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Paraskevi Malea*, Theodoros Kevrekidis, Athanasios Mogias and Ioannis-Dimosthenis S. Adamakis

Kinetics of cadmium accumulation and occurrence of dead cells in leaves of the submerged angiosperm *Ruppia maritima*

Abstract: Cadmium accumulation and leaf cell death in the brackish water-submerged angiosperm *Ruppia maritima* L. were investigated under laboratory conditions exposed to increasing metal concentrations (2.22–355.88 μM). The Michaelis-Menten equation satisfactorily described accumulation kinetics in plant compartments (leaves, rhizome-stems, roots). Equilibrium concentration and uptake rate generally tended to increase, whereas bioconcentration factor at equilibrium decreased, as exposure concentration increased. The relationship between tissue concentration and the set of exposure concentrations and times was adequately described by multiple regression equations. Leaf cell death was observed after 3 or 5 days depending on dosage, but dead cell percentage was small after 9 days, suggesting a rather slow progress of cell death. The lowest leaf cadmium concentration associated with the onset of cell death was within the range of cadmium concentrations reported for seagrasses from various locations, implying that cadmium poses a risk to submerged angiosperms in coastal waters. However, toxicity appeared to be related to the rate of metal uptake rather than to total tissue concentration; an earlier onset of cell death at the highest exposure concentration was associated with the highest uptake rate, and dead cell percentage on the ninth day tended to increase with uptake rate. The data presented provide insights into metal accumulation by, and their effects on, submerged angiosperms colonizing coastal waters.

Keywords: *Ruppia*; toxicity; trace metal; uptake kinetics; uptake rate.

***Corresponding author: Paraskevi Malea,** Department of Botany, School of Biology, Aristotle University of Thessaloniki, GR-541 24 Thessaloniki, Greece, e-mail: malea@bio.auth.gr
Theodoros Kevrekidis and Athanasios Mogias: Laboratory of Environmental Research and Education, Democritus University of Thrace, Nea Hili, GR-68100 Alexandroupolis, Greece
Ioannis-Dimosthenis S. Adamakis: Department of Botany, School of Biology, Aristotle University of Thessaloniki, GR-541 24 Thessaloniki, Greece

Introduction

In estuarine environments, seagrasses are often found growing in contact with brackish water-submerged angiosperms, such as *Ruppia* species (Hemminga and Duarte 2000). Among the *Ruppia* species, *Ruppia maritima* L. has the widest salinity tolerance known for any submerged angiosperm (Kantrud 1991) and occurs worldwide in a variety of coastal habitats. This species usually occurs at low intertidal elevations in estuaries, but mixes with seagrasses up to at least 1.5 km offshore in large oceanic bays (Kantrud 1991). In the Mediterranean Sea, *Ruppia* species [*R. maritima*, *Ruppia cirrhosa* (Petagna) Grande] are almost entirely restricted to brackish lagoons and salt marshes, but some open sea *Ruppia* stands were also observed in very sheltered and shallow bays (see Boudouresque et al. 2009 and references therein).

Seagrass and brackish water-submerged angiosperm communities are recognized as one of the most important biotic communities in shallow coastal waters. Populations of these angiosperms exhibit high primary production and serve as habitats and nurseries for a variety of invertebrates, fish, turtles, and mammals; this aquatic vegetation also functions in nutrient cycling, stabilizing fine sediments, and mitigating shoreline erosion dynamics (see Davis et al. 2000 and references therein). Monitoring seagrasses and brackish water-submerged angiosperms is rapidly becoming one of the foremost methods to determine the overall health and condition of coastal and estuarine environments (Bortone and Turpin 2000). In particular, these angiosperms have been considered to be a valuable tool in the evaluation of metal contamination in shallow coastal waters (see among others Pergent-Martini and Pergent 2000). Metals, particularly mercury, cadmium, and lead, are of greatest environmental concern because of their toxicity to biota above a threshold availability and their persistence in the environment (Kennish 2000).

Several field studies have been carried out concerning the use of a wide diversity of seagrasses as bioindicators

of metal contamination (see synthesis in Ferrat et al. 2003). In addition, a number of studies have addressed the kinetics of metal accumulation in these angiosperms through laboratory experiments in order to support their use as bioindicators in field situations (e.g., Faraday and Churchill 1979, Fabris et al. 1982, Lyngby and Brix 1984, Malea 1994, Malea et al. 1995a,b, Warnau et al. 1996). Information on metal accumulation issues is also available for submerged aquatic angiosperms occurring in brackish waters such as *Vallisneria* spp. and *Potamogeton* spp. (e.g., Di Giulo and Scanlon 1985, Greger et al. 1995, Fritioff et al. 2005, Lafabrie et al. 2011), as well as for salt marsh plants usually growing in the intertidal zones of estuaries, such as *Spartina* species (e.g., Williams et al. 1994, Windham et al. 2003, Cacador et al. 2009, Duarte et al. 2011, Redondo-Gómez 2013). In recent years, research efforts have focused on the capacity of aquatic plants to act as biomonitors of metal contamination through the use of early symptoms termed biomarkers (e.g., Ferrat et al. 2003, Bucalosi et al. 2006, Alvarez-Legorreta et al. 2008, Singh et al. 2010, Malea et al. 2013a); biomarkers, defined as "cellular, molecular and biochemical changes induced by chemical pollutants, measurable in biological systems such tissues, cells and biological fluids" (Depledge et al. 1995), are especially useful as an early warning signal of emerging environmental problems. On the other hand, data on the relationship between metal accumulation and the occurrence of toxic effects in aquatic organisms, particularly seagrasses and brackish water-submerged angiosperms, are still scarce (see Lewis and Devereux 2009, Adams et al. 2010, Malea et al. 2013b); metal concentrations in key biomonitors associated in some way with ecotoxicological effects, which were identified using biomarkers, have the potential to be used for detecting the presence of ecotoxicologically significant metal pollution (Rainbow 2006).

Concerning *Ruppia* species, scientific knowledge on trace element accumulation is very limited; in particular, very few studies have been carried out, mainly with *R. cirrhosa* (Sanchiz et al. 1999, 2000), *Ruppia megacarpa* Mason (Kilminster 2013), and *R. maritima*. Most of the few field studies that provide information for *R. maritima* determined concentrations of trace elements, most commonly copper, zinc, and lead, in whole plant tissues, while in some cases, sediment element concentrations were also assessed (see Di Giulo and Scanlon 1985, Pulich 1987, Lacerda et al. 1992, Falandyz 1994, Wu and Guo 2002, Lewis et al. 2004). Malea et al. (2008) studied the monthly variation and distribution of metal (iron, zinc, copper, lead, cadmium) contents in compartments of *R. maritima* and suggested that this brackish water-submerged angiosperm is a strong accumulator of metals. However, we are not aware of any published data concerning the kinetics of metal accumulation and toxic effects induced by metal stress in *Ruppia* species, particularly *R. maritima*. Such information is needed in order for this submerged angiosperm to be potentially used as a valuable tool in biomonitoring programs for the protection and management of a variety of brackish water coastal environments worldwide.

The present study aims to provide information on metal accumulation and toxic effects in submerged angiosperms colonizing shallow coastal waters. Using increasing cadmium concentrations under laboratory conditions, we investigated (a) the kinetics of cadmium accumulation in leaves, rhizomes-stems, and roots of the brackish water-submerged angiosperm *R. maritima* for 11 consecutive days, (b) the toxic effects (leaf cell death) of cadmium on this angiosperm, and (c) the relationships between exposure concentration, accumulation kinetics, and toxic effects.

Materials and methods

Plant collection

Ruppia maritima was collected from Monolimni Lagoon (Evros River Delta, Northern Aegean Sea, Mediterranean Sea). Evros River is the largest flowing water body on the Balkan Peninsula; it arises on Mt. Rila in Bulgaria and flows southward. Before reaching the Aegean Sea, the Evros River divides into two branches, the Eastern Branch, which is the natural border between Greece and Turkey and the Western Branch inside Greece, and forms a delta. Three islets and several lagoons occur in the part of the delta inside Greece. Monolimni Lagoon occupies an area of approximately 112 ha, communicating with the mouth of the Western Branch Estuary and the coastal section of the delta through an opening 15 m wide. The vegetation composition in the main part of Monolimni Lagoon (the innermost section) is rather homogeneous, consisting mainly of a perennial population of *R. maritima*, which grows from April to October and reproduces during summer (Malea et al. 2004).

Plants were collected in the central part of the innermost section of Monolimni Lagoon (40°46′N, 26°03′E) at 0.6 m depth in late August 2011 with a 20 cm diameter acrylic corer, which penetrated to a depth of 30 cm; all the above- and below-substrate plant material that was rooted within the 20-cm area was collected. All plants

were rinsed in lagoon water at the collection site and transported to the laboratory in plastic containers containing lagoon water.

Treatments

Fresh green plants without epiphytes were kept for 24 h in lagoon water under laboratory conditions in order to equilibrate. Plants were incubated in plastic aquaria containing 10 l of cadmium sulfate hydrate ($3CdSO_4 \cdot 8H_2O$ 98.7%, insoluble matter ≤0.005%, chloride ≤0.001%, total nitrogen ≤0.0005%, Ca≤0.005%, Cu≤0.0005%, Fe≤0.0005%, K≤0.01%, Na≤0.005%, Pb≤0.002%, Zn≤0.002%; lot number: 1.02027.0100; Merck, Darmstadt, Germany) dissolved in filtered (Whatman GF/C) lagoon water at one of the following Cd concentrations: 2.22, 4.44, 44.48, 88.95, 177.94, and 355.88 μM, corresponding to 0.25, 0.5, 5, 10, 20, and 40 mg l^{-1}, respectively. At a salinity 8.6, these total cadmium concentrations were calculated to correspond to 0.33, 0.65, 6.52, 13.04, 26.09, and 52.17 μM (or 0.04, 0.07, 0.73, 1.47, 2.93, and 5.86 mg l^{-1}) of free cadmium ion, respectively, using the equation proposed by Sunda et al. (1978). Lower exposure concentrations might have resulted in toxic effects only after several days of incubation, and thus, these effects would most probably have been obscured by leaf deterioration. A control treatment, with no added metal, was included in the experiments. A similar set of exposure concentrations has also been used previously in a similar study (Malea et al. 2013b). Plant material of about 12 g dry wt was incubated in each aquarium; no sediment and no complexing agents were added. The lagoon water used for the experiments was also collected from the sampling site. The lagoon water used in the experiments had: salinity 8.5–8.7, pH 7.5–7.6, dissolved O_2 7.4–7.6 mg l^{-1}, NO_3^- 0.88 mg l^{-1}, and NH_4^+ 0.48 mg l^{-1}, cadmium 0.5 μg l^{-1} (or 0.045 μM). The solutions in the aquaria were changed every 2 days in order to maintain the original levels. Salinity, pH, and dissolved O_2 were measured every 2 days. The aquaria were aerated constantly using aquarium pumps and covered with plastic film (Sanitas, Sarantis S.A., Athens, Greece) in order to prevent evaporation. The experiments were conducted under a constant 16:8 h day:night regime at an ambient temperature of 21±1°C and an irradiance of 120 μmol m^{-2} s^{-1}. After 0, 3, 5, 7, 9, and 11 days, at least three plant subsamples, each one including about 250 leaves (approximately 400 mg dry wt) and the corresponding stems, rhizomes (both approximately 200 mg dry wt), and roots (approximately 30 mg dry wt), were removed at random from each aquarium. Plant material was separated into leaves, rhizomes-stems, and roots. Similar procedures have been used in previous studies (e.g., Malea 1994, Malea et al. 1995a,b).

Cadmium determination

The materials from each plant compartment of the subsamples from a single aquarium collected on the same day were pooled. The samples were washed in double-distilled water, dried to a constant weight (60°C) and ground in an agate mill. Three subsamples of each powdered plant sample were wet digested in $HNO_3/HClO_4$ (4:1) at 50°C for 1 h and then at 130°C for 3 h. Similar methods have been frequently used in previous studies (e.g., Malea 1994). In several cases, the quantity of powdered root was insufficient for metal analysis; hence, the kinetics of cadmium accumulation in roots at 4.44, 88.95, and 177.94 μM Cd were not examined. Cadmium concentrations were measured using graphite furnace atomic absorption spectrophotometry (AAS, AANALYST 700 Perkin-Elmer, MA, USA). Pro-analysis-grade reagents (Merck, Darmstand, Germany) were used, and reagent blanks were run concurrently. Standards were prepared by serial dilution of stock solutions. The accuracy of the technique was checked by analysis of standard sea lettuce reference material (*Ulva lactuca* no 279, Community Bureau of Reference BCR, Brussels, Belgium); one sample of the standard reference material was included in each analytical batch. Results were in agreement with certified values (certified value, mean±SD:0.274±0.032 μg g^{-1} dry wt; measured value, mean±SD: 0.280±0.020 μg g^{-1} dry wt; recovery: 102%).

Evans Blue staining

In order to check the occurrence of dead leaf cells of *Ruppia maritima*, samples were stained with Evans Blue following the protocol of Malea et al. (2013a) adapted from Chen et al. (2008). After 0, 3, 5, 7, 9, and 11 days of incubation, at least 20 leaves from each aquarium were randomly collected and incubated for 15 min at room temperature in a 0.25% Evans Blue (Sigma, Taufkrichen, Germany) solution in lagoon water collected from the collection site. After several washes with lagoon water, the leaf segments were observed under a Zeiss (Berlin, Germany) Axiostar Plus light microscope equipped with a Canon (New York, NY, USA) PowerShot A640 camera, and the occurrence of dead cells (stained blue) was checked. For the 88.95-μM treatment on days 0, 3, 5, 7, 9, and 11, and for each treatment on day 9, segments from the middle section of at least 10 leaves (one segment per leaf) were photographed,

the dead cells from a total of about 260 cells per leaf were counted, and the percentage of dead cells was calculated.

Data analysis

The kinetics of cadmium accumulation were fitted to the Michaelis-Menten equation (Eq. 1), which is frequently used in metal kinetic studies, where C represents the tissue metal concentration reached in time *t*, C_{max} is the maximum or saturation concentration, and K_m is the time taken to reach half of the value of C_{max} (see among others Fernández et al. 2006, Costa et al. 2011, Diaz et al. 2012).

$$C=(C_{max}\times t)/(K_m+t) \quad (1)$$

The data were fitted using IBM Statistics SPSS® (New York, NY, USA) 19, by means of nonlinear regression, and with sequential quadratic programming as the estimation method (Diaz et al. 2012). In order for the information on accumulation kinetics to be completed, the rate of the initial uptake (found by dividing half of the value of C_{max} by K_m), the time required to reach equilibrium (T_{eq}), the equilibrium concentration (C_{eq}), and the mean rate of uptake (V_c) were calculated. C_{eq} was estimated from the Michaelis-Menten equation as the tissue concentration at which the daily increase in concentration was <1% of that of the previous day, T_{eq} was estimated as the time required to reach C_{eq}, and V_c by dividing C_{eq} by T_{eq} (Fernández et al. 2006, Diaz et al. 2012). Bioconcentration factors (BCFs) at equilibrium were also calculated (Eq. 2), where C_{eq} is the equilibrium concentration, C_i is the initial tissue metal concentration (at day 0), and C_w is the metal concentration in the water (Martins and Boaventura 2002, Diaz et al. 2012).

$$BCF=(C_{eq}-C_i)/C_w \quad (2)$$

The Wilcoxon matched-pairs signed-rank test was used to compare metal concentrations in different plant compartments. Spearman's rank correlation coefficient (ρ) was calculated to identify correlations.

A multiple regression model with log-transformed exposure concentration and time as predictors and tissue concentration as the dependent variable was additionally tested in each plant compartment.

The time period required for 10% of leaf cells to die (LT_{10}) in the 88.95-μM treatment and the 10% cell death effect concentration (LC_{10}) on the ninth incubation day and their 95% confidence limits were estimated by probit analysis with XLSTAT 2013 (New York, NY, USA).

Results

Accumulation kinetics

Cadmium concentrations in leaves, rhizome-stems, and roots of *Ruppia maritima* analyzed immediately after collection in the field (day 0, mean±standard error) were 0.335±0.003, 0.305±0.003, and 1.780±0.037 μg g^{-1} dry wt, respectively.

The kinetics of cadmium accumulation into leaves, rhizome-stems, and roots of *R. maritima* were rapid during the first days of exposure; this initial rapid accumulation was generally followed by a slower accumulation phase and/or a steady state (Figure 1). A marked increase in leaf cadmium content was observed on the last incubation day in the 355.88-μM treatment. Cadmium concentrations reached during the incubation period differed to some extent among *R. maritima* compartments (Figure 1). In particular, at 2.22, 4.44, 44.48, and 177.94 μM, leaves showed significantly higher cadmium contents than rhizome-stems and at 2.22 μM, roots had significantly higher concentrations than either leaves or rhizome-stems (Wilcoxon test, $p<0.05$); cadmium concentrations reached in roots were also highest in the 44.48 and 355.88 μM treatments (Figure 1). In leaves and rhizome-stems, the fit to the Michaelis-Menten equation was adequate for all of the treatments, but for leaves, the initial five points only were fitted at 355.88 μM because of the marked increase in tissue metal content on the last day of incubation (Figure 1, Table 1). In roots, the Michaelis-Menten model provided a good fit for the 44.48- and 355.88-μM treatments (Figure 1, Table 1).

In leaves, the values of the maximum concentration (C_{max}) and the equilibrium concentration (C_{eq}) generally tended to increase as the exposure concentration (C_w) increased up to 88.95–177.94 μM, while the rate of initial uptake ($C_{max}/(2\times K_m)$) and the mean rate of uptake (V_c) increased with increasing C_w up to 355.88 μM (Table 1); in particular, C_{eq}, $C_{max}/(2\times K_m)$ and V_c were significantly and positively correlated with C_w (ρ=0.829, 0.829, and 0.943, respectively; n=6). In general, the opposite was found for the time taken to reach half of the value of C_{max} (K_m) and the equilibrium time (T_{eq}); both parameters displayed the highest value at 4.44 μM and the lowest at 355.88 μM (Table 1). In rhizome-stems, C_{max} and C_{eq} increased with increasing C_w up to 355.88 μM (Table 1), and a significant positive correlation was found between both these concentrations and C_w (ρ=1, n=6). The bioconcentration factor (BCF) at equilibrium in both leaves (range: 17.0–1009.1) and rhizome-stems (range: 21.7–431.0) decreased as C_w increased from 4.44 to 355.88 μM (Table 1); in both

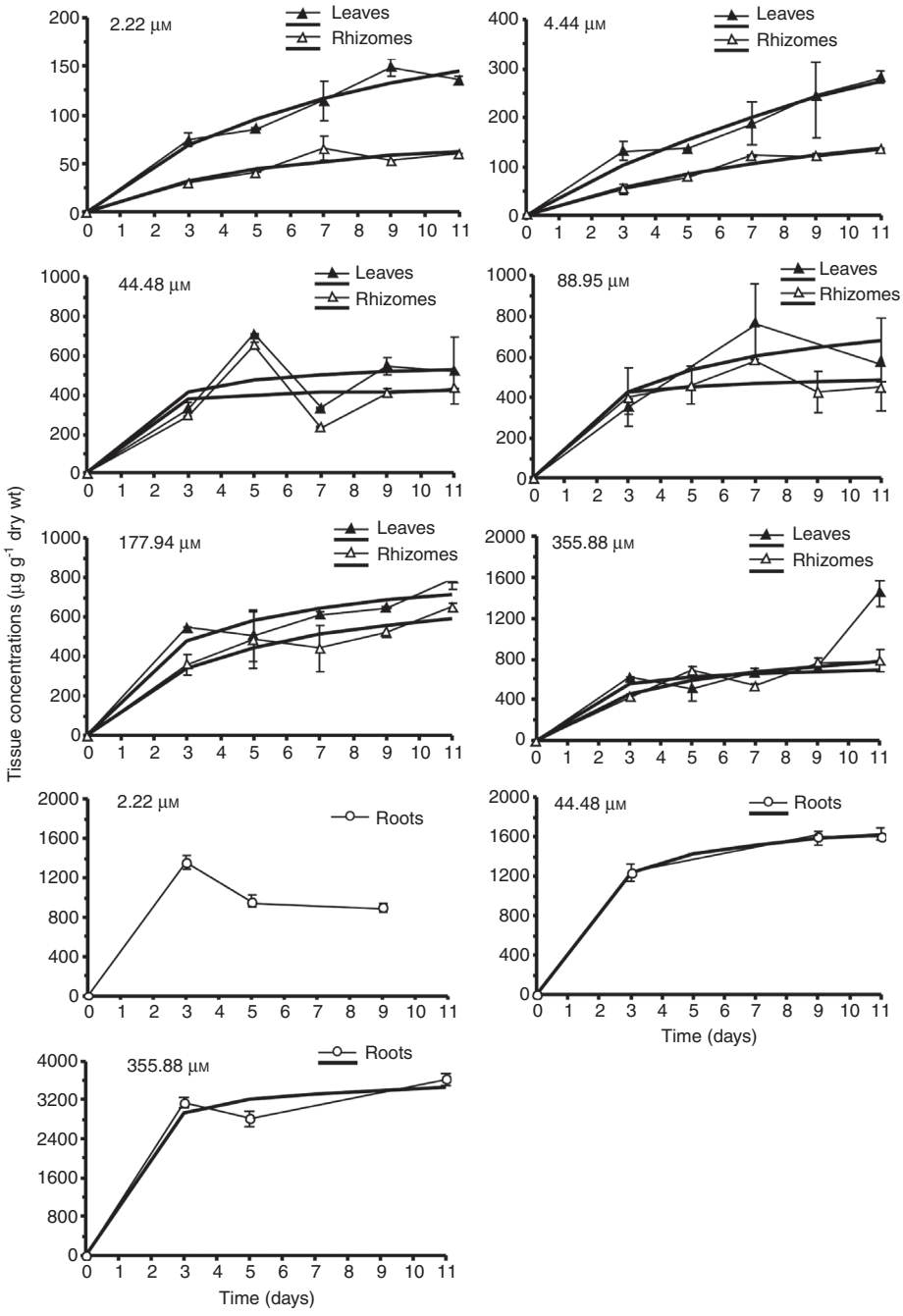

Figure 1 Kinetics of cadmium accumulation in leaves, rhizome-stems and roots of *Ruppia maritima* at different concentrations of cadmium in water. Values plotted are mean tissue concentration±standard deviation (n=3). Bold lines are the accumulation kinetics calculated using a Michaelis-Menten equation.

compartments, BCF at equilibrium showed a significant negative correlation with C_w (ρ=-0.943, n=6).

The relationship between tissue concentration and the combination of exposure concentration and time was adequately described by multiple regression equations. Time was found to be more significant than exposure concentration in predicting tissue concentration in leaves and rhizome-stems, but had no significant effect in roots (Table 2).

Leaf cell death

Some of the leaf cells died upon cadmium treatment as confirmed by Evans Blue staining, in contrast to the control where no positive Evans Blue staining was ever observed. The dead cells were stained blue as shown in Figure 2 and were commonly observed in groups. Dead cells appeared at 2.22–177.94 µM from the fifth day onward,

Table 1 Kinetics of cadmium accumulation in leaves, rhizome-stems, and roots of *Ruppia maritima* exposed to different concentrations of cadmium in water.

Exposure concentration (μM)						
	2.22	**4.44**	**44.48**	**88.95**	**177.94**	**355.88**
Leaves						
C_{max}	247.3 (±60.5)	779.89 (±79.2)	593.28 (±58.2)	882.56 (±62.4)	880.17 (±60.4)	747.44 (±38.2)
K_m	7.78 (±3.7)	20.15 (±3.8)	1.26 (±2.5)	3.22 (±1.3)	2.52 (±1.2)	0.97 (±0.8)
$C_{max}/(2\times K_m)$	15.90	19.35	235.43	137.04	174.57	386.07
r^2	0.966[a]	0.969[a]	0.691[b]	0.867[c]	0.947[a]	0.942[b]
T_{eq}	25 (±5)	37 (±7)	12 (±4)	17 (±4)	16 (±3)	10 (±2)
C_{eq}	188.62 (±35)	504.88 (±60)	536.90 (±59)	742.01 (±81)	760.36 (±75)	681.47 (±48)
V_c	7.55	13.65	44.74	43.65	47.52	68.15
BCF	753.12	1009.09	107.31	74.24	38.00	17.03
Rhizome-stems						
C_{max}	94.31 (±21.2)	299.74 (±56.7)	440.02 (±45.4)	512.05 (±51.3)	818.17 (±59.1)	1047.02 (±87.4)
K_m	5.63 (±2.7)	12.79 (±5.4)	0.50 (±0.2)	0.62 (±0.3)	4.10 (±2.1)	3.96 (±2.1)
$C_{max}/(2xK_m)$	8.38	11.72	440.90	412.28	99.85	132.20
r^2	0.921[a]	0.974[a]	0.575[c]	0.916[a]	0.955[a]	0.929[a]
T_{eq}	22 (±6)	33 (±8)	8 (±2)	9 (±2)	19 (±5)	19 (±6)
C_{eq}	75.10 (±19)	216.03 (±41)	414.19 (±61)	479.00 (±59)	673.04 (±67)	866.44 (±91)
V_c	3.41	6.55	51.77	53.22	35.42	45.60
BCF	299.18	431.00	82.70	47.87	33.64	21.65
Roots						
C_{max}			1847.11(±115.4)			3673.54 (±137.9)
K_m			1.46(±0.9)			0.76 (±0.5)
$C_{max}/(2xK_m)$			631.71			2426.38
r^2			0.999[b]			0.973[b]
T_{eq}			12 (±5)			9 (±4)
C_{eq}			1646.5 (±92)			3388.53 (±121)
V_c			137.21			376.50
BCF			328.95			84.67

The fits correspond to a Michaelis-Menten equation: $C=(C_{max}\times t)/(K_m+t)$.
C, tissue concentration (μg g^{-1} dry wt) reached in a given time; C_{max}, maximum tissue concentration; K_m, time (in days) to reach half of the value of C_{max}; *t*, time (in days); T_{eq}, time (in days) to reach equilibrium; C_{eq}, equilibrium concentration (μg g^{-1} dry wt); V_c, mean rate of uptake (in concentration/day); BCF, bioconcentration factor at equilibrium; standard errors are given in parentheses.
[a]$p<0.001$; [b]$p<0.01$; [c]$p<0.05$.

Table 2 Results of multiple regression analysis performed using log-transformed exposure concentration (C_w) and time (T) as predictors and tissue concentration (C) as the dependent variable ($\log_{10} C=a+b_1\cdot\log_{10}C_w+b_2\cdot\log_{10}T$)

Variables	Std. coefficients	*t*	Significance	Model summary
Leaves				
$\log_{10} C_w$	0.210	2.544	<0.01	R^2=0.888,
$\log_{10} T$	0.866	14.600	<0.001	p<0.001
Rhizome-stems				
$\log_{10} C_w$	0.292	3.590	0.001	R^2=0.782,
$\log_{10} T$	0.835	10.277	<0.001	p<0.001
Roots				
$\log_{10} C_w$	0.846	4.854	0.002	R^2=0.727,
$\log_{10} T$	0.082	0.469	ns	p<0.001

ns, not significant.

while they appeared from the third day in the highest concentration tested (355.88 μM). In the 88.95-μM treatment, the percentage of dead cells (D, mean,%) increased from 0.3% and 2.5% on days 5 and 7 to 7.6% on day 9 and 33. 5% on day 11 (Figure 3A); the estimated period of time required for 10% of cells to die (LT_{10}) was 8.9 (95% confidence limits: 8.7–9.1) days. On the ninth day of incubation, this percentage increased with increasing exposure concentration (C_w), and a significant positive correlation was found between D and C_w (ρ=1, n=6,). The 10% cell death effect concentration (LC_{10}) on the ninth incubation day was 268.8 (95% confidence limits: 245.1–297.1) μM (Figure 3B).

The time period before the first occurrence of dead leaf cells (5 days at 2.22–177.94 μM, 3 days at 355.88 μM)

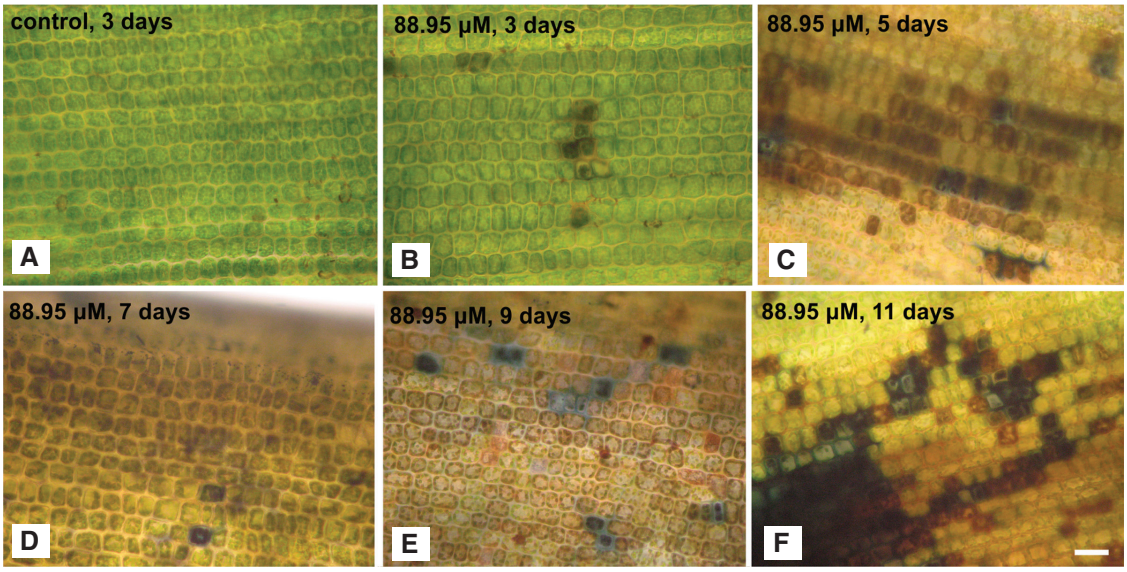

Figure 2 Photomicrographs of *Ruppia maritima* leaf cells stained with Evans Blue. Control (A) and after treatment with 88.95 µM of cadmium for 3, 5, 7, 9 and 11 days (B–F); dead cells appear blue; scale bar=100 µm.

was shorter than the estimated time period taken to reach the equilibrium cadmium concentration (T_{eq}) in leaves (Table 1) in all of the treatments; hence, cell death was generally first detected during the initial phase of cadmium accumulation. The lowest experimental tissue concentration associated with the first occurrence of cell death was 86.2 µg g^{-1} dry wt (2.22 µM treatment, day 5); the corresponding concentration value predicted using the Michaelis-Menten model was 96.8 µg g^{-1} dry wt (Figure 1). When *Ruppia maritima* was treated at cadmium concentrations of 4.44–355.88 µM, these values had been exceeded by the third incubation day (see Figure 1); however, as already noted, cell death was first detected on the fifth incubation day in the 4.44–177.94 µM treatments.

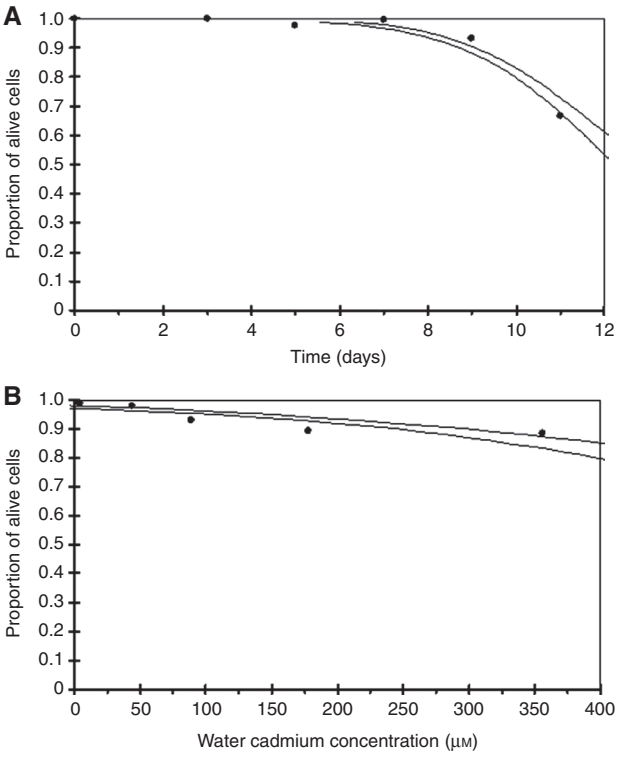

Figure 3 Leaf cell death in *Ruppia maritima* in response to incubation with cadmium. Proportion of live cells (A) after different times of incubation in 88.95 µM of cadmium in water and (B) after 9 days incubation in different concentrations of cadmium. Symbols indicate observed mean values; lines indicate the lower and upper bounds (95%) estimated by probit analysis with XLSTAT 2013.

The highest values of the mean rate of uptake (V_c) and of the initial rate of uptake in leaves (355.88 µM treatment; Table 1) were associated with the earliest onset of cell death (third incubation day). On the ninth incubation day, the percentage of dead cells (D,%) tended to increase as the rate of initial uptake and V_c increased (Table 1, Figure 3B); in particular, D showed a significant positive correlation with $C_{max}/(2\times K_m)$ (ρ=0.829, n=6) and V_c (ρ=0.943, n=6).

Discussion

Accumulation kinetics

The initial concentrations of cadmium (day 0) in leaves and rhizome-stems of *Ruppia maritima* were lower, and that for roots was higher than those previously reported for whole plant tissues of *R. maritima* from other geographical areas

(Di Giulio and Scanlon 1985, Lewis et al. 2004). In addition, these values were among the lowest of those earlier measured all year round in the corresponding compartments of *R. maritima* at the same sampling area (Malea et al. 2008).

The kinetics of cadmium accumulation into compartments of *R. maritima* were rapid during the first days of exposure and then became gradually slower. This trend is generally consistent with previous observations concerning cadmium accumulation in compartments of seagrass species (e.g., Brinkhuis et al. 1980, Fabris et al. 1982, Lyngby and Brix 1984, Malea 1994). Cadmium accumulation may have involved three successive processes, namely, a first stage that corresponds to rapid adsorption of the metal ions onto the surface of the plant, a second stage that represents the diffusion across the plasma membrane into the protoplasm, and a third stage that corresponds to the active accumulation of metal within the plant (e.g., Pickering and Puia 1969, Malea 1994, Martins and Boaventura 2002, Andrade et al. 2006, Fernández et al. 2006). A potential release by the plant into the medium of organic ligands, capable to complex dissolved metals, may have played a role in controlling the metal uptake (e.g., Naidu and Harter 1998, Vasconcelos and Leal 2001, Karavoltsos et al. 2013).

A substantial amount of cadmium is probably bound to the cell wall (e.g., Vogel-Mikuš et al. 2010), which seems to function as a barrier protecting the protoplast from metal toxicity; Sousa et al. (2008) observed that the salt marsh plant *Halimione portulacoides* (L.) Allen mostly retains cadmium in the cell wall (70% in roots, 64% in stems, and 54% in leaves), while intracellular metal content is much lower (19% in roots, 25% in stems, and 35% in leaves). The steady state may correspond to an equilibrium attained between the metal in solution and the metal adsorbed to active sites on the cell surface (Sunda and Huntstman 1998, Kola and Wilkison 2005). Complexation of metal ions inside the cell by organic molecules and compartmentalization, particularly in the vacuole, may be also included in the array of the cellular defense mechanisms (e.g., Hall 2002, Wang et al. 2008, Conn and Gillihan 2010). Overall, cadmium is expected to be accumulated in different cellular compartments, and the observed accumulation kinetics were most probably the net result of several different processes. The marked increase in cadmium content in *R. maritima* leaves on the last day of incubation at the highest exposure concentration may have been the result of extensive cell death; dead cells have been suggested to display an elevated metal absorption capacity (Lyngby and Brix 1984, Bond et al. 1985, Malea 1994).

Cadmium concentrations reached during the incubation period appeared to differ to some extent among *R. maritima* compartments. Roots, which present the highest surface/volume ratio (Fabris et al. 1982), generally accumulated more cadmium than leaves and rhizome-stems. Following root and/or leaf uptake, cadmium may have been transported to other plant compartments, mainly rhizome-stems, as it is considered that metal uptake in submerged angiosperms can follow two pathways – from surrounding water to leaves and then rhizomes, or from interstitial water into roots to rhizomes and leaves (e.g., Pulich 1987, Ralph et al. 2007). Hence, cadmium accumulation in rhizome-stems of *R. maritima* may have resulted from both a direct uptake from the surrounding medium and some internal transport. Under laboratory conditions, Faraday and Churchill (1979) found basipetal transport, while Brinkhuis et al. (1980) observed both basipetal and, at a slower rate, acropetal transport of cadmium in the seagrass *Zostera marina* L.

In field situations, a variability in the distribution pattern of cadmium among plant compartments of *R. maritima* might be expected due to differences in the bioavailability of this element in the water column and sediments (see Lewis and Devereux 2009 for factors affecting metal bioavailability in water and sediments). Nevertheless, our findings are consistent with previous field observations, where it has been found that *R. maritima* roots accumulated the highest cadmium loads and, rhizome-stems, the lowest ones during most of the annual cycle (Malea et al. 2008). Higher cadmium accumulation in roots than in other compartments has been also observed in salt marsh species such as *Spartina maritima* (Curtis) Ferland and *Halimione portulacoides* (e.g., Reboreda and Cacador 2007, Sousa et al. 2008). As previously reported (e.g., Sousa et al. 2011), roots may act as a barrier, preventing metal toxicity in leaves.

The maximum and equilibrium concentrations in leaves and rhizome-stems, as well as the rate of cadmium uptake into leaves, generally tended to increase with the concentration in water indicating that *R. maritima* displays a high absorption capacity of cadmium and an abundance of cell wall or intracellular binding sites (Sanchiz et al. 1999). This observation also indicates that cadmium in the plant is correlated with that in the surrounding medium over a wide range of exposure concentrations (see also Costa et al. 2011). However, the bioconcentration factors at the steady-state condition generally tended to decrease with increasing exposure concentration suggesting a gradual reduction of available binding sites (see Fabris et al. 1982). These findings are generally consistent with those of earlier studies concerning

cadmium accumulation by seagrasses (Faraday and Churchill 1979, Fabris et al. 1982, Malea 1994). Concerning leaves, in particular, the observation that the uptake rate tended to increase as cadmium concentration in water (C_w) increased up to 355.88 μM, while the maximum and equilibrium concentrations as C_w increased only up to 88.95–177.94 μM suggests that, at very high exposure concentrations (>177.94 μM), leaves of *R. maritima* initially accumulate cadmium very rapidly, but then, the active sites at their surface become saturated.

The multiple regression equations obtained permit the metal concentrations accumulated in plant compartments to be rigorously related to metal concentration in the surrounding medium and exposure time. Time was found not to have a significant effect on tissue concentration in roots as the initial fast metal accumulation was rapidly followed by a slower accumulation phase or a steady state.

The maximum experimental cadmium concentration in leaves of *R. maritima* (281.5 μg g^{-1} dry wt) incubated in lagoon water (8.6 salinity) containing cadmium at a concentration of 4.44 μM (or 0.5 mg l^{-1}) was comparable to that in leaves of the seagrass *Heterozostera tasmanica* (Martens *ex* Ascherson) den Hartog (approximately 300 μg g^{-1} dry wt) exposed for 8 days to seawater containing 0.4 mg l^{-1} of cadmium (Fabris et al. 1982). The experimental cadmium concentrations in *R. maritima* leaves (579–772 μg g^{-1} dry wt) during the steady-state phase at 88.95 μM were also comparable to those accumulated in leaves of the seagrass *Halophila stipulacea* (Forsskål) Ascherson (526–721 μg g^{-1} dry wt) incubated for 16 days in seawater (35 salinity) containing 100 μM of cadmium (Malea 1994). The maximum and equilibrium concentrations and the rate of uptake estimated for cadmium accumulation in leaves of *R. maritima* in the 4.44- to 88.95-μM treatments were higher, while those of the 177.94- and 355.88-μM treatments were lower, than the corresponding values found for the accumulation of this metal in intermediate-juvenile leaf blades of the seagrass *Cymodocea nodosa* (Ucria) Ascherson exposed to seawater (36.5 salinity) containing cadmium concentrations of 4.44–355.88 μM (Malea et al. 2013b).

Leaf cell death

Cadmium caused leaf cell death in *Ruppia maritima*, as was also shown for the seagrasses *Halophila stipulacea* and *Cymodocea nodosa* (see Malea 1994, Malea et al. 2013b). The observation that in the 88.95-μM treatment dead leaf cells were first detected on the fifth incubation day but the time period required for 10% of cells to die (LT_{10}) was estimated to be 8.6 days suggests a rather slow progress of cell death. The corresponding values reported for leaf blades of the seagrass *C. nodosa* exposed to seawater (36.5 salinity) containing 88.95 μM of cadmium were 7 and 7.2 days (Malea et al. 2013b). That the progress of cell death appeared to be slow is also corroborated by the observation that the percentage of dead leaf cells of *R. maritima* on the ninth incubation day at 355.88 μM was only 11.5%, while the corresponding percentage in *C. nodosa* was 28.7%. This suggests that the first occurrence of dead leaf cells in *R. maritima* could be used as a warning signal of developing cadmium contamination in coastal brackish water environments. Because the cadmium concentrations to which *R. maritima* was exposed in this study would be reached only in cases of extreme contamination, further controlled experiments are needed to provide additional results for lower exposure concentrations.

The lowest experimental tissue concentration of cadmium associated with the onset of cell death (86.2 μg g^{-1} dry wt, 2.22 μM treatment, fifth incubation day) is within the wide range of reported cadmium concentrations in leaves of seagrass species (0.1–266 μg g^{-1} dry wt) from various geographical areas (see review in Lewis and Devereux 2009). This finding may imply that cadmium poses a risk to submerged angiosperms colonizing shallow coastal waters at worldwide locations. The observation that the lowest tissue concentration associated with the onset of cell death was exceeded up to the third day at higher exposure concentrations but cell death was first detected at a later time, however, may suggest that toxicity is not strongly related to a threshold total tissue concentration of the toxic metal. Hence, our findings seem not to be compatible with the critical body residue (CBR) approach, which assumes that toxicity occurs when a threshold total body (or tissue) concentration of accumulated metal is exceeded (McCarty and Mackay 1993).

As suggested earlier, cadmium is thought to be accumulated in different cellular compartments. Metal that is bound as exchangeable forms to binding sites in cell walls or plasma membranes (extracellular metal) is not in direct contact with the cytoplasm and, thus, cannot be regarded to have an immediate influence upon metabolism (Sidhu and Brown 1996). Metal taken up into the cell (intracellular metal) can be stored safely in a detoxified form or remain metabolically available (e.g., Pergent-Martini and Pergent 2000, Conn and Gilliham 2010). Hence, the onset of toxic effects may depend only on the concentration of accumulated metal in a metabolically available form; in particular, toxicity may occur when the rate of intracellular metal uptake exceeds the rate of detoxification of metabolically available metal (see Rainbow 2002). As an increase in the rate of total metal uptake most probably involves both

an increase in the rate of extracellular uptake and that of intracellular uptake (see Fernández et al. 2006), this explanation is corroborated by the observations that (1) the earlier onset of cell death in the 355.88-μM treatment was associated with the highest values of the initial rate of uptake and the mean rate of uptake, and (2) the percentage of dead cells on the ninth incubation day tended to increase as these kinetic parameters increased.

This interpretation is in agreement with the arguments put forward by Rainbow (2002) for aquatic invertebrates, namely, that the factor that determines the effects is not whole-body concentration *per se*, but the rate of metal uptake in relation to the metabolic capacity for detoxification and storage. Our findings are also consistent with those concerning the effects of cadmium accumulation on microtubule integrity and cell viability in leaf blades of the seagrass *C. nodosa* (Malea et al. 2013b). That this explanation is corroborated by data obtained from the 355.88-μM treatment shows the usefulness of the set of exposure concentrations used in this study.

Overall, the onset and progress of leaf cell death appeared to be a function of the rate of cadmium accumulation in leaves rather than of the total tissue cadmium concentration, suggesting that, in the interpretation of tissue residues in submerged angiosperms colonizing coastal brackish water environments, the time frame of the exposure must be taken into account. This finding also suggests that the estimation of kinetic parameters of total metal uptake (rate of initial uptake, mean rate of uptake) in these angiosperms could be utilized for predicting metal effects.

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