.K. 1995. Detection of *Vibrio vulnificus* and *Vibrio cholerae* O1 in oyster tissue using immunoelectron microscopy. J. Shellfish Res. 14:493-99.

Alison, M.R., Sarraf, C.E., 1995. Apoptosis: Regulation and relevance to toxicology. Hum. Exp. Toxicol. 14**,** 234-247.

Almroth, B.C., Sturve, J., Berglund, Å., Förlin, L., 2005. Oxidative damage in eelpout (*Zoarces viviparus*), measured as protein carbonyls and TBARS, as biomarkers. Aquat. Toxicol. 73:171–180.

Auffret, M., Oubella, R., 1997. Haemocyte aggregation in the oyster *Crassostrea gigas*: *in vitro* measurement and experimental modulations by xenobiotics. Comp Biochem Physiol. 118:705-712.

Baden, S.P., Loo, L-O., Pihl, L., Rosenberg, R., 1990. Effects of eutrophication on benthic communities including fish: Swedish west coast. Ambio 3, 113-122.

Baden, S.P., Neil, D.M., 1998. Accumulation of manganese in the haemolymph, nerve and muscle tissue of *Nephrops norvegicus* (L.) and its effect on neuromuscular performance. Comp. Biochem. Physiol. 119, 351-359.

Baden, S.P., Eriksson, S.P., Gerhardt, L., 1999. Accumulation and elimination kinetics of manganese from different tissues of the Norway lobster *Nephrops norvegicus* (L.). Aquat. Toxicol. 46:127-137.

Baden, S.P., Eriksson, S.P., 2006. Role, routes and effects of manganese in Crustaceans. Oceanogr. Mar. Biol. Ann. Rev. 44:61–83.

Bukau, B., Horwich, A.L., 1998. The Hsp70 and Hsp60 Chaperone Machines. Cell. 92(3): 351-366.

Candida Carnevali, M.D. 2006. Regeneration in Echinoderms: repair, growth, cloning. ISJ. 3: 64-76.

Canesi, L., Gallo, G., Gavioli, M., Pruzzo, C. 2002. Bacteria- hemocyte Interactions and Phagocytosis in Marine Bivalves. Micro Res and Tech. 57:469-476.

Casini, M., Lövgren, J., Hjelm, J., Cardinale, M., Molinero, J-C., Kornilovs, G. 2008. Multi-level trophic cascades in a heavily exploited open marine ecosystem. Proc. R. Soc. B. doi:10.1098/rspb.2007.1752. Published online.

Cerenius, L., Söderhäll, K. 2004. The prophenoloxidase-activating system in invertebrates. Imm. Rev. 198(1):116-126.

Cerenius, L., Lee, B.L., Söderhäll, K. 2008. The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol. 29(6):263-71.

Chaga, O., Lignell, M., Söderhäll, K., 1995. The haematopoietic cells of the freshwater crayfish, *Pacifastacus leniusculus*. Anim. Biol. 4**:**59-70.

Cheng, T.C., A.S. Thakur, and E. Rifkin. 1969. Phagocytosis and an internal defense mechanism in the mollusca: With an experimental study of the role of leucocytes in the removal of inc particles in *Littorina Scabra* Linn. Proc. Symp. Mollusca, Bangalore II: 546-566.

Cheng TC. 1983. The role of lysosomes in molluscan inflammation. Am. Zool. 23:129-44.

Chia, F.S., Xing, J., 1996. Echinoderm haemocytes. Zool. Studies. 35:231-254.

Cizewski Culotta V, Yang M, Hall MD. 2005. Manganese transport and trafficking: Lessons learned from Saccharomyces cerevisiae. Euc cell 4(7):1159-1165.

Coffman, J. A. 2003. Runx transcription factors and the developmental balance between cell proliferation and differentiation. Cell Biol. Int. 27(4):315-324.

Colwell, R., Hug, A., 2001. Marine ecosystems and cholera. Hydrobiologia. 460: 141-145.

Coteur, G, DeBecker, G., Warnau, M., Jangoux, M., Dubois, Ph., 2002. Differentation of immune cells challenged by bacteria in the common European starfish, *Asterias rubens* (Echinodermata). European J Cell Biol. 81:413-418.

Coteur, G., Corriere, N., Dubois, Ph., 2004. Environmental factors influencing the immune responses of the common European starfish (*Asterias rubens*). Fish Shellfish Immunol. 16:51-63.

Coteur, G., Gillian, D., Pernet, Ph., Dubois, Ph., 2005. Alteration of cellular immune responses in the seastar *Asterias rubens* following dietary exposure to cadmium. Aquat Toxicol. 73:418-421.

Crozatier M, Meister M. 2007. Drosophila haematopoiesis. Cell Microbiol. May;9(5):1117-26. Epub 2007 Mar 29. Review. PubMed PMID: 17394559.

De Paola, A., Motes, M.L., Chan, A.M., Suttle, C.A., 1998. Phages infecting Vibrio vulnificus are abundant and diverse in oysters (*Crassostrea virginica*) collected from the Gulf of Mexico. Appl Environ Microbiol. 64(1):346-351.

Diaz, R.J., Rosenberg, R., 1995. Marine benthic hypoxia. A review of its ecological effects and the behavioural responses of benthic macrofauna. Oceanogr. Mar. Biol. Ann. Rev. 245-303.

Diaz, R.J., Rosenberg, R., 2008. Spreading dead zones and consequences for marine ecosystems. Science. 321:926-929.

Doyle, RJ., Dziarski, R. 2001. The bacterial cell: peptidoglucans. In: Sussman, M. (Ed.) Molecular Medical Microbiology. Academic Press, London, pp. 137-153.

Dybas, L., Frankboner, P.V., 1986. Holothurian survival strategies: Mechanisms for the maintenance of the bacteriostatic environment in the coelomic cavity of the sea cucumberm *Parastichopus californicus*. Dev. Comp. Immunol. 10:311-330.

Dyrynda EA, Pipe RK, Ratcliff NA. 1997. Sub-populations of haemocytes in the adult and developing marine mussel, *Mytilus edulis*, identified by use of monoclonal antibodies. Cell Tissue Res. 289:527-36

Dziarski, R. 2004. Peptidoglycan recognition proteins (PGRPs). Mol Immunol. 40:877-886.

Eiler, A., Johansson, M., Bertilsson, S., 2006. Environmental influences on Vibrio populations in northern temperate and boreal coastal waters (Baltic and Skagerrak Seas). Appl Environ Microbiol. 72(9):6004-6011.

Field, R. H., Appelton, P. L. 1995. A *Hematodinium*-like dinoflagellate infection of the Norway lobster *Nephrops norvegicus*: observations on pathology and progression of infection. Dis Aquat Org 22, 115-128.

Flajnik, M. F., Du Pasquier, L. 2004. Evolution of innate and adaptive immunity: can we draw a line? Trends Immunol. 25:640-644.

Fontaine, C.T., Lightner, D.V. 1974. Observations on the phagocytosis and elimination of carmine particles injected into the abdominal musculature of the white shrimp, *Penaeus setiferus*. Invertebr. Pathol. 24:141-148.

Gavin, C.E., Gunter, K.K., and Gunter, T.E., 1999. Manganese and calcium transport in mitochondria: implications for manganese toxicity. Neurotoxicol. 20, 445-453.

Gurther, G.C., Werner, S., Barrandon, Y., Longaker, M.T. 2008. Wound repair and regeneration. Nature. 453:314-321.

Haine ER, Moret Y, Siva-Jothy MT, Rolff J. 2008 Antimicrobial defense and persistent infection in insects. Science. 322(5905):1257-9.

Hall, I.R., Hydes, D.J., Statham, P.J. & Overnell, J. 1996. Dissolved and particulate trace metals in a Scottish Sea Loch: an example of a pristine environment? Mar Poll Bull. 32:846-854.

Hernroth, B.E, Baden, S.P., Holm, K., Andrén, T., Söderhäll, I. 2004. Manganese induced immune-suppression in Norway lobster. Aquat. Toxicol. 70, 223-231.

Hirata, Y., 2002. Manganese-induced apoptosis in PC12 cells. Neurotoxicol. Teratol. 24:639-653.

Hoffmann, J.A., Reichhart, J.M., 2002, *Drosophila* innate immunity: an evolutionary perspective. Nat. Immunol. 3:121-126.

Holm, K., Dupont, S., Sköld, H., Stenius, A., Thorndyke, M., Hernroth, B. 2008. Induced cell proliferation in putative haematopoietic tissues of the sea star, *Asterias rubens* (L). J. Exp. Biol. 211(16):2551-2558.

Holm, K., Hernroth, B., Thorndyke, M. 2008. Coelomocyte numbers and expression of HSP70 in wounded sea stars during hypoxia. Cell Tissue Res. 334(2):319-325.

Holmes, J.M., Gräns, A-S., Neil, D.M., Baden, S.P., 1999. Effects on the metal ions Mn^{2+} and Co^{2+} on muscle contraction in the Norway lobster, *Nephrops norvegicus*. J. Comp. Physiol. B. 169:402-410.

Iregren, A., 1990. Physiological test performance in foundry workers exposed to low levels of manganese. Neurotoxicol. Teratol. 12:673-675.

Jacobson, M.D., Weil, M., Raff, M.C., 1997. Programmed cell death in animal development. Cell 88:347-354.

Jangoux, M. 1990. Diseases of Echinodermata. In: Kinne, O. (Ed.), Diseases of Marine Animals. Biologisch Anstalt Helgoland. Hamburg, pp. 439-567.

Jiravanichhpaisal, P., Lee, B.L., Söderhäll K. 2006. Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization. Immunology 211(4):213-36.

Johansson, M.W., Keyser, P., Sritunyalucksana, K., Söderhäll, K., 2000. Crustacean haemocytes and haematopoiesis. Aquaculture. 191:45-52.

Johansson M.W., Söderhäll K., 1989. Cellular immunity in crustaceans and the proPO system. Parasitology Today. 5:6.

Kan H, Kim CH, Kwon HM, Park JW, Roh KB, Lee H, Park BJ, Zhang R, Zhang J, . Söderhäll, K. and Lee, B.L. 2008. Molecular control of phenoloxidase-induced melanin synthesis in an insect. J. Biol. Chem. 283(37):25316-23.

Kim, C.H., Kim, S.J., Hongnan K., Kwon, H.M., Roh, K.B., Jiang, R., Yang,Y., Park, J.W., Lee, H.H., Ha, N.C., Kang, H.J., Nonaka, M., Söderhäll, K., Lee, B.L. 2008. A three-step proteolytic cascade mediates the activation of the peptidoglycan-induced toll pathway in an insect. J. Biol. Chem. 283(12):7599-607.

Kurata, S., Ariki, S., Kawabata, S., 2006. Recognition of pathogens and activation of immune responses in *Drosophila* and horseshoe crab innate immunity. Immunobiology. 211:237-249.

Lackie, A.M., 1988. Hemocyte behavior. Adv. Insect Physiol. 21:85-178.

Leclerc, M., Luquet, B., Brillouet, C., 1987. In vitro effect of rabbit anti sea star lymphocyte serum on axial organ cells. Cell. Biol. Int. Rep. 11(11):819-23.

Lightner D.V., Redman R. 1977. Histochemical demonstration of melanin in cellular inflammatory processes of *Penaeid* shrimp. J. Inver. Path. 30(3):298-302.

Luk, E., Jensen, L.T., Culotta, V.C., 2003. The many highways for intracellular trafficking of metals. J. Biol. Inorg. Chem. 8(8):803-9.

Magnusson, K., Ekelund, R., Dave, G., Granmo, Å., Förlin, L., Wennberg, L., Samuelsson, M.O., Berggren, M., Brorström-Lundén, E., 1996. Contamination and correlation with toxicity of sediment samples from Skagerrak and Kattegat. J. Sea Res. 35, 223-234.

Matranga, V., Toia, G., Bonaventura, R., Müller, W.E.G. 2000. Cellular and biochemical responses to environmental and experimentally induces stress in sea urchin coelomocytes. Cell Stress Chaperones. 5:113-120.

Mayland, E., St-Jean, S.D., Courtenay, S.C., 2005. Haemocyte responses of blue mussels (*Mytilus edulis*) transferred from a contaminated site to a reference site: can the immune system recuperate? Aquacult Res. 36:926-971.

Medizhitov, R., Janeway, Jr., C.A., 2002. Decoding the patterns of self and nonself by the innate immune system. Science. 296:298-300.

Messick, G.A., Shields, J.D., 2000 Epizootiology of the parasitic dinoflagellate *Hematodinium* sp. in the American blue crab *Callinectes sapidus*. Dis Aquat Org. 43:139-152.

Michel, T., Reichhart, J-M., Hoffmann, J.A., Royet, J., 2001. *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglucans recognition protein. Nature 414:756-759.

Mitta, G., Vandenblucke, F., Hubert, F., Roch, P. 1999. Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. J. Cell Sci. 112:4233-4242.

Mosmann, T., 1983. Rapid colorometric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Met. 65, 55-63.

Muñoz-Chápuli, R., Carmona, R., Guadix, J.A., Macías, D., Pérez-Pomares, J.M., 2005. The origin of the endothelial cells: an evo-devo approach for the invertebrate/vertebrate transition of the circulatory system. Evol Dev. 7(4):351-358.

Olafsen, J. A., Mikkelsen, H.V., Glæver, H.M., Høvik Hansen, G., 1993. Indigenous Bacteria in Hemolymph and Tissues of Marine Bivalves at Low Temperatures. Appl. Environ. Microbiol. 59(6):1848-1854.

Pancer, Z., Rast, J.P., Davidson, E.H., 1999. Origins of immunity: transcription factors and homologues of effector genes of the vertebrate immune system expressed in sea urchin haemocytes. Immunogenetics. 49:773-786.

Pinsino, A., Thorndyke, M.C., Matranga, V. 2007. Coelomocytes and post-traumatic response in the common sea star, *Asterias rubens*. Cell Stress Chaperones. 12:331-341.

Pihl, L., Baden, S.P., Diaz, R.J., 1991. Effects of periodic hypoxia on distribution of demersal fish and crustaceans. Mar. Biol. 108, 349-360.

Pipe, R.K., 1990. Differential binding of lectins to haemocytes of the mussel *Mytilus edulis*. Cell Tissue Res. 261:261-268.

Pipe RK. 1992. Generation of reactive oxygen metabolites by the heamocytes of the mussel, *Mytilus edulis*. Dev Comp. Immunol.16:111-22.

Pipe, R.K., Farley, S.R., Coles, J.A., 1997. The separation and characterisation of haemocytes from the mussel *Mytilus edulis*. Cell Tissue Res. 289:537-545.

Raff, M., 1998. Cell suicide for beginners. Nature 396, 119-122.

Raffray, M., Cohen, G.M., 1997. Apoptosis and necrosis in toxicology: A continuum or distinct modes of cell death? Pharmacol. Ther. 75, 153-177.

Ra Londe, R. 2006. *Vibrio parahaemolyticus* in Alaska: An aggressive response to a potential crisis. J. Shellfish Res. 25(2):764

Ratcliffe, N.A. & Rowley, A.R., 1979. A comparative synopsis of the structure and function of the blood cells of insects and other invertebrates. Dev Comp Imm. 3:189.

Ratcliffe, N.A., Leonard, C.M., Rowley, A.F., 1984. Prophenoloxidase activation: non-self recognition and cell co-operation in insect immunity. Science. 226:557-559.

Rennert, J., Coffman, J. Mushegian, A., Robertson, A. 2003. The evolution of Runx genes I. A comparative study of sequences from phylogenetically diverse model organisms. Evol. Biol. 3(1): 4.

Rosenthal, R S., Dzarski, R., 1994. Isolation of peptidoglucans and soluble peptidoglucan fragments. Meth. Enzymol. 235:253-285.

Sahoo, P.K., Pillai, B.R., Mohanty, J., Kumari, J., Mohanty, S., Mishra, B.K. 2007. *In vivo* humoral and cellular reactions, and fate of injected bacteria *Aeromonas hydrophila* in fresh water prawn *Macrobrachium rosenbergii*. Fish Shellfish Immunol. 23:327-40.

Simkiss, K., Taylor, M.G., 1989. Metal fluxes across the membranes of aquatic organisms. Aquat. Sci. 1:173-188.

Sminia T., 1974. Haematopoiesis in the freshwater snail Lymnaea stagnalis studied by electron microscopy and autoradiography. Cell Tissue Res. 150(4):443-54.

Smith, V.J., 1981. The Echinoderms. In: N. A. Ratcliffe, A.F. Rowley (eds.): Invertebrate Blood Cells. Academic Press, London. 2:514-558.

Smith, V.J., Söderhäll, K., 1983. Induction of degranulation and lysis of haemocytes in the freshwater crayfish, Astacus astacus by components of the prophenoloxidase activating system in vitro. Cell Tissue Res. 233(2):295-303.

Smith, V.J., Crisholm, J.R., 2001. Antimicrobial proteins in crustaceans. Adv. Exp. Med. Biol. 484:95-112.

Steller, H., 1995. Mechanisms and genes of cellular suicide. Sci. 267**:**1445-1449.

Sterling, K.M., Mandal, P.K., Roggenbeck, B.A., Ahearn, S.E., Gerencser, G.A., Ahearn, G.A. 2007. Heavy metal detoxification in crustacean epithelial lysosomes: role of anions in the compartmentalization process. J. Exp. Biol. 210:3484-493.

Steiner, H., 2004. Peptidoglugan recognition proteins: on and off switches for innate immunity. Imm Rev. 198:83-96.

Stricker, S., Poustka, A.J., Wiecha, U., Stiege, A., Hecht, J., Panopoulou, G., Vilcinskas, A., Mundlos, S., Seitz, V. 2003. A single amphioxus and sea urchin runt-gene suggests that runt-gene duplications occurred in early chordate evolution. Dev. Comp. Imm. 27(8):673-684.

Söderhäll, I., E. Bangyeekhun, Mayo, S., Söderhäll, K. 2003. Hemocyte production and maturation in an invertebrate animal; proliferation and gene expression in hematopoietic stem cells of Pacifastacus leniusculus. Dev. Comp. Imm. 27(8):661-672.

Söderhäll, K., 1982. Prophenoloxidase activating system and melanization- a recognition mechanism of arthropods? A review. Dev Comp Immunol. 6:601-611.

Söderhäll, K., Aspán, A., Duvic, B., 1990. The proPO-system and associated proteins; role in cellular communication in arthropods. Res Immunol. 141(9):896-907.

Söderhäll K., Cerenius, L. 1992. Crustacean immunity. Annual rev. Fish Dis. 2:3-23.

Söderhäll K., Cerenius L. 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. Curr. Op. Immunol. 10:23-28.

Temara, A., Aboutboul, P., Warnau, M., Jangoux, M, Dubois, P. 1998 Uptake and fate of lead in the common Asteroid *Asterias rubens* (Echinodermata). Water Air Soil Pol. 102:201-08.

Tracey, W. D., Speck, N. A. 2000. Potential roles for RUNX1 and its orthologs in determining hematopoietic cell fate. Semin. Cell Dev. Biol. 11(5):337-342.

Trefry, J.H., Presley, B.J., Keeney-Kennicutt, W.L., Trocine, R.P., 1984. Distribution and chemistry of manganese, iron, and suspended particulates in Orca Basin. Geo-Marine Letters 4:125-130.

Verity, A.M., 1999. Manganese neurotoxicity: A mechanistic hypothesis. Neurotoxicol. 20:489-498.

Viarengo, A. 1985. Biochemical effects of trace metals. Mar Pol Bul. 16:153-158.

Wang, Y., J.D. Gu. 2005. Influence of temperature, salinity and pH on the growth of environmental Aeromonas and Vibrio species isolated from Mai Po and the inner Deep Bay Nature reserve Ramsar site of Hong Kong. J. Basic Microbiol. 45(1):83-93.

Weber, A.N., Tauszig-Delamasure, S., Hoffmann, J.A., Lelievre, E., Gascan, H., Ray, K.P., Morse, M.A., Imler, J.L., Gay, N.J., 2003. Binding of the *Drosophila* cytokine Spätzle to Toll is direct and establishes signaling. Nat. Immunol. 4:794-800.

Werner, T., Borge-Renberg, K., Mellroth, P., Steiner, H., Hultmark, D. 2003. Functional diversity of the *Drosophila* PGRP-LC gene cluste in response to lipopolysaccaride and peptidoglucan. J. Biol. Chem. 278:26319-26322.

Williams, H.R., Macey, B.M., Burnett, L.E., Burnett, K.G. 2009. Differential localization of bacteriostasis of *Vibrio campellii* among tissues of Eastern oyster, *Crassostrea virginica*. Dev. Comp. Immunol. 4:592-600.

Winston, G.W., M.N. Moore, M.A. Kirchin, and C. Soverchia. 1996. Production of reactive oxygen species by hemocytes from the marine mussel, *Mytlius edulis*: lysosomal localization and effect of xenobiotics. Comp. Biochem. Physiol. 113: 221-229.

Wootton, E.C., Dyrynda, E.A., and Ratcliff, N.A. 2003. Bivalve immunity: comparison between the marine mussel (*Mytilus edulis*), the edinle cockle (*Cerastoderma edule*) and razor-shell (*Ensis siliqua*). Fish. Shell. Imm. 15:195-210.

Wright, R.K., 1981. Urochordates: Invertebrate blood cells. Ratcliffe, N and Rowley, A. (Eds.) AP. London 2:565-626.

Wu, C., Söderhäll, I., Kim Y.A., Liu, H., Söderhäll, K. 2008. Hemocyte-lineage marker proteins in a crustacean, the freshwater crayfish, Pacifastacus leniusculus Proteomics. 8(20):4226-4235.

Wyllie, A.H., 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284, 555-556.

Yokoo, S., Goetz, P., Tojo, S., 1995. Phagocytic activities of haemocytes separated by two simple methods from larvae of two different lepidopteran species, *Agrotis segetum* and *Galleris mellonella*. Appl. Entomol.Zool. 30:343-350.

Zann, L., Brodie, J., Vuki V., 1990. History and dynamics of the crown-of-thorns starfis *Acanthaster planci* (L.) in the Suva area, Fiji. Coral Reefs. 9(3):135-144.

ZoBell, C.E., Feltham, C.B., 1938. Bacteria as food for certain marine invertebrates. J. Mar. Res. 1:312-327.

SVENSK SAMMANFATTNING

Eutrofiering, eller övergödning av näringsämnen, tillsammans med överfiske resulterar i att växternas primärproduktion ökar och det blir algblomningar. När de sedan bryts ned leder detta i sin tur till att mängden syre i vattnet minskar och följden blir att flertalet metaller löses ut från bottensediment och kan orsaka problem för levande organismer i dessa miljöer. Eutrofiering är ett ökande problem längs våra kuster.

Mangan (Mn) är en metall som är mycket riklig i mjuka havsbottnar, eftersom mangan förekommer i många av våra vanliga bergarter. Mangan är normalt bundet som MnO_2, men vid hypoxi, minskad syrehalt, reduceras MnO_2 till den biotillgängliga jonformen Mn^{2+}, som lätt tas upp av levande organismer. Hypoxi kan förekomma under kortare eller längre perioder, dagar till månader, men eftersom Mn^{2+} kan ta lång tid att återoxideras kan förhöjda mängder mangan finnas i bottenvattnet under en period även efter en tid av hypoxi. Mängden biotillgänglig mangan kan i extrema förhållanden öka upp till 1000 gånger så mycket som vid normal syretillförsel. Mangan i låga doser är en essentiell, livsnödvändig, metall som främst behövs under ämnesomsättningen för att kunna producera och aktivera vissa viktiga enzymer i både djur och växter. Förhöjd halt av mangan är däremot toxisk, giftig. Metallen stör då jonbalansen och kan interagera med kalcium, Ca^{2+}. Att mangan kan påverka det centrala nervsystemet har länge varit känt och ett symtom kallat manganism, som påminner om nervsjukdomen Parkinson, kan utvecklas.

De ryggradslösa djuren, evertebraterna, har inte samma typ av immunförsvar som ryggradsdjuren, vertebraterna, med specifika antikroppar och ett långtidsminne, men de har blodceller som påminner om människans vita blodkroppar. Dessa blodceller kommer från stamceller som finns i depåer i vissa vävnader. Där delar sig stamcellerna och genomgår en mognadsprocess som omvandlar dem till aktiva blodceller som används vid försvar mot angripande mikroorganismer, som bakterier till exempel. Deras immunförsvar är baserat på aktivitet i deras blodceller i blodet. Det finns flera typer av blodceller. Vissa har förmåga att fagocytera, sluka, inkräktande mikroorganismer och partiklar för att döda dem inne i cellen när andra blodceller kan frigöra giftiga ämnen som förgör angripande organismer utanför cellen. På grund av de öppna blodsystem som de flesta evertebrater har, har de behov av ett effektivt direkt försvar. Sårläkning och koagulering är viktiga processer för att minska blodförlust. Detta är generellt i alla evertebrater, däremot är det

skillnader mellan dem i hur de mobiliserar immunförsvaret. En rad fundamentala immunmekanismer har bevarats genom evolutionen, som till exempel blodcellernas roll, förmågan att känna igen främmande molekyler och att producera och frigöra cytotoxiska ämnen.

I den här avhandlingen har jag undersökt hur celldelningen och utmognaden av stamceller påverkas av mangan och om deras överlevnad påverkas i tre olika arter; havskräfta, blå mussla samt vanlig sjöstjärna. Vidare har jag undersökt om aktiveringen av blodcellernas påverkas och om det ger effekt på djurens förmåga att försvara sig mot sjukdomsalstrande bakterier som kan vara vanligt förekommande i kustnära vatten. Detta har jag gjort genom ett flertal experiment då jag i akvarier har utsatt djuren för sådana koncentrationer av mangan som man kan finna på våra havsbottnar och jämfört dem med kontrolldjur som inte exponerats för mangan. Jag har kunnat se att mangan kan starta en process som göra att de blodbildande stamcellerna dör genom så kallad programmerad celldöd, apoptosis. Det kan vara en förklaring till varför havskräftans blodceller minskar i antal då de utsätts för mangan. När sjöstjärnorna däremot utsattes för mangan blev resultatet det omvända. I dessa djur hade samma koncentration av metallen en stimulerande effekt som gjorde att stamcellerna delade sig mer och antal blodceller ökade. Emellertid kunde jag se att deras förmåga att fagocytera mikroorganismer minskade och de visade också tecken på stress. Detta kunde jag studera genom att analysera särskilda stressproteiner. Ökningen av antalet cirkulerande blodceller tycks kompensera för att de fagocyterar mindre och dessa djur behöll även sin förmåga att eliminera bakterier då dessa injicerades direkt i deras blod. Både kräftor och blåmusslor fick däremot svårare att avlägsna bakterier då de utsattes för mangan vilket tyder på att de blir mer känsliga för infektioner än vad sjöstjärnorna blir. Jag kunde även se att både kräfta och mussla ansamlade mer mangan i vävnaderna än vad sjöstjärnorna gjorde. Däremot återhämtade manganbehandlade djur sig i rent vatten efter att de hade varit utsatta för manganexponering. Under tre dagar renades de från nästan allt mangan och deras förmåga att eliminera bakterier återhämtades nästan till fullo.

I den här avhandlingen har jag kunnat se att mangan i koncentrationer som är realistiska att hitta längs våra kuster under perioder påverkar de ryggradslösa djurens immunförsvar på olika vis, men effekter har påträffats hos alla testade arter. Havskräftor och musslor påverkades mest och deras infektionskänslighet ökade medan sjöstjärnornas bakteriedödande förmåga förblev oförändrad. Trots att

mangans effekter inte tycks ge kvarstående påverkan, kan det innebära att under de perioder då mangan är tillgängligt har sjukdomsalstrande mikroorganismer en ökad chans att etablera infektioner vilket kan vara av stor betydelse för djurets överlevnad. Nyligen har det kommit rapporter om att syrebrist och därmed tillgängligt mangan har ökat i kustområden världen över vilket gör studien särskilt relevant.