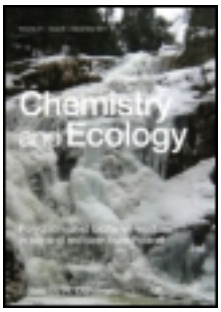

Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gche20

Bioconcentration of Cd and Ni in various tissues of two marine bivalves living in different habitats and exposed to heavily polluted seawater

Olga Chalkiadaki[ab], Manos Dassenakis[b] & Nikos Lydakis-Simantiris[a]
[a] Laboratory of Environmental Chemistry and of Biochemical Processes, Department of Environmental and Natural Resources Engineering, Technological Educational Institute of Crete, PO BOX 89, Chania, Crete 73135, Greece
[b] Laboratory of Environmental Chemistry, Faculty of Chemistry, National and Kapodistrian University of Athens, Panepistimioupoli Zografou, Athens 15771, Greece
Published online: 21 May 2014.

To cite this article: Olga Chalkiadaki, Manos Dassenakis & Nikos Lydakis-Simantiris (2014): Bioconcentration of Cd and Ni in various tissues of two marine bivalves living in different habitats and exposed to heavily polluted seawater, Chemistry and Ecology, DOI: 10.1080/02757540.2014.917172

To link to this article: http://dx.doi.org/10.1080/02757540.2014.917172

Chemistry and Ecology, 2014
http://dx.doi.org/10.1080/02757540.2014.917172

Taylor & Francis
Taylor & Francis Group

Bioconcentration of Cd and Ni in various tissues of two marine bivalves living in different habitats and exposed to heavily polluted seawater

Olga Chalkiadaki[a,b], Manos Dassenakis[b] and Nikos Lydakis-Simantiris[a]*

[a]*Laboratory of Environmental Chemistry and of Biochemical Processes, Department of Environmental and Natural Resources Engineering, Technological Educational Institute of Crete, PO BOX 89, Chania, Crete 73135, Greece;* [b]*Laboratory of Environmental Chemistry, Faculty of Chemistry, National and Kapodistrian University of Athens, Panepistimioupoli Zografou, Athens 15771, Greece*

(*Received 26 August 2013; final version received 18 March 2014*)

Two marine bivalves, *Mytilus galloprovincialis* and *Callista chione*, were exposed to various concentrations of cadmium and nickel (0.5, 1.0, 2.5 and 20 ppm), for 20 days, plus 10 days' depuration period, in a laboratory experiment. Animals from each experimental condition were dissected and the bioaccumulation and distribution of Cd and Ni were determined in their gills, mantles and remaining bodies. The concentrations of Fe and Zn were also measured. Heavy metal tolerance, bioconcentration and distribution of heavy metals in tissues were considerably different in *M. galloprovincialis* and *C. chione*: (i) both animals were tolerant to Ni pollution, even at the highest concentration used; (ii) *C. chione* was more tolerant to Cd; (iii) *M. galloprovincialis* was a better Cd and Ni accumulator, with the exception of the highest Cd concentration tested, where *C. chione* accumulated more Cd; (iv) Fe and Zn levels were much more affected in *M. galloprovincialis*; (v) in general, accumulation and distribution of Ni and Cd in the tissues were metal-, species- and time of exposure- dependent; (vi) significant amounts of heavy metals remained in the tissues after 10 days' depuration. Our results support a hypothesis for a two competing processes mechanism for metal accumulation and detoxification.

Keywords: *Mytilus galloprovincialis*; *Callista chione*; cadmium; nickel; marine pollution; bioaccumulation

1. Introduction

Trace metals exist naturally in the earth's crust. Some of them (e.g. copper, zinc, iron), are essential for biological systems, as they participate in numerous biochemical enzymatic processes and have important structural and functional roles on metal-related biological molecules, e.g. metallothioneins.[1–4] On the other hand, trace metals such as cadmium, lead, or mercury are generally toxic for living organisms, even at low concentrations in the environment.[5,6] The interactions of the trace metals with various organisms are very complex. In many cases, these interactions lead to the accumulation of toxic trace metals in different parts of the organisms with unpredicted results on their health. Trace metals that are introduced and biomagnified through the food chain may affect human health.[7–10]

*Corresponding author. Email: lydakis@chania.teicrete.gr

Trace metal pollution is one of the most important environmental problems in areas affected by intense anthropogenic activities.[11–13] The scientific research on this topic is of high priority as well as the establishment of effective monitoring networks that shall provide the information needed for minimising this environmental threat. Regarding aqueous environments, the use of bioindicators and biomarkers has recently become very wide as it can provide information about the ecotoxicological activity of pollutants.[9,14–21]

In this work, the accumulation of Cd and Ni in different parts of *Mytilus galloprovincialis* and *Callista chione* is studied in laboratory conditions. Cadmium is considered as a toxic metal [22,23] even at low concentrations (although it can be useful for some diatoms [24,25]) and its accumulation in several bioindicators has been studied. Nickel is an essential micronutrient for organisms, which, however, can be converted to toxic at high concentrations.[26–29] Relatively few studies exist about Ni accumulation and its impact in aquatic organisms.

In this study we compare the behaviour of two bivalves *(Mytilus galloprovincialis and Callista chione)* living in different habitats, in seawater containing Cd and Ni in concentrations higher than those usually measured in polluted environments. The purpose of the use very high concentrations of pollutants was on one hand the study of the behaviour of these organisms when exposed to very high levels of contamination (which may be caused e.g. by an industrial accident) and on the other hand to examine the possibility of the existence of a biochemical 'record' in these organisms showing a short but intense pollution of a coastal area. The tolerance of the two species under examination in several levels of Cd and Ni pollution, as well as the accumulation of the metals in gills, mantle and the remaining body, and the depuration of the organisms after they return in a heavy metal-free environment were investigated. We also studied the effects of the exposure of bivalves to Cd and Ni on the behaviour, variations and distribution of other, biologically related metals (Fe and Zn) in order to examine possible synergistic or antagonistic phenomena.

The Mediterranean mussel *Mytilus galloprovincialis*, has been widely used as bioindicator for trace metals contamination of coastal areas. It has been studied in natural and in laboratory conditions for its behaviour in trace metal-contaminated environments, but there is not enough information on the distribution and the biochemical role of metals in its various parts.[13,16,17,22,23,27,30–38] Other mollusks have also been studied for their capacity to accumulate heavy metals.[4–6,9,15,39–43] However, much more work needs to be done towards this direction, as it has been shown that the accumulation of heavy metals in different organisms is metal- and organism-dependent,[32,44,45] and there are many aquatic organisms, some of them with high commercial value, with almost unknown behaviour in heavy metal polluted environments. One of the less studied bivalves, very common in the dietary tradition of many Mediterranean countries, is *Callista chione*, a clam which leaves in the coastal mud. This work adds knowledge on the behaviour of this commercially important animal in heavy metal polluted environments.

2. Materials and methods

Mussels (*Mytilus galloprovincialis*) were collected from an aquaculture in Elefsis bay, SE from the city of Athens. The culture was 7–8 m under sea surface. Smooth clams (*Callista chione*) were hand-collected by scuba divers from populations (about 10 m under sea surface) inhabiting in the coastline of Aegina island, in Saronikos gulf, Greece. Both species were brought alive in the laboratory, where they were put in 70 L aquaria. The aquaria were filled with natural seawater collected from an unpolluted area with 36‰ ± 1‰ salinity. Air was continuously pumped in the seawater. *Callista chione* were placed in a sand layer at the bottom of the aquaria. Before use, the sand was washed thoroughly with deionised water and kept in deionised water overnight. Finally, it was washed three times with deionised water. Care was taken to use animals with the same shell

length (6–8 cm), as size is related with the age of the organisms. The age of the animals used in this study was estimated 12–15 months for *M. galloprovincialis* and about 2 years for *C. chione*. Animals were not collected during spawning periods as the organisms' nutrition (filtering rate) is altered at that period of time and the metal uptake rate is affected.[46,47]

Immediately after their arrival in the laboratory, 10 animals were dissected on ice, and the gills and mantles were removed from the remaining bodies. The three separated tissues were washed thrice in deionised water and kept at −24°C until use. The metal levels in these tissues are designated as 'Day 0'.

The organisms were acclimated to the laboratory conditions in constantly aerated seawater for five days and then were transferred to 70 L aquaria (2.5 animals/L seawater for *M. galloprovincialis* and 1 animal/L seawater for *C. chione*) and were exposed to 0.5 mg/L, 1.0 mg/L, 2.5 mg/L and 20.0 mg/L Cd or Ni for 20 days. These amounts correspond to 4.4, 8.9, 22.2 and 177.9 μmol Cd/L and to 8.5, 17.0, 42.6, 340.7 μmol Ni/L, respectively. Half of the water volume was replaced by fresh seawater every second or third day, depending on the number of animals in the aquaria. Fresh seawater was the only food source for the animals throughout the experiment. Temperature was kept at 17–18°C. After the five-day acclimation period, the corresponding trace metal concentration was added to each of the aquaria. After five days of exposure, 30 animals from each experimental condition were removed. They were divided to three groups of 10 animals for preparation of three composite samples ('Day 5'). The animals were dissected on ice and the gills, mantle and the remaining body were separated. The tissues were washed quickly in deionised water and stored at −24°C until heavy metal analysis. This procedure was repeated at the 10th, 15th, and 20th day of the experiment. After the 20th day, the remaining animals were transferred to fresh, heavy metal-free seawater for a 10-day depuration experiment. Seawater in the aquaria was replaced with fresh every two days. These animals were then dissected as described above. Control animals were kept in metal-free seawater throughout the experiment. The aquaria were inspected very frequently (several times a day) for dead animals, which were removed immediately, and if they were still intact they were dissected as described above for later analysis. After removing dead organisms, the seawater in the aquarium was immediately replaced by fresh (with the addition of the appropriate amount of the heavy metal stock solution).

Tissue samples used for the determination of metals concentrations were lyophilised (Lab-Congo, Freezone 4.5 lyophiliser) and homogenised to fine powder. 0.3–0.5 g of the dry tissues were digested overnight with 7 mL concentrated HNO_3 in closed PTFE beakers on a hot plate at 80°C. The digests were diluted to 25 mL and stored in polyethylene bottles at 4°C until analysis. Control samples, blanks and reference materials (Quasimeme QTM 044 BT, *Mytilus edulis* and IAEA 436, tuna fish) were also digested and measured as described above.

For the determination of the background metal levels, heavy metal analysis was performed on the natural seawater used in the aquaria and on the sand used in the aquaria of *Callista chione.* Seawater samples were filtered through 0.45 μm, Millipore filters in order to collect particulate heavy metals. The filters were treated overnight with concentrated HNO_3 in closed PTFE beakers on a hot plate.[48] Dissolved trace metals were pre-concentrated on Chelex-100 resin columns [49] and eluted by 2N HNO_3. The sand samples were frozen, lyophilised, homogenised and sieved through 1 mm sieve. The total cadmium and nickel content of the sediments was determined after acid digestion with concentrated acids (HNO_3 – $HClO_4$ – HF) in Teflon beakers according to ISO 14869-1:2000 method.[50]

Concentrations of Cd, Ni, Zn and Fe in tissue digests and of Cd and Ni in seawater samples and the sediments were determined by Atomic Absorption Spectrometry. Flame atomisation (FAAS, Varian SpectraAA 200) was used for Cd, Zn and Fe, whereas electrothermic atomisation (Graphite Furnace Atomic Absorption Spectrometry with background correction based on the Zeeman Effect, Varian SpecraAA-640Z GTA 100) was used for Ni determination. Three measurements were carried out on each sample. Differences more than 10% between values were

not acceptable and in these cases atomic absorption measurements were repeated. The relative standard deviations of all the triplicate analyses ranged between 5 and 25%.

The detection limits (LOD) of the atomic absorption methods using the above mentioned equipment for Cd were 0.01, 0.001 and 4 ppb for dissolved, particulate and sand samples respectively. For Ni the detection limits were 0.03 ppb for dissolved and particulate samples whereas for sand samples the LOD was 280 ppb. The quantification limits (LOQ) for both metals were approximately three times higher than the detection limits.

The accuracy of the procedures was tested by certified reference materials, Quasimeme QTM 044 BT (*Mytilus edulis*), IAEA 436 (tuna fish) and PACS-2 sediment. The recovery for all metals was between 97% and 102% with very low standard deviation values.

3. Results and discussion

Seawater samples from the areas of *Mytilus galloprovincialis* cultures and *Callista chione* harvesting sites were analysed for their heavy metal content. Similar analyses were carried out for the seawater used for the experiments presented in this paper. The background concentration for dissolved cadmium varied between 0.01 and 0.06 μg/L, whereas for dissolved nickel it varied between 0.27 and 0.88 μg/L, far below the USEPA limits for chronic toxicity to aquatic life in saltwater (8.8 μg/L and 8.2 μg/L for Cd and Ni, respectively,[51]). Particulate cadmium and nickel were below detection limits for all the sampling stations. The values measured in seawater samples for both metals were in agreement with corresponding measurements from these sites, performed on a regular basis in our laboratory.[52]

The Cd concentrations found in the digested sand samples were below detection limits, whereas Ni concentrations were very low (data not shown). According to USEPA and the criteria set for sediment concentrations of metals, Cd and Ni concentrations in sediments used in *Calllista chione* aquaria were in the category of the non-polluted (below detection limit for cadmium and <20 mg/kg for nickel).[53]

3.1. *Heavy metal accumulation in the tissues of* M. galloprovincialis *and* C. chione

Table 1 contains the Cd and Ni concentrations in the three tissues dissected from animals immediately after their arrival in the laboratory. These values are represented as 'Day 0' in Figures 1–3. The Cd and Ni values measured in the tissues of both animals the Day 0 are low. The background nickel concentration in *C. chione* is higher than the corresponding concentration in *M. galloprovincialis* (mainly in the mantle and the body). The Cd and Ni concentrations in the three tissues of *M. galloprovincialis* and *C. chione* for all the examined conditions and the days of exposure are presented in Figures 1 and 2, along with ± SD for all the conditions examined.

Table 1. Concentrations of Cd and Ni (μg/g dry weight) in gills, mantle and the remaining body of *M. galloprovincialis* and *C. chione* at the Day 0.

	Cd		Ni	
	M. galloprovincialis	*C. chione*	*M. galloprovincialis*	*C. chione*
Gills	3.4	1.1	2.9	3.5
Mantle	2.2	1.1	0.6	6.4
Body	2.2	4.5	1.8	20.6

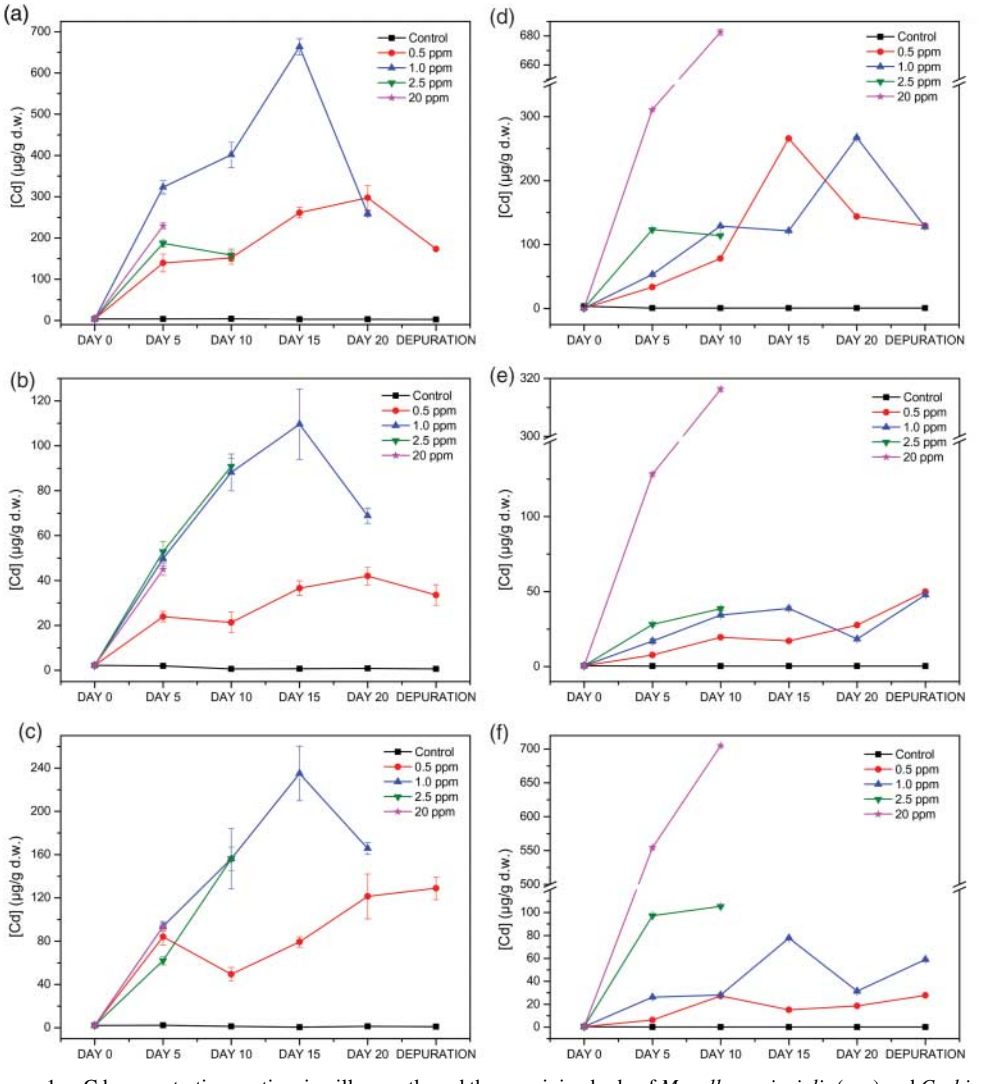

Figure 1. Cd concentration vs. time in gills, mantle and the remaining body of *M. galloprovincialis* (a–c) and *C. chione* (d–f) in different Cd concentrations in seawater. (a) and (d) gills, (b) and (e) mantle, (c) and (f) remaining body.

3.1.1. *Cd*

Both species were exposed to 0.5, 1.0, 2.5, and 20.0 mg/L Cd for 20 days, corresponding to 4.4, 8.9, 22.2, and 177.9 μmol Cd/L, respectively. The lack of points at higher Cd exposure levels in Figure 1 is due to high mortality of the organisms in these conditions and, consequently, the decrease of the available populations for the continuation of the experiment (see below). Exposure of *M. galloprovincialis* to 2.5 mg Cd/L seawater resulted in the extinction of the population within 10 days, whereas exposure to 20 mg Cd/L accelerated the lethal effects (all the organisms died in 5 days, Table 2). *C. chione* was more tolerant to Cd contamination: organisms survived in both 2.5 mg and 20 mg Cd/L seawater for 10 days (Figure 1).

In general, the accumulation of Cd in the three tissues of *M. galloprovincialis* followed the order gills > body > mantle (Figure 1(a)–(c)). This trend for *M. galloprovincialis* has been observed in other studies [23,31,54] as well. The Cd concentrations in the gills at 'Day 5' presented statistically

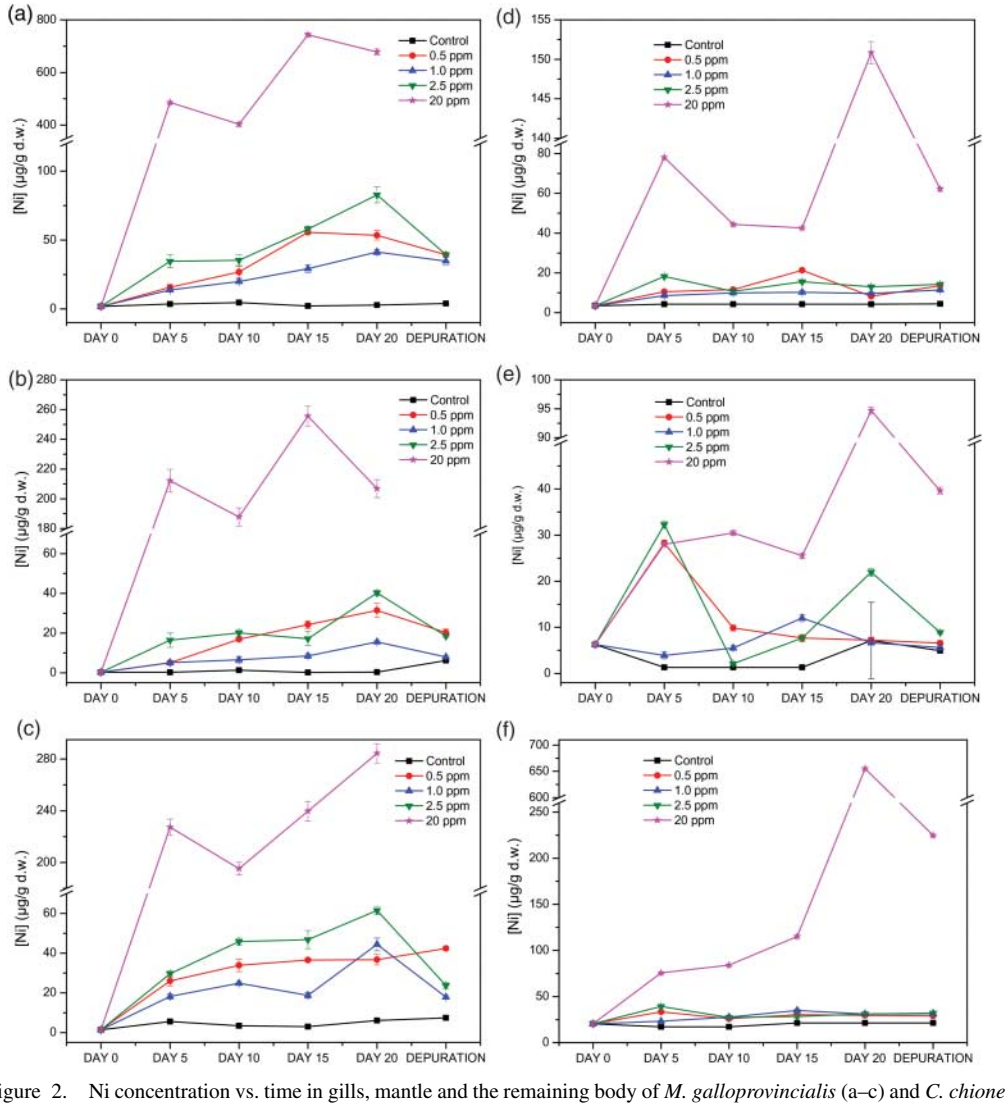

Figure 2. Ni concentration vs. time in gills, mantle and the remaining body of *M. galloprovincialis* (a–c) and *C. chione* (d–f) in different Ni concentrations in seawater. (a) and (d) gills, (b) and (e) mantle, (c) and (f) remaining body.

significant differences (independent samples t-test, $p < 0.05$). Interestingly, there was a significant decrease in Cd concentration in all *M.galloprovincialis* tissues after the 15th day of the experiment in 1.0 mg Cd/L. Gills accumulate Cd more effectively in 1.0 mg Cd/L (Figure 1(a)). Higher Cd concentrations in seawater cause a decrease in Cd concentration in mussels' gills. These observations could be due to either a reduction in the feeding rate through water filtering when animals are in stress conditions, or due to a triggering of a defensive mechanism, which occurs earlier (i.e. the 5th day of exposure) when the animals are exposed to concentrations higher than 1.0 mg Cd/L seawater or later (i.e. the 15th day of exposure) when they are exposed to lower Cd concentrations. This hypothesis is supported by the observation that there was a slowdown in the uptake of Cd in the gills after 5 days of exposure to 2.5 mg Cd/L seawater: the additional Cd, accumulated through filtering, could be removed effectively by this defensive mechanism. Eventually, the toxic effects of Cd prevailed and the animals died. At even higher Cd levels, the toxicity of the heavy metal was acute and the defensive mechanism was not able to protect the

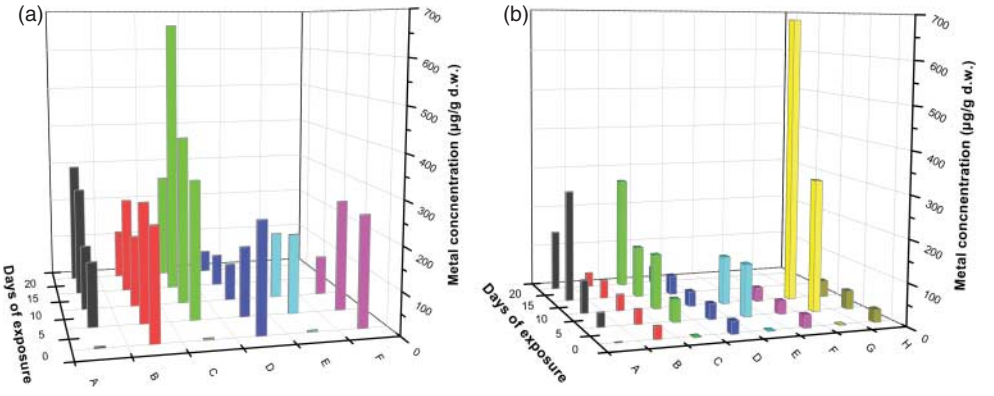

Figure 3. (a) Cd (A, C, E) and Zn (B, D, F) concentrations in the gills of *M. galloprovincialis* after exposure in 0.5 (A, B), 1.0 (C, D), and 2.5 mg Cd/L seawater (E, F). (b) Cd (A, C, E, G) and Zn (B, D, F, H) concentrations in the gills of *C. chione* after exposure in 0.5 (A, B), 1.0 (C, D), 2.5 (E, F) and 20 mg Cd/L seawater (G, H).

Table 2. Cd and Ni bioconcentration factors for *M. galloprovincialis* and *C. chione*[a].

	Cd						Ni					
	M. galloprovincialis			*C. chione*			*M. galloprovincialis*			*C. chione*		
	Gills	Mantle	Body	Gills	Mantle	Body	Gills	Mantle	Body	Gills	Mantle	Body
4.4 µmol/L							8.5 µmol/L					
Day 5	292	44	164	65	15	12	22	10	40	12	52	33
Day 10	295	41	96	155	38	54	42	31	48	14	15	18
Day 15	517	72	158	530	34	29	96	47	54	27	11	18
Day 20	590	82	240	286	55	37	102	61	59	1.0	10	16
8.9 µmol/L							17.0 µmol/L					
Day 5	319	48	91	52	17	26	10	4.8	13	4.2	1.5	6.2
Day 10	397	88	155	128	34	27	15	5.2	12	5.6	3.1	11
Day 15	661	109	234	121	38	77	27	8.3	16	2.5	10	13
Day 20	256	68	164	267	18	31	39	15	38	1.9	4.6	10
22.2 µmol/L							42.6 µmol/L					
Day 5	73	20	24	49	11	39	12	6.5	9.6	5.6	12	8.9
Day 10	62	36	62	45	15	42	12	7.5	13	2.6	0.3	4.0
Day 15	–	–	–	–	–	–	22	6.8	18	4.5	2.5	2.8
Day 20	–	–	–	–	–	–	32	16	22	3.5	8.3	3.8
177.9 µmol/L							340.7 µmol/L					
Day 5	11	2.1	4.6	15.5	6.4	28	24	11	11	3.7	1.3	2.9
Day 10	–	–	–	34	16	35	20	9	9	2.0	1.4	3.4
Day 15	–	–	–	–	–	–	37	13	12	1.7	1.2	4.7
Day 20	–	–	–	–	–	–	34	10	14	7.2	4.6	32

[a]- indicates no more alive organisms.

animals, whereas at the lowest Cd concentration examined in seawater, the accumulated Cd in the gills probably did not reach the necessary threshold for the proposed defensive mechanism to be triggered.

The Cd concentrations in the mantle and the body at 'Day 5' were almost the same and independent of the exposure level (independent samples *t*-test, $p > 0.05$), whereas at the other experimental periods, the cadmium concentrations measured in the mantle and body of the animals exposed to 0.5 mg Cd/L seawater were statistically different (independent samples *t*-test, $p < 0.05$) than the other two exposure levels which were statistically same ($p > 0.05$). These

observations probably show that the rate of the translocation of Cd from the gills (the first destination of the metal in the organism) to the other tissues is steady and independent of the levels of exposure. Owing to mortality (see below) and to an underestimation of the number of animals that would be required, depuration data were available only for the lowest exposure level (Figure 1(a)–(c)). During the 10 days of depuration, Cd concentration levels in the mussels exposed to 0.5 mg Cd/L seawater for 20 days were reduced by 46% in the gills, whereas mantle lost only 18% of the accumulated Cd and the body retained the total amount of the metal. Longer depuration experiments are needed in order to examine the behaviour of each tissue of *M. galloprovincialis*, but at least for the conditions we used, we can conclude that depuration occurs rather slowly. Our results indicate that Cd contamination of these species, is most likely due to the accumulation in the part of the animal we call 'remaining body'. This is consistent with total metal content determination in the three tissues under examination (see below).

Figure 1(d)–(f) presents the plots of Cd accumulation in the tissues of *C. chione* vs. exposure time in the examined seawater concentrations. In general, the accumulation in the three different tissues follows the same order as in the mussels' tissues (gills > body > mantle). However, the Cd concentrations in the clams' tissues have two profiles: for the first three concentrations in the seawater (namely 0.5, 1.0 and 2.5 mg Cd/L), the accumulated Cd was considerably lower than the corresponding in mussels. At the highest concentration tested, (20 mg Cd/L seawater), the concentrations of the accumulated Cd in all three tissues were higher than the corresponding in mussels, and also, the accumulation of the metal in the body becomes higher than in the gills changing the order mentioned above. Thus, per gram of dry weight, *M. galloprovincialis* is a much more effective Cd accumulator than *C. chione* at relatively lower pollution levels, but the opposite is true for higher pollution levels. Higher Cd concentrations in seawater result in higher Cd concentrations in all tissues, in general. The 'saturation' of the Cd accumulation which is observed in mussels' mantle and body above 1.0 mg/L seawater is absent in clams. In contrast, the accumulation of Cd in the body of clams increases dramatically when exposed to higher Cd concentrations (Figure 1(f)) until the bivalves die. Cd accumulation in *C. chione* gills seemed to slow down after 15 days of exposure at 0.5 mg Cd/L, after 10 days of exposure at 1.0 mg Cd/L, and after 5 days of exposure at 2.5 mg Cd/L. This behaviour is similar to what we observed for *M. galloprovincialis* gills, and it could be rationalised again considering two competing processes taking place, namely the uptake of the metal through water filtering and a biological defensive mechanism, the triggering of which depends on the pollution level and/or time of exposure. Mantle and body exhibit a decrease in Cd concentration after 15 days in 1.0 mg Cd/L seawater (Figure 1(e) and (f)). In general, *C. chione* survives better compared to *M. galloprovincialis* in higher Cd concentrations in seawater (see below).

The depuration experiments carried out on *C. chione* exposed to the two lower Cd concentrations in seawater are also presented in Figure 1(d)–(f). Interestingly, *C. chione* shows a different behaviour than *M. galloprovincialis*: gills show a quite significant depuration (especially at 1.0 mg Cd/L seawater, where the depuration of the gills is 54%) whereas a concomitant increase of Cd levels in the other two tissues was observed. It is possible that these two processes are related, and more experiments are in progress to clarify this issue. In any case, considerable amounts of the pollutant are still present in the tissues of the animals after 10 days of depuration, a result which resembles findings by Freitas *et al.*[6] and Hédouin *et al.*[43] in other bivalve species.

The trend lines, and their correlation coefficients, of Cd accumulation in the three tissues of the two bivalves were calculated for the two lower exposure levels (data not shown). For both animals, all trend lines showed a statistically significant positive trend (the correlation coefficients of the corresponding trend lines were higher than the theoretical values of 0.902 for degrees of freedom $(n-2) = 2$ and 0.773 for degrees of freedom $= 3$), except for the Cd concentration data in the body of animals exposed to the lowest Cd level (0.5 mg Cd/L seawater). Positive trends have also been reported in other studies of *M. galloprovincialis* and other bivalves.[5,9,23]

3.1.2. *Ni*

Exposure of the two species under study in Ni-contaminated seawater, even at high concentrations (20 mg/L Ni, 340.7 μmol Ni/L), did not cause any mortality. On the other hand, Ni accumulation in both species is lower than Cd accumulation. Nickel accumulation in the tissues of the two species under study is shown in Figure 2. Figure 2(a)–(c) contains data for Ni concentration in *M. galloprovincialis* tissues. As for Cd, in all concentrations of Ni in seawater and in all tissues, there is an increase in the accumulation of Ni with the time of exposure to Ni-polluted environment. However, this accumulation is higher when organisms are exposed to lower concentrations of Ni (0.5 mg Ni/L seawater) as compared to the intermediate concentration (1.0 mg Ni/L). This behaviour has also been observed in other studies.[27] At higher seawater concentration (2.5 mg Ni/L), the accumulation becomes higher in all three tissues, and this is much more profound at the highest concentration used (20 mg Ni/L). The accumulation of Ni follows the order gills > body > mantle, same as Cd, however the concentrations of Ni in *M. galloprovincialis* tissues are much lower compared to corresponding Cd concentrations in this organism, with the exception of the highest Ni concentration in seawater (20 mg Ni/L), where all three tissues of the mussels contain much higher levels of Ni compared to the highest concentration of Cd used in our study, 20 mg/L 177.9 μmol/L seawater (see Figures 1 and 2). This observation, however, needs to be evaluated taking into account the much higher toxicity effects of Cd on the mussels (e.g. mortality rates, see below). Nickel depuration experiments on *M. galloprovincialis* are also shown in Figure 2(a)–(c). The amounts of Ni removed after 10 days of depuration varied with the experimental conditions: gills depuration ranged between 17 and 52%, whereas mantle depuration between 35–54% and body depuration between 0–64%. It is important to note that the Ni concentrations after 10 days of depuration do not return to the control levels; instead they remain relatively high in most of the experimental conditions. Longer depuration experiments are needed to study the behaviour of this species.

C.chione exhibits a quite different behaviour than *M. galloprovincialis* regarding Ni accumulation (Figure 2(d)–(f)). In general, Ni accumulation in *C. chione* tissues is not linear and not as straightforward as for *M. galloprovincialis*. Our results indicate that *C. chione* behaves as if it tries to resist to Ni accumulation. Higher amounts of Ni are accumulated in the tissues of this species mainly when the animals are exposed for several days to very high levels of Ni in seawater, whereas the tendency of the accumulation follows the order body > gills ≈ mantle. As in mussels, 0.5 mg Ni/L seawater results in higher accumulation as compared to 1.0 mg Ni/L seawater. The highest Ni concentration in seawater results in much higher accumulation in all tissues (Figure 2). In gills, Ni accumulation reaches a maximum in the 5th day when animals are exposed to 2.5 mg Ni/L seawater and in the 15th day when exposed to 0.5 or 1.0 mg Ni/L seawater, followed by a decrease the following days. These results could imply a defensive mechanism, similar to the mechanism described above for Cd accumulation, which is triggered at relatively higher Ni concentrations after some days of exposure, and it is capable for removing Ni. This defensive mechanism could be balanced, or even outcompeted, at higher Ni concentrations.

Nickel accumulation in *C. chione* tissues was lower than the accumulation in *M. galloprovincialis* tissues. However, *M. galloprovincialis* exhibits better depuration activity than *C. chione*: as mentioned above, in a 10-day depuration experiment, Ni concentrations in all mussel tissues were reduced in almost all the conditions examined. Nickel levels in *C. chione* tissues seem unaffected for most of the conditions examined, after 10 days of depuration (Figure 2).

The calculation of the trend lines of Ni accumulation and their correlation coefficients resulted in a statistically significant positive trend in all tissues of *M. galloprovincialis* with only one exception (Ni concentration in the body of animals exposed to the lower Ni level of 0.5 mg/L). The trend line plots for *C. chione* showed a significant increasing trend only in the gills exposed to 0.5 and 1.0 mg Ni/L seawater and the body exposed to 0.5 mg Ni/L.

3.1.3. *Bioconcentration factor analysis*

The bioconcentration factors of the three tissues under examination, for both organisms and in all conditions used, are presented in Table 2. The bioconcentration factor (BCF) is the ratio of the concentration of a substance in a tissue over the concentration of it in the aqueous environment.[55] The BCF is a measure of the tendency of a substance in water to accumulate in aquatic organisms.[56] The BCFs for the three tissues under examination were calculated from the concentrations of Cd and Ni in all the tissues of the two animals under study, in all the conditions used. The equation which was used is

$$\mathrm{BCF} = \frac{C_{\text{tissue}} - C_{\text{tissue control}}}{C_{\text{water}}}$$

where C_{tissue} is the concentration of the heavy metal in the specific tissue at the specific conditions, $C_{\text{tissue control}}$ is the concentration of the heavy metal in the same tissue from control animals which remained in heavy metal-free seawater for the same period of time as the exposed animals, and C_{water} is the nominal concentration of the heavy metal in the seawater. The results in Table 2 verify the observations about the accumulation of Cd and Ni in the gills, mantles and remaining bodies of *M. galloprovincialis* and *C. chione*: (i) Both animals show a much higher tendency for Cd than Ni accumulation. (ii) In general, *M. galloprovincialis* is a better accumulator for Cd in the three lower concentrations, but *C. chione* accumulates more of this metal at the highest exposure level, at least the first five days of exposure. (iii) BCF values for Ni are much higher for *M. galloprovincialis* than for *C. chione* in almost all the conditions tested, showing that, in general, *M. galloprovincialis* is a better accumulator than *C. chione*. (iv) All BCF values for Cd followed the order gills > body > mantle for corresponding conditions, showing the tendency of this metal to accumulate more in the gills of both organisms. This order is retained for Ni in *M. galloprovincialis* tissues, but is changed in *C. chione*, becoming body > gills, mantle. Between gills and mantle of *C. chione* the BCF trend for Ni is not clear.

The hypothesis presented in previous paragraphs of two competing processes taking place for the accumulation of the heavy metals in the tissues (mainly in the gills) of the two bivalves is also supported by the BCF values: BCF value for Cd in gills of *M. galloprovincialis* exposed to 1.0 mg Cd/L seawater for 20 days is 256, whereas for 15 days of exposure it is 661. For 2 mg Cd/L seawater, the BCF values are 73 after 5 days of exposure and 62 after 10 days. Similar observations can be made for several conditions, already referred previously in this article. Finally, it needs to be noted that, in general, BCF values vary with the time of exposure, showing that the accumulation of the metals in the tissues of the animals did not reach equilibrium until the end of the experiment. This is more obvious for Cd accumulated in the gills of both animals.

3.2. *Correlation of Zn and Fe levels with accumulated Cd and Ni*

The potential correlations of the naturally existing Zn and Fe concentrations in the tissues of the two animals with the levels of Cd and Ni were investigated for all the conditions used in this study. For this reason, atomic absorption measurements for Fe and Zn were carried out in the same samples used for the determination of Cd and Ni. Figure 3(a) and (b) presents, in 3D plots, the alteration of Zn concentrations in the gills of *M. galloprovincialis* and *C. chione* vs. the Cd concentration in seawater and the days of exposure. For comparison, the levels of Cd in the gills are also shown. From these plots several observations can be made: first, the control levels (Day 0) of Zn in the gills of the two animals are completely different, with the gills of *M. galloprovincialis* containing much higher concentrations of Zn than the gills of *C. chione*. Secondly, the levels of Zn in the gills of *M. galloprovincialis* appear, in general, to decrease as the levels of the accumulated Cd increase. This is true for all the pollution levels examined. On the other hand, the Zn levels in the

Table 3. Correlation coefficients for the correlation trend lines of Cd and Ni with Zn and Fe in the gills, mantles and bodies of *M. galloprovincialis* and *C. chione*.

	M. galloprovincialis		*C. chione*	
	$R_{\text{experimental}}$	n^a	$R_{\text{experimental}}$	n^a
Cd-Zn gills	−0.683	13	+0.532	16
Cd-Zn mantle	−0.662	13	−0.395	16
Cd-Zn body	−0.668	13	+0.087	16
Ni-Zn gills	−0.226	20	+0.321	20
Ni-Zn mantle	−0.607	20	+0.356	20
Ni-Zn body	−0.391	20	+0.138	20
Ni-Fe gills	−0.776	20	+0.028	20
Ni-Fe mantle	−0.504	20	+0.081	20
Ni-Fe body	−0.564	20	−0.032	20

Note: The statistically significant correlations are marked with grey.
[a]denotes the number of samples included in the calculations. The theoretical values for the correlation coefficients were: $|R_{\text{theoretical}}| = 0.553 (n = 13)$, 0.497 ($n = 16$), 0.444 ($n = 20$).

gills of *C. chione* do not seem to vary significantly in the different conditions examined, showing that the Zn concentrations are not affected by the accumulation of Cd in this tissue. In Table 3 the correlation coefficients of the trend lines between the studied metals are presented. The positive or negative correlation between the concentrations of the two metals is designated by the sign of the corresponding slope value. The statistically significant correlations are marked with grey shade. For Cd and Zn, these data show a negative correlation in all three tissues of *M. galloprovincialis*, and a positive correlation in the gills of *C. chione*. Also, negative correlations are shown for Ni and Zn in the mantle of *M. galloprovincialis* and for Ni and Fe in all three tissues of this species. Interestingly, corresponding correlations are absent for the tissues of *C. chione*. In general, data in Table 3 show that accumulation of Cd and Ni in the tissues of *M. galloprovincialis* affects the biologically important metals much more compared to *C. chione*. Maybe this is one of the reasons of the higher tolerance of *C. chione* to increased levels of Cd pollution presented in this study.

3.3. *Cd accumulation in the tissues of dead mussels*

The mussels and clams-containing aquaria were inspected several times a day for dead organisms. While possible, and with the requirement that they were completely intact, recently died organisms were collected and dissected on ice, immediately after they were identified. Unfortunately, this was not possible for all the conditions, and certainly it was not possible for *C. chione*, as this bivalve lives buried in the sand, and when it was identified dead it already was seriously damaged (decomposed). Figure 4 contains the concentrations of Cd in gills, mantles and bodies dissected from dead mussels, the third and the fifth day after their exposure to 20 mg Cd/L seawater. The corresponding Cd concentrations in the tissues from alive animals at the fifth day of their exposure to Cd-contaminated seawater are also given for comparison. These results show clearly that there is an excessive Cd accumulation in the gills and the mantles of the dead animals compared to the concentrations in the tissues of alive animals. There was a two-fold increase of Cd in gills and a four-fold increase in mantle. Bodies from dead animals did not show considerable increase in Cd concentration. Most likely, the defensive system of some animals was not as effective as needed in an environment heavily contaminated with Cd, and the consequent excessive accumulation of the metal in the animals, and especially in their gills, caused their death. Possibly, right after the exposure of the mussels to high Cd concentrations there is an acute accumulation of the metal in the gills, which gradually is reduced through the distribution of the metal to other tissues, or

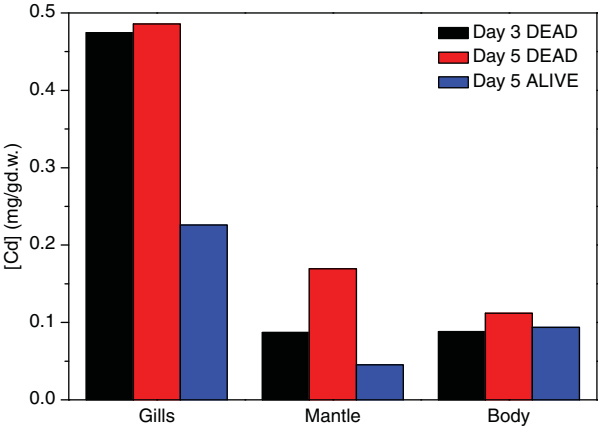

Figure 4. Concentrations of Cd in the tissues of dead and alive *M. galloprovincialis* exposed to 20 mg Cd/L seawater.

through the secretion of it via the digestion system. The death of the animals could be caused by the inefficient removal of the excess Cd from the gills.

3.4. *Heavy metal distribution in different tissues*

The distribution of the heavy metals under examination in the three different parts of the organisms was estimated and the data are presented in Figures 5 (Cd) and 6 (Ni). In general, *M. galloprovincialis* and *C. chione* exhibited quite different behaviour regarding the distribution of the accumulated metals in their tissues. Also, there are differences between the two metals regarding their distribution in the tissues of each species.

Starting with the background levels of the two metals in the tissues of the bivalves (Day 0), *M. galloprovincialis* accumulates most of Cd in gills (40%) rather than in the mantle (32%) and the body (28%), whereas *C. chione* stores background Cd mainly in the body (57%) and mantle (35%) rather than the gills (8%) (Figure 5). Nickel is accumulated in control *M. galloprovincialis* (Day 0) more evenly between gills and body (40 and 41%, respectively) with less amount being present in mantle (19%), whereas in *C. chione* Ni is stored almost exclusively in the body (91%), with the remaining being present in the mantle (Figure 6).

Within the first five days of exposure to the lower concentrations of the metals in seawater a rather abrupt increase of the relative amount of both Cd and Ni in *M. galloprovincialis* body was observed, which was not obvious at higher concentrations. Contrarily, exposure of *C. chione* to the lower concentration of Cd in seawater results in a steep increase of the percentage of the metal in gills, whereas exposure of this species to Ni causes an initial increase of its percentage in mantle.

The first five days, the relative accumulation of Cd in *M.galloprovincialis* gills increases with the concentration of the metal in seawater (from 29 to 59%). Similar behaviour but at a smaller extent (from 16 to 33%) is also observed for the first three Ni concentrations. The % Cd in the remaining body decreases accordingly (from 55 to 26%). On the other hand, the % Cd content in the body of this animal shows a moderate increase with time at the same Cd concentration in seawater, for all concentrations examined.

Ni content is higher in *M.galloprovincialis* bodies (36 to 75%) compared to the other two tissues. This is more profound for the first three Ni concentrations in seawater, whereas at the highest Ni concentration the distribution between gills and body becomes more even.

Cd distribution in *Callista chione* tissues showed the opposite behaviour than in *Mytilus galloprovincialis*. In general, the Cd content in gills decreases as the Cd concentration in seawater

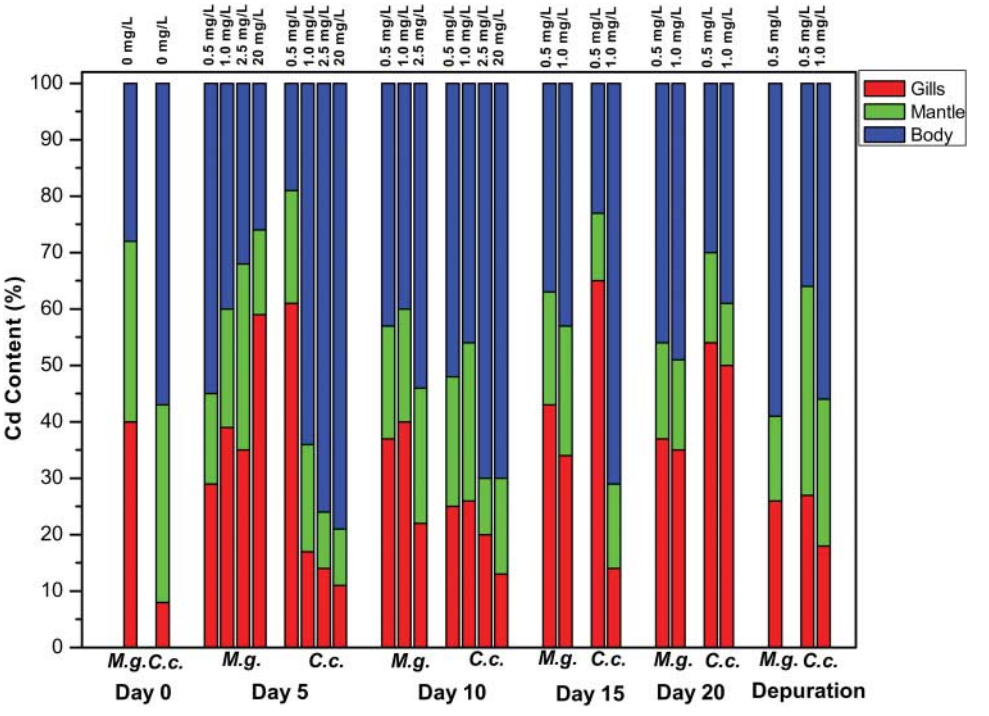

Figure 5. % Cd content in gills, mantle and the remaining body of *M.galloprovincialis* and *C. chione* in the time course of the experiment.

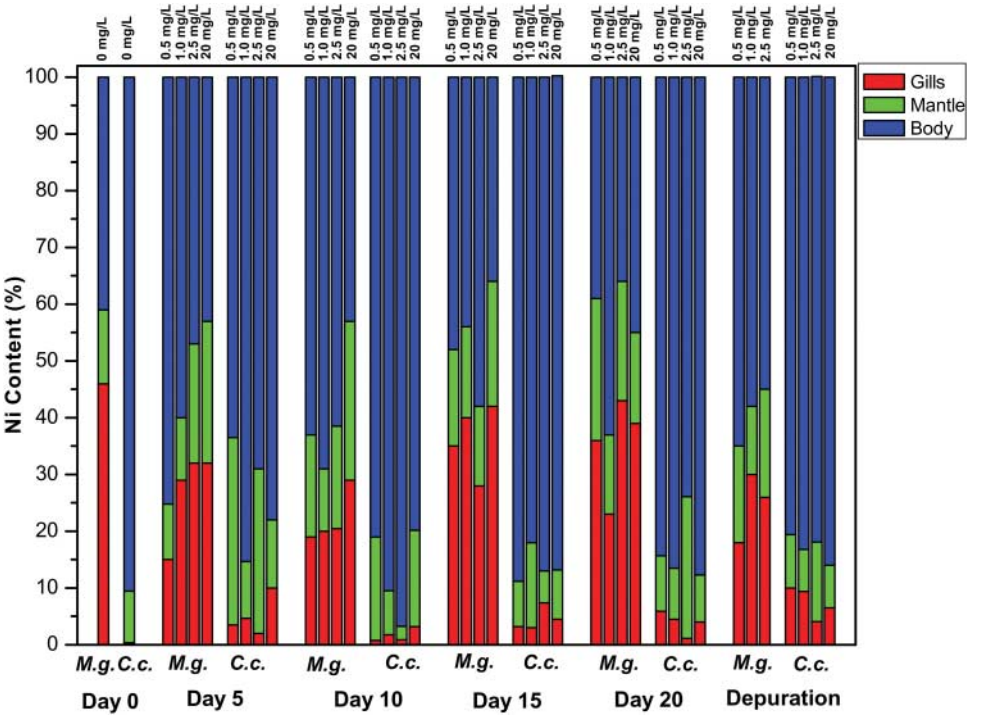

Figure 6. % Ni content in gills, mantle and the remaining body of *M.galloprovincialis* and *C. chione* in the time course of the experiment.

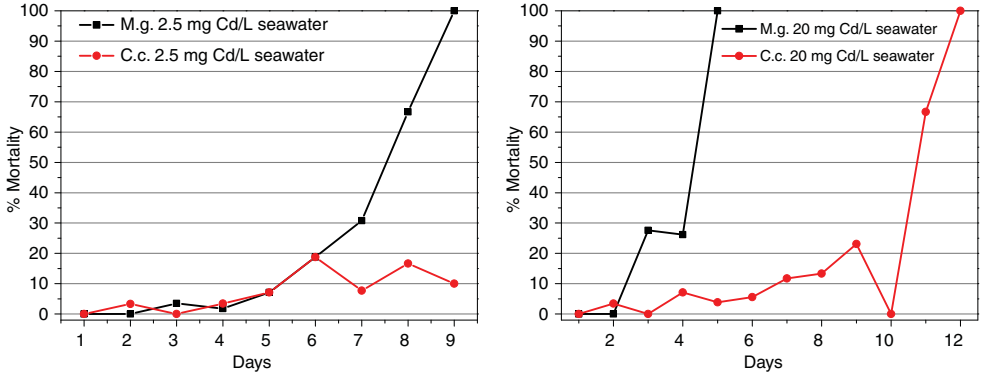

Figure 7. Mortality rates of *M.galloprovincialis* and *C.chione* exposed to 2.5 and 20 mg Cd/L seawater.

increases, whereas the Cd content in the body increases accordingly. This is more profound in the fifth day of the experiment where the Cd content in gills decreases from 62 to 11% and in the body it increases from 19 to 79% as the Cd concentration in seawater increases from 0.5 to 20 mg/L. The percentage of Cd in mantle also decreases in higher Cd concentrations in seawater.

Ni accumulates mainly in the body of *Callista chione*, a behaviour which is similar with that of mussels but at higher percentages (64 to 97%), independently of the experimental conditions. In contrast with *M. galloprovincialis*, Ni did not exceed 10% in gills of *C. chione* in any of the conditions examined in this work.

3.5. *Tolerance to high Cd and Ni contamination*

For mussels and clams exposed to nickel, even to 20 mg/L, no deaths occurred until the end of the experiments. On the other hand, when both animals were exposed to cadmium, relatively high mortality was observed.

The mortality rates of the two species were calculated as the ratio of the number of the dead animals over the total number of animals (dead and alive) the day of the measurement × 100. In Figure 7, data are presented for the % mortality rates of the two organisms in the two higher Cd concentrations in seawater.

Mussels exposed to 0.5 μmol/L and 1.0 mg/L Cd survived until the end of the experiment with zero losses, whereas clams at the same concentrations showed a small mortality (3–6%) in the first 2–3 days, but afterwards the cultures stabilised and no deaths were observed in the time course of the experiment.

Both species exposed to 2.5 mg Cd/L survived until the 10th day with a constantly increasing mortality rate for mussels (for mussels, mortality increased almost exponentially after the fifth day). Mussels exposed to the highest cadmium concentration, 20 mg/L Cd, exhibited moderate mortality rates the first 3–4 days of the experiment (26–28%) and finally survived until the fifth day of the experiment, whereas clams survived until the 12th day, with low mortality rates the first 10 days. Our data indicate clearly that *Callista chione* is more tolerant to Cd and Ni heavy contamination in seawater than *Mytilus galloprovincialis*.

4. Conclusions

Studies on the behaviour of organisms used as bioindicators and/or as food, in high or very high pollution levels are of particular significance as it is very important to know the survival

capacity, the rates and the levels of the pollutants accumulation in the animals' tissues etc. Extreme environmental pollution conditions of short duration and high intensity can occur mainly in coastal areas influenced by various anthropogenic activities and may cause serious impacts on the local ecosystems. Owing to their incidental appearance and short duration these pollution events are difficult to be studied in the field so such studies are possible mainly under laboratory conditions.

M. galloprovincialis is used as a biomarker for marine pollution but has not been studied exposed to high concentrations of heavy metals for relatively long time.[3,57,58] On the other hand, there are very few studies about the effects of heavy metals on *C. chione*, a smooth clam with high commercial value and very common seafood in many countries. In this study, we present significant differences on the levels of accumulation and distribution of Cd and Ni in gills, mantles and the remaining bodies of the two organisms, and on their tolerance in high concentrations of Cd and Ni. In summary:

- *M. galloprovincialis* is a more effective accumulator of Cd and Ni than *C. chione*. However, *C. chione* becomes a better Cd accumulator than *M. galloprovincialis* when exposed to very high concentrations of Cd.
- A negative correlation between Zn and accumulated Cd and between Fe and accumulated Ni was observed in *M. galloprovincialis*, but not in *C. chione* tissues. This could be one of the reasons of increased tolerance of *C. chione* to elevated heavy metal pollution levels, as shown in this study.
- Cd and Ni are distributed differently in the tissues of the two bivalves. For pollution assessment, the right tissue needs to be chosen for analysis.
- The depuration of the two animals varied significantly for the two metals and for the different tissues examined. After 10 days of depuration, significant amounts of metals remained in the tissues, showing that the depuration of these species is a slow process.
- Cd exhibited much higher toxicity than Ni. *C. chione* is a more tolerant organism against high concentrations of Cd in seawater than *M. galloprovincialis*.
- After some time of exposure of the bivalves in Cd or Ni-contaminated seawater a decrease of the accumulation of the metals is observed, depending on the metal, its concentration and the tissue. This result is consistent with a hypothesis we make about two competing processes taking place regarding the behaviour of the two bivalves in a heavy metal-contaminated environment: in one process, Cd or Ni accumulates first in the gills through filter-feeding and then it is distributed in other tissues of the organism. When accumulation reaches a critical point, either after longer time of exposure to relatively lower concentrations or shorter time of exposure to higher concentrations of the metal in seawater, a second biochemical procedure is triggered, by which removal (sequestration, excretion etc.) of the metal takes place. Work is in progress to validate this hypothesis and to elucidate this detoxification mechanism.

Acknowledgements

Excellent technical assistance on the atomic absorption spectrometry by Dr. Vicky Paraskevopoulou is acknowledged. This research has been co-financed by the European Union (European Social Fund – ESF) and Greek national funds through the Operational Program 'Education and Lifelong Learning' of the National Strategic Reference Framework (NSRF) – Research Funding Program: Heracleitus II. Investing in knowledge society through the European Social Fund.

References

[1] Simkiss K, Taylor A. Cellular mechanisms of metal ion detoxification and some new indices of pollution. Aquat Toxicol. 1981;1:279–290.
[2] Viarengo A, Nott JA. Mechanisms of heavy metal cation homeostasis in marine invertebrates. Mini-Review. Comp Biochem Phys C. 1993;104:355–372.

[3] Amiard JC, Amiard-Triquet C, Barka S, Pellerin J, Rainbow PS. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers, Review. Aquat Toxicol. 2006;76:160–202.
[4] Pellerin J, Amiard JC. Comparison of bioaccumulation of metals and induction of metallothioneins in two marine bivalve (*Mytilus edulis* and *Mya arenaria*). Comp Biochem Physiol C. 2009;150:186–195.
[5] Erk M, Ruus A, Ingebrigtsen K, Hylland K. Cadmium accumulation and Cd-binding proteins in marine invertebrates-A radiotracer study. Chemosphere. 2005;61:1651–1664.
[6] Freitas R, Ramos Pinto L, Sampaio M, Costa A, Silva M, Rodrigues AM, Quintino V, Figueira E. Effects of depuration on the element concentration in bivalves: comparison between sympatric *Ruditapes decussatus* and *Ruditapes philippinarum*. Estuar Coast Shelf Sci. 2012;110:43–53.
[7] Geffard A, Sartelet H, Garric J, Biagianti-Risbourg S, Delahaut L, Geffard O. Subcellular compartmentalization of cadmium, nickel and lead in *Gammarus fossarum*: comparison of methods. Chemosphere. 2010;78:822–829.
[8] Figueira E, Lima A, Branco D, Quintino V, Rodrigues AM, Freitas R. Health concerns of consuming cockles (*Cerastoderma edule* L.) from a low contaminated coastal system. Environ Int. 2011;37:965–972.
[9] Chandurvelan R, Marsden ID, Gaw S, Glover CN. Impairment of green-lipped mussel (*Perna canaliculus*) physiology by waterborne cadmium: relationship to tissue bioaccumulation and effect of exposure duration. Aquat Toxicol. 2012;124–125:114–124.
[10] Stankovic S, Jovic M. Health risks of heavy metals in the mediterranean mussels as seafood. Environ Chem Lett. 2012;10:119–130.
[11] Pacyna JM, Scholtz MT, Li YF. Global budget of trace metal sources. J Environ Rev. 1995;3:145–159.
[12] Dudka S, Adriano DC. Environmental impacts of metal ore mining and processing: a review. J Environ Qual. 1997;26:590–602.
[13] Fernández B, Campillo JA, Martínez-Gómez C, Benedicto J. Assessment of the mechanisms of detoxification of chemical compounds and antioxidant enzymes in the digestive gland of mussels, *Mytilus galloprovincialis*, from mediterranean coastal sites. Chemosphere. 2012;87:1235–1245.
[14] Viarengo A, Canesi L. Mussels as biological indicators of pollution. Aquaculture. 1991;94:225–243.
[15] Chong K, Wang WX. Comparative studies on the biokinetics of Cd, Cr, and Zn in the green mussel *Perna viridis* and the Manila clam *Ruditapes philippinarum*. Environ Pollut. 2001;115:107–121.
[16] Domouhtsidou GP, Dailianis S, Kaloyianni M, Dimitriadis VK. Lysosomal membrane stability and metallothionein content in *Mytilus galloprovincialis* (L.), as biomarkers combination with trace metal concentrations. Mar Pollut Bull. 2004;48:572–586.
[17] Tsangaris K, Papathanasiou E, Cotou E. Assessment of the impact of heavy metal pollution from a ferro-nickel smelting plant using biomarkers. Ecotox Environ Safe. 2007;66:232–243.
[18] Serafim A, Lopes B, Company R, Cravo A, Gomes T, Sousa V, Bebianno MJ. A multi-biomarker approach in cross-transplanted mussels *Mytilus galloprovincialis*. Ecotoxicology. 2011;20:1959–1974.
[19] Gomiero A, De Biasi AM, Da Ros L, Nasci C, Spagnolo A, Scarcella G, Fabi G. A multidisciplinary approach to evaluate the environmental impact of offshore gas platforms in the western Adriatic Sea. Chem Ecol. 2011;27:1–13.
[20] Solaum O, Rodriguez JG, Borja A, Franco J, Larreta J, Valencia V. Background metal levels determination in bivalves – quality assessment of the European water framework directive. Chem Ecol. 2013;29:11–27.
[21] Shilla D, Qadah D, Kalibbala M. Distribution of heavy metals in dissolved, particulate and biota in the Scheldt estuary, Belgium. Chem Ecol. 2008;24:61–74.
[22] Viarengo A, Palmero S, Zanicchi G, Capelli R, Vaissiere R, Orunesu M. Role of metallothioneins in Cu and Cd accumulation and elimination in the gill and digestive gland cells of *Mytilus galloprovincialis*. Lam. Mar Environ Res. 1985;16:23–36.
[23] Odžak N, Martinèiæ D, Zvonariæ T, Branica M. Bioaccumulation rate of Cd and Pb in *Mytilus galloprovincialis* foot and gills. Mar Chem. 1994;46:119–131.
[24] Lee JG, Roberts SB, Morel FMM. Cadmium: a nutrient for marine diatom *Thalassiosira weissflogii*. Limnol Oceanogr. 1995;40:1056–1063.
[25] Lane TW, Morel FMM. A biological function for cadmium in marine diatoms. PNAS. 2000;97:4627–463.
[26] Beyersmann D, Hartwig A. Carcinogenic compounds: a recent insight into molecular and cellular mechanisms. Arch Toxicol. 2008;82:493–512.
[27] Attig H, Dagnino A, Negri A, Jebali J, Boussetta H, Viarengo A, Dondero F, Banni M. Uptake and biochemical responses of mussels *Mytilus galloprovincialis* exposed to sublethal nickel concentrations. Ecotoxicol Environ Saf. 2010;73:1712–1719.
[28] Clancy HA, Costa M. Nickel: a pervasive carcinogen. Future Oncol. 2012;8:1507–1509.
[29] Millward GE, Kadam S, Jha AN. Tissue-specific assimilation, depuration and toxicity of nickel in *Mytilus edulis*. Environ Pollut. 2012;162:406–412.
[30] Bebianno MJ, Machado LM. Concentrations of metals and metallothioneins in *Mytilus galloprovincialis* along the South coast of Portugal. Mar Pollut Bull. 1997;34:666–671.
[31] Serra R, Isani G, Tramontano G, Carpenè E. Seasonal dependence of cadmium accumulation and Cd-binding proteins in *Mytilus galloprovincialis* exposed to cadmium. Comp Biochem Phys C. 1999;123:165–174.
[32] Saavedra Y, González A, Fernádez P, Blanco J. Interspecific variation of metal concentrations in three bivalve mollusks from Galicia. Arch Environ Con Tox. 2004;47:341–351.
[33] Szefer P, Kim BS, Kim CK, Kim EH, Lee CB. Distribution and coassociations of trace elements in soft tissue and byssus of *Mytilus galloprovincialis* relative to the surrounding seawater and suspended matter of the southern part of the Korean Peninsula. Environ Pollut. 2004;129:209–228.

[34] Amiard JC, Journel R, Bacheley H. Influence of field and experimental exposure of mussels (*Mytilus* sp.) to nickel and vanadium on metallothionein concentration. Comp Biochem Physiol C. 2008;147:378–385.
[35] Gorbi S, Virno Lambetri C, Notti A, Benedetti M, Fattorini D, Moltedo G, Regoli F. An ecotoxicological protocol with caged mussels, *Mytilus galloprovincialis*, for monitoring the impact of an offshore platform in the Adriatic Sea. Mar Environ Res. 2008;65:34–49.
[36] Dailianis S. Production of superoxides and nitric oxide generation in haemocytes of mussel *Mytilus galloprovincialis* (Lmk.) after exposure to cadmium: a possible involvement of Na+/H+ exchanger in the induction of cadmium toxic effects. Fish Shellfish Immunol. 2009;27:446–453.
[37] Deudero S, Box A, Tejada S, Tintoré J. Stable isotopes and metal contamination in caged marine mussel *Mytilus galloprovincialis*. Mar Pollut Bull. 2009;58:1025–1031.
[38] Mićović V, Bulog A, Kučić N, Jakovac H, Radošević-Stašić B. Metallothioneins and heat shock proteins 70 in marine mussels as sensors of environmental pollution in Northern Adriatic Sea. Environ Toxicol Phar. 2009;28:439–447.
[39] Doherty FG, Failla ML, Cherry DS. Metallothionein-like heavy metal binding protein levels in Asiatic clams are dependent on the duration and mode of exposure to cadmium. Water Res. 1988;22:927–932.
[40] Duquesne SJ, Coll JC. Metal accumulation in the clam *Tridacna crocea* under natural and experimental conditions. Aquat Toxicol. 1995;32:239–253.
[41] Roméo M, Gnassia-Barelli M. Metal distribution in different tissues and in subcellular fractions of the mediterranean clam *Ruditapes decussatus* treated with cadmium, copper, or zinc. Comp Biochem Physiol C. 1995;111:457–463.
[42] Serra R, Carpenè E, Marcantonio AC, Isani G. Cadmium accumulation and Cd-binding proteins in the bivalve *Scapharca inaequivalvis*. Comp Biochem Phys. 1995;111:165–174.
[43] Hédouin L, Pringault O, Metian M, Bustamante P, Warnau M. Nickel bioaccumulation in bivalves from the New Caledonia lagoon: Seawater and food exposure. Chemosphere. 2007;66:1449–1457.
[44] Wang WX, Rainbow PS. Influence of metal exposure history on trace metal uptake and accumulation by marine invertebrates. Ecotox Environ Safe. 2005;61:145–159.
[45] Wang WX, Rainbow PS. Comparative approaches to understand metal accumulation in aquatic animals. Review. Comp Biochem Phys C. 2008;148:315–323.
[46] Cossa D, Bourget E, Piuze J. Sexual maturation as a source of variation in the relantionship between cadmium concentration and the body weight of the *Mytilus edulis* L. Mar Pollut Bull. 1979;10:174–178.
[47] Orren MJ, Eagle GA, Hennig B, Green A. Variation in trace metal content of the mussel *Choromytilus meridionalis* with season and sex. Mar Pollut Bull. 1980;11:253–257.
[48] Dassenakis M, Scoullos M, Rapti K, Pavlidou A, Tsorova D, Paraskevopoulou V, Rozi E, Stamateli A, Siganos M. The distribution of copper in Saronikos Gulf after the operation of the wastewater treatment plant of Psitalia. Global Nest. 2004;5:133–143.
[49] Scoullos M, Dassenakis M. Determination of dissolved metals in seawater using the resin Chelex-100. Proceedings of the 1st Symposium on Oceanography and fisheries; 1984; Athens, Greece, 302–309.
[50] ISO 14869-1:2000. Soil quality- Dissolution for the determination of total element content-Part 1: dissolution with hydrofluoric and perchloric acids.
[51] USEPA, Office of Water, Office of Science and Technology. National recommended water quality criteria [Internet]; 2009. [cited 2012 Aug 18]. Available from: http://www.epa.gov/ost/pc/revcom.pdf.
[52] Scoullos M, Sakellari A, Giannopoulou K, Paraskevopoulou V, Dassenakis M. Dissolved and particulate trace metal levels in the Saronikos Gulf, Greece, in 2004. The impact of the primary wastewater treatment plant of Psittalia. Desalination. 2007;210:98–109.
[53] Nichols SJ, Manny BA, Schloesser DW, Edsall TA. Heavy metal contamination of sediments in the upper connecting channels of the great lakes. Hydrobiologia. 1991;219:307–315.
[54] Vlahogianni T, Dassenakis M, Scoullos M, Valavanidis A. Integrated use of biomarkers (superoxide dismutase, catalase and lipid peroxidation) in mussels *Mytilus galloprovincialis* for assessing heavy metals' pollution in coastal areas from the Saronikos Gulf of Greece. Mar Pollut Bull. 2007;54:1361–1371.
[55] Chiou CT. Bioconcentration of organic contaminants. In: Partition and adsorption of organic contaminants in environmental systems. Hoboken, NJ: John Wiley & Sons; 2002.
[56] IUPAC. Chemistry and human health division, glossary for toxokinetics of chemicals, IUPAC recommendation, 2003. [cited 2012 Aug 18]. Available from: http://iupac.org/reports/provisional/abstract03/nordberg_prs.pdf
[57] Alves Maranho L, Seabra Pereira CD, Choueri RB, Cesar A, Gusso-Choueri PK, Torres RJ, Abessa DMD, Morais RD, Mozeto AA, DelValls TA, Martin-Diaz ML. The application of biochemical responses to assess environmental quality of tropical estuaries: field surveys. J Environ Monit. 2012;14:2608–2615.
[58] Gorbi S, Bocchetti R, Binelli A, Bacchiocchi S, Orletti R, Nanetti L, Raffaelli F, Vignini A, Accoroni S, Totti C, Regoli F. Biological effects of palytoxin-like compounds from Ostreopsis cf. ovata: a multibiomarkers approach with mussels *Mytilus galloprovincialis*. Chemosphere. 2012;89:623–632.