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Interactive effects of metal contamination and pathogenic organisms on the introduced marine bivalve *Ruditapes philippinarum* in European populations

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Co-infection by opportunistic pathogens affects metal accumulation and some defense-related activities in the Manila clam Ruditapes philippinarum.

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ABSTRACT

In natural environment, marine organisms are concomitantly exposed to pollutants and multiple disease agents resulting in detrimental interactions. The present study evaluated interactive effects of metal contamination (cadmium) and pathogenic organisms (trematode parasites *Himasthla elongata* and pathogenic bacteria *Vibrio tapetis*) singularly and in combination on the bivalve *Ruditapes philippinarum*, an introduced species to Europe, under laboratory controlled conditions. After 7 days, metal bioaccumulation and pathogen load were analyzed as well as metallothionein (MT) response and hemocyte concentrations and activities. Results showed that infection by opportunistic pathogens affects metal accumulation, leading to maximal Cd accumulation in co-infected clams. Among stressors only *V. tapetis* induced significant effects on immune parameters whereas a particular interaction "trematode-bacteria" was shown on MT responses. Despite low trematode infection in agreement with the resistant status of *R. philippinarum* to these macroparasites, significant interaction with bacteria and metal occurred. Such results highlight the necessity of taking pathogens into account in ecotoxicological studies.

1. Introduction

Coastal marine organisms are at risk of exposure to a wide variety of both natural and anthropogenic stressors. Coastal zones are endangered by anthropogenic inputs of contaminants due to their extensive use in agricultural, chemical and industrial processes. Metals such as cadmium (Cd) have long been recognized as major pollutants of the marine environment, constituting a hazard to marine organisms (Cheung et al., 2003; Roesijadi, 1994).

There is growing concern of the environmental effects of metal pollution in terms of disease and disease susceptibility (Morley, 2010; Pipe and Coles, 1995). In many invertebrates, immunity is carried out by effector molecules as well as proliferation and activities of non-specific cells, the hemocytes. These components are contained in the hemolymph, an open circulatory system in direct contact with nervous and endocrine systems maintaining homeostasis in invertebrate (Pipe and Coles, 1995). This interdependence makes the immune processes particularly sensitive to environmental stressors, including metals. Pollutants could also directly affect pathogenic organisms and consequently their occurrence, distribution and virulence in a positive or negative way depending on an unknown number of interactive variables, e.g. host pollutant metabolism or parasite exposure (Sures, 2008). In contrast, infectious agents could interfere with the bioaccumulation of toxic compounds (Cross et al., 2003; Evans et al., 2001). Therefore, concomitant exposure to metal and pathogens may lead to different kinds of interactions and are more complex than a simple sum of both of them (Sures, 2008).

Exploited marine bivalves are often used as sentinel organisms in contaminant monitoring programs due to their ability to filter large quantities of water leading to the accumulation of contaminants from seawater and food (Beliaeff et al., 2002; Nicholson, 2003). During feeding, bivalves may ingest many species of bacteria, providing substantial carbon and nitrogen contribution (McHenery and Birkbeck, 1986). Some species of bacteria are pathogenic. Among those, species from the genus *Vibrio* are an important cause of diseases in cultured fish and shellfish (Jeffries, 1982; McGladdery, 1999). Numerous reports of Vibriosis and associated mass mortalities have been reported in the Manila clam *Ruditapes philippinarum* in Japan, Korea and the European Atlantic

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coastline (England, Ireland, Italy, Spain and Norway) (Paillard et al., 2008). The disease was caused by the Gram-negative, non-sporulating motile bacterium *Vibrio tapetis* (Paillard, 2004). In a context of stressful environmental condition, several studies demonstrated an increase of susceptibility to *Vibrio* spp. infections in different hosts in relation to environmental perturbation such as modifications of temperature or salinity (Paillard et al., 2004; Reid et al., 2003). Finally, co-infection with parasites could also increase the susceptibility of the host to *Vibrio* spp. infections (Rajkumar et al., 2007; Soudant et al., 2004). Thus, in the context of metal pollution, we could wonder if such portals of entry, locally increasing bacterial load, might interfere with metal accumulation in host tissues.

The aim of this work was thus to assess potential interactions between cadmium contamination, bacterial challenge (*V. tapetis*) and trematode parasite infestation (*Himasthla elongata*) on the clam *R. philippinarum* through a three factors experimental design. Previous studies showed that the introduced species Manila clam was resistant to trematodes (Dang et al., 2009). However, there are some reports of such infestation in the native area (Lee et al., 2001; Ngo and Choi, 2004) with high prevalence of infested clams (7–42.9%) (Park et al., 2008). Particularly, *H. elongata* is not encountered in the native area of *R. philippinarum* (Japan, Korea, Philippines), but is sympatric in this species in European ecosystems and is considered as an opportunistic trematode species with many potential bivalve second intermediate hosts (=euryxenic parasite) (Lauckner, 1983). The status of introduced species gives this study another dimension. Indeed, many of these species were deliberately introduced due to their competitive performance in terms of growth, survival and resistance. In a context of multiple stressors, particular sensitivity could be present in comparison to that observed in native species (Karatayev et al., 2009; Morley, 2008; Paul-Pont et al., 2010a).

Thus, it is relevant to assess whether the apparent resistance of Manila clams to native trematode remained the same in association with the highly pathogenic bacteria *V. tapetis* and/or metal exposure. Laboratory experiment was performed with these sources of stress applied in single, double and triple combinations during seven days. At the end of the experiment, Cd accumulation, bacterial load, parasite infestation and several physiological parameters were assessed. MT concentration was determined in gills and visceral mass in relation to its role in detoxification of toxic metals and its ability to respond to inflammatory processes (Gagné et al., 2007). Finally, analysis of hemocytes concentration and activities (phagocytosis, oxidative burst, viability, adherence capability) were performed in order to assess the modulation of immune function in relation to multiple environmental stressors.

2. Material and methods

2.1. Bivalve treatments

Manila Clams *Ruditapes philippinarum* (38.5 ± 0.6 mm shell length, mean $\pm$ SD) were collected in March 2007 at Andernos, Arcachon Bay, France (44°42'N, 1°8'W). The sampling site consists of an intertidal *Zostera noltii* seagrass bed that is under the influence of freshwater input (Leyre River) (Lassalle et al., 2007). Salinity ranges from 22 to 32 psu and temperature ranges from 1 °C in winter to 25°C in summer. The median grain size is 142 μm corresponding to fine sand (Lassalle et al., 2007).

The experimental procedure followed a three-factor design with three treatments (C: Cadmium; H: *Himasthla elongata*; V: *Vibrio tapetis*) and the four possible interactions (CH; CV; HV; CHV). Clams were acclimatized for three days prior to the beginning of the experiment. This experiment was conducted in laboratory controlled conditions using glass aquaria of $12 \times 12 \times 24$ cm filled with 1 L of synthetic seawater (Instant Ocean, salinity: 30.3 ± 0.2 psu, mean $\pm$ SD), aerated by a diffuser system. Plastic coverings were placed over the inside walls to avoid cadmium contamination and to minimize cadmium adsorption on walls. The temperature was fixed at 15 °C ($\pm$0.7 °C, mean $\pm$ SD), pH was regularly monitored and remained stable (8.1 ± 0.3, mean $\pm$ SD) and the photoperiod was fixed at 12 h using a timer and artificial light sources. No sand was added in order to minimize bacterial contamination as the bacteria may adsorb to the sediment surface and so may bias bacterial load calculation at the end of the experiment. Four clams were introduced into each experimental unit (EU) and three EU replicates were carried out for each experimental condition. Therefore a total of 24 EU were running simultaneously for a period of seven days.

2.2. Contamination protocol

Vibrio tapetis (CTC4600) was isolated from diseased *R. philippinarum* (Borrego et al., 1996). Bacterial contamination was achieved by adding a culture of *V. tapetis* to the water column to reach a concentration of 1×10^9 bacteria mL^{-1} at T_0. This recreation of natural conditions was selected rather than injection of the culture into the palleal cavity or directly into the muscle to minimize stress. In order to keep a relatively constant load of *V. tapetis*, concentration of 1×10^9 bacteria mL^{-1} was also adjusted after 3 days.

Himasthla elongata cercariae were collected from infected periwinkles (*Littorina littorea*). Infected snails were kept at 15 °C and fed with macro algae (*Ulva* spp.). For the experiment, periwinkles were placed at 20 °C under artificial light source. This condition induces cercariae release into the water from which batches of ten were immediatly transferred (cercariae age < 1 h) into the EU until each EU contained 100 cercariae. To maintain a constant pressure of infection, cercariae were added at T_0 and after 1, 3 and 4 days.

A single nominal concentration of Cd at 133 nM (corresponding to 15 μg Cd L^{-1} added as $CdCl_2$) was selected. After the first contamination (T_0), metal quantification in the water of the experimental units was performed everyday to ensure that metal concentration remained constant throughout the experimental period. Any decrease in metal concentration due to adsorption and absorption mechanisms was compensated for daily by the addition of aqueous Cd solution.

2.3. Sampling procedure

After seven days of exposure (T_7), one clam per EU was removed. Hemolymph was withdrawn from the adductor muscle of each individual and was observed under a stereomicroscope to ensure the quality of samples (no gamete, no dilution with seawater, no cell debris, etc…). Hemolymph was kept on ice until immunological analyses. Foot and mantle were dissected and squeezed between two sterilized glass slides under a stereomicroscope. Then, *H. elongata* metacercariae were rapidly counted. The remaining tissues were dissected to separate gills and visceral mass. A small section (>50 mg) of both organs was used for MT quantification and was placed into polyethylene bags (Whirl-Pak) under N_2 atmosphere at −80 °C to minimize MT oxidation. The rest of both organs were kept at −80 °C until cadmium quantification.

2.4. Metals and metallothionein quantification

Cadmium quantification was carried out in the water and the different organs of each clam. The Sampledwater was acidified at 2% with nitric acid (Fluka; Buchs, Switzerland. 65% HNO_3) before Cd analysis. Tissues were digested in 1–1.5 mL (depending on weight of tissue) of nitric acid at 100 °C for 3 h to dissolve metals in the liquid for quantification. After a six-fold dilution of digestates with ultrapure water (MilliQ®, Bedford, MA, USA), Cd concentrations were determined by electrothermic atomic absorption spectrophotometry with Zeeman correction, using a graphite furnace (M6 Solar AA spectrometer, Thermoptec). The detection limit was 0.1 μg Cd L^{-1}. Analytical methods were simultaneously validated for each sample series by the analysis of standard biological reference materials (Tort-2: Lobster hepatopancreas and Dolt-3: Dogfisher liver from National Research Council of Canada, Ottawa). Throughout our cadmium analyses, mean values of Tort-2 and Dolt-3 were 27.3 ± 0.4 μg g^{-1} and 18.9 ± 0.6 μg g^{-1}, respectively. These values were in certified ranges of Tort-2 and Dolt-3.

To quantify MT, an inorganic mercury saturation method followed by total mercury determination using flameless atomic absorption spectrometry (LECO AMA 254, ALTEC, Prague, Czech Republic) was used. This technique was first described by Baudrimont et al. (1997b). Inorganic mercury saturation relies on mercury's affinity to MT and its ability to displace other heavy metals which can also fix to MT. The amount of mercury detected in each sample is proportional to MT concentration. As the exact quantity of Hg-binding sites per MT molecule is unknown for *R. philippinarum*, MT concentration cannot be expressed in mol g^{-1} (wet weight, ww). MT concentrations were expressed as nmol Hg-binding sites per g (ww): [(ng Hg in sample)/(mL of supernatant)] × [(tissue dilution)/(Hg molar mass)]. The detection limit was estimated at 1 ng Hg.

Three reference samples or "blanks" were prepared to monitor the Hg complexation efficiency of the hemoglobin. The mean of the three blank values at each analytical run was deduced from the Hg burdens measured in each sample.

A recovery percentage from purified rabbit liver MT (Alexis biochemicals ALX-202-071) was systematically determined. This "internal standard" enabled us to determine the ratio between the binding sites measured after Hg saturation and the potential binding sites indicated by the supplier and previously verified by Cd and Zn determinations on purified MT solution samples. Throughout our MT analyses, the mean recovery percentage was 94.3 ± 2.8%. This value was consistently within the certified ranges (100 ± 20%) of the method.