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In situ assessment of *Ulva australis* as a monitoring and management tool for metal pollution

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Abstract We investigated physiological responses of *Ulva australis* to metals and their implications for biomonitoring and management tool. To determine the capacity of *Ulva* to accumulate metals over the short-term, we undertook an in situ experiment where we transplanted thalli to sites with different levels of metal pollution. After 12 days, arsenic, copper, lead, and zinc accumulation was observed. Zinc was significantly greater ($p = 0.001$) at the most polluted site and was highly correlated ($r = 0.87$) with seawater total Zn concentration. We also assessed whether metal exposure can compromise *U. australis* performance by evaluating physiological responses and changes in thalli ultrastructure. We observed an increase in electron-dense bodies in the cell walls and vacuoles, which clearly indicates metal accumulation. However, there was no change in physiological performance (i.e. growth rate, F_v/F_m, $rETR_{max}$, or in photosynthetic pigments content) between the control and transplanted thalli ($p > 0.05$). Bioaccumulation capacity of *U. australis* was assessed by deploying thalli at a highly polluted site for 45 days, where zinc in *Ulva* markedly increased over time and was highly correlated with the environmental concentrations (total Zn in seawater, $r = 0.85$). The metal uptake rate increased steadily over time, confirming that *Ulva* is clearly capable of bioaccumulation. However, visual examination of the thalli suggested degradation over time, which might limit deployment time (20 days). Clearly, *U. australis* has potential as a biomonitor/management tool, particularly for zinc, but the results suggest it may be a useful tool for removing metals from the environment.

Keywords Chlorophyta · Bioaccumulation · Photosynthetic performance · Physiological performance · Ultrastructure · Zinc

Introduction

Heavy metal pollution is a worldwide concern, with a broad range of anthropogenic activities contributing metals to our environment, resulting in long-lasting impacts on aquatic ecosystems (Bird et al. 1998). As the human population has increased exponentially, the impact of contaminants on the environment has also increased (Kumar et al. 2011). Heavy metals are toxic, non-degradable contaminants, which can accumulate in living organisms (Fu and Wang 2011). Metal contamination can arise from both natural sources and human activities (Hurd et al. 2014). Estuaries are areas where contaminants accumulate (Guisti 2001), mainly because there are attractive places for human settlement. The Derwent Estuary in Tasmania, Australia, is heavily polluted by metals, largely as a result of the legacy of past industry. The estuary has been monitored for more than 15 years (Whitehead et al. 2010; Coughanowr et al. 2015), and this clearly shows that despite

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the marked reductions in metal inputs, some areas remain highly contaminated, particularly in the middle estuary, whilst the lower estuary has much lower levels (Whitehead et al. 2010).

Where metal pollution is a concern, there is the potential for bioaccumulation, and this can have adverse implications for both the ecosystem and human health. Biomonitoring is a technique that provides information on the contaminants that my affect ecosystems (Phillips and Rainbow 1994; Zhou et al. 2008). There are two ways that this can be achieved, the first is the 'active' approach that relies on native organisms to monitor environmental conditions; the second is the 'passive' approach which relies on transplanted organisms placed in the selected study area for a fixed period of time (Lacroix et al. 2015; Ronci et al. 2016). Active biomonitoring (ABM) is the approach most commonly used to evaluate metal impacts in the environment and is the focus of most environmental monitoring programs. However, a combination of the ABM and passive biomonitoring (PBM) can provide a better understanding of the impacts on the broader ecology (Brown et al. 2012) as this approach would provide an appreciation of both the short-term (PMB) and longer-term impacts (ABM). Where pollution exists, it may be desirable to find a way to remediate the affected areas and reduce detrimental impacts to the aquatic environment. Bioremediation is a process that utilises living organisms which have the ability to accumulate or degrade chemical contaminants into less toxic forms to clean up polluted environments (Vidali 2001; Juwarkar et al. 2010; Kumar et al. 2011). Heavy metal removal is a cost-effective alternative where bioremediation has been demonstrated in a range of organisms such as bacteria, fungi, plants and algae (Vieria and Volesky 2000; Juwarkar et al. 2010).

Many seaweed species (Phyla Ochrophyta (brown), Rhodophyta (red) and Chlorophyta (green)), have been shown to have the potential to accumulate metals in ABM (Luoma et al. 1982; Ho 1990; Brown et al. 1999; Zbikowski et al. 2007; Boubonari et al. 2008; Ryan et al. 2012), but there is little information on the potential of algae to be used for PMB. There have been many studies looking at the types of seaweeds that would be most suitable for bioremediation of nutrient-enrichment, with many of these focussed on the interactions of aquaculture and Integrated Multi-trophic Aquaculture (IMTA) (Troell et al. 1997, 1999; Chopin et al. 1999; Zhou et al. 2006; Buschmann et al. 2009; Grote 2016), but there is less information about the specific applications of seaweeds for bioremediation of metal-polluted environments.

Some studies have evaluated heavy metal effects in different species of seaweed, looking at physiological responses and ultrastructural alterations under laboratory or controlled conditions (Pellegrini et al. 1991; Baumann et al. 2009; Zakeri and Abu Bakar 2012; Gouveia et al. 2013; Felix et al. 2014; Santos et al. 2014). However, although laboratory experiments can provide useful information, there is still need to understand how such species might perform under real-world, field conditions. Field experimentation, particularly information obtained from transplant experiments, should complement laboratory experiments (Rainbow 1995) and provide a much better overall understanding of bioaccumulation and remediation potential. Although there are some studies that have looked at in situ metal uptake or metal release in seaweeds (Myklestad and Eide 1978; Ho 1984, 1990; Malea et al. 1995; Malea & Haritonidis 2000; Boubonari et al. 2008; Ryan et al. 2012), others have examined in vitro how heavy metal pollution affects the physiological performance and ultrastructure of species such as *Ulva* sp., *Ulva laetevirens* and *Ulva intestinalis* (Schiavon et al. 2012; Vecchia et al. 2012; Andrade et al. 2004). A field study of *Ulva lactuca* and *Sargassum stenophyllum* found them to be relatively pollution-tolerant but showed that in relation to an increasing urban pollution gradient, the physiological performance of *S. stenophyllum* declined while *U. lactuca* improved along an urban pollution gradient (Scherner et al. 2012). In general, the cosmopolitan ulvacean species are good indicators of pollution (Rainbow and Phillips 1993). We selected *Ulva australis* for the focus of this study as recent research has shown that it takes up metals and is widely distributed throughout of the Derwent Estuary (D. Farias, unpublished data).

There is evidence that *Ulva* can tolerate metal pollution and that under controlled conditions may be useful for metal remediation, but it is still not clear how effective these algae might be as in situ remediation tools in highly metal-polluted systems. Under highly polluted conditions, the remediation potential of seaweeds may actually be limited by their ability to survive the complex mix of anthropogenic stressors that are present at these locations not causing mortality (Volesky 1990; Pinto et al. 2003). In this study, we assess in situ the biomonitoring and bioaccumulation potential of *U. australis* to determine whether it might be a suitable management tool in a highly metal-impacted environment. The aims of this study were to use deployed thalli to evaluate whether metal uptake can compromise the physiology of *U. australis*, to determine to what extent *U. australis* can accumulate metals in contaminated areas and assess the management implications for using *U. australis* as a biomonitor.

Methods

Study sites

The study sites were located to reflect a range of metal-impacted levels. An unpolluted control site was located 73 km southeast of Hobart city (42°52′S/147°19′E) on Bruny Island (BI), well beyond the influence of any metal contamination from the Derwent Estuary (Fig. 1 and Table 1). Two sites with historical "metal pollution" were

Fig. 1 Study area indicating control site and experimental sites, transplanted site 1 (TS1) and transplanted site (TS2) in the Derwent estuary, Tasmania
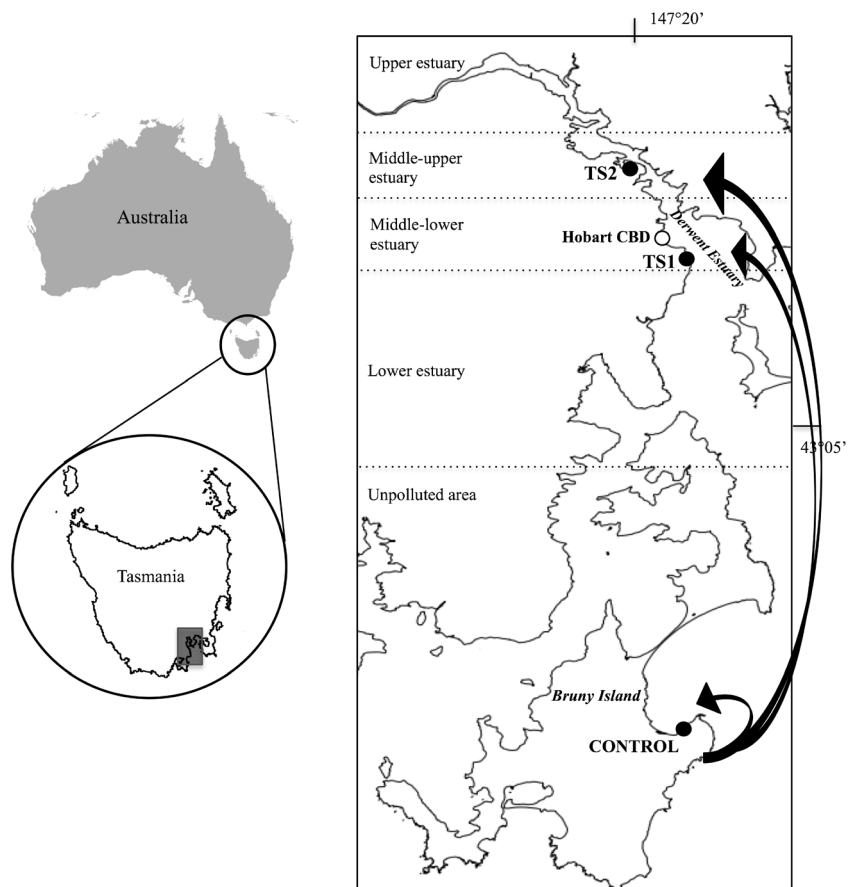
selected from the Derwent Estuary (Coughanowr et al. 2015), to receive transplanted seaweeds collected from control site. Transplant site 1 (TS1) is a recreational area located in the middle-lower estuary and transplant site 2 (TS2) is an enclosed bay, situated in the middle estuary (Fig. 1). TS2 is mainly affected by contaminants derived from urbanisation, recreational activities and industrial discharge and is characterised by high levels of metal such as cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) identified in seawater and sediment. This region also has the highest levels of ammonia + ammonium and nitrate-nitrite in the estuary (Whitehead et al. 2010; Coughanowr et al. 2015).

Metal content in *U. australis* and physiological and ultrastructural performance To evaluate metal accumulation capacity and whether metals can compromise *U. australis* performance, we used passive biomonitoring approach to analyse physiological and ultrastructural responses.

Thalli were collected from the control site, on 19 August 2015, and transplanted back into the same site and to sites TS1 and TS2. Forty-five individuals of 3.1 ± 1.5 g were haphazardly collected from the shoreline at low tide and transported in an insulated container with seawater to be transplanted elsewhere at the control site and at TS1 and TS2 the same day.

Ulva thalli were weighed and after thalli placed in each of five plastic mesh (0.6 cm mesh pore) baskets that were 18 cm height × 13 cm diameter ($n = 5$). Each of the five baskets was attached to a 1.5 m long × 2.5 cm diameter steel pole: the baskets containing *Ulva* plus the pole comprised one experimental unit (Fig. 2). Three experimental units ($n = 3$) were pushed into the sediment by snorkelling ensuring the top of the baskets were deployed at 2-m depth at high tide. The poles at each site were positioned to ensure they were 5 m away from one another. Limited replication ($n = 3$) was tested due to practical deployment conditions on the field. After 12 days, the *Ulva* thalli at each site were collected and metal content, growth rate, photosynthetic performance and concentration of photosynthetic pigments were assessed (see below). The ultrastructure of each thallus was examined (see below) and any differences noted. Additionally, 'ambient' *Ulva* plants (i.e. those that had grown naturally at each site) were collected from control site, TS1 and TS2, from the shoreline during low tide for metal comparison with the metal content in transplanted thalli.

Metal content in thalli

Metal content in *U. australis* was measured at the start and end of the experiment on both the transplanted thalli and ambient

Table 1 Environmental conditions for study sites and metal content (mg kg^{-1}) in *Ulva australis*

Parameter/site	Control		TS1		TS2	
	Experiment	FRDC[a]	Experiment	DEP 2015[b]	Experiment	DEP 2015[b]
Salinity	34.9 ± 0.1		33.1	30 ± 2	26	19 ± 4
Temperature (T°C)	15 ± 0.1		15.5	14 ± 2	14.8	14 ± 2
O$_2$ (mg L^{-1})	9.08		7.9	8 ± 2	7.4	9 ± 2
Ammonia/Ammonium (µg L^{-1})		0.59 ± 0.3		15	57	74
Nitrite/Nitrate (µg L^{-1})		1.50 ± 0.3		15	43.8	50
Nitrogen (µg L^{-1})				280		360
Total phosphorus (µg L^{-1})				40		56
Dissolve phosphate		0.23 ± 0.1				
Total Zn in surface water (µg L^{-1})	3.16 ± 0.8			12 ± 2	28.2	21 ± 2

Parameter/site	TS2			
	t_0	t_1**	t_2	t_3**
Salinity		26.78 ± 0.4		29.6 ± 1.2
Temperature (T °C)	20.1 ± 0.6	20.7 ± 0.1	20.5 ± 0.1	21.8 ± 0.2
Light (µmol m^{-2} s^{-1})	271.9 ± 0.1	105 ± 22.8	88 ± 20.4	216 ± 90.6
O$_2$ (mg L^{-1})		8.44 ± 0.04		7.03 ± 0.26
Total Zn in surface water (µg L^{-1})	20	21	24.5	31
Zn in *Ulva australis* (mg kg^{-1})	18.8 ± 2.4	217 ± 38.8	485 ± 73.1	731 ± 110.5

Control site, transplanted site 1 (TS1) and transplanted site 2 (TS2)

[a] Data courtesy of Storm Bay project, FRDC project 2014/031. Values expressed as dissolved concentrations

[b] Data courtesy of Derwent Estuary Program (DEP). Values expressed as total concentrations

thalli. *Ulva* samples were transported to the laboratory in an insulated container, and then rinsed to remove epiphytes and sand following the methods of Gledhill et al. (1998). To avoid contamination, all plastic ware were washed with alkaline detergent Decon 90.2% solution and 5% HNO$_3$ acid bath for at least 24 h. All material was then rinsed with Milli-Q water (18 MΩ).

Initial wet weight (W_i) was recorded prior to freezing at −24 °C for up to a week until analysis. Frozen samples were then freeze-dried for 48 h until constant weight and the final weight (W_f) recorded. Samples were then ground in a coffee grinder to <2-mm particle size. Ground freeze-dried samples were sent to Analytical Services Tasmania (AST), a National Association of Testing Authorities, Australia (NATA) certified laboratory, for analysis of As, Cu, Pb and Zn using an inductively coupled plasma atomic emission spectroscopy (ICP-AES). A certified reference material (CRM), dogfish muscle CRM DORM-2, was used to test the accuracy and precision of the analysis. The metal detection limit of this analysis was 0.1 mg kg^{-1} dry matter basis (DMB) for As, Cu, Pb and Zn. Any evidence of damage to the thalli was also noted as a potential response to the experiment.

Fig. 2 Schematic diagram of experimental design of transplanting thalli of *Ulva australis* for in situ assessment at the study sites

Thallus

Baskets

Experimental unit
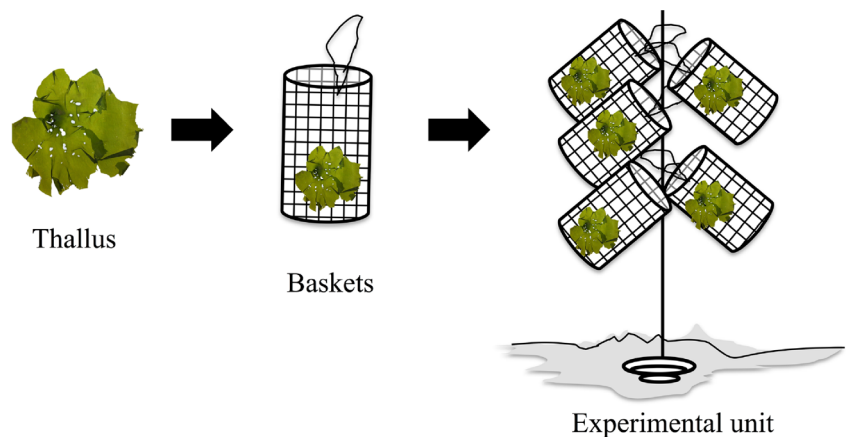
Growth rate

Growth rate (Gr) of *U. australis* was calculated by comparing the initial and final wet weight, following Yong et al. (2013). Gr (% day^{-1}) = ((W$_t$/W$_i$)$^{1/t}$ − 1) × 100, where W_t = final wet weight (g), W_i = initial wet weight (g) and t = time (days).

Photosynthetic performance

Chlorophyll *a* fluorescence in *U. australis* was measured using a pulse-amplitude modulated (PAM) diving fluorometer (Walz, Germany). The PAM was configured at Gain 4 according to Scherner et al. (2013). Before every pulse measurement, seaweeds were dark-adapted for 20 min using Walz underwater clips. Measurements were performed in situ on each replicate ($n = 3$) transplant sample before the experiment was started and at the end of the experiment and on the ambient *Ulva* thalli from every study site ($n = 3$). Photosynthetic parameters measured were maximum quantum yield of photosystem II (PSII) = F$_v$/F$_m$ and maximum relative electron transport rate (rETR$_{max}$), which is the maximal relative rate of electron transport activity (μmol electrons m^{-2} s^{-1}) (Longstaff et al. 2002; Schreiber et al. 2011). rETR$_{max}$ was calculated from the rapid light curves (RLCs) as described in Ralph and Gademann (2005) using the exponential curve model of Platt et al. (1980). All parameters were calculated by the PAM processor version 1.0 (PAM-Processor 2015) using R (R Core Team 2013).

Photosynthetic pigments

Chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) contents were analysed by collecting 0.5 g of *Ulva* at the start and end of the experiment. Samples were frozen at −20 °C until analysis. For pigment extraction, thalli were placed in a 15-mL polypropylene tube with 3 mL of dimethyl sulfoxide 99.9% (DMSO; Sigma-Aldrich) for 1 h in an oven at 40 °C. Then, 200 μL of solution was added to a 6.4-mm diameter 96-well microplate (IWAKI) and absorbance was read using a micro-plate reader (Biotek synergy HT) to detect Chl *a* (663 nm) and Chl *b* (645 nm). Pigments were quantified according to Wellburn (1994) and the results of photosynthetic pigments expressed in μg g^{-1} wet weight.

Ultrastructure

Samples for ultrastructure analysis were collected at the end of the transplant experiment. Individual *Ulva* were sampled to collect 1-mm length sections. These were placed in a vial and fixed in 2 mL of 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH = 7.2) and 2% paraformaldehyde overnight. Samples were post-fixed with 1% osmium tetroxide and 0.05 M sodium cacodylate for 8 h followed by dehydration in an ethanol gradient series. Fixation time and reagent concentrations were modified from those previously described by Bouzon et al. (2011). Finally, samples were embedded in Spurr's resin (Low viscosity, ProSciTech) and 60-nm sections cut with an ultramicrotome (Leica EM UC7) before viewing in a transmission electron microscope (JEOL JEM-1011 TEM). Block cutting and TEM analysis were undertaken at the Central Laboratory of Electron Microscopy, Federal University of Santa Catarina, Florianópolis, Brazil.

Environmental data

The Derwent Estuary Program (DEP) provided details on the total Zn (μg L^{-1}) and nutrient (i.e. ammonium, nitrate and total phosphorus) concentrations in surface seawater (0.1 m depth) at TS1 and TS2 at the time of sampling. In addition, surface seawater samples, at 0.1 m depth (250 mL), were collected for total metal analysis from the control site at the start of the experiment (Table 1), and nutrient data (i.e. ammonium, nitrate and phosphate) were provided by the Storm Bay project (FRDC project 2014/031) for the control site. Total Zn metal analysis and nutrients in water were analysed by AST. The water detection limit for Zn was 1 μg L^{-1}.

Bioaccumulation assessment To address the bioaccumulation capacity of *U. australis*, we conducted an in situ experiment in a highly metal-polluted site. The metal accumulation capacity in a long-term deployment and bioaccumulation rate were assessed as follows.

To determine metal uptake rate and metal bioaccumulation, individual thalli of *U. australis* (14.5 ± 1.2 g) were deployed on 6 January 2016 in individual baskets as described above. In this case, four mesh baskets each containing one weighted *Ulva* individual were attached to each pole, giving 6 experimental units, which were deployed at TS2. Samples were monitored for growth and metal accumulation (see above) at the start of the experiment (t$_0$), and at regular intervals (t$_1$ = 15 days, t$_2$ = 30 days and t$_3$ = 45 days) thereafter, with one basket being removed from each experimental unit each time. Any evidence of damage to the plants was also noted as a potential response to the treatment.

To evaluate the association between metal accumulated in thalli and metals available in the environment, surface water samples were collected with a 50-mL syringe and a filtered through a Sartorius Stedim 0.45-μm filter (Minisart), into 50-mL plastic centrifuge tubes, and kept at −24 °C until analysis. Seawater samples were analysed by AST using an ICP-AES. Metal concentrations in seawater are expressed as μg L^{-1}. Data loggers (HOBO pendant) were attached to three of the experimental units ($n = 3$) to provide details of any differences in the temperature (°C) and light (μmol photons m^{-2} s^{-1}) throughout the 45 days of experiment.

Zinc bioaccumulation in *U. australis* is expressed as the concentration factor (CF), which is the ratio of the metal (Zn) content in seaweed (C$_o$) and the metal concentration detected in seawater (C$_{sw}$), CF = C$_o$ mg kg^{-1}/C$_{sw}$ μg L^{-1} (Conti

and Cecchetti 2003). Additionally, the metal accumulation rate (Mr) provides an estimate of the metal (Zn) content in the thalli at experimental time (At_f) less the metal content in the thalli at the beginning (At_i) divided by time (t):
$$Mr = (At_f - At_i)/t.$$

Statistical analysis

A one-way ANOVA (95% confidence interval) was used to evaluate differences in *Ulva* metal content, Gr, photosynthesis performance and photosynthetic pigments content between transplanted thalli. Levene's test was used prior to analysis to assess normality and homogeneity of the data; no transformation was required. Analysis was carried out with SPSS IBM Statistics version 22.

Photosynthesis performance, changes in photosynthetic pigment contents and changes in metal concentration of ambient versus transplanted plants were assessed using a two-way ANOVA (95% confidence interval, factors: site and treatment). Again, Levene's test was used to assess normality and homogeneity of variance, with variance heterogeneity corrected using weighted least square regression (WLS). A Tukey post hoc test was conducted where significant differences were observed, in order to further clarify the difference between ambient and transplanted thalli. Analysis was conducted in R studio statistical software (R Core Team 2013).

The relationship between Zn accumulated in *U. australis* and total Zn in surface waters was assessed using a Pearson Correlation test, conducted in R studio statistical software (R Core Team 2013).

Results

Metal content in *U. australis* and physiological and ultrastructural performance At the end of the 12-day experiment, As, Cu, Pb and Zn were detected in the transplanted *U. australis* at each of the three study sites, with Zn content being consistently higher than other metals (Fig. 3). However, As, Cu and Pb were not accumulated to any significant level in *Ulva* and so were not considered further in this analysis. For Zn, there were significant differences between control and transplanted sites ($p = 0.001$, F = 11.908, df = 2) with a strong interaction between site (contaminant level) and treatment (transplanted plants) ($p = 0.018$, F = 12.148, df = 3) (Fig. 4). Post hoc comparison revealed significantly higher levels of Zn accumulated in thalli transplanted to TS2 compared to control plants ($p = 0.001$), Zn content was greater in transplanted thalli at TS2 than at TS1 ($p = 0.001$), and Zn was greater in transplanted thalli at TS1 in relation to the control ($p = 0.015$).

Ulva australis collected at the control sites had approximately 12.6 mg kg^{-1} of Zn, compared with ambient plants that were grown at TS1 and TS2, which had 30.5 and 113.7 mg kg^{-1}
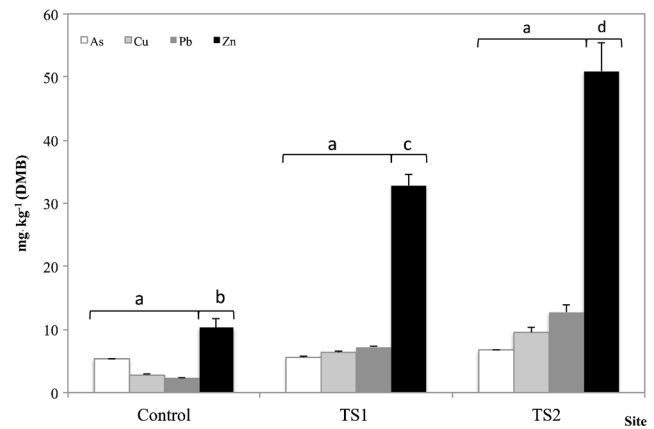
Fig. 3 Metal content (mg kg^{-1} dry matter biomass, DMB) in *U. australis*, arsenic (As), copper (Cu), lead (Pb) and zinc (Zn) after 12 days of transplantation. Control site, transplanted site 1 (TS1) and transplanted site 2 (TS2). Values expressed on mean (±SE, $n = 3$). *Letters above the bars* indicate differences between control, TS1 and TS2 shown as *a*, *b*, *c* and *d* (transplanted plants only). *Same letters denote* no significant differences

DMB, respectively (Fig. 4). After 12 days, the levels of Zn in transplanted thalli at the control site were not markedly different ($p > 0.05$) from that observed in the control ambient plants (i.e. in 12 days, plants accumulated 81.5% of the initial content or 10.3 mg kg^{-1}). However, at TS1, the Zn content in the transplanted *U. australis* had increased to 107.3% of ambient levels or 32.8 mg kg^{-1}. Interestingly, transplanted *Ulva* at the most polluted site (TS2) took up only 44.8% of the ambient loads (50.9 mg kg^{-1}) (Fig. 4).

The concentration factor (CF) results show that Zn bioaccumulation was measurable over 12 days of deployment; CF was lower at control and semi-polluted sites (TS1) than at TS2, the more polluted site (Table 2). There was a high correlation
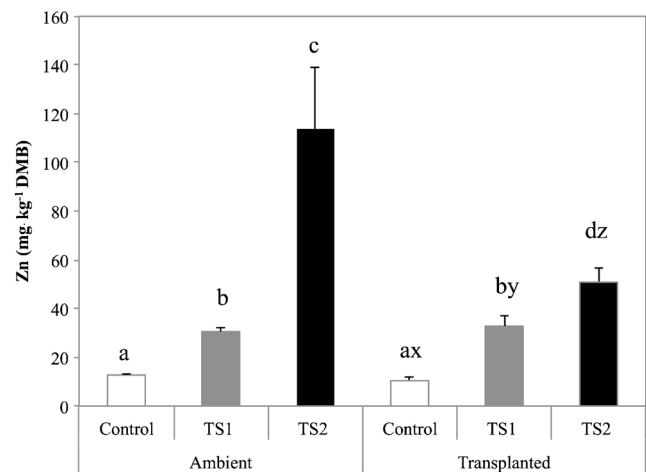
Fig. 4 Zinc (Zn) content (mg kg^{-1} dry matter biomass, DMB) in *U. australis* at control site, transplanted site 1 (TS1) and transplanted site 2 (TS2). Metal levels expressed as mean concentration (±SE, $n = 3$). *Letters above the bars* indicate how the transplanted thalli compare to ambient plants within each sites shown as *a*, *b*, *c* and *d*, and differences between control, TS1 and TS2; shown as *x*, *y* and *z* (transplanted plants only). *Same letters* denote no significant differences

Table 2 Concentration factor (CF) for *Ulva australis* experiment 1 ($n = 3$) and experiment 2 ($n = 6$), and metal accumulation rate (Mr) for experiment 2

Physiological and ultrastructural assessment		
Site	Treatment	CF
Control	Transplanted	5.2 ± 0.6
	Ambient	6.3 ± 0.1
TS1	Transplanted	5.5 ± 0.7
	Ambient	7.5 ± 1
TS2	Transplanted	5.1 ± 0.6
	Ambient	11.4 ± 2.5
Bioaccumulation assessment		
	CF	Mr (mg kg^{-1} day^{-1})
TS2	t_0	0.9 ± 0.1	–
	t_1	10.4 ± 1.8	13.3 ± 3
	t_2	19.8 ± 3	17.9 ± 4
	t_3	23.6 ± 3.3	24.3 ± 8

Values expressed as mean (±SE)

between the total Zn concentration in surface seawater and Zn accumulated in *Ulva* ($r = 0.87$, $p = 0.001$, df = 7).

Physiological performance

Growth rate, maximum quantum of yield (F_v/F_m), the maximum relative electron transport rate (rETR$_{max}$) and photosynthetic pigment content did not appear to be affected by the different metal contents at the transplant locations. No significant differences ($p > 0.05$) in growth rate, F_v/F_m, rETR$_{max}$ (Table 3) or photosynthetic pigments (Chl *a* and Chl *b*) between control and transplanted thalli at either of the contaminated sites were observed (Table 3). Similarly, there was no significant difference ($p > 0.05$) in growth rate, F_v/F_m, rETR$_{max}$ or photosynthetic pigments when transplanted plants were compared with ambient plants (Table 3).

Ultrastructure

The cytology of the control cells shows that the internal organelles are well organised, and the key cellular components,

such as chloroplast, starch grains, lipid bodies, Golgi body and vacuoles, are clearly visible (Fig. 5a). Chloroplasts are delimited by vacuoles and the thylakoids are parallel enclosing starch granules (Fig. 5b), a mitochondrion is visible near the chloroplast (Fig. 5b, c), and the cell wall shows organised cellulose microfibrils sitting parallel with each other (Fig. 5c, d). A few mitochondria were visible associated with the chloroplast and Golgi (Fig. 5e).

However, 12 days after being transplanted to a semi-polluted site (TS1), there were marked changes in the cell cytology. Cells showed evidence of electron-dense material in the vacuole and an increase in the number of mitochondria in relation to the control (Fig. 6a–d). Thylakoids of the chloroplast appear to be organised in groups and slightly disarranged enclosing a starch grain (Fig. 6b, c). Cellulose fibrils of the wall are irregularly positioned and electron-dense material deposited that was not observed in the control (Fig. 6c, d). Thalli transplanted to the high-polluted site (TS2) showed a higher level of damage within the cell. The cytoplasm is retracted and modified vacuoles show that metals have affected plasmolysis; the amount of starch grains is also noticeably reduced (Fig. 7a) in relation to the control. In a number of cells, electron-dense material was observed inside the vacuoles (Fig. 7a, b) and similarly on the cell wall (Fig. 7c). Although the thylakoids were not strongly affected, an increase in mitochondria number suggests increased of mitochondrial activity (Fig. 7d).

Bioaccumulation assessment

There was clear evidence of an increase in Zn content in *U. australis* over the time (Table 1), with a significant increase in the Zn content of the transplanted thalli ($p = 0.001$, F = 22.632, df = 3). Although there was no significant difference between Zn content in control and transplanted thalli after 15 days ($p > 0.05$), the levels were significantly greater at the transplanted sites after 30 days, and levels continued to increase up to 45 days ($p = 0.001$). There was a strong correlation between total Zn concentration in seawater and Zn content in the *Ulva* ($r = 0.85$, $p = 0.001$, dg = 22). Bioaccumulation was evident after 15 days at the highly polluted site (TS2).

The CF differed depending on length of exposure, increasing as metal exposure time increased. The metal accumulation

Table 3 Physiological and photosynthetic responses of *Ulva australis* in situ assessed after 12 days of transplantation

Parameter	GR (% day^{-1})	F_v/F_m		rETR$_{max}$ (μmol electrons m^{-2} s^{-1})		Chlorophyll *a* (μg g^{-1})		Chlorophyll *b* (μg g^{-1})	
Site/Treatment	12 days	12 days	Ambient	12 days	Ambient	12 days	Ambient	12 days	Ambient
Control	4.6 ± 1.4	0.79 ± 0.03	0.79 ± 0.1	8.3 ± 0.2	12.3 ± 2.3	78.5 ± 3.3	71.6 ± 10.5	36.2 ± 1.7	33.9 ± 5.0
TS1	3.01 ± 1.2	0.83 ± 0.01	0.77 ± 0.06	14.6 ± 1.4	10.2 ± 0.1	105.7 ± 46.6	80.3 ± 6.2	65.1 ± 18.9	38.1 ± 3.7
TS2	6.1 ± 0.9	0.82 ± 0.02	0.86 ± 0.0	17.3 ± 2.9	20.8 ± 4.03	77.03 ± 7.9	61.8 ± 8.3	40.7 ± 7.1	32.6 ± 4.2

Control site, transplanted site 1 (TS1) and transplanted site 2 (TS2). Values expressed as mean of measured parameter (±SE, $n = 3$)
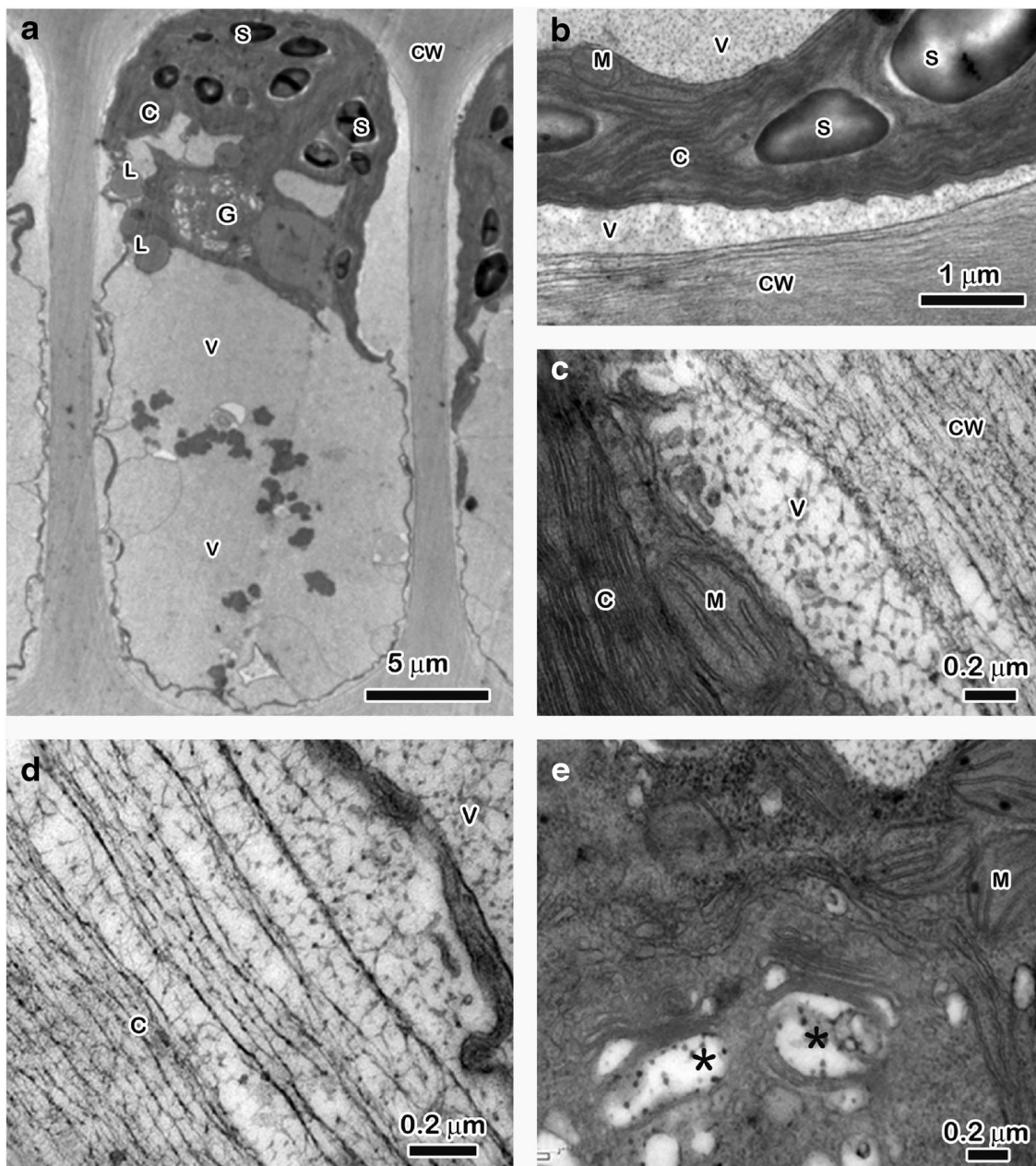
Fig. 5 Transmission electron microscopy (TEM) micrographs of *U. australis* ultrastructure from control thalli. **a** Cell general view with internal components well organised; **b** cell wall, starch granules and chloroplast; **c** cell wall, vacuole and thylakoids of chloroplast parallel organised; **d** cellulose fibrils of cell wall and **e** mitochondria on chloroplast. *C* chloroplast, *CW* cell wall, *G* Golgi, *L* lipid droplets, *M* mitochondria, *S* starch granules, *V* vacuole and *internal Golgi material

rate (Mr) showed a similar pattern, also increasing over time (Table 2), from 13.3 mg kg^{-1} day^{-1} after the first 15 days increasing to 17.9 and 24.3 mg kg^{-1} day^{-1} after 30 and 45 days, respectively. Zn content started at 18.8 ± 1.5 mg kg^{-1} (t_0) and reached the highest concentrations, 863 mg kg^{-1}, in the transplanted thalli after 45 days (Table 1). The transplanted thalli appeared visibly degraded after 15 days, and the damage in the thalli continued to increase over the time, being markedly deteriorated after 30 and 45 days (Fig. 8).

Discussion

This study provides evidence of metal accumulation in *U. australis*, confirming that in situ deployment of this species can be an effective means to monitor metal pollution but also suggesting that *Ulva* deployment may have potential as a means to remove metals under certain conditions.

Passive biomonitoring (PBM) assessment showed that transplanted *U. australis* thalli accumulate metals to levels
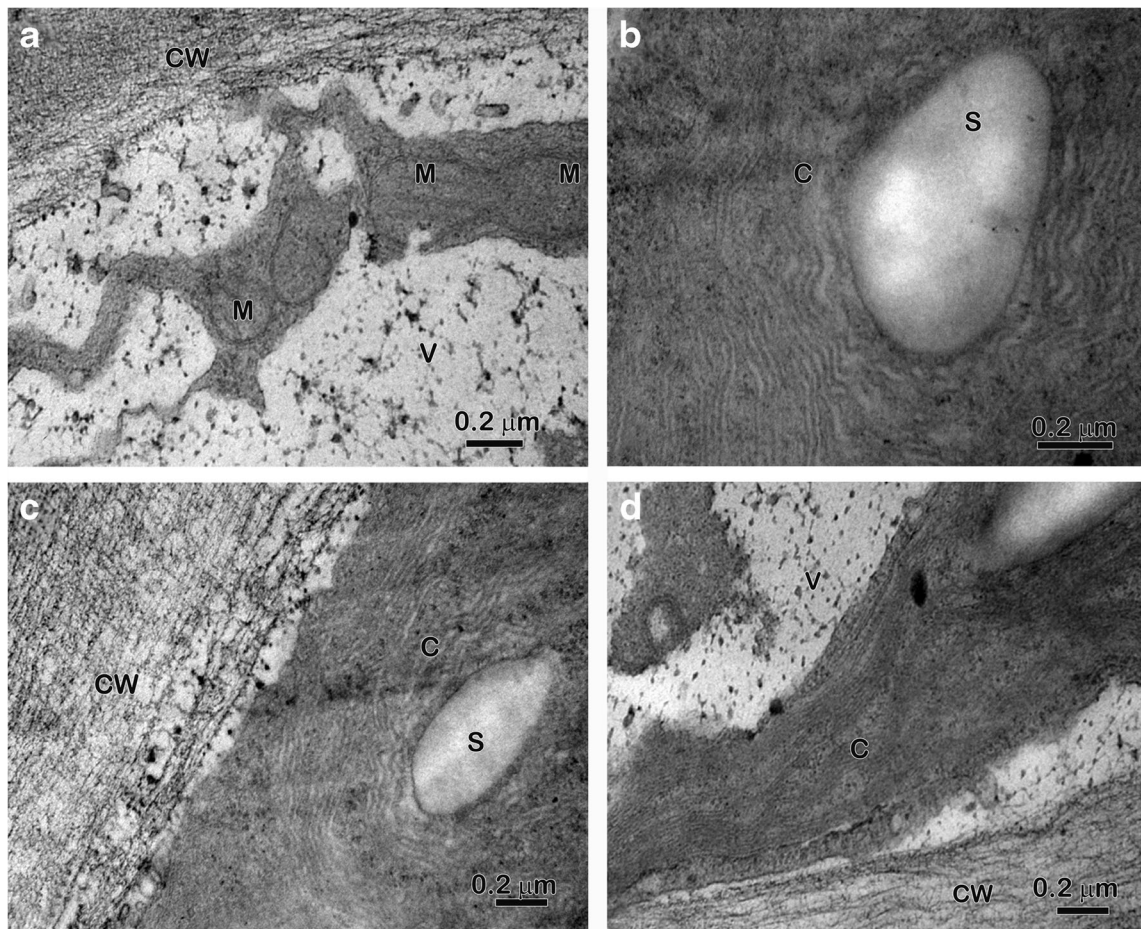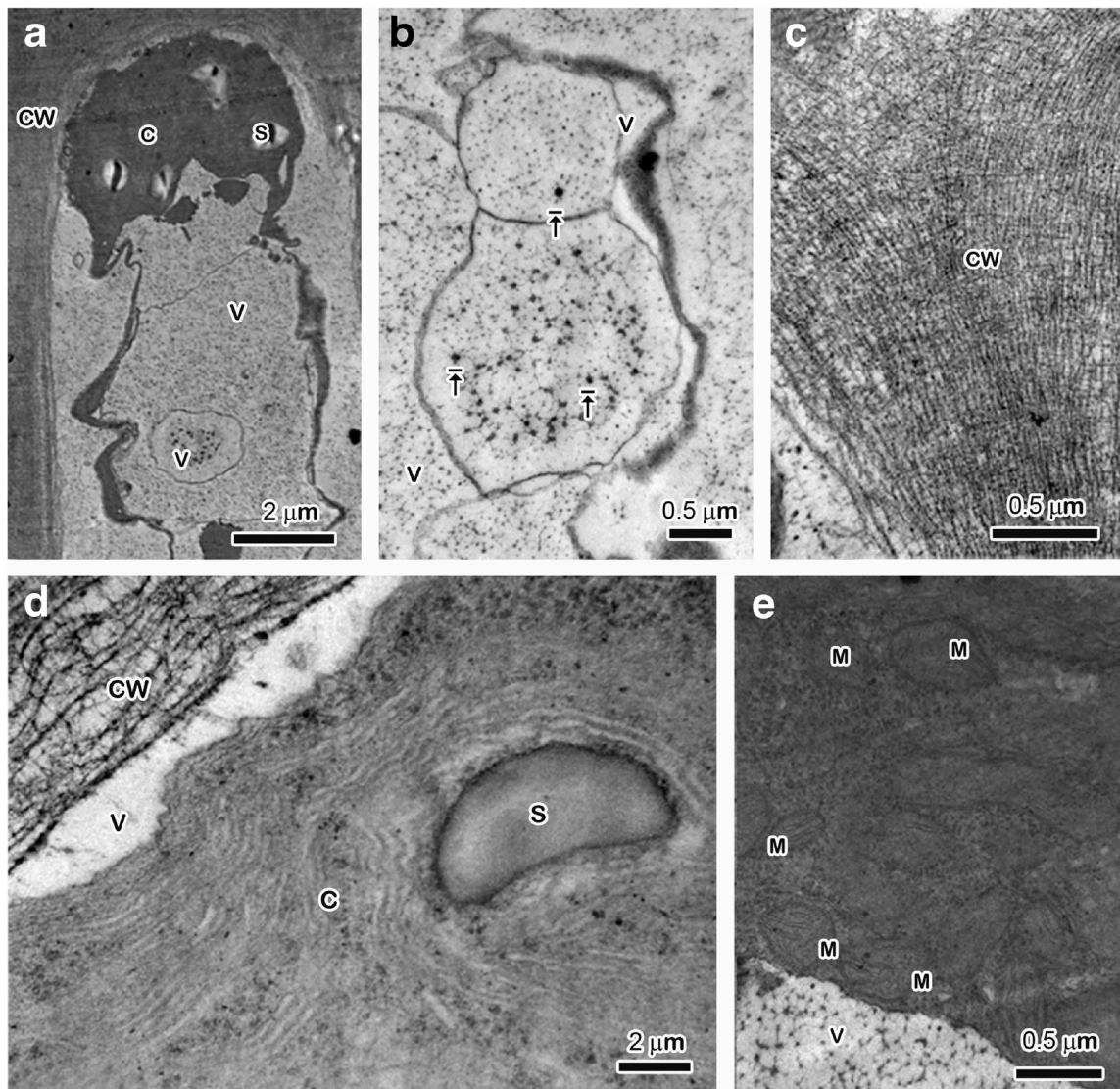
Fig. 6 Transmission electron microscopy (TEM) micrographs of *U. australis* ultrastructure transplanted to a semi-polluted site (TS1). **a** Electron-dense bodies deposited on vacuole; **b** thylakoids of the chloroplast slightly altered; **c** electron-dense bodies deposited on the cell wall; **d** cell wall cellulose fibrils slightly unorganised. *C* chloroplast, *CW* cell wall, *M* mitochondria, *S* starch granules, *V* vacuole

similar to those detected in ambient plants, with the final Zn content reflecting background contamination levels. Metal content in algae clearly increased as metal levels in the environment increased, which is consistent with previous observations (Mehta and Gaur 2005). In order to utilise seaweeds effectively as a bioindicator, it is important to know how long it takes to achieve a level of contamination that would be equivalent to that observed in seaweed under ambient conditions. In this case, equilibrium with ambient metal content was achieved relatively in a short period of time: 12 days. After 12 days, there was a linear correlation between the level of metals accumulated in the transplanted thalli and the metal available in the environment. It has been suggested that a linear relation between these parameters is an indication of a good bioindicator (Rainbow and Phillips 1993; Hurd et al. 2014). Long-term deployment, 45 days, showed that *U. australis* could accumulate Zn at very high concentrations, and that accumulation increased linearly over time. The mean Zn concentration at 45 days (731.4 mg kg^{-1}) was 58 times higher than that observed at the control site. This was considerably higher than levels previously reported for *U. lactuca*

66 mg kg^{-1} from polluted sites (Ho 1990), and 10 mg kg^{-1} in *Ulva* sp. from unpolluted areas (Ryan et al. 2012). These results are consistent with those previously reported for Zn in transplanted *Ascophyllum nodosum* and *Fucus vesiculosus*, where Ho (1984) in a seminal study found that Zn had increased 19 and 95%, respectively, after 2 months compared to the concentration detected in ambient plants.

Zn content in *Ulva* assessed over 45 days was markedly higher than that in plants deployed for only 12 days, suggesting that a longer exposure time enhanced bioaccumulation. Bioaccumulation is the active process by which an organism uptakes metals; in this process, the organisms can resist/tolerate particular toxicants (Oliveira et al. 2011). However, we observed that the longer the plants were deployed, the greater the thallus deterioration was observed. Whilst this could be just natural deterioration related to the seasonal life-cycle of *Ulva* (Hurd et al. 2014), it could also be related to experimental conditions (e.g. experimental baskets). Consequently, we would suggest limiting the deployment time for *U. australis* no longer than 20 days, as this timeframe allowed plants to accumulate high concentrations of Zn

Fig. 7 Transmission electron microscopy (TEM) micrographs of *U. australis* ultrastructure transplanted to a high-polluted site (TS2). **a** Cell general view demonstrating irregularity on the cytoplasm delimitation; **b** in a number of cells electron-dense materials was observed inside of vacuoles (*arrows*); **c** cell wall fibrils and electron-dense bodies; **d** chloroplast is not visible affected and **e** increment of mitochondria on chloroplast. Repetitive results were identified in all cell and samples analysed making the data robust. *C* chloroplast, *CW* cell wall, *M* mitochondria, *S* starch granules, *V* vacuole

(~300 mg kg^{-1}) without visual deterioration. This strategy would enable to uptake high amount of metals and quickly "clean-up" of toxicants in the environment.

Metal accumulation was greatest at TS2, which is one of the most polluted areas, both in terms of metals and nutrients, in the Derwent Estuary (Coughanowr et al. 2015). Several studies have suggested that ambient concentrations of nutrients such as nitrate, ammonium and phosphate can influence metal accumulation (Lee and Wang 2001), with an increasing metal uptake capacity when nitrogen is widely available (Pinto et al. 2003). In a related species, *U. fasciata*, it has been shown that under controlled conditions, the rate of Cd accumulation increased with increasing nitrate concentration (Lee and Wang 2001). Therefore, it might be suggested that high

levels of nutrients (i.e. ammonium and nitrite) and total inorganic nitrogen (360 μg L^{-1}) available at TS2 (Prince of Wales Bay) (Coughanowr et al. 2015) may have facilitated the metal uptake, providing the optimum environmental conditions for *U. australis* to accumulate metals.

Salinity has been also shown to affect metal uptake, increasing when salinity decreases. A previous study showed that metal concentration in *U. australis* sampled as a part of ABM was higher at less saline areas (D. Farias unpublished data). This may be a function of the fresh water having higher concentrations of metals than seawater (Hurd et al. 2014). The PBM in the current study showed that major ultrastructural changes were apparent at lower salinities. The effect of salinity on uptake has been shown in a laboratory experiment

Fig. 8 *Ulva australis* thalli transplanted in high-polluted site (TS2) to evaluate metal uptake rate. **a** Initial thallus condition; **b** transplanted thallus at 15, **c** 30 and **d** 45 days
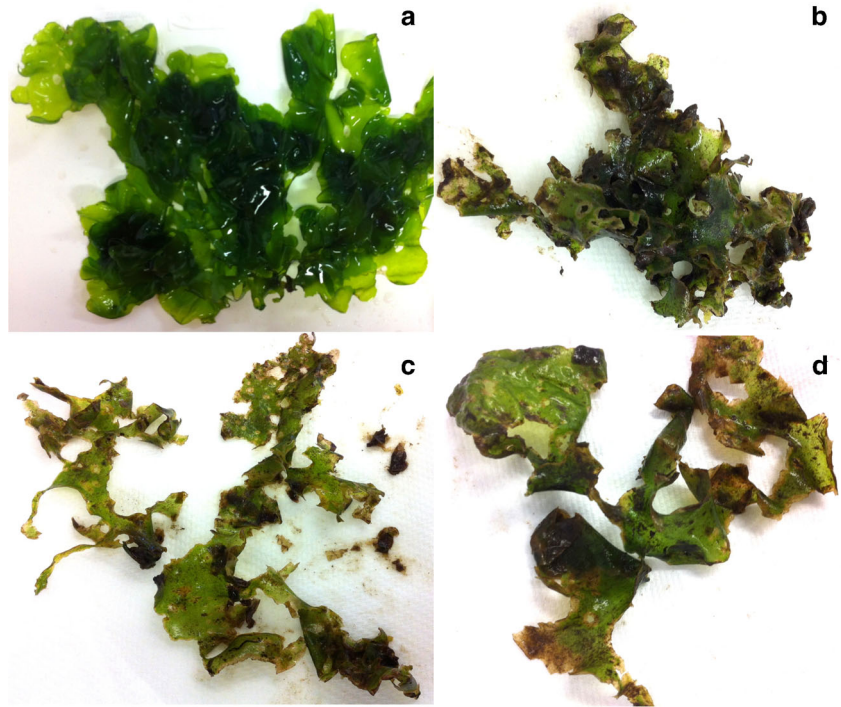
(Felix et al. 2014), who showed that Cd absorption in the red algae *Pterocladiella capillacea* increased when salinity decreased resulting in damage to the ultrastructure and negatively affect the physiological performance. Salinity has been recommended as a relevant parameter that should be considered when using seaweeds in estuarine biomonitoring programs (Munda 1984; Mamboya et al. 2009). The results suggest that the combined effects of low salinity, high nutrients levels and high metal loads at site TS2 would make this site an optimal location for biomonitoring, both ABM and PBM, as well as good location for evaluating the in situ efficiency of *U. australis* as a potential bioremediation tool.

Internal cellular changes showed the effects of metal accumulation. Whilst cellular changes were observed in transplanted *Ulva* plants from both sites, TS1 and TS2, there was a greater level of change (i.e. incorporation of electron-dense bodies and the increment of mitochondria and vacuoles) in thalli from TS2 site. The presence of electron-dense bodies clearly indicate metal deposited within the cell, a feature previously observed in laboratory experiments (D. Farias unpublished data) and confirming the role of the cell wall in metal accumulation in *U. australis*. Previous laboratory experiments had demonstrated metal deposits in *Hypnea musciformis* exposure to Cd (Bouzon et al. 2011), in *P. capillacea* (Felix et al. 2014) and in *U. flexuosa* (formerly *Enteromorpha flexuosa*) exposed at 50 μg L^{-1} of Cu (Andrade et al. 2004). The increase in the number of vacuoles could be explained by the increment in the Golgi activity. The Golgi is an active organelle that forms new vesicles (Lanubile et al. 1997), and cytoplasm vesiculation has been

related to the association between cell wall and metals ions in *U. laetevirens* exposed to Cd as a metal tolerance mechanism (Vecchia et al. 2012). This alteration has been observed in the microalga *Chlorella vulgaris* exposed to Cd, where it was also proposed to be a mechanism to improve metal tolerance and maintain low cytosolic ion concentrations (El-Naggar and Sheikh 2014). There were more vacuoles observed in the transplanted thalli from TS2, suggesting that there was a higher level of cellular processes involved in managing metal accumulation. Interestingly, despite the marked changes observed in both metal accumulation and ultrastructure, there was no evidence of any significant effects on the growth rate, maximal photochemical yield (F_v/F_m), maximum relative electron transport rate (rETR$_{max}$) or pigment content of transplanted *U. australis* thalli. This could suggest acclimation of transplanted plants.

Most of the investigations of the physiological effects of metal contamination on seaweed have been undertaken under controlled conditions. Unfortunately, the results of these lab-based experiments are highly variable and may not necessarily reflect the complexity of natural situation (Hurd et al. 2014). For instance, the concentrations of Zn and Cu can affect the Fv/fm in Ulvacean (Andrade et al. 2004; Han et al. 2008; Baumann et al. 2009) and similarly, mercury (Hg) has been shown to severely affect the F_v/F_m of *Caulerpa racemosa*, *C. lentillifera* and *U. reticulata* (Zakeri and Abu Bakar 2012). While Pb increased the electron transport rate ETR in *S. cymosum*, Cu inhibited ETR after 7 days of exposure (Costa et al. 2015). The current study was specifically developed to evaluate metal accumulation in situ, under the complex range

of conditions that really occur in nature, as it was demonstrated in *Padina gymnospora* and *S. stenophyllum* (Amado Filho et al. 1999) and therefore is better placed to characterise the applicability of *U. australis* as a monitoring and management tool under actual field conditions. The results show that *U. australis* can be transplanted to metal-polluted areas for a short period of time without affecting the photosynthesis performance.

In a recent study, Zakeri and Abu Bakar (2012) suggested the maximum quantum yield (F_v/F_m) as the most effective parameter to evaluate metal toxicity in algae. Using this approach Scherner et al. (2012) showed in situ that the F_v/F_m in *U. lactuca* improved 26 days after transplantation from clean to polluted areas, whilst F_v/F_m declined in the brown seaweed *S. stenophyllum* subject to the same conditions. This clearly demonstrated that there are differences in tolerance between species exposed to contamination. However, in this research, we showed that the photosynthetic performance is not only a relevant method to evaluate in situ metal effects; the assessment of the ultrastructure is a valuable and novel tool that can clearly demonstrate metal accumulation effects in seaweeds, and one which would add valuable to in situ assessment.

Whilst the results of this study clearly show the potential value of *U. australis* as a bioindicator of metal pollution and the effectiveness of this species in metal accumulation, the levels obtained at the more polluted sites do raise some concerns with respect to the potential for adverse environmental interactions. Concentrations of Zn accumulated in *U. australis* at the most polluted site (TS2) were high (above 700 mg kg^{-1} after 45 days) and as such may provide a mechanism for transfer of metals into the food web (Young 1975; Huang et al. 2008). There are no specific guidelines for acceptable levels of metals in seaweeds, either in terms of seafood safety or environmental acceptability. However, it is suggested that levels of Zn in sediments should be below 410 mg kg^{-1} to protect environmental values within aquatic ecosystems (ANZECC 2000). Whilst these values cannot be directly compared, the fact that the levels obtained in seaweeds are almost double that recommended for sediments might suggest that there is a risk here and further research might be needed to better characterise this before employing *Ulva* in the natural environment. The levels of Zn which are considered toxic for human consumption in crustaceans (<40 mg kg^{-1}), molluscs (130–290 mg kg^{-1}) and fish (<15 mg kg^{-1}) vary markedly (FSANZ 2006) but in all cases are considerably lower than those observed in *U. australis* at the most contaminated site after 45 days. As explained above, these levels cannot be directly compared and clearly if *Ulva* is used for remediation would never be used for human consumption. But, the very high levels observed in *U. australis* at the most contaminated site might suggest that there could be an indirect risk of toxicity and/ or trophic accumulation.

In conclusion, passive biomonitoring showed that in 12 days, *U. australis* uptake metal rates that reflected background loads, with higher uptake at the most polluted sites, showing the potential of this species as a bioindicator of metal pollution. Metal accumulation did not affect *U. australis* physiology as growth rate, photosynthesis performance (F_v/F_m and rETR$_{max}$) or photosynthetic pigment content. However, there was evidence of internal cellular changes (incorporation of electron-dense bodies) that could be related to metal load.

The bioaccumulation assessment, 45 days, demonstrated that accumulation increased over the time. However, there was deterioration of the thalli over time and as a result, it is suggested that 20 days is the most appropriate time for deployment *U. australis*. This timeframe would allow the plant to accumulate high levels of metals in a heavy metal-impacted environment whilst still remaining healthy and viable. These recommendations will enable *U. australis* to be more effectively used as a biomonitor to provide better understanding for managing metal pollution.

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