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Freshwater *Ulva* (Chlorophyta) as a bioaccumulator of selected heavy metals (Cd, Ni and Pb) and alkaline earth metals (Ca and Mg)

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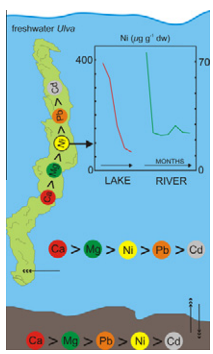
HIGHLIGHTS

- ► Freshwater *Ulva* are characterized by an elevated ability to accumulate Ni.
- ► *Ulva* can be used as bioaccumulator for Ni and Pb.
- ► Significant differences in the level of Ni accumulation were found between the river and lake *Ulva*.

GRAPHICAL ABSTRACT



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ABSTRACT

We analyzed the ability of freshwater taxa of the genus *Ulva* (Ulvaceae, Chlorophyta) to serve as bioindicators of metal in lakes and rivers. Changes in heavy metal (Ni, Cd and Pb) and alkaline earth metal (Ca and Mg) concentrations in freshwater *Ulva* thalli were investigated during the period from June to August 2010. The study was conducted in two ecosystems in Western Poland, the Malta lake (10 sites) and the Nielba river (six sites). Three components were collected for each sample, including water, sediment and *Ulva* thalli. The average concentrations of metals in the water sample and in the macroalgae decreased in the following order: Ca > Mg > Ni > Pb > Cd. The sediment revealed a slightly altered order: Ca > Mg > Pb > Ni > Cd. Ca and Mg were found at the highest concentrations in thalli due to the presence of carbonate on its surface. Among the examined heavy metals in thalli, Ni was in the highest concentration, and Cd found in the lowest concentration. There were statistically significant correlations between the levels of metals in macroalgae, water and sediment. Freshwater populations of *Ulva* exhibited a greater efficiency to bioaccumulate nickel as compared to species derived from marine ecosystems.

1. Introduction

Macroalgae are among the most complex and reliable organisms in studies of heavy metal pollution due to their rapid rate of metal accumulation from aqueous solutions (Phillips, 1977). Biological monitoring of marine water primarily focuses on the red algae (Muse et al., 1995), brown algae (Bryan and Hummerston, 1973) and green algae (Villares et al., 2002). The uptake of heavy metals by these algae proceeds as follows: Chlorophyta > Phaeophyta > Rhodophyta (Al-Shwafi and Rushdi, 2008). Thus, green algae, particularly from the genus *Ulva* (Ulvophyce, Ulvaceae), have been a most commonly used in these studies (Haritonidis and Malea, 1999). However, in most studies on the accumulation of heavy metals by *Ulva* only concentrations of these elements in the organisms, and not of the sample site (water, sediments) have been characterized. The complete analysis of the heavy metal concentrations in the entire natural environment from which these

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organisms are sampled may provide information on the extent of contamination and shall reveal possible applications of using *Ulva* as -bioindicator (Villares et al., 2002).

Most studies on the bioaccumulation and bioindication of heavy metals by *Ulva* have neglected freshwater species growing in rivers, ponds, lakes and canals. This reflects that fact that only a few hundred inland–water locations exists around the world, where *Ulva* has been reported (Rybak and Messyasz, 2010), and only a few of these sites are documented to be permanent habitats for these macroalgae. The sites documenting freshwater populations of *Ulva* to date are in the United States (Reinke, 1981), Czech Republic (Mareš, 2009) and Poland (Messyasz and Rybak, 2009). All of these locations document only the freshwater taxa of the macroalgae with monostromatic tubular thalli (e.g., *U. flexuosa*). Species with the distromatic frondose thalli (e.g., *U. lactuca*) have not been reported in freshwater ecosystems (Messyasz and Rybak, 2009).

Within the Western Poland, the massive development of *Ulva* populations is observed in the Nielba River and Malta Lake. Permanent and long-term (since 1995) occurrence of macroalgae in these sites was the basis to use them for metal accumulation ability examinations in these freshwater ecosystems.

The objective of the present study is to provide new data on the accumulation capacity of freshwater *Ulva* for selected heavy metals and alkaline earth metals, with a particular focus on using freshwater *Ulva* as a bioaccumulator for nickel, cadmium and lead contamination in water. We present a comprehensive study by analyzing changes in the concentration of these heavy metals in freshwater *Ulva* during the growing season, and compared these data with reference samples of the surrounding water and sediment.

2. Materials and methods

2.1. Sampling

The studies were conducted in June to August, 2010 during the optimum period of growth for freshwater populations of *Ulva*. Thalli samples of *Ulva* were collected from two different aquatic ecosystems in Western part of Poland. One was Malta Lake (10 sites) and the other was at Nielba River (six sites) (Fig. 1).

Located in the center of Poznan, Malta Lake is an artificial reservoir that was built in 1952. The surface of the lake is 0.64 km^2, with a length of 2.2 km, width of 0.46 km and an average depth of 3 m. In the western part of the reservoir, the Cybina River flows into the lake, supplying it with nutrient rich water. Freshwater *Ulva* were found in the northern part of Malta Lake (sites M1–M4) and along the southern shore (M5–M10) (Fig. 1A). *Ulva* mats developed only in the littoral zone of lake in close proximity to vascular plants, particularly stands of *Phragmites australis*, *Potamogeton perfoliatus* and *P. pectinatus*. At the end of May, isolated *Ulva* thalli from the lake were observed as small filaments with lengths of a few centimeters and width of 0.5–1.0 mm in small qunatities lying and creeping on the surface of the sediment. The largest, free-standing freshwater *Ulva* mats appeared at the end of June and in July; thalli were not found in August. The *Ulva* developed in a diversified bottom structure containing organic, sandy and rocky gravel, and where a varying inclination of substrate was present (Table A1). The water depth varied from 0.20 to 1.50 m at sites M1–M10 from which the samples of water, thalli and sediment were collected. In water containing *Ulva*, the conductivity was 607.0 to 804.0 $\mu S\ cm^{-1}$, the pH varied from 6.57 to 8.99, and the Cl^- concentrations did not exceed 65.50 $mg\ L^{-1}$).

The second collections of freshwater *Ulva* came from the Nielba River, located in the city of Wągrowiec. The Nielba River has a length of approximately 27 km and reaches a width of 2.0–2.5 m at locations where the samples were collected. Freshwater *Ulva* (N1–N6) were collected from the river before it crossed at a right angle with the Wełna River (a rarely observed example of bifurcation) (Fig. 1B). Thalli from the freshwater *Ulva* in the Nielba River occurred from the end of June until the beginning of August. The large amounts of *Ulva* biomass generated at the river sites caused frequent river blockage and made the water flow rather difficult. The river bottom in places of *Ulva* development was organic with deposit thicknesses of approximately 0.5 m (Table A1). The water depth at the sites N1–N6 fluctuated from 0.7 to 1.3 m. The conductivity varied between 719.0 and 860.0 $\mu S\ cm^{-1}$, the pH ranged between 7.40 and 9.97, and concentrations of Cl^- were below110 $mg\ L^{-1}$).

From each site, three types of test samples were obtained: water, sediment and the *Ulva* thallus (Table A1).

Samples of water, sediment, and thalli were collected parallel at each site. Water samples were placed in a 0.5 L plastic container and transported over 3 h at 4 °C to the laboratory. Water samples were preserved in the field using 15% HNO_3. In the laboratory, the samples were filtered through a nitrocellulose microbiological filter, pore size of 0.45 μm. Filtered water samples were poured into 100 mL containers and deposited in a freezer at −20 °C.

Sediment samples were collected directly from underneath the *Ulva* mat using a plastic tube sediment sampler (diameter of 5 cm and length of 1 m). Only the surface layer of the sediment, approximately 1 cm thick, was collected for analysis. The sediment was placed in a plastic container and transported to the laboratory in cold storage. The sediment was then sieved through a nylon sieve (mesh diameter of 1 mm–500 μm) to remove plant debris, sand, stones and other impurities. The resulting sediment fraction was dried for 2 h at 105 °C and placed in 100 mL plastic containers.

Thalli samples were collected manually from the center of the macroalgae mats formed by *Ulva*. Approximately 500 g of thalli was collected and flushed with water from the given site. Thalli were then placed in a 1 L plastic container and transported to the laboratory at 4 °C. Next, *Ulva* thalli were rinsed five times with distilled water to remove the attached filamentous algae, vascular plants and other pollutants. Thalli were dried for 30 min on cellulose filter paper at room temperature. Later, thalli were dried for 2 h at 105 °C, and the dry substance obtained was placed into a 100 mL capacity plastic container.

Basic physicochemical parameters of the water (temperature, conductivity, concentration of oxygen, Cl^- and pH) at the *Ulva* sites were measured in the field using a YSI Professional Plus multimeter.

2.2. Samples mineralization

The metal extraction from *Ulva* thalli was carried out by digesting 0.5 g of algae with a mixture of 15 mL of 65% HNO_3 and 5 mL of 30% H_2O_2 in Teflon bombs in a MarsX5 microwave oven. All samples were mineralized in two steps: I – 300 s and 400 W; II – 300 s and 800 W.

The labile fraction of sediment was extracted with 1 M HCl. The samples were stored overnight to allow the complete removal of generated CO_2. Subsequently, the samples were shaken mechanically at room temperature for 1 h. The extract was centrifuged at 5000 rpm for 2 min. Metal extraction was carried out by digesting 0.4 g of the sediment. The other steps followed the procedure for macroalgae mineralization (see above).

Metal extraction from water was carried out by digesting 25 mL and following the same protocol as described for thalli and sediment.

2.3. Analysis of metal ion concentration

The content of Ca, Mg, Ni, Cd and Pb ions in the investigated samples were determined, after previous mineralization in a

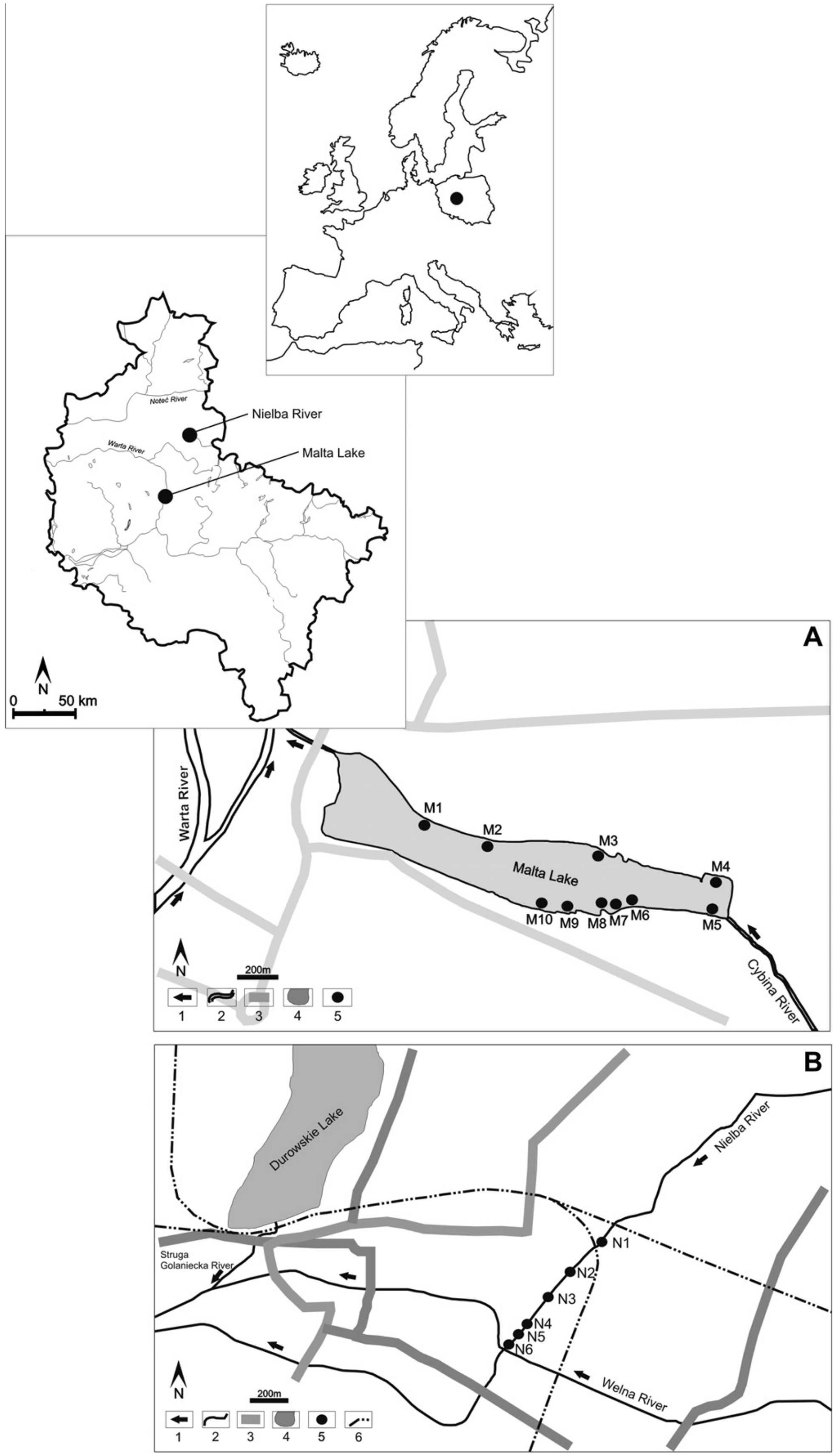

Fig. 1. Location of sampling sites along (A) Malta Lake and (B) Nielba River. 1 – water-flow direction, 2 – river, 3 – main road, 4 – lake, 5 – research stations, 6 – railway.

microwave oven Mars Xpress, on an inductively-coupled plasma emission spectrometer VISTA-MPX produced by VARIAN ICP. Calibration was performed using aqueous standard solutions. The mineralization was conducted in Teflon bombs in a mixture of 65%HNO_3 + H_2O_2 (3:1), at a power of 800 W and 400 W during 10 min. After the mineralization, the samples were quantitatively transferred into 10 mL flasks, filled with redistilled water and then, Ca, Mg, Ni, Cd and Pb ions were determined.

2.4. Statistical analysis

The software STATISTICA 9.0 was used for data analysis. The correlation between metal concentrations in algae, sediment and water was defined using Pearson's linear correlation coefficient. Differences in the average concentrations of metals between sites were estimated using the F test (ANOVA).

3. Results

3.1. Metal concentrations – Malta Lake

The highest concentrations of alkaline earth metals were noted in the *Ulva* thalli developing in Malta Lake. The average concentrations of calcium and magnesium in the algal thalli were 231881.5 $\mu g\ g^{-1}$ and 7025.7 $\mu g\ g^{-1}$ respectively. The lowest metal concentrations in the thallus were observed for cadmium, which did not exceed 1.0 $\mu g\ g^{-1}$ (Table A2). The average concentrations of metals in the algae decreases in the following order: Ca > Mg > - Ni > Pb > Cd. In the sediment, lower concentrations of alkaline earth metals and heavy metals were present compared to those in the thalli. In the sediment, the average concentrations of calcium and magnesium were 52542.6 of $\mu g\ g^{-1}$ and 1406.5 $\mu g\ g^{-1}$, respectively. The lowest metal concentrations were for cadmium, which did not exceed 1.0 $\mu g\ g^{-1}$, and were similar to that observed in the *Ulva* thalli. Moreover, the average metal concentrations in the sediment decreased in the same order as in *Ulva* thalli. In the water of the *Ulva* habitats, the concentrations of cadmium and lead were below the detection limit. On the other hand, the water samples contained the highest average concentrations of calcium and magnesium (83.0 $\mu g\ g^{-1}$ and 14.8 $\mu g\ g^{-1}$, respectively). The average metal concentrations in water decreased in the following order: Ca > Mg > Ni.

Temporal fluctuations in the metal concentration were observed in *Ulva* thalli collected from Malta Lake. The lowest concentrations of calcium were noted at M5, M6, M8 and M10 in June, and the highest concentrations were found in the second half of July, e.g. at the end of the vegetative *Ulva* season. This trend was especially noticeable in thalli samples collected from M4, M5 and M6, where the average concentration of calcium was approximately 5–7 times higher in July compared to June (Fig. 2A).

In thalli, there was a gradual decrease in magnesium concentration over time for 7 of the 10 sites. The highest concentrations of magnesium in *Ulva* thalli were in June (14179.0 $\mu g\ g^{-1}$), and the lowest in July (3740.36 $\mu g\ g^{-1}$) at the same site (M8; Fig. 2B).

The highest concentrations of nickel were observed in *Ulva* thalli in mid-July at M6 (417.06 $\mu g\ g^{-1}$). High concentrations of nickel also appeared in June at M5 (388.09 $\mu g\ g^{-1}$) and M10 (339.50 $\mu g\ g^{-1}$). A fluctuation in the concentration of nickel in thalli was noted at M6, M8 and M10. In thalli from M2, M3 and M4 there was an increase in the nickel concentration In contrast, there was a decrease in the nickel concentration in *Ulva* thalli occurred at M1 and M5 over time. Specifically, the nickel concentrations decreased from 388.09 $\mu g\ g^{-1}$ to 64.96 $\mu g\ g^{-1}$at M5 over a period of 5-week (Fig. 2C).

The concentrations of lead in *Ulva* thalli from Malta Lake fluctuated at different sites. No distinct trends were observed. The highest Pb concentrations occurred in thalli at M10 (14.73 $\mu g\ g^{-1}$) and M9 (10.50 $\mu g\ g^{-1}$), while the lowest concentrations were at M2 (0.75 $\mu g\ g^{-1}$), M4 (0.91 $\mu g\ g^{-1}$) and M5 (0.94 $\mu g\ g^{-1}$) (Fig. 2D).

The cadmium concentrations of in the thalli samples were generally below the detection limit (BDL < 0001). Only 10 samples from seven sites had detectable cadmium concentrations. The highest concentration of cadmium was found in thalli sampled in July at M6 (1.03 $\mu g\ g^{-1}$) (Fig. 2E).

3.2. Metal concentrations – Nielba River

High levels of alkaline earth metals (Ca and Mg) were detected in thalli from the Nielba River. The average concentrations of calcium and magnesium were 235253.26 $\mu g\ g^{-1}$and 6168.01 $\mu g\ g^{-1}$, respectively. The lowest concentrations of cadmium did not exceed1.5 $\mu g\ g^{-1}$ (Table A3). The average concentrations of metals in *Ulva* thalli decreased in the following order: Ca > Mg > - Ni > Pb > Cd. In the organic deposits obtained from river sediment beneath the algal mats, lower metal concentrations were detected, as compared to those in thalli and in water samples. The average concentrations of calcium and magnesium in the sediment samples were 38057.60 $\mu g\ g^{-1}$ and 2888.16 $\mu g\ g^{-1}$, respectively. Similar to the *Ulva* thalli the sediment samples contained the lowest cadmium concentration of all metals studied, with a maximum of 0.42 $\mu g\ g^{-1}$. The average metals concentrations in the river sediment decreased in the following order: Ca > Mg > Pb > Ni > Cd. In water sampled from the *Ulva* habitat, the average concentrations of alkaline earth metals were the highest of all the metals analyzed, with concentrations of 122.04 $\mu g\ g^{-1}$ and 18.40 $\mu g\ g^{-1}$ for calcium and magnesium, respectively. The cadmium concentrations were below the detection limit (BDL < 0.001) in all water samples from the *Ulva* sites in the Nielba River. The average concentrations of detectable metals in the water samples decreased in the following order: Ca > Mg > Ni > Pb (Table A2).

Concentrations of calcium in *Ulva* thalli from the river varied over time, and an increasing trend was observed only at N3, while a decreasing trend was observed at N1 and N2. At the remaining sites, the metal concentrations were subject to fluctuations without a clear trend. The highest concentrations of calcium in algae thalli were found in July at N1 (305885.09 $\mu g\ g^{-1}$), and the lowest was found in June at N4 (74428.43 $\mu g\ g^{-1}$) (Fig. 3A).

Our results show that monthly changes in magnesium concentrations in thalli fluctuated near the mean over time. An increase in the magnesium concentration at the end of June was only at N4, with a maximum concentration of 15557.94 $\mu g\ g^{-1}$. Concentrations of magnesium in the *Ulva* thalliat this site were approximately 2.5 times higher comparedto the average concentration from all sites (Fig. 3B).

The changes in nickel concentration in the macroalgae at individual sites and in time were not significant. In two cases, a significant increase in nickel concentration was recorded in the *Ulva* thalli. In June, the beginning of the vegetative period for river *Ulva,* the nickel concentration at N4 was 75.87 $\mu g\ g^{-1}$. Equally high nickel concentrations were found in August at N1, with a value of 55.96 $\mu g\ g^{-1}$. At the remaining sites values of nickel ranged between 22.99 and 28.98 $\mu g\ g^{-1}$ (Fig. 3C).

In *Ulva* thalli at the three river sites (N1–N3), an increase in lead concentration was observed in samples over time. The highest concentrations of lead appeared in thalli during August at N2 (13.25 $\mu g\ g^{-1}$) and N1 (9.96 $\mu g\ g^{-1}$). The lowest Pb concentrations in *Ulva* thallus were recorded in July at N4 (1.09 $\mu g\ g^{-1}$) (Fig. 3D).

Concentrations of cadmium during all periods and sites from the Nielba River were above BDL. In the majority of thalli samples, the concentrations of cadmium fluctuated from 0.09 to 0.18 $\mu g\ g^{-1}$. An elevated cadmium concentration was observed at only two sites. In June at N4, the cadmium concentration reached

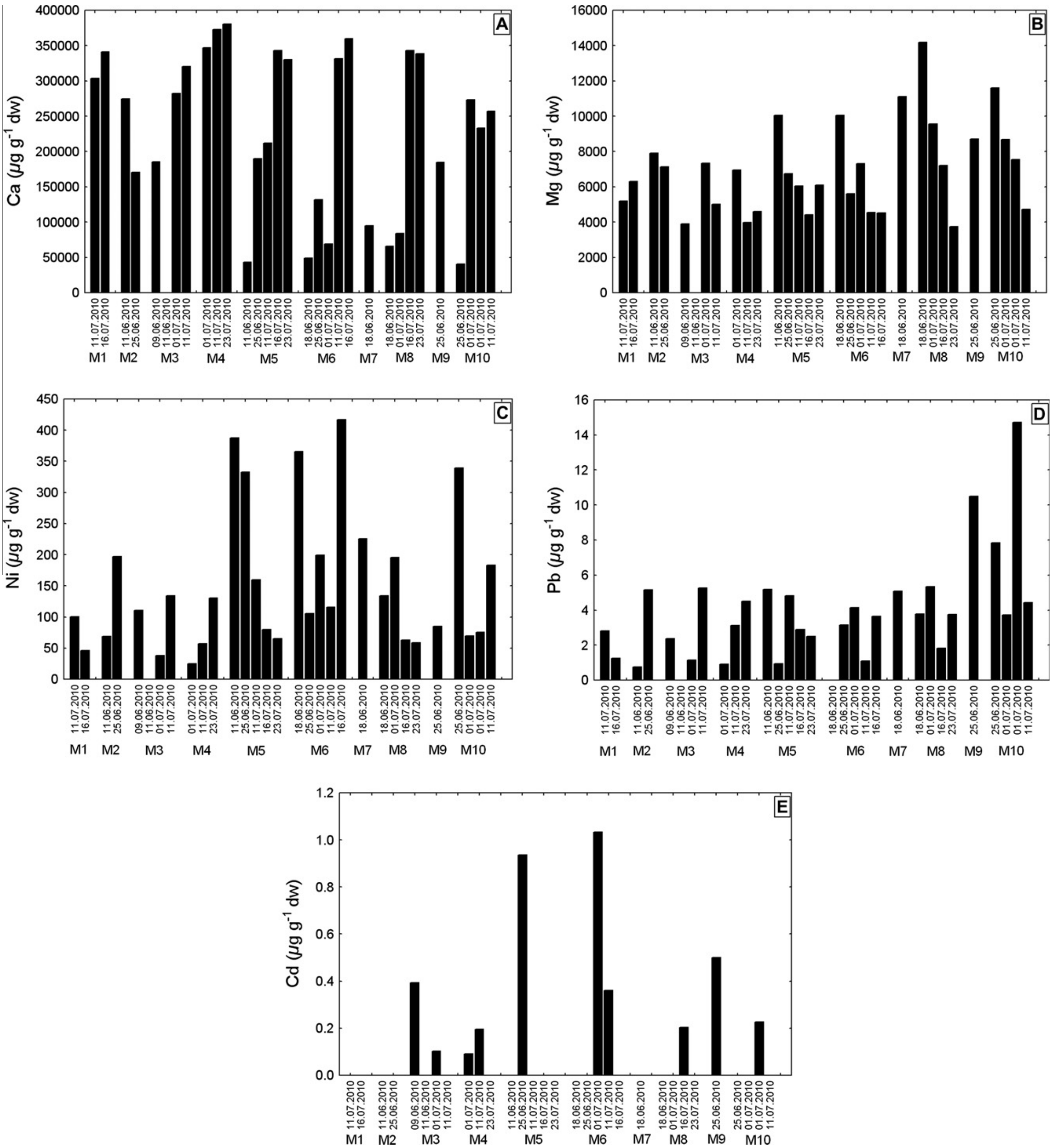

Fig. 2. Variation in metal levels in *Ulva* during the period from June to July 2010 at each sampling site at Malta Lake. M1–M10 – sampling sites in the lake.

0.48 µg g^{-1},and in August at N2, the concentration reached 0.27 µg g^{-1} (Fig. 3E). In the case of N3, concentrations of cadmium in *Ulva* thallus remained constant at 0.09 µg g^{-1} from mid-July until the beginning of August.

3.3. Comparison the metals concentration in Ulva from two freshwater ecosystems

No statistically significant differences in the average concentration of the alkaline earth metals (Ca and Mg) wereobserved when comparing *Ulva* from the river and lake sites. An *F* test (ANOVA) performed for the mean values of calcium and magnesium in thalli from the lake and river gave $F_{(1;49)} = 0.0157$; ($P = 0.90$) and $F_{(1;48)} = 1.2764$; ($P = 0.26$), respectively (Fig. 4A and D). Similarly, the average concentrations of cadmium and lead in *Ulva* thalli did not differ between samples from the different water ecosystems (Fig. 4B and C). In the case of nickel, the differences between concentrations in thalli from the river and lake were statistically significant with values of $F_{(1;49)} = 25.0$ for $P = 0.00001$ (Fig. 4E). Moreover, the average nickel concentration in thalli from the lake was 152.2 µg g^{-1} while that in thalli from the river was 28.90 µg g^{-1}. Our results show that in the *Ulva* thalli collected from Malta Lake, the concentrations of nickel were on average five times higher than in thalli coming from the Nielba River.

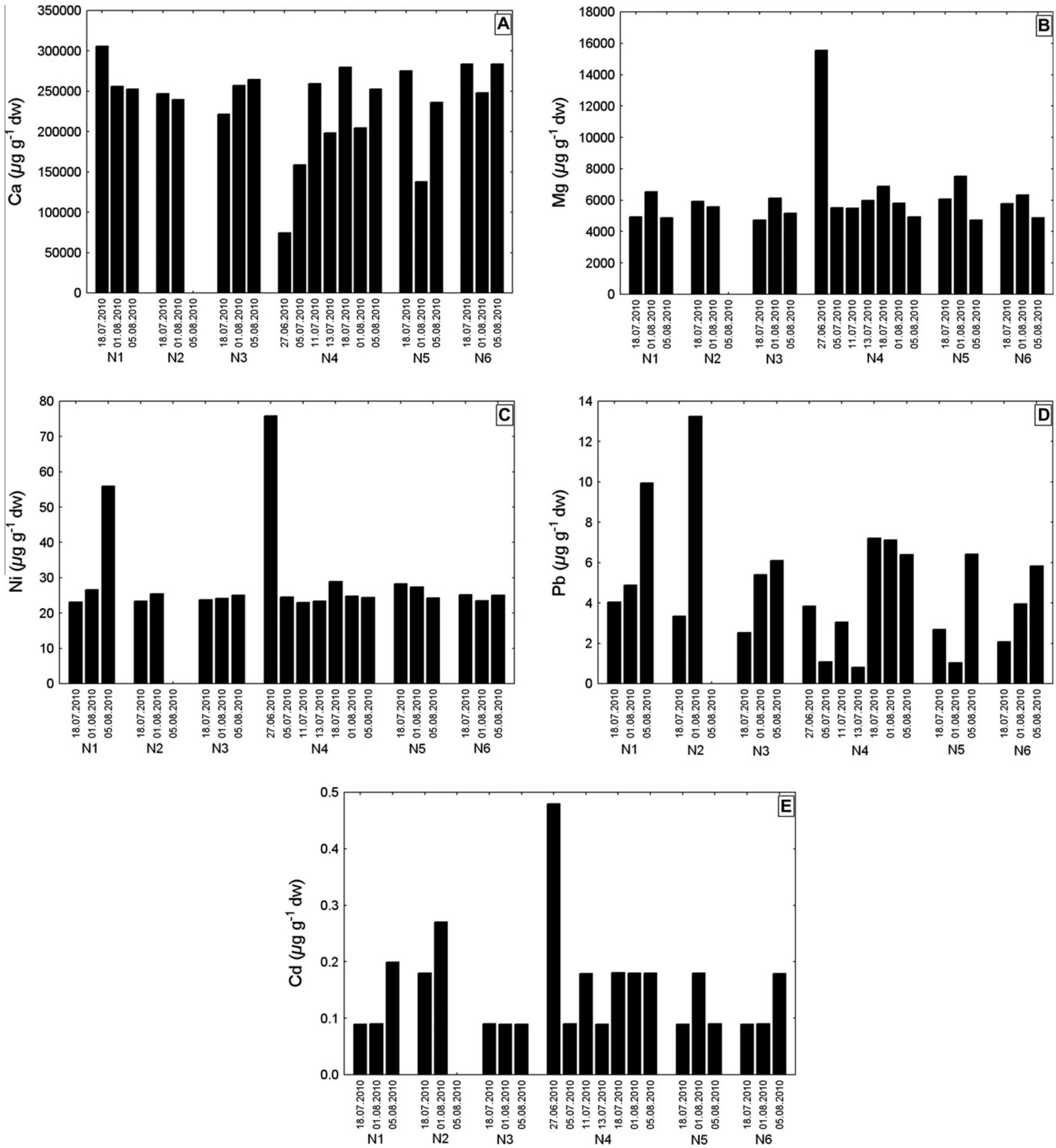

Fig. 3. Variation in metal concentration in *Ulva* during the period from June to August 2010 for each sampling site in the Nielba River. N1–N6 – sampling sites in the river.

3.4. Correlations

A Pearson's linear correlation was performed between the recorded concentrations of heavy metals and alkaline earth metals in *Ulva* thalli, water and sediment from each site along the lake and river.

In Malta Lake, a series of statistically significant correlations between concentrations of particular metals in water and thalli, as well as between concentrations found in the sediment, were observed at all sites (M1–M10). A negative correlation between Mg and Ca concentrations ($r = -0.78$; $P < 0.001$) was noticed in thalli (Fig. 5). Moreover, a strong correlation between the concentrations of Mg and Cd ($r = 0.76$; $P < 0.001$) was recorded in the lacustrine sediment collected from beneath macroalgae mats. Strong positive correlations between concentrations of Pb and Ni ($r = 0.68$; $P < 0.001$) and between concentrations of Mg and Ca ($r = 0.60$; $P < 0.001$) were obtained in the water samples. Many of the observed correlations, however, were not statistically significant (Tables 1 and A4).

Statistically significant correlations were found in samples from the Nielba River at N1–N6 (Table 2). The strongest correlation was observed between Pb and Cd ($r = 0.97$; $P < 0.001$) (Fig. A1A), Pb and Mg ($r = 0.94$; $P < 0.001$), Mg and Cd ($r = 0.95$; $P < 0.001$), and Mg and Ca ($r = 0.93$; $P < 0.001$) concentrations in the sediment.

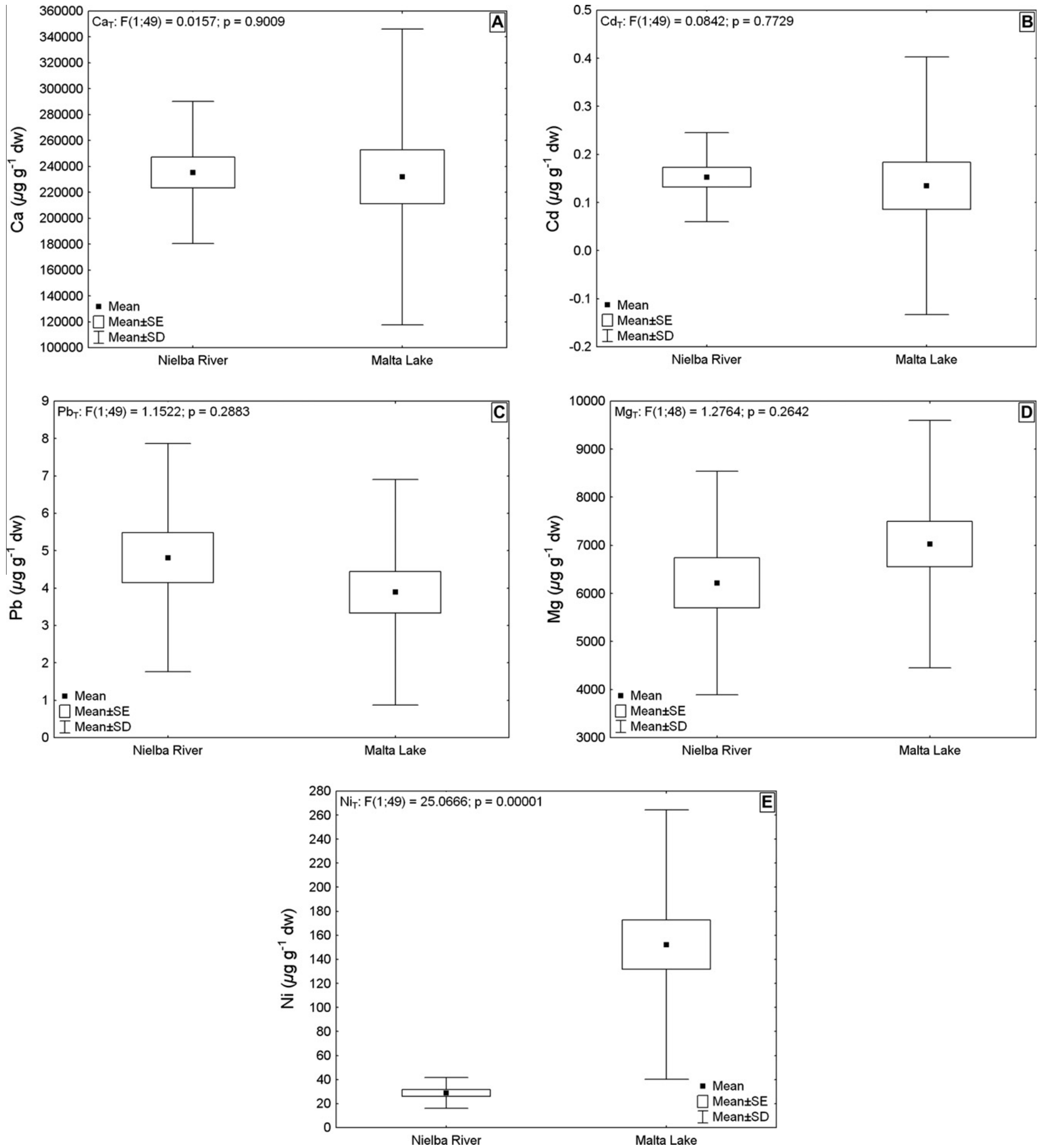

Fig. 4. Comparison of the mean concentrations of metals in freshwater *Ulva* (μg g^{-1} dw) between populations from Malta Lake and the Nielba River. $_T$ – *Ulva* thalli, SD – standard division, SE – standard error.

Statistically significant negative correlations between concentrations of Mg and Ca ($r = -0.71$; $P < 0.001$) and between Ni and Cd ($r = 0.77$; $P < 0.001$) were noted in the *Ulva* thalli obtained from Nielba River. The remaining correlations are shown in Table 2.

Pearson's correlations carried out for data from all sites in the lake and river ($N = 50$) gave the strongest correlations for the concentrations of Mg and Cd in the sediment ($r = 0.88$; $P < 0.001$) (Fig. A1B) and between Mg and Ca ($r = -0.73$; $P < 0.001$) in *Ulva* thallus.

4. Discussion

Organisms used as bioindicators of pollution in aquatic environments have been characterized by Phillips (1990) and Rainbow and Phillips (1993). Such organisms should reflect only contaminants specific to a particular site and found in many different locations, ensuring a wide geographic relevance (Melville and Pulkownik, 2007). Bioindicators should also be sensitive to specific pollutants and tolerate large concentrations of these pollutants in the

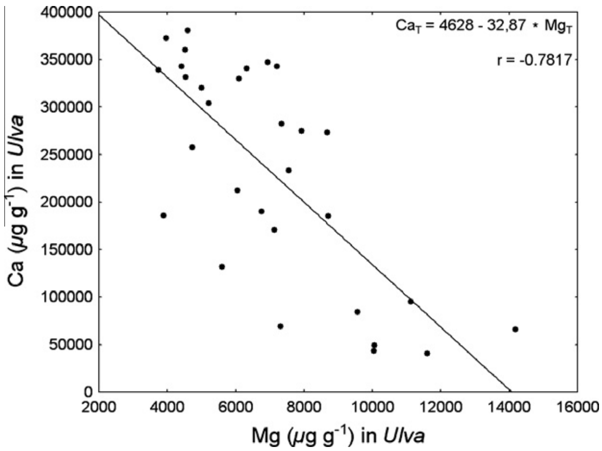

Fig. 5. Correlation between Ca and Mg concentration in *Ulva* from Malta Lake. Pearson's coefficient of correlation: $r = -0.7817$, $P < 0.001$, $N = 29$, $_T$ – *Ulva* thalli.

environment (Rainbow and Phillips, 1993). In this study, the freshwater *Ulva* populations represent a wide-spread class of algae that can be found in waters with varying salinity in all areas of the earth, except the Arctic (Bäck et al., 2000).

Determining the identity of this algal genus is simple and irrespective of whether the thallus sample is monostromatic tubular or distromatic frondose. *Ulva* species may alsoto develop and reproduce in heavily polluted waters with high concentrations of heavy metals and other nutrients (Reed and Moffat, 2003). In the light of this information, freshwater *Ulva* populations, similar to their marine counter parts, are fulfilling the required guidelines for use as bioindicators.

Universally, species of macroalgae of the genera *Ulva* are regarded as good bioindicators of heavy metal contamination in sea water (Villares et al., 2002; Ustunada et al., 2010). However, the legitimacy of using these organisms for monitoring contaminated waters requires having complete information about metal concentrations in the macroalgae habitat. Such information is obtained through the analysis of sediment and water samples, plus different organisms that grow with the *Ulva* species (Phillips, 1977). The concentration of many elements (including heavy metals) in algae fluctuate with seasons due to changes in their bioaccumulation during development and independent of ion concentrations in the environment (Villares et al., 2002). It was also noticed that reliable monitoring of heavy metal pollution requires *Ulva* to cover a large area and be found in at least a dozen sites (Say et al., 1990). Thorough biomonitoring using *Ulva* assumes that samples will be taken with great frequency during the entire algal life cycle. For marine *Ulva* populations, it is possible to sample thalli over a longer period during the year, particularly in areas with a warm climate. Unfortunately, in the case of freshwater *Ulva* appearing in the center of Europe (areas dominated by a varying temperate climate), it is only possible to carry out examinations from spring to early autumn (May–September). Freshwater *Ulva* populations are only found in water bodies during this time (Messyasz and Rybak, 2009; 2010; Rybak and Messyasz, 2010). Other circumstances also limit the ability to collect thalli samples from freshwater *Ulva* in ecosystems of flowing waters, including sudden intense rainfall, floods and river channel dredging. In our work, two freshwater *Ulva* populations from Malta Lake and the Nielba River were examined. Thalli of freshwater *Ulva* appeared at these sites during the warmest months of the year (June–August) and disappeared when cooler weather and periods of intense, sustained rainfall arrived.

Research concerning *Ulva* for water monitoring has been described for littoral sea ecosystems (Malea and Haritonidis, 1999), the oceanic coast (Orduña-Rojas and Langoria-Espinoza, 2006) oceanic islands (Tabudravu et al., 2002) and estuaries (Say et al., 1990; Daka, 2005). Knowledge of *Ulva* populations appearing in freshwater ecosystems (<0.5‰ according to the Venetian system of water salinity classification; Dethier, 1992) remains limited. The use of macroalgae for monitoring metal contamination in fresh waters is quite limited when compared with other methods using species of vascular plants, such as *Elodea canadensis*, *E. nuttallii* (Thiébaut et al., 2010; Hansen et al., 2011) or *Myriophyllum spicatum* (Li et al., 2010). Macroalgae, however, have up to 10 times greater ability to bioaccumulate metals when compared with vascular plants (Michalak and Chojnacka, 2008). These results highlight the potential of using freshwater *Ulva* taxa as biomonitors, particularly for river and lake waters, which are exposed mostly to heavy metal contamination.

In the present study, the highest metal concentrations were for calcium and magnesium in thalli of freshwater *Ulva*, both in the lake and river populations. In examination of metal concentrations from thalli of the marine forms of *Ulva*, Ca and Mg are rarely analyzed because their levels do not provide essential information about water contamination. The presence of considerable concen-

Table 1
Coefficients of linear Pearson's correlation (r) between metal concentrations in water, sediment and alga, considering all samples from Malta Lake.

	Ca_T	Ca_W	Ca_S	Cd_T	Cd_W	Cd_S	Mg_T	Mg_W	Mg_S	Ni_T	Ni_W	Ni_S	Pb_T	Pb_W	Pb_S
Ca_T	1.0	$-0.25^{n.s.}$	$-0.24^{n.s.}$	$-0.15^{n.s.}$	$-0.15^{n.s.}$	$-0.09^{n.s.}$	$-\underline{0.78}^{***}$*	$-0.07^{n.s.}$	$-0.04^{n.s.}$	$-\underline{0.57}^{**}$	0.57^{**}	$0.05^{n.s.}$	$-0.25^{n.s.}$	$0.31^{n.s.}$	$0.09^{n.s.}$
Ca_W		1.0	$0.24^{n.s.}$	$-0.03^{n.s.}$	$\underline{0.37}^{*}$	$0.06^{n.s.}$	$0.13^{n.s.}$	$\underline{0.60}^{***}$	$0.08^{n.s.}$	$0.19^{n.s.}$	$0.06^{n.s.}$	$0.08^{n.s.}$	$-0.17^{n.s.}$	$0.28^{n.s.}$	$-0.11^{n.s.}$
Ca_S			1.0	$0.34^{n.s.}$	$-0.13^{n.s.}$	$0.10^{n.s.}$	$0.14^{n.s.}$	$0.07^{n.s.}$	$\underline{0.49}^{**}$	$0.26^{n.s.}$	$-0.30^{n.s.}$	$0.13^{n.s.}$	$-0.22^{n.s.}$	$-0.18^{n.s.}$	$0.33^{n.s.}$
Cd_T				1.0	$-0.17^{n.s.}$	$-0.16^{n.s.}$	$-0.04^{n.s.}$	$0.15^{n.s.}$	$-0.02^{n.s.}$	$0.10^{n.s.}$	$-0.22^{n.s.}$	$-0.23^{n.s.}$	$0.04^{n.s.}$	$-0.14^{n.s.}$	$-0.17^{n.s.}$
Cd_W					1.0	$0.31^{n.s.}$	$0.07^{n.s.}$	$0.29^{n.s.}$	$0.13^{n.s.}$	$0.23^{n.s.}$	$-0.02^{n.s.}$	$0.07^{n.s.}$	$0.09^{n.s.}$	$0.13^{n.s.}$	$0.17^{n.s.}$
Cd_S						1.0	$0.09^{n.s.}$	$-0.10^{n.s.}$	$\underline{0.76}^{***}$	$0.16^{n.s.}$	$-0.13^{n.s.}$	$0.21^{n.s.}$	$-0.07^{n.s.}$	$-0.02^{n.s.}$	$0.31^{n.s.}$
Mg_T							1.0	$-0.19^{n.s.}$	$0.05^{n.s.}$	$0.31^{n.s.}$	$-\underline{0.44}^{*}$	$0.06^{n.s.}$	$0.20^{n.s.}$	$-0.37^{n.s.}$	$-0.02^{n.s.}$
Mg_W								1.0	$-0.13^{n.s.}$	$0.04^{n.s.}$	$-0.09^{n.s.}$	$0.10^{n.s.}$	$0.14^{n.s.}$	$0.21^{n.s.}$	$-0.16^{n.s.}$
Mg_S									1.0	$0.21^{n.s.}$	$-0.11^{n.s.}$	$0.04^{n.s.}$	$-0.03^{n.s.}$	$0.07^{n.s.}$	$\underline{0.46}^{*}$
Ni_T										1.0	$-0.14^{n.s.}$	$-0.13^{n.s.}$	$0.04^{n.s.}$	$-0.16^{n.s.}$	$-0.09^{n.s.}$
Ni_W											1.0	$0.03^{n.s.}$	$-0.19^{n.s.}$	$\underline{0.68}^{***}$	$-0.17^{n.s.}$
Ni_S												1.0	$-0.13^{n.s.}$	$-0.26^{n.s.}$	$0.20^{n.s.}$
Pb_T													1.0	$-0.20^{n.s.}$	$-0.06^{n.s.}$
Pb_W														1.0	$-0.13^{n.s.}$
Pb_S															1.0

n.s., Not significant; T, *Ulva* thalli; W, water; S, sediment; $N = 29$.
[*] Significant correlation ($P < 0.05$).
[**] Significant correlation ($P < 0.01$).
[***] Significant correlation ($P < 0.001$).

Table 2
Coefficients of linear Pearson's correlation (*r*) between metal concentrations in water, sediment and alga, considering all samples from the Nielba River.

	Ca_T	Ca_W	Ca_S	Cd_T	Cd_W	Cd_S	Mg_T	Mg_W	Mg_S	Ni_T	Ni_W	Ni_S	Pb_T	Pb_W	Pb_S
Ca_T	1.0	$-0.03^{n.s.}$	$0.12^{n.s.}$	$\underline{-0.57}^{**}$	$0.02^{n.s.}$	$0.21^{n.s.}$	$\underline{-0.71}^{***}$	$-0.33^{n.s.}$	$0.20^{n.s.}$	$\underline{-0.54}^{*}$	$0.08^{n.s.}$	$-0.34^{n.s.}$	$0.28^{n.s.}$	$-0.09^{n.s.}$	$0.22^{n.s.}$
Ca_W		1.0	$-0.34^{n.s.}$	$0.06^{n.s.}$	$\underline{0.65}^{**}$	$\underline{-0.50}^{*}$	$-0.07^{n.s.}$	$0.29^{n.s.}$	$-0.34^{n.s.}$	$-0.07^{n.s.}$	$0.05^{n.s.}$	$-0.28^{n.s.}$	$0.26^{n.s.}$	$-0.42^{n.s.}$	$-0.42^{n.s.}$
Ca_S			1.0	0.004	$-0.17^{n.s.}$	$\underline{0.90}^{***}$	$-0.20^{n.s.}$	$-0.27^{n.s.}$	$\underline{0.93}^{***}$	$-0.20^{n.s.}$	$-0.04^{n.s.}$	$0.18^{n.s.}$	$0.10^{n.s.}$	$0.18^{n.s.}$	$\underline{0.90}^{***}$
Cd_T				1.0	$0.32^{n.s.}$	$-0.01^{n.s.}$	$\underline{0.77}^{***}$	$0.30^{n.s.}$	$-0.04^{n.s.}$	$\underline{0.77}^{***}$	$-0.15^{n.s.}$	$\underline{0.51}^{**}$	$0.30^{n.s.}$	$-0.07^{n.s.}$	$-0.06^{n.s.}$
Cd_W					1.0	$-0.27^{n.s.}$	$0.07^{n.s.}$	$0.09^{n.s.}$	$-0.18^{n.s.}$	$0.26^{n.s.}$	$-0.18^{n.s.}$	$0.05^{n.s.}$	$0.21^{n.s.}$	$\underline{-0.47}^{*}$	$-0.24^{n.s.}$
Cd_S						1.0	$-0.20^{n.s.}$	$-0.36^{n.s.}$	$\underline{0.95}^{***}$	$-0.18^{n.s.}$	$-0.02^{n.s.}$	$0.26^{n.s.}$	$0.07^{n.s.}$	$0.19^{n.s.}$	$\underline{0.97}^{***}$
Mg_T							1.0	$0.30^{n.s.}$	$-0.24^{n.s.}$	$\underline{0.77}^{***}$	$0.04^{n.s.}$	$\underline{0.53}^{*}$	$-0.16^{n.s.}$	$0.11^{n.s.}$	$-0.23^{n.s.}$
Mg_W								1.0	$-0.29^{n.s.}$	$0.20^{n.s.}$	$\underline{0.46}^{*}$	$0.15^{n.s.}$	$0.12^{n.s.}$	$0.04^{n.s.}$	$-0.36^{n.s.}$
Mg_S									1.0	$-0.20^{n.s.}$	$-0.01^{n.s.}$	$0.24^{n.s.}$	$0.09^{n.s.}$	$0.11^{n.s.}$	$\underline{0.94}^{***}$
Ni_T										1.0	$-0.04^{n.s.}$	$0.42^{n.s.}$	$0.15^{n.s.}$	$-0.04^{n.s.}$	$-0.22^{n.s.}$
Ni_W											1.0	$-0.12^{n.s.}$	$0.01^{n.s.}$	$\underline{0.48}^{*}$	$0.04^{n.s.}$
Ni_S												1.0	$-0.22^{n.s.}$	$0.12^{n.s.}$	$0.25^{n.s.}$
Pb_T													1.0	$-0.33^{n.s.}$	$0.06^{n.s.}$
Pb_W														1.0	$0.16^{n.s.}$
Pb_S															1.0

n.s., Not significant; T, *Ulva* thalli; W, water; S, sediment; $N = 21$.
* Significant correlation ($P < 0.05$).
** Significant correlation ($P < 0.01$).
*** Significant correlation ($P < 0.001$).

trations of these elements in freshwater *Ulva* thalli is a result of the rich encrustations on the outside and interior of the thallus. These crusts are formed by crystals of calcium or magnesium carbonate, which constitute up to 48% the dry biomass of the algae (Messyasz et al., 2010). It was observed that calcification of thalli from the freshwater taxa of *Ulva* is related to an increase in the brittleness of thalli under the influence of physical factors (e. g. waves action), resulting in a change of the thallus surface from smooth to rough and uneven to the touch. In cells of *U. rigida* collected from Thermaikos Bay in Greece, Ca concentrations fluctuated in the range from 530 to 3930 $\mu g\ g^{-1}$ (Malea and Haritonidis, 2000). In the freshwater *Ulva* thalli from Malta Lake and the Nielba River, concentrations of calcium were 40 898.09–380 420.9 $\mu g\ g^{-1}$. However, Mg concentrations in the *U. rigida* thallus were 11 600 to 38 300 $\mu g\ g^{-1}$ (Malea and Haritonidis, 2000), while levels found in the freshwater *Ulva* thallus were lower, ranging from 3740.36 to 15 557.94 $\mu g\ g^{-1}$dw. Elevated magnesium ion concentrations in thalli samples of marine *Ulva* species relative to the freshwater forms are due to the presence of higher concentrations of this element in salt water, where magnesium is found mainly in the form of $MgCl_2$ and $MgSO_4$ (Dojlido, 1995). The abundance of Ca ions in the freshwater *Ulva* thallus results from the presence of $CaCO_3$ crystals formed on the surface of the thallus as a result of calcification to preserve the carbonate balance during photosynthesis (Siong and Asaeda, 2009), and as a result of the chemical composition of fresh waters, where Ca ions are present in greater concentrations relative to Mg ions (Dojlido, 1995).

In thalli of freshwater *Ulva* from both aqueous ecosystems, nickel was present in the highest concentrations ($\sim$101.43 $\mu g\ g^{-1}$) of all heavy metals examined, while the average lead and cadmium concentrations were relatively low at 4.27 $\mu g\ g^{-1}$ and 0.14 $\mu g\ g^{-1}$, respectively. During the present study, nickel concentrations in freshwater *Ulva* thalli were as high as 417.06 $\mu g\ g^{-1}$. To date, the highest concentrations of nickel have been in monostromatic tubular thalli of *Ulva* species (identifiedas *Enteromorpha* sp.) from the coast of N.W. Spain, ranging from 7.64 to 339.0 $\mu g\ g^{-1}$ (Puente, 1992). The most common species from the Baltic Sea, *U. intestinalis,* collected from the coast of Sweden, revealed thalli nickel concentrations below 70.0 $\mu g\ g^{-1}$ (Hägerhäll, 1973). Increased concentrations of this metal have been observed in *U. clathrata* (34.55 $\mu g\ g^{-1}$) and *U. reticulata* (39.06 $\mu g\ g^{-1}$) collected from littoral waters of Goa Bay in India (Agadi et al., 1978). Taking into account the natural background levels, our finding that freshwater thalli accumulate large amounts of nickel, independent of the type of ecosystem (lake, river), demonstrates the potential importance of using freshwater *Ulva* as a biomonitor of heavy metal pollution in water.

It was found that concentrations of lead in *Ulva* thalli from the lake and river were between 0 to 14.73 $\mu g\ g^{-1}$. Interestingly, previous studies have shown different levels of lead in marine species of macroalgae, including values as high as 87.4 $\mu g\ g^{-1}$for *U. linza* (Malea and Haritonidis, 1999), 62 $\mu g\ g^{-1}$for *U. flexuosa* (Tabudravu et al., 2002) and approximately 15.0 $\mu g\ g^{-1}$ for *U. intestinalis* (Daka, 2005). These values are up to two times higher than the average Pb concentrations found in freshwater *Ulva* thalli. This supports the concept that freshwater populations of macroalgae have a far lower ability to accumulate lead than species from littoral and oceanic zones, such as *U. flexuosa and U. lactuca.* As described in previous findings, the average Pb concentrations in thalli of marine species (e.g., *U. crinita*) were below 3 $\mu g\ g^{-1}$in only a few cases (Hornung et al., 1992) (Table A5).

The Cd concentration in thalli of freshwater *Ulva* were very low, ranging from 0–1.03 $\mu g\ g^{-1}$. At the lake sites, Cd concentrations were below the detection limit (DL = 0001). Low concentrations of Cd ($\sim$0.10 $\mu g\ g^{-1}$) have been recorded in other *Ulva* species, including *U. compressa* from Israel (Hornung et al., 1992) and *U. linza* from Thermaikos Bay (Malea and Haritonidis, 1999). These had similar values to the average concentrations recorded here for the freshwater *Ulva* thalli. In another study (Wahbeh, 1985), cadmium concentrations in the thalli of *U. clathrata* were observed to be 8.1 $\mu g\ g^{-1}$. Other marine populations, including *U. compressa* and *U. lactuca*, had Cd concentrations of 7.8 and 5.3 $\mu g\ g^{-1}$, respectively (Abdallah et al., 2005; Andrade et al., 2006). These observed values of Cd are 40–50 times higher than the concentrations – in samples of the freshwater *Ulva* thalli (Table A5).

Heavy metals concentrations in thalli of marine *Ulva* species appear dependent on local conditions. Fluctuations in the concentration of heavy metals (Ni, Cd, Pb) in freshwater *Ulva* were also noted. In freshwater *Ulva* thallus, sudden increases in the concentrations of Ni or Pb were observed at individual sites, which differed from mean values (Fig. 3). The order of metal concentrations detected in freshwater *Ulva* thalli both for the river and the lake populations are as follows: Ca > Mg > Ni > Pb > Cd. For *U. rigida* from Tharmaikos Bay in Greece, the order of metal concentrations in the algae was as follows: Mg > Ca > Pb > Ni > Cd (Malea and Haritonidis, 2000). In *U. rigida* populations from the Paludedella Rosa Bay (Lagoon Venice), the concentrations showed the following trend: Ni > Pb > Cd (Favero et al., 1996). Also, for *U. compressa* from the Gulf of Aden, the order of metal concentrations was: Ni > Pb > Cd (Al-Shwafi and Rushdi, 2008). The order of heavy metal concentrations, for marine species such as *U. rigida* from the Lagoon Venice or *U. compressa* (Favero et al., 1996), correlated closely with the order observed for freshwater *Ulva*. Moreover, Cd had the lowest concentrations for both thalli from marine and freshwater *Ulva* species. The poor accumulation of cadmium seems to be a feature typical for all species of *Ulva,* including species with monostromatic tubullar and distromatic frondose thalli. As presented above, the order of metal concentrations indicate a diversification in the accumulation potential of individual species (e.g., for *U. lactuca* and *U. linza*) and show us differences among populations of the same species (e.g., *U. rigida*) including similarities across species (e.g., *U. rigida–U. lactuca*).

According to Phillips (1990), a good biomonitor should demonstrate strong linear correlations between metal concentrations recorded in its cells and water from the environment. Such relationships are particularly apparent in laboratory tests concerning the bioaccumulation and biosorption of metals by species from the genus *Ulva* (Chan et al., 2003; Chojnacka, 2008). Tabudravu et al. (2002) observed a significantly high linear correlation between concentrations of Cu, Zn and Pb in thalli of *U. flexuosa* and their levels in aquaculture. In this experiment, positive correlations were also observed for levels of metals in the sediment and in the *U. flexuosa* thalli, especially with respect to Pb concentrations. Strong correlations between values of heavy metals in the thallus and experimentally enriched media were observed for *U. crinita* (Chan et al., 2003). A number of relationships have been recorded between heavy metal concentrations in marine *Ulva* thalli and in the sediment and water from the surroundings; however, the correlations were not as clear as in experiments conducted *ex situ* under cultivation conditions. In a study by Malea and Haritonidis (2000), *U. rigida* collected from the Thermaikos Bay showed weak correlation between Pb in the thallus and in marine water. With respect to the other examined metals, it was not observed, so that the levels found in the algal thalli depended directly on the concentration of metals in the salt water. Moreover, in samples of *U. rigida* thalli from the Evros river outflow to the Aegean Sea, negative correlations between Pb concentrations in the algae and in water with positive correlations between Pb values in *U. rigida* and sediment (Boubonari et al., 2008). Although there were positive correlations between Pb concentrations in the thallus and sea water and between the thallus and the sediment as well as negative correlations for Cd concentration between the thallus and sea water and between the thallus and sediment in *U. linza,* none of these results were statistically significant (Malea and Haritonidis, 1999).

The present study shows that a few weakly significant correlations between the concentrations of different metals in the freshwater algal thalli and the water could be determined. Negative correlations were found for Ni and Ca and also between Ni and Mg. With respect to biomonitoring with the *Ulva* species, the correlations between concentrations of metals in thalli and the sediment are also important (Villares et al., 2002). In the majority of research findings connected with marine algae species (*U. linza* and *U. lactuca*), strong correlations between the concentrations of metals in the thalli and the sediment have not been observed (Malea and Haritonidis, 1999; Kamala-Kannan et al., 2008). However, positive correlations of copper and lead concentrations from *U. rigida* and the sediment were reported. The value of the Sperman's rank coefficient for Cu and Pb were: $rs = 0.67$ and $rs = 0.61$, respectively, while the remaining correlations examined for Zn and Cd were positive, although statistically not significant (Boubonari et al., 2008). Our investigation showed significant correlation between the concentrations of nickel in the thalli and of calcium in the sediment in the case of freshwater *Ulva* populations both at Malta Lake and the Nielba River sites.

Overall, many positive and negative relationships between the levels of different heavy metals in algae thalli are known for the marine *Ulva* species (Favero et al., 1996). In thalli of *U. lactuca, U. compressa* and *U. flexuosa* from the coast of the Red Sea had a positive correlations between concentrations of Mn–Ni; Pb–Cr and Pb–Cd, while at the other site (Mars Alam) the strongest correlations were between Cr–Pb and Ni–Cd (Abdallah et al., 2005). Statistically significant correlations were also observed between Cd and Pb concentrations in thalli of *U. rigida* from the Lagoon of Venice and for Cu–Ni and Fe–Pb (Favero et al., 1996). In the case of freshwater *Ulva* populations, a significant negative correlation between magnesium and calcium concentrations was recorded in the algal thalli. In addition, the value of the coefficient for the Pearson's linear correlation taking all samples into consideration ($n = 50$) was $r = -0.73$ for $P < 0.001$. We suggest that this negative correlation between the Mg and Ca results from the mutual exclusion in the process of precipitating calcium and magnesium carbonate on the surface of the thallus.

In fact, the natural processes connected with the history of the species and taxa diversification fundamentally influence the heavy metal absorption by *Ulva* species (Abdallah et al., 2005). Environmental conditions dominating in the chlorophyte macroalgae habitat, including physical factors, the chemical specificity of dissolved heavy metals in the water, the salinity and concentrations of nutrients, all play a role in the process of metal accumulation (Tabudravu et al., 2002). Therefore, the above-mentioned habitat parameters should be taken into consideration and measured at the same time as the level of heavy metals in all samples (i.e., thallus, sediment, water).

Under typical conditions, the concentrations of heavy metals in salt water are very low and because of that, they are difficult to monitor without using special instruments with high sensitivity. Additionally, high concentrations of salt in marine water interfere with the detection level of heavy metals dissolved in the water. Therefore, the monitoring of heavy metal contamination using *Ulva* species is a better choice than exclusive analysis of water and sediment samples (Butterworth et al., 1972; Bryan and Hummerstone, 1973). Yet, the metals concentration in algal thalli can be related to concentrations in the water or in the sediment. Control of heavy metal concentrations in the thalli of species from the genus *Ulva* is essential, especially with respect to the proper functioning of the trophic pyramid in aqueous ecosystems. A number of studies reported that many marine species of *Ulva* are the first producers in the food chain for herbivores in the littoral zones, especially snails and crustaceans (Rogers et al., 2000; Kamermans et al., 2002). Thus, in light of information on increasing concentrations of metals at consecutive trophic levels (biomaginification), metals accumulated by marine *Ulva* species can cause a disturbance in the development of herbivores and their predators (Romano et al., 2003). Many marine *Ulva* species are also consumed by people, suggesting that monitoring metal concentrations in algae is beneficial for maintaining global health, particularly in areas of the world with centuries-old traditions of eating algae. However, with respect to freshwater *Ulva* populations, thalli consumption was only observed by pond snails. By increasing *Ulva* populations in the freshwater ecosystems of Europe, the native organisms of lakes and rivers have begun to use algae thalli as a source of food or refuge (Rybak and Messyasz, 2010). In this way, freshwater *Ulva* populations have become a significant component of several food chains in inland ecosystems.

The new trophic relationships developing between populations of marine macroalgae and native freshwater communities require more complete analyses. Work connected with monitoring contaminant concentrations in thalli of freshwater *Ulva* should also be expanded, especially in situations where the algae are collected for specific economic needs or consumed by animals.

5. Conclusions

- Freshwater *Ulva* accumulate alkaline earth metals (Ca and Mg) and heavy metals such as Ni, Cd and Pb.
- Freshwater *Ulva* populations are characterized by an elevated ability to accumulate nickel compared with marine species from this genus.
- Concentrations of alkaline earth metals in the freshwater *Ulva* thallus are a few hundred to a few thousand times higher than levels in water from the same sites.
- Statistically significant correlations between concentrations of heavy metals in the freshwater *Ulva* thallus and water were not observed.
- Significant differences in the level of nickel accumulation were recorded between the river and lake *Ulva* populations.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2012.05.071.

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