Fish & Shellfish Immunology 27 (2009) 500–507

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

A comparative study on the influence of manganese on the bactericidal response of marine invertebrates

Carolina Oweson [a], Bodil Hernroth [b,c,*]

[a] Inst. of Marine Ecology, University of Gothenburg, Kristineberg 566, 450 34 Fiskebäckskil, Sweden
[b] The Royal Swedish Academy of Sciences, Kristineberg 566, 450 34 Fiskebäckskil, Sweden
[c] Dept. of Biomedicine, Kristianstad University, 291 88 Kristianstad, Sweden

ARTICLE INFO

Article history:
Received 24 March 2009
Received in revised form
4 July 2009
Accepted 7 July 2009
Available online 15 July 2009

Keywords:
Manganese
Immunotoxicology
Asterias rubens
Nephrops norvegicus
Mytilus edulis
Vibrio parahaemolyticus
Bactericidal
Haemocytopenia

ABSTRACT

Manganese, Mn, is a naturally abundant metal in marine sediments. During hypoxic conditions the metal converts into a bioavailable state and can reach levels that have been shown immunotoxic to the crustacean *Nephrops norvegicus*. For this species it has previously been shown that exposure to 15 mg L^{-1} of Mn decreased the number of circulating haemocytes while it for the echinoderm *Asterias rubens* increased the number of coelomocytes. Here, we compared if five days of exposure to the same concentration of Mn affects the bactericidal capacity of these two species and the mollusc *Mytilus edulis* when inoculated with the bacterium *Vibrio parahaemolyticus*. Viable counts of the bacteria were investigated at a time-course post-injection in the blood and the digestive glands of Mn-exposed and unexposed (controls) animals. Accumulation of Mn was also analyzed in these tissues. When exposed to Mn the haemocyte numbers were significantly reduced in *M. edulis* and it was shown that the bactericidal capacity was impaired in the mussels as well as in *N. norvegicus*. This was most obvious in the digestive glands. These two species also showed the highest accumulation of the metal. In *A. rubens* the bactericidal capacity was not affected and the metal concentration was similar to the exposure concentration. After a recovery period of three days the concentration of Mn was significantly reduced in all three species. However, in *M. edulis* and *N. norvegicus* it was still double that of *A. rubens* which could explain the remaining bactericidal suppression observed in *N. norvegicus*. This study pointed out that exposure to such Mn-levels that are realistic to find in nature could have effects on the whole organism level, in terms of susceptibility to infections. The effect seemed associated to the accumulated concentration of Mn which differed on species level.

1. Introduction

Manganese (Mn) is an essential trace metal, involved in metabolic processes in both animals and plants. It acts as a cofactor or activator of different enzymatic reactions, e.g. electron transfer reactions, anti-oxidative defence and phosphorylation [1]. On the other hand an excess of the ionic Mn is known as neurotoxin since it interferes with chemical synapse functions and is able to cross the blood–brain barrier affecting the central nervous system [2]. This metal is particularly abundant in the sediments of the soft bottoms of the oceans where it is generally enclosed in a four-valent colloid state, MnO_2. However, during hypoxic conditions often occurring during periods of days to several weeks [3–5] this state is converted into bioavailable ions, Mn^{2+}. In previous studies on the crustacean *Nephrops norvegicus* it was recognized that the number of haemocytes and their immune activity are suppressed [6,7] when the organism is exposed to such levels of Mn that are relevant to find during hypoxic conditions. Contrary, the number of coelomocytes of the echinoderm *Asterias rubens* has repeatedly been shown to increase [8] indicating that the bactericidal capacity of these two species may differ when the animals are exposed to Mn.

Invertebrates are lacking the adaptive immunity and are restricted to the innate immune system for the defence in which the blood cells are the key players. In aquatic invertebrates the open coelom or semi-open haemal circulatory systems continuously expose them to potential pathogens [9,10]. The mobilization of circulating coelomocytes or haemocytes, respectively [11,12] is thus necessary for an immediate action against invaders. The immune response is based on humoral and cellular actions which are proven exceptionally efficient in pathogen elimination as witnessed by the invertebrates' evolutionary success [13].

* Corresponding author at: The Royal Swedish Academy of Sciences, Kristineberg 566, 450 34 Fiskebäckskil, Sweden. Tel.: +46 523 18513; fax: +46 523 18502.
E-mail address: Bodil.Hernroth@marecol.gu.se (B. Hernroth).

1050-4648/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2009.07.001

Although the previous cellular investigations have revealed immune modulation in response to Mn the relevance on the organism level has not been explored. The aim of the present study was to investigate the influence of Mn on the capacity of clearance of bacteria, comparing species from three different marine invertebrate phyla, *A. rubens* representing the Deuterostomia (phylum Echinodermata; sub-phylum Asterozoa), *Mytilus edulis* (phylum Mollusca; sub-phylum Bivalvia) and *N. norvegicus* (phylum Arthropoda; sub-phylum Crustacea) belonging to the Protostomia. The Gram-negative bacterium *Vibrio parahaemolyticus* was used for the study since *Vibrios* have been reported as fish and invertebrate pathogens [14–17]. In addition, a recovery study was conducted to examine the persistence of the influence of Mn on the bactericidal capacity.

The immune defence strategies of these species share several common mechanisms but they also differ. For example the coelomocytes of echinoderms [18] and haemocytes of bivalves [19,20] are mainly based on phagocytic cells with a subsequent killing of bacteria in the phagolysosomes [21–23]. In contrast the arthropods show a higher activity of phenoloxidase [24] which is an intermediary product of their pre-dominating defence, the pro-phenol-activating system (Pro-PO-AS) [25–27]. The different immune strategies as well as detoxification mechanisms have certainly developed to cope with the surrounding threats and thus their susceptibility to environmental stressors may differ.

The accumulation and elimination kinetics of Mn in *N. norvegicus* have been extensively investigated [28–31] but similar studies on *A. rubens* [32] and *M. edulis* [33] are few. Here, we hypothesize that the bactericidal capacity in the blood and the digestive glands of these marine invertebrates is affected by the concentration of manganese accumulated in the tissues.

2. Material and methods

2.1. Animal handling and Mn exposure

The three studied species, the common sea star, *A. rubens,* the blue mussel, *M. edulis*, and the Norway lobster, *N. norvegicus*, were all collected (Sept–Nov 2008) either at the mouth of or right outside the Gullmar Fjord, adjacent to Sven Lovén Centre for Marine Science (former Kristineberg Marine Research Station), situated at the Swedish west coast (tidal amplitude 0.2 m). Animals were maintained in basins supplied with running seawater of stable temperature and salinity, 12 °C and 33 PSU, and were fed regularly until used for the experiments. Specimens of *A. rubens* and *M. edulis* were collected by scuba divers, *A. rubens* at 5–15 m depth and *M. edulis* at 1–2 m. *N. norvegicus* was 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 the most distant arm tip, *M. edulis* 5–7 cm length of shells and *N. norvegicus* 5–8 cm length of carapax. The groups were randomly chosen without considering sex.

During time of experiment the animals were kept in containers with seawater allowing sea stars 1.5 L, lobsters 50 L and mussels 0.5 L per individual, at 12 °C and 33 PSU. Equal numbers of animals were exposed to 15 mg Mn L^{-1} seawater, achieved by using manganese(II) chloride tetrahydrate (GR, Merck, Germany) and un-exposed in seawater without Mn additive (controls). The water, gently stirred for *A. rubens* and continuously aerated for *M. edulis* and *N. norvegicus*, was exchanged daily and the animals were not fed during the experiment. Plastic tubes were placed in the containers for lobster to allow them to hide. The animals were exposed to Mn for 5 days before being inoculated with bacteria and then continuously throughout the experiment. In order to study recovery of the bactericidal capacity animals where initially exposed to Mn for 5 days and thereafter the water was exchanged to seawater without Mn additive for 3 days before inoculating with bacteria.

2.2. Handling of bacteria and determination of appropriate doses

The bacterium, *V. parahaemolyticus*, used for studying the bactericidal capacity, was isolated from mussels, *M. edulis*, collected in June 2007, from the same area as the experimental animals. The bacteria were isolated by homogenizing mussel tissue in 0.1% peptone (pH 8.6), 1:1. The suspension was serially diluted in 25% NaCl (pH 8.5) and 100 μL of the dilutions were spread on Thiosulphate Citrate Bile Sucrose (TCBS; Oxoid Ltd. CM0333, Cambridge, UK) agar plates. These were incubated at 37 °C for 24 h and blue–green coloured colonies were isolated. The colonies were biochemically verified to belong to the species *V. parahaemolyticus* with the API 20NE® strip system (bioMérieux Inc., Hazelwood, MO). The bacteria were multiplied in 1% peptone with 25% NaCl (pH 8.5) at 37 °C for 3 h and aliquots were cryopreserved and stored at −80 °C, until used.

Prior to the experiment a pilot-study was performed to investigate the virulence of *V. parahaemolyticus* to the different species and to identify an appropriate sub-lethal dose to be used. When injecting *A. rubens* ($n = 15$) with10^6 bacteria per g of wet weight (wwt) it affected their ectoderm showing necrotic spots and several individuals died. The same dose inoculated to *N. norvegicus* ($n = 15$) highly affected their mobility and approximately 50% of the animals died. For *M. edulis* ($n = 15$) this dose was not lethal and 5×10^7 bacteria per g wwt were required to see any effects. At this dose they did not produce enough byssus-filament to attach to the surface of the basin and the adductor muscle was seemingly weakened as it was possible to produce a gap between the shells by hand. When injecting 10^5 bacteria per g wet weight (wwt) in *A. rubens* and *N. norvegicus*, respectively, and 5×10^6 bacteria per g wwt in *M. edulis* there was no mortality or other visual effects observed while these doses were considered sub-lethal and chosen for the experiment. In time for injection the bacteria were incubated over night in 37 °C on a shaking table in the peptone solution, to reach a pre-stationary phase. This growth phase of the injected bacteria was chosen because previous pilot studies at our lab have shown that it strongly limits the variability when recovering viable counts in the tissues of mussels and lobsters. The cultured bacteria were harvested through centrifugation and washed twice in phosphate buffer solution isotonic to seawater (PBS–NaCl; 2 mM KH_2PO_4, 10 mM Na_2HPO_4, 3 mM KCl, 600 mM NaCl, pH 7.4) before being diluted. Optical density (OD_{600nm}) of 1.0 corresponded to approximately 10^9 bacteria per mL according to the determination of colony forming units (CFU).

In order to spread the bacteria within the coelomic fluid/haemolymph of the animals the sea stars were injected in the cavity of three of their five arms, mussels in the posterior adductor muscle and lobsters were injected at three different places on the ventral side of abdomen.

2.3. Bactericidal response

Viable counts of *V. parahaemolyticus* were investigated in coelomic fluid from *A. rubens* and haemolymph from *M. edulis* and *N. norvegicus* (here called blood) as well as from the pyloric caeca of *A. rubens* and haepatopancreas of *M. edulis* and *N. norvegicus* (here called digestive glands). Samples were collected from both Mn-exposed and control animals after 8, 24 and 48 h ($n = 5$) post-injection (p.i.). Viable counts of *V. parahaemolyticus* were analyzed in an additional control group (not injected; $n = 5$) for each of the