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Cross-effects of nickel contamination and parasitism on zebra mussel physiology

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Abstract Aquatic organisms are exposed to pollution which may make them more susceptible to infections and diseases. The present investigation evaluated effects of nickel contamination and parasitism (ciliates *Ophryoglena* spp. and intracellular bacteria Rickettsiales-like organisms), alone and in combination, on biological responses of the zebra mussel *Dreissena polymorpha*, and also the infestation abilities of parasites, under laboratory controlled conditions. Results showed that after 48 h, more organisms were infected in nickel-exposed groups, which could be related to weakening of their immune system. Acting separately, nickel contamination and infections were already stressful conditions; however, their combined action caused stronger biological responses in zebra mussels. Our data, therefore, confirm that the parasitism in *D. polymorpha* represents a potential confounding factor in ecotoxicological studies that involve this bivalve.

Keywords *Dreissena polymorpha* · Parasites · Nickel · Cellular biomarkers · Immune defense

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Introduction

Environmental studies using biomarkers to evaluate molecular, biochemical and cellular effects induced by pollutants usually take into account some biotic and abiotic factors known to influence biological responses, e.g., the season, the food availability or the stage of development (Domouhtsidou and Dimitriadis 2001; Bochetti and Regoli 2006; Guerlet et al. 2007). Whereas parasitism has often been neglected in such studies before, recent field and laboratory investigations often report infections in vertebrates and invertebrates as confounding factors in ecotoxicological studies (Sures 2004; Marcogliese et al. 2005; Baudrimont et al. 2006; Morley et al. 2006; Minguez et al. 2009). Among alterations identified in physiological systems, chemically induced immunological disorders have been well documented in an increasing number of species (review in Auffret et al. 2006; Ellis et al. 2011). Such effects are particularly important as the immune system defends an organism against potential pathogens and parasites (Auffret et al. 2006). Pollutants may thus involve higher susceptibility to infection and associated diseases, and so may increase the probability to sample infected organisms (reviewed in Sures 2004; Morley 2010). However, the interaction between environmental contamination and parasite infection remains complex, since parasites can also be sensitive to pollution (Morley et al. 2003; Sures 2004). Bivalves have physiological and behavioral characteristics (e.g. sedentarity, filtration of large water volume, bioaccumulation of xenobiotics) that make them good sentinel organisms in water quality assessment (Kraak et al. 1991). The zebra mussel, *Dreissena polymorpha*, a widespread invasive species in Europe and North America, is an important bioindicator of freshwater environments reflecting the site specific pollution (Claudi and Mackie 1994;

McMahon 1996; Guerlet et al. 2007). Like all organisms, *D. polymorpha* can be infected by a variety of parasites. Over 45 species of endosymbionts have been reported to use *D. polymorpha* as intermediate or the only host in their life cycle (Molloy et al. 1997; Karatayev et al. 2002; Mastitsky 2004; Mastitsky and Gagarin 2004). Among these, we focused on two parasites common in our study area: (1) parasitic ciliates *Ophryoglena* spp. found within the digestive gland lumina and whose pathogenicity remains unknown and, (2) Rickettsiales-like organisms (RLOs), intracellular bacteria in digestive cells known to cause inflammatory reactions (Molloy et al. 1997, 2001, 2005). Our previous studies carried out under field conditions and related to the interactions between parasitism in *D. polymorpha* and freshwater pollution has shown that (1) infection is a source of data distortion modifying physiological responses of organisms also exposed to environmental pollution (Minguez et al. 2009), and (2) the presence of RLOs can be a sign of heavy metal pollution such as nickel (Minguez et al. 2011). Nickel is of high environmental importance and is considered as one of the priority metals in the European water framework directive (WFD). It is introduced into water systems both by human activities and natural ways (e.g. surface run-off by industrial and domestic waste discharge, or following natural erosion of soils and rocks). Values up to 27,200 µg/l have been reported in wastewater from Ni mining, smelting and refinery operations (Eisler 1998). Several authors reported a nickel-related depression of immune system both in vertebrates and invertebrates (Eisler 1998; Harkin et al. 2003; Vijayavel et al. 2009).

As field conditions allow neither control of environmental factors nor situations totally free of contamination, we conducted a study under controlled laboratory conditions. The aim of the present work was to assess the infestation ability of ciliates *Ophryoglena* spp. and RLOs during a short-time nickel exposure, and the cross-effect of nickel and/or parasitism on the biological responses of *D. polymorpha*. We hypothesized that the nickel-exposed mussels would be more susceptible to parasitism due to a weakened physiological status. To test this hypothesis, several immunological (i.e. hemocyte phagocytic capacity, the production of reactive oxygen species, and lysosome activity in hemocytes) and cellular biomarkers (i.e. the digestive lysosomal and peroxysomal systems, and the accumulation of neutral lipids and lipofuscin granules) commonly used in ecotoxicological studies (Giamberini and Cajaraville 2005; Auffret et al. 2006; Guerlet et al. 2007) were analysed depending on the exposure condition and infection status. The hemocytes of bivalve molluscs play a prominent role in the defence against potential pathogens. The immune response mainly involves phagocytosis and is complemented by an array of killing mechanisms, which may include lysosomal activity and generation of highly reactive oxygen metabolites (Anderson et al. 1995; Wootton and Pipe 2003). Four cellular parameters related to the general physiological status of *D. polymorpha* were assessed. (1) The lysosomal system involved both in normal functions, such as food uptake/digestion, and detoxification/excretion processes of pollutants. Herein, we looked for one particular type of lysosomal alteration, i.e. variation in their structural parameters (number and/or size) (Cajaraville et al. 1995). (2) The peroxisomal system, localized in the enzyme-containing organelles. It is mainly involved in the β-oxidation of fatty acids, but also participates in oxidative reactions that use dioxygen and generate reactive oxygen species that are then detoxified by catalase and other peroxisomal antioxidant enzymes (Cancio and Cajaraville 2000). (3) Neutral lipids, which represent an important energy source for bivalve molluscs (Calvaletto and Gardner 1999) and whose accumulation in tissues is often associated with lysosomal system damages (Moore 1988; Domouhtsidou and Dimitriadis 2001). (4) Lipofuscin granules, which appear to be the product of oxidative and non-oxidative catabolism of lipids, and may reveal cellular damages (Moore 1988).

Materials and methods

Animals and treatments

Specimens of *D. polymorpha*, ranging in length from 20 to 25 mm, were sampled in April 2010 in Commercy, a site having low trace-metal concentrations on the Meuse River (North-East of France) (Minguez et al. 2009). The bivalves were transferred into the laboratory and acclimatized during 48 h before the experiment in continuously aerated spring water (Ca^{2+}: 106; Mg^{2+}: 4.2; Na^{+}: 3.5; K^{+}: 1.5; HCO_3^{-}: 272; SO_4^{2-}: 50; Cl^{-}: 3.8; F^{-}: 0.9). After this period (i.e. t0), 80 individuals were chosen to assess the background parasitological parameters of the sampled population of *D. polymorpha* at the beginning of the experiment. The remaining zebra mussels were then randomly divided into three experimental groups of 150 individuals each, kept in separate aquaria. One was the control group and the other two were exposed to nickel concentration of 20 µg Ni/l (European legal limit of concentration in drinking water) (CD 98/83/EC 1998) or 500 µg Ni/l (French legal limit of concentration in industrial discharge) (Decree of 2nd February 1998 on the classified installations, Article 32). The chosen nickel concentrations although high were considered environmentally relevant, since in polluted environments such as areas of nickel mining, smelting, and effluents may lead to high concentrations of Ni. Both control and exposure

groups were kept under identical conditions, with 12 l of well-aerated spring water maintained at constant temperature (12°C) and photoperiod (16:8 light:dark ratio). After 24 h, 50 mussels from each group were examined for parasites. The exposure was stopped after 48 h, and both infection, immunological, and cellular parameters were assessed on 70 individuals from each group. The concentration of nickel was measured in the exposure water at each sampling time-point (i.e. t0, t24 and t48) to estimate the true concentration of exposure.

Hemolymph analysis

After 48 h of experiment, hemolymph was withdrawn from the adductor muscle sinus of each zebra mussel individual using 1 ml syringe with a 27G ¾ needle. Hemolymph from each mussel was transferred into an individual eppendorf tube held on ice to limit hemocyte aggregation for further flow cytometry analysis, and then diluted in anti-aggregant solution slightly modified from Auffret and Oubella (1994) to an osmolarity of 74 mOsmol. Three hemocyte parameters, i.e. phagocytosis, reactive oxygen production and lysosome presence, were determined using a flow cytometer (FACScalibur™, BD Biosciences). For phagocytosis, hemocytes were mixed with yellow-green latex beads (Fluosphere® Fluorescent Microspheres, Invitrogen), diameter 2 µm, at a ratio of 1:30 (hemocyte: beads). After 4 h of incubation at 18°C in the dark, the reaction was stopped on ice and the percentage of phagocytic cells was evaluated as the percentage of hemocytes that had engulfed at least three beads (Delaporte et al. 2003). ROS production by hemocytes was measured using 2′7′-dichlorofluorescein diacetate, DCFH-DA (final concentration = 0.01 mM) (Lambert et al. 2003). All tubes were incubated in the dark at 18°C for 2 h and then transferred to ice for 5 min to stop the reaction. Lysosome presence was analysed with a commercial kit (LysoTracker® Green DND-26, 1 mM in DMSO, Molecular Probes) which consists of a fluorophore linked to a weak base that is partially protonated at neutral pH. The LysoTracker® is freely permeant to cell membranes and typically concentrates in lysosomes. LysoTracker marker was added to hemocyte suspensions at final concentration of 75 nM. Samples were incubated 10 min in the dark at 18°C and then the reaction was stopped on ice (5 min). The oxidative activity and the presence of lysosomes correspond to the mean geometric fluorescence detected in the total haemocyte population and are expressed in arbitrary units (AU).

Parasite inventory

A part of digestive gland from each mussel was excised to measure biological responses by histochemistry, and prepared as described in Giambérini and Cajaraville (2005) (see below). The remaining tissues were fixed in Bouin's fixative to perform the parasite inventory. The protocol of this procedure can be found in Minguez et al. (2009). Briefly, after classical histological techniques, 30–40 sections per zebra mussel (5 µm) were studied microscopically for the presence of parasites. Two epidemiological parameters were assessed: the prevalence and the mean intensity of infection (Bush et al. 1997).

Histochemistry and stereology

The removed digestive glands were used to measure four biological responses: the structural changes of the lysosomal system, the peroxisomal catalase activity, and the accumulation of neutral lipids and lipofuscin granules. The digestive lysosomal system was located by the revelation of β-glucuronidase activity in unfixed cryostat sections according to Cajaraville et al. (1991) and following the adapted protocol for freshwater organisms by Giambérini and Cajaraville (2005). Unsaturated neutral lipids were demonstrated by oil red O staining (Moore 1988), and the lipofuscin granules were stained by the Schmorl reaction (Pearse 1972). The histochemical revelation of the peroxisomal catalase in unfixed cryostat sections was adapted from Cajaraville et al. (1993), as described in Guerlet et al. (2006). Cellular biomarkers were quantified on digestive tissue sections (8 µm) by image analysis (Cell*, Olympus) using a Sony DP 50 color video camera connected to an Olympus BX 41 microscope wit a 100× objective. Five fields of view were randomly selected and examined on one section per individual. Areas not belonging to the digestive gland tissues were discarded from the analysis. The only stereological parameter considered herein to simplify the analysis is the surface density of all biomarkers ($Sv_L = S_L/V_C$; $Sv_P = S_P/V_C$; $Sv_{NL} = S_{NL}/V_C$; $Sv_{LF} = S_{LF}/V_C$, where C = digestive cell cytoplasm, L = lysosomes, P = peroxisomes, NL = neutral lipids, LF = lipofuscin, S = surface, V = volume).

Data analysis

All the statistical analyses were performed using Statistica software version 7.1. (Statsoft, USA). Differences were considered significant at $p < 0.05$. The χ^2 test was used to evaluate differences in prevalence rates. Mean infection intensities and physiological responses were examined with the two-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc tests after testing for normality and homogeneity of the data. Thus, measurements of the intensity of infection and the biological responses were log-transformed to meet these assumptions. The correlation between biological responses was assessed with the Spearman correlation coefficient. To evaluate an integrated

impact of parasitism and nickel exposure, we applied the "Integrated Biomarker Response", which combines all the measured biomarker responses into one general "stress index" (IBR, Beliaeff and Burgeot 2002). Three types of IBRs were calculated: (1) a general index with all tested biomarkers except the lysosome activity in hemocytes to not over-represent its response in the final index value since it was correlated with all other biomarkers, (2) an IBR with only the three immunological parameters, and (3) an IBR with the four physiological markers measured in the digestive gland.

Results

Epidemiological parameters

The variations of epidemiological parameters during the experiment, i.e. the prevalence rates and the mean infection intensities of *Ophryoglena* spp. and RLOs, are shown in Fig. 1. The percentages of zebra mussels infected by ciliates and intracellular bacteria tended to continuously increase over the 2-day exposure period, with higher prevalence rates detected in mussels exposed to the highest Ni concentration. The mean infection intensity with *Ophryoglena* spp. increased by 24 h and then decreased by the end of the experiment (ANOVA, time effect, $p < 0.05$); however, no clear trends were observed for the mean infection intensity of RLOs. The exposure concentration and interaction with time did not significantly influence the prevalence rates and mean infection intensities of ciliates or bacteria (χ^2 and ANOVA, respectively, concentration effect, $p > 0.05$).

The following results concerning biological responses will consider only females (i.e. at least 5 organisms per group) since not enough males were found to be infected with parasites (random sampling).

Biological responses

Immunological parameters were modulated by both nickel exposure and parasitism (Table 1). The mussels infected with RLOs displayed higher levels of ROS in hemolymph than the non-infected congeners, whereas the ciliate infection tended to induce more ROS production only in zebra mussels exposed to 500 µg Ni/l. In contrast, nickel exposure alone tended to induce a lower ROS content, irrespective of the metal concentration (Table 2). No significant effect was observed for phagocytosis activity (Table 1). However, nickel tended to decrease the ability of hemocytes to phagocytose, and this was more pronounced in infected organisms (Table 2). Nickel exposure, infection, and their interaction affected the lysosome activities in hemocytes (Table 1), with a reduction of the number of cells marked with the lysotracker probe in all cases (Table 2).

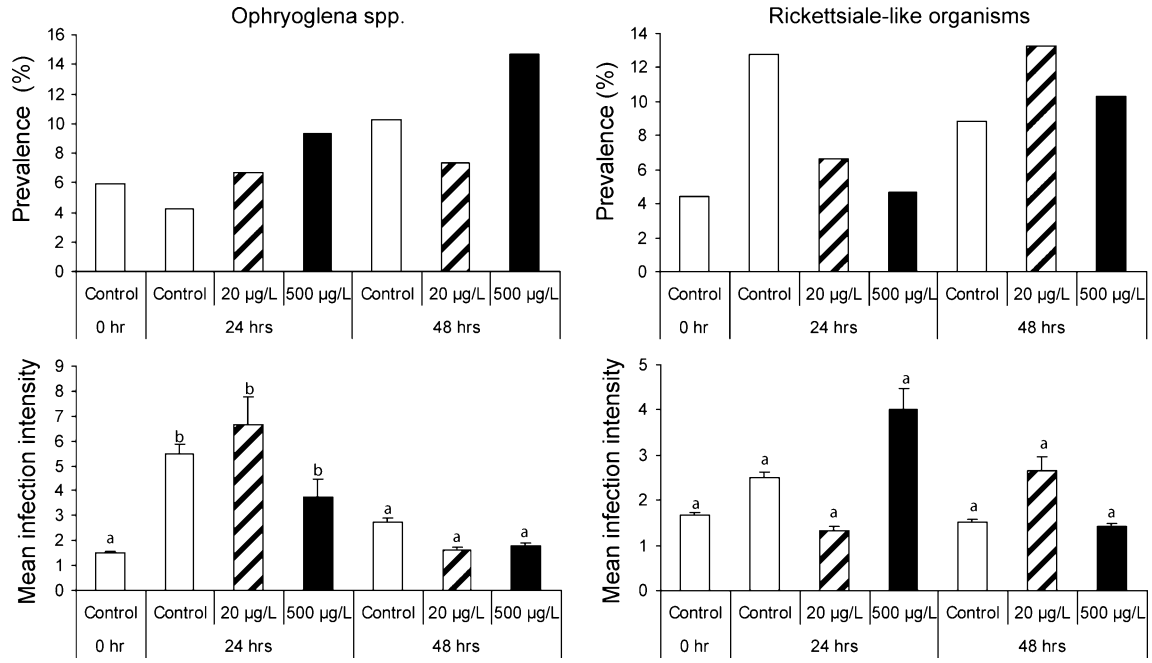

Fig. 1 Prevalence rates and the mean infection intensities (±SD) of ciliates *Ophryoglena* spp. and intracellular bacteria, Rickettsiales-like organisms, measured at three time points (0, 24 and 48 h of exposure) in three experimental groups (control, exposed to 20 or 500 µg Ni/l). *Different letters* indicate significant differences between groups

Table 1 Results of the two-way ANOVA comparing the effect of nickel exposure and parasite infection on biomarker responses measured in zebra mussels after 48 h of experiment

Variables	Source of variation	df	*F*	*p*
Immunological parameters				
ROS production	Ni exposure	2	21.367	**<0.001**
	Infection (*Parasite* sp.)	2	3.861	**<0.05**
	Ni exposure × Infection	4	2.064	0.104
Phagocytosis	Ni exposure	2	2.377	0.11
	Infection (*Parasite* sp.)	2	0.475	0.63
	Ni exposure × Infection	4	0.295	0.95
Lysosome	Ni exposure	2	35.776	**<0.001**
	Infection (*Parasite* sp.)	2	16.682	**<0.001**
	Ni exposure × Infection	4	3.986	**<0.01**
Cellular parameters				
Catalase	Ni exposure	2	3.67	**<0.05**
	Infection (*Parasite* sp.)	2	1.25	0.30
	Ni exposure × Infection	4	1.02	0.41
Lysosome	Ni exposure	2	11.43	**<0.001**
	Infection (*Parasite* sp.)	2	38.25	**<0.001**
	Ni exposure × Infection	4	3.77	**<0.05**
Neutral lipid	Ni exposure	2	1.85	0.17
	Infection (*Parasite* sp.)	2	57.24	**<0.001**
	Ni exposure × Infection	4	6.79	**<0.001**
Lipofuscin	Ni exposure	2	2.34	0.11
	Infection (*Parasite* sp.)	2	0.01	0.98
	Ni exposure × Infection	4	0.47	0.75

Bold values indicate significant *p*-values

Concerning the cellular parameters measured in the digestive gland, variations were observed depending on the nickel exposure and/or parasitism, except for the lipofuscin content which was not affected by any of these stressors (Table 1). The catalase activity seemed to be higher in organisms exposed to nickel compared with control ones, irrespective of the concentration. Parasitism tended also to stimulate the peroxisomal activity, particularly in mussels infected by RLOs (Table 2). Nickel exposure activated the digestive lysosomal system in non-infected organisms. Infected organisms displayed a more developed system, as revealed by a larger number of lysosomes. This was particularly true for the mussels parasitized by ciliates. The development of the digestive lysosomal system was higher in organisms exposed and infected, as indicated by the significant results of the two-way ANOVA (Table 2). Higher levels of neutral lipids were induced in organisms infected by *Ophryoglena* spp. and in mussels subjected both to RLOs and nickel (Table 2).

Integrated Biomarker Responses

The Integrated Biomarker Responses are shown in Fig. 2. In the first IBR (Fig. 2a), all the tested biomarkers were taken into account to synthesize the effects of exposure, infection, and both stressors together on the general physiological status of *D. polymorpha*. The two other IBR were calculated to closer examine the stress induced in the immune system (Fig. 2b) or in cellular responses measured in the digestive gland (Fig. 2c).

Results showed that the infection alone (control group) induced more stress (i.e. higher IBR values), and this was particularly true for the zebra mussels infected by RLOs (Fig. 2a). The exposure to nickel alone (non-infected organisms, white bars) also involved higher responses with increasing nickel concentration (Fig. 2a), visible in both the immune system and cellular responses, without dose-dependency (Fig. 2b, c). Combined together, the two stress factors had a synergic effect only at the highest Ni concentration of 500 µg Ni/l, due likely to strong immunological responses (Fig. 2b).

Discussion

The aim of the present 2-day experimental study was to assess the interactive effects of nickel exposure and parasitism on the physiology and defence-related activities (immunity, lysosomal and peroxisomal responses, and energy reserves) of the zebra mussel. Relatively few

Table 2 Immunological and cellular biomarker values in *D. polymorpha* (mean ± SD)

		ROS production AU	Phagocytosis %	H. Lysosome AU	Catalase 10^{-5} µm^2/µm^3	DG. Lysosome 10^{-3} µm^2/µm^3	Neutral Lipid 10^{-2} µm^2/µm^3	Lipofuscin 10^{-4} µm^2/µm^3
Control	Non-infected	139.66 ± 56.90	25.12 ± 4.33	55.64 ± 12.38^{a}	2.84 ± 0.67	0.76 ± 0.33	0.39 ± 0.23	5.52 ± 3.00
	Ophryoglena	121.51 ± 23.57^{a}	24.30 ± 3.44	50.90 ± 30.90^{a}	2.98 ± 1.60	1.79 ± 0.81^{a}	3.20 ± 0.01*	4.23 ± 0.43
	RLOs	171.09 ± 8.18 ^{a}	21.60 ± 6.18	44.92 ± 4.15^{oa}	4.06 ± 1.38	0.99 ± 0.33^{a}	0.62 ± 0.30^{a}	5.78 ± 2.48
20 µg/l	Non-infected	73.19 ± 17.34	21.55 ± 4.79	20.60 ± 2.62^{b}	4.27 ± 1.84	0.92 ± 0.27	0.84 ± 0.41	6.21 ± 1.92
	Ophryoglena	49.35 ± 6.24^{b}	19.58 ± 1.42	16.39 ± 4.06^{b}	2.46 ± 1.05	4.00 ± 1.17*^{b}	2.96 ± 0.16*	7.17 ± 3.00
	RLOs	71.07 ± 20.81^{b}	20.52 ± 6.18	23.37 ± 6.46^{b}	3.67 ± 1.93	1.38 ± 0.74^{ab}	0.55 ± 0.23^{a}	6.46 ± 1.42
500 µg/l	Non-infected	106.49 ± 35.80	19.67 ± 6.60	53.46 ± 9.71^{a}	4.35 ± 1.89	1.20 ± 0.39	0.33 ± 0.19	5.46 ± 1.27
	Ophryoglena	138.41 ± 32.34^{oa}	18.44 ± 8.11	24.76 ± 5.64*^{b}	4.75 ± 1.41	3.35 ± 0.52*^{b}	3.11 ± 0.28*	5.52 ± 0.24
	RLOs	181.56 ± 54.30*^{a}	19.18 ± 1.26	37.46 ± 10.10*^{a}	5.68 ± 0.10	2.16 ± 0.39*^{b}	2.28 ± 0.53*^{b}	5.09 ± 0.46

Results of the ANOVA: (1) significant differences between infected versus non-infected organisms in each exposure group were illustrated by * or ° (2) different small letters illustrated significant differences between organisms with the same infection status but exposed to different nickel concentration

* $p < 0.05$, ° $p < 0.10$

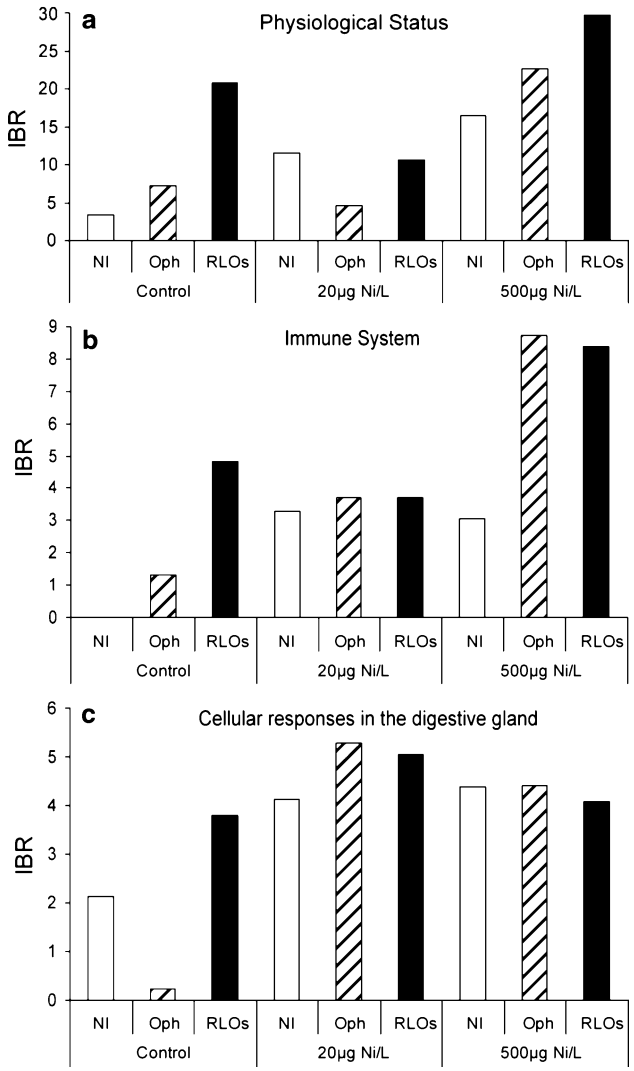

Fig. 2 Integrated Biomarker Response (IBR) of each infection status × exposure modality couple, with **a** immunological and cellular biomarkers (physiological status), **b** immunological markers only and **c** cellular markers. *NI* non-infected, *Oph* infected by *Ophryoglena* spp., *RLOs* infected by Rickettsiales-like organisms

investigations have examined the interaction between ecotoxicology and parasitology using freshwater organisms (Heinonen et al. 2001; Marcogliese et al. 2005; Minguez et al. 2009). Moreover, most studies in this framework focused on a single biological marker, such as the heat shock proteins or metallothioneins (Baudrimont et al. 2006; Sures and Radszuweit 2007). Only recently, studies conducted in multistress conditions have simultaneously analysed different biological responses (Paul-Pont et al. 2010a, b). The interest of our study was twofold: (1) to examine a set of biomarkers in a freshwater organism, *Dreissena polymorpha*, widely used in ecotoxicological studies, and (2) to evaluate the effect of microparasites (i.e. ciliates and bacteria) on physiological responses in mussels exposed to nickel pollution. Thus far, most investigations have

focused on macroparasites, e.g. trematodes (reviewed in Sures 2004; Marcogliese and Pietrock 2011).

From an initial prevalence rate of 6%, the number of zebra mussels infected by *Ophryoglena* spp. increased throughout the experiment in all the exposure groups. This was associated with an increase of the mean infection intensity after 24 h and a decrease after 48 h, while the average ciliate abundance was constant. These results suggest a rapid and direct infection of non-infected zebra mussels from the infected individuals during the first 24 h of the experiment. The increase in prevalence of *Ophryoglena* infection after 48 h has already been observed by us in another laboratory experiment with zebra mussels kept in tanks (unpublished data). Karatayev et al. (2002) have also reported *Ophryoglena* trans-infections between individual *D. polymorpha* from two different populations; however, in their experiment such transinfections were much slower, ca. 8 days. Little information exists on the life cycle of *Ophryoglena* spp. infecting zebra mussels. Further studies are necessary to learn the biology of these ciliates and better understand this host-parasite system. Concerning the RLOs, epidemiological parameters tended to follow the same patterns as those of ciliates, with an increase of prevalence rates and a rise followed by a decrease of the mean intensities over the 48-h experiment. Our study is the first to report such variations in epidemiological parameters of RLOs in a short-time experiment; the mechanisms of infection, however, remain unclear. It should be noted that the prevalence rates of both parasites were higher at the highest nickel concentration, suggesting a weakening of zebra mussels due to the contamination to such a point when the infection could have been promoted.

Among the first biological compartments coming into play in the fight against stressors, the immune system provides an important line of defence (Nott 1993; Anderson et al. 1995). In bivalve molluscs, it is based on innate mechanisms where cellular and humoral processes together ensure cytotoxic and antimicrobial functions (Auffret et al. 2006). Contaminants like heavy metals are well known to impair immune response of organisms and thus to interfere with the host susceptibility to infections and associated diseases (Pipe and Coles 1995). Herein, nickel at sublethal concentrations seemed to affect all the tested immunological responses. The phagocytosis by hemocytes of *D. polymorpha* tended to decrease with exposure concentration. The number of active lysosomes was also lower in exposed organisms. These observations seem to be consistent with an immunosuppressive activity of nickel (Eisler 1998), and could explain the higher prevalence of infection observed in zebra mussels exposed to 500 µg Ni/l. Vijayavel et al. (2009) have observed a significant decrease of phagocytosis in the mud crab *Scylla serrata* exposed to sublethal concentrations of nickel. Phagocytosis is dependent on cell membrane functioning and thus any metal-induced disruption of the cell membrane stability may significantly impair this process (Vijayavel et al. 2009). In our study, the decrease in lysosomes in hemocytes could be directly related to instability of membranes and/or the cell death caused by nickel exposure, since the lysotracker probe labels only lysosomes in live cells. The impairment of phagocytosis activity is the most widely observed immune response in organisms exposed to metals and/or hydrocarbons (reviewed in Auffret et al. 2006). However, immunological effects of pollutants strongly depend on both the nature and concentration of contaminants (Cajaraville et al. 1996).

Parasites may be treated by the mussels in the same way as foreign particles, and therefore parasite infestations might also involve special biological responses, such as hemocytic microbicidal activity (Anderson et al. 1995; Svärdh and Johannesson 2002). Our study showed that infection, and particularly the infection with intracellular RLOs, induced a decrease of phagocytosis ability and lysosome activity, as well as an increase of ROS production. These intracellular bacteria seemed to be able to modulate defence mechanisms of their host, such as the lysosomal activity in hemocytes. In vertebrates, some bacteria of the Rickettsiales order have the ability to suppress the innate host immune response in order to avoid inflammatory reaction and early elimination during the infection (Zhang et al. 2004; Walker 2007). This respiratory burst would be related to immune defences, with a major role in microbicidal activity (Vijayavel et al. 2009). However, the oxidative stress seemed not to be sufficient to cause visible cellular damages. In our experiment, no significant differences were observed in the lipofuscin contents. It appears that the antioxidant systems, such as the catalase activity, would not be over-exceeded.

Concerning the digestive cellular biomarkers, the exposure to nickel was found to cause the development of the digestive lysosomal system with more numerous lysosomes. Metals are known to activate this system through an increased exocytosis of enlarged lysosomes associated with the synthesis of new ones (Marigómez et al. 1996; Guerlet et al. 2006). No variations in the neutral lipid contents induced by nickel exposure were observed in our experiment. This result was in agreement with previous work showing that neutral lipids mainly respond to organic pollutions (Marigómez and Baybay-Villacorta 2003). In the present study, infections in *D. polymorpha* were also associated with an activation of the lysosomal system. The lysosomes were particularly larger and more numerous in the mussels parasitized by *Ophryoglena* spp. Moreover, this infection seems to enhance the effect of nickel exposure on the digestive lysosomal system: the parasitized zebra mussels exposed to nickel displayed a better developed

lysosomal system compared to the non-infected congeners. This kind of lysosomal response has also been observed in the field and laboratory studies where mussels from polluted sites or exposed to contaminants displayed an activation of the digestive lysosomal system compared to their congeners in control treatments. Activation of the lysosomal system is considered as a general stress response in bivalves (Marigómez et al. 1996). In addition, we found zebra mussels parasitized by *Ophryoglena* spp. to display higher levels of neutral lipids. Several authors have observed such an increased lysosomal neutral lipid accumulation in mussels collected from polluted sites, and have attributed it to lysosomal autophagy (Moore 1988; Krishnakumar et al. 1994). Another interesting result of our study was that the infection by RLOs seemed to trigger even higher lipid content than the nickel exposure did. In line with Minguez et al. (2009), our results thus suggest that the RLOs and *Ophryoglena* spp. infections can be confounding factors in ecotoxicological studies that use *D. polymorpha* as sentinel organisms.

The use of an integrative index combining several biomarkers has been reported from a number of ecotoxicological studies that involved fishes and mussels as sentinels. In such studies, the differences in biological responses are summarized in the form of a multivariate dataset (Beliaeff and Burgeot 2002; Broeg and Lehtonen 2006; Damiens et al. 2007; Pytharopoulou et al. 2008). As biomarkers respond to different stressors, this kind of index brings an advantage to assess the condition of target species. Herein, we used the integrated biomarker response index to combine the immunological and cellular markers (i.e. Total IBR). Two other IBRs were also calculated, including an 'immunological' one with the three responses measured in hemocytes (i.e. ROS production, phagocytosis, lysosomes activity), and a 'cellular' index with the four biological responses quantified in the digestive gland (i.e. catalase activity, lysosomal system, neutral lipid, and lipofuscin contents). The values of the total IBR were strongly related to immunological parameters, particularly to the ROS production. Bocquené et al. (2004) classified IBR values into four classes and suggested that the values higher than 9 indicated greater effects. Our results indicate that the exposure to sublethal nickel concentrations involved certain stress in zebra mussels, seen in both the immune system and digestive cellular responses. The infection with RLOs and, to a lesser extent with *Ophryoglena* spp., seemed to induce stressful conditions for the host. In groups exposed to the lowest nickel concentration, the digestive compartment responded more strongly, with IBR values being higher than 4 (correspond to the 3rd class of Bocquené et al. 2004). In the zebra mussels exposed to the highest concentration of nickel, the IBR reached values well above 9, indicating a clear effect of nickel. Situations where infected zebra mussels were exposed to nickel presented even more stressful conditions. Beliaeff and Burgeot (2002) reported that the application of IBR in stress assessments depended on a relevant choice of biomarkers in relation to the specific objectives of research or monitoring. Our investigation aimed to determine if zebra mussels exposed to nickel were immunodepressed and, if so, more susceptible to infections. The 'immunological' IBR would be sufficient to illustrate the stressful conditions of organisms. However, Damiens et al. (2007) highlighted the importance of the number of biomarkers included in the calculation of IBR. Indeed, when a set of biomarkers is relatively large (e.g. over 6), the weight of an individual factor is markedly reduced compared to the cases with less biomarkers. Even though each of the IBRs used in our study delivered self-sufficient pieces of interesting information, the one that best illustrated the physiological stress associated with exposure to nickel and/or parasite infection was likely the IBR calculated on the basis of all biomarkers pooled together (i.e. immunological and cellular responses).

Conclusion

The specific goal of the present study was to jointly assess the infestation ability of RLOs and *Ophryoglena* spp. and the potential interactions between nickel contamination and parasitism on immunological and cellular responses of zebra mussels during a short-time experiment. This kind of study under controlled conditions are needed in order to update the interactions between pollutants, parasites and hosts, difficult to identify on the field because of the presence of a multitude of parameters coming into play. Our results indicate that the infection with microparasites is likely to be promoted due to a weakening of the immune system in zebra mussels exposed to nickel, and that a 48-h period is enough for this effect to manifest itself. Parasitism by definition affects physiological conditions of the host, and we have clearly observed it in the control group; however, in the presence of pollution, infected organisms are more likely to be stressed since they have to defend themselves against both stressors. The interaction between nickel and parasite infection is supposedly much more complex than the direct single effects of pollution and infection alone. In our study, these two factors had a significant synergic effect on most of the tested physiological responses. This effect, however, depended on the parasite species, with RLOs being more harmful than *Ophryoglena* spp.

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