Accumulation of selenium in Ulva sp. and effects on morphology, ultrastructure and antioxidant enzymes and metabolites

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A R T I C L E   I N F O

Article history:
Received 15 May 2012
Accepted 24 June 2012

Keywords:
Ulva
Selenium
Sulfur
Antioxidants
Ultrastructure
Photosynthetic activity

A B S T R A C T

The impact of selenium (Se) on Ulva sp., a green macroalga naturally growing in the Venice Lagoon, was investigated. The alga was provided for 10 days with concentrations of selenate (Na2SeO4) ranging from 0 to 100 μM. Se accumulation in the algal biomass was linearly related to the selenate dose and this relationship was not affected by the high sulfate concentration measured in the seawater. The amount of Se measured in the alga was always relatively low and not hazardous to algal consumers. However, Se induced the formation of hydrogen peroxide (H2O2) in Ulva sp. and, as a result, the activity of antioxidant enzymes (superoxide dismutase, SOD, and catalase, CAT) and the amount of antioxidant metabolites (phenols, flavonoids and carotenoids) increased, even when selenate was supplied to the macroalga at low concentration (2.5 μM). This indicated that different components of the antioxidant defence system played a pivotal role in overcoming oxidative damage by Se in the macroalgae, and explained the lack of morphological and ultrastructural alterations in Ulva sp. exposed to selenate.

1. Introduction

Selenium (Se) is a very important element from an ecotoxicological point of view due to the narrow concentration range existing between its essentiality and toxic effect to human and animal health (Pilon-Smits and LeDuc, 2009; Zhu et al., 2009).

In the aquatic environments, Se occurs principally in two oxidation states, Se3+ (selenite) and Se6+ (selenate) (Plant et al., 2004). The ratio between selenite and selenate depends on the water pH and on the presence of complexing agents and organic matter (Pyrzynska, 1998). Generally, selenite dominates under reducing conditions, while selenate is mainly found in oxidizing alkaline waters. Furthermore, selenate is highly soluble and thus more bioavailable than selenite to aquatic organisms (Chapman et al., 2010; Plant et al., 2004). Organic selenides can also exist in natural waters, although at lower concentration than inorganic Se compounds (Fan et al., 2002).

Uptake studies indicate that selenite and selenate can be incorporated into algal cells (De Alcantara et al., 1998; Wheeler et al., 1982) and affect growth in a dose-dependent manner (Umisová et al., 2009). At low concentration, Se acts as beneficial element by promoting normal cell growth and function, as observed in plants (Pilon-Smits et al., 2009; Reunova et al., 2007). For several marine unicellular algae, including the green alga Chlamydomonas reinhardtii, Se has even been recognized as an essential nutrient, being a component of important seleno-enzymes similar to those identified in mammals (Fu et al., 2002; Harrison et al., 1988; Novoselov et al., 2002). However, at high dose Se is toxic to algae, leading to reduction of growth rate or alterations in the levels of reactive oxygen species (ROS) that may cause cellular damage (Fournier et al., 2010; Pelah and Cohen, 2005; Umisová et al., 2009; Wheeler et al., 1982). In the freshwater microalga Chlorella zofingiensis, the treatment with selenite caused an increase in activity of antioxidant enzymes, including superoxide dismutase (SOD) isoforms (Pelah and Cohen, 2005). In a recent study, Chlorella vulgaris was shown to produce higher amount of phytochelatins and glutathione (GSH) in response to toxic selenate concentrations (Simmons and Emery, 2011).

The toxic effects of Se on marine algae depend on the alga species (Abdel-Hamid and Schulberg, 2006; Dazhi et al., 2003; Wheeler et al., 1982). Se concentration, and also on the oxidation state of the element (Pastierova et al., 2009; Umisová et al., 2009). Indeed,
although high cellular concentration of either selenite or selenate may cause oxidative stress or cell apoptosis, selenite was found to be less toxic than selenate in many cases, at least in microalgae (Wheeler et al., 1982).

The uptake of inorganic Se species (selenite and selenate) is known to vary as a function of pH over the range 5–9 (Riedel and Sanders, 1996; Tuzen and Sari, 2010). In C. reinhardtii the maximum uptake of selenate occurred at pH 8, whereas selenite uptake increased significantly at the lower pH values (Riedel and Sanders, 1996).

Selenium accumulation in algae can also be affected by the presence of certain macronutrients, like phosphorus (P) and sulfur (S) (Lee and Wang, 2001). Sulfate, in particular, is a well-known antagonist of selenium (Fournier et al., 2010; Simmons and Emery, 2011; Williams et al., 1994). In C. reinhardtii the toxicity of selenate appeared to be directly correlated to intracellular Se accumulation, which was directly dependent on the ambient concentration of sulfate that may compete with selenate for the transport proteins (Fournier et al., 2010). In the same microalga and in Selenastrum capricornutum, increasing sulfate concentration in the growth substrate resulted in a substantial decrease of selenate (Riedel and Sanders, 1996; Williams et al., 1994) and selenite (Morlon et al., 2006) uptake, and the green microalga Scenedesmus quadricauda was found to be more sensitive to selenite and selenate under S deficient conditions (Umisová et al., 2009). Since S. capricornutum cells supplied with different concentrations of selenate and sulfate exhibited different capacity to take up selenate even though the S:Se molar ratio was maintained, the existence of different permease affinities for sulfate and selenate and/or of more permease systems for these ions in algae has been hypothesized (Williams et al., 1994).

Macroalgae may have a great potential as Se bioindicators, due to their wide distribution and large sizes (Lee and Wang, 2001). Furthermore, a number of species, including Ulva sp., can be introduced in the human and animal diet, especially in the form of dietary supplements, being considered a rich source of natural antioxidants (Duan et al., 2006; Fleurence, 1999; Kuda et al., 2005; Zhang et al., 2003).

To our knowledge data concerning the effects of Se in microalgae are well documented, while no studies have been performed to elucidate in details the cellular response to this element by macroalgae. On this account, the current research is aimed at investigating the capability to accumulate and tolerate Se by a green laminar seaweed Ulva sp., growing naturally in the Venice Lagoon.

Sulfur content in the alga and in the seawater was determined as a potential factor affecting Se accumulation of Ulva. The effects of Se accumulation in the alga were assayed measuring the activity of antioxidant enzymes and quantifying antioxidant non-enzymatic metabolites. Additionally, analyses of ultrastructure, morphology and photosynthetic efficiency were performed.

2. Materials and methods

2.1. Algal material and experimental conditions

Thalli of Ulva sp. were collected in March 2010 from the Venice Lagoon (Italy). Species belonging to this genus show a very simple morphology and a certain degree of phenotypic plasticity, heavily influenced by environmental conditions, making difficult the delineation of species, based only on morphological features (Loughnan et al., 2008). For this reason, we prefer to refer to Ulva sp., rather than a specific species.

Once collected, thalli were thoroughly rinsed in seawater and cleaned using a soft brush to eliminate the epiphytes present on their surface. Subsequently, thalli were cut in 15 mm diameter disks and weighed. Disks of same weight (±5% variation) were placed in flasks containing 1 L of filtered seawater (Millipore GF/C, 1–2 μm pore size), and kept for 3 days to acclimate inside a climatic chamber with a 14 h light/10 h dark cycle, at a temperature of 16 °C and a photon flux density of 80 μmol m⁻² s⁻¹ according to Dalla Vecchia et al. (2007, 2012). The initial pH of the seawater in the flasks was 7.2. In each flask 100 disks were cultivated.

After acclimation, Se in the form of sodium selenite (Na₂SeO₃, Sigma–Aldrich, Steinheim, Germany) was added to the seawater at the following concentrations: 0 (control), 2.5, 10, 50 or 100 μM. The level of Se in the seawater before selenite addition was undetectable, being below the limit determined via ICP-AES. The wide range of selenite concentrations was used to determine the relationship between physiological and ultrastructural changes with increasing selenite doses. For each Se concentration five replicates were performed.

Disks and seawater were sampled at the beginning of the experiment and at the 10th day of treatment. Before analyses, thalli were carefully washed with distilled water to remove any Se bound to surface. For dry weight measurements, 20 disks from each flask were used.

2.2. Elemental analysis of Se and S

Seaweed thalli were dried for 48 h at 80 °C, and 100 mg of thalli dry weight per each treatment were then digested in nitric acid (95%, v/v) as described by Zarcinas et al. (1987). Inductively coupled plasma atomic emission spectroscopy (ICP-AES, Spectrum CiosCCD, Kleve, Germany) was used as described by Fassel (1978) to determine each digest’s Se and S concentrations. The obtained values were expressed in mg element kg⁻¹ dry weight.

The determination of Se and S was performed in the seawater: (1) before the addition of selenate; (2) immediately after the addition of selenate; (3) after 10 days from the addition of selenate either in the presence or absence of Ulva sp. thalli. Se and S were directly quantified in 10 mL of filtered (0.2 μm) seawater samples using ICP-AES as described by Fassel (1978). No preliminary digestion procedure was performed before the analysis. Results were expressed in mg L⁻¹.

2.3. Sulfate and selenite content

Seaweed thalli (500 mg) were ground in liquid nitrogen and then 10 mL of distilled water were added. The samples were incubated for 2 h in a heating block at 85 °C. The obtained extracts were filtered onto 0.45 μm (Millipore) and analyzed for sulfate content by HPLC using a Dionex IonPac AS11 4 mm column, coupled to guard column AG 14 and a CD20 Conductivity Detector. The column was eluted over a period of 18 min with 3.5 mM Na₂CO₃/1 mM NaHCO₃ in H₂O, at a flow rate of 0.9 mL/min and at 1400 PSI pressure.

For the measurement of water sulfate concentration, samples of seawater were first filtered onto 0.45 μm. Then the samples were analyzed via HPLC using the same procedure described above. To check the consistency of ambient selenite concentrations during experiments, culture medium samples were analyzed by HPLC as reported for sulfate. Sulfate contents in seaweed and in seawaters, as well as selenite content in seawater, were expressed in mg kg⁻¹ fresh weight and mg L⁻¹, respectively.

2.4. Quantification of pigments and photosynthetic oxygen evolution

Chlorophyll and carotenoids were determined in thalli of Ulva sp. after 3 and 10 days of treatment, using N,N-dimethylformamide (1:1) (Moran and Porath, 1980). The extracts were kept in the dark for 1 day at 4 °C (Wellburn, 1993) and then analyzed...
spectrophotometrically (124 Perkin-Elmer; Norwalk, CT, USA) at 644 nm for Chl a, 647 nm for Chl b, and 480 nm for carotenoids. The concentrations of chlorophylls and carotenoids were calculated using the extinction coefficients according to Inskipp and Bloom (1985) and expressed in mg g\(^{-1}\) fresh weight.

Photosynthesis was measured as oxygen release in control and Se-treated seaweeds using an oxygen monitor (Ysi Model 53, Yellow Spring Instrument Co., OH, USA). For the analysis, single disks were cut into 3–4 mm long segments to improve the efficiency of the method, according to Ishii et al. (1977) and Rascio et al. (1991). Seaweed disks were carefully rinsed with distilled water and the suspension medium used for the assay was the seawater used as the culture medium in the flasks. To verify the possible effects of Se on the integrity of photosynthetic apparatus, the oxygen evolution was also determined in control and Se-treated Ulva disks at the end of the experimental period (10 days) using fresh filtered seawater (pH 7.2) as suspension medium.

The measurements were carried out under saturating light (1400 µmol photon m\(^{-2}\) s\(^{-1}\) PAR) using a 150 W lamp (Philips, Aachen, Germany) as light source. The tubes were placed inside a thermostatic bath at 20 °C and kept stirred. The oxygen evolution rate was expressed as µmol O\(_2\) mg\(^{-1}\) chlorophyll h\(^{-1}\).

2.5. Light and electron microscopy

Samples from control and selenate-treated thallus disks of Ulva sp. were fixed overnight at 4 °C in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.9) and post-fixed at 4 °C for 2 h in 1% osmium tetroxide in the same buffer. The specimens were dehydrated in a graded series of ethyl alcohol and propylene oxide and embedded in araldite. Sections were cut using an ultramicrotome (Ultracut S, Reichert-Jung, Wien, Austria). For transmission electron microscopy, ultrathin sections (600 Å) stained with uranyl acetate and lead citrate were observed with a transmission electron microscope (TEM 300, Hitachi, Tokyo, Japan) operating at 75 kV. For light microscopy, thin sections (1 µm) stained with toluidine blue (1% basic toluidine and 1% Na tetraborate, 1:1, v/v) were observed by a DMR 5000 Leica (Sweden) microscope, equipped with a digital image acquisition system.

2.6. In situ determination of hydrogen peroxide

Intracellular production of hydrogen peroxide (H\(_2\)O\(_2\)) in Ulva sp. was measured using dichlorofluorescin diacetate (H\(_2\)DCF-DA, Molecular Probes, Leiden, The Netherlands) as fluorescent dye. Once inside cells, H\(_2\)DCF-DA can be oxidized into highly fluorescent 2′,7′-dichlorofluorescin (DCF) by intracellular H\(_2\)O\(_2\) and other peroxides. Cells of control and selenate-treated algae were observed using an epifluorescence microscope (LEICA DMR) at an excitation wavelength of 480 nm. A total of 300 cells for each sample were analyzed for DFC positivity. Data are expressed as percent.

2.7. Antioxidant enzyme activity measurement

Superoxide dismutase (SOD) and catalase (CAT) activities were measured in both control and selenate-treated thallus disks of Ulva sp. Samples were homogenized in HCl 10 mM, KCl 0.15 M, sucrose 0.5 M pH 7.6 as extraction buffer, using an Ultraturrax T8 (IKA). The homogenates were sonicated for 10 s at 4 °C, centrifuged at 13,400 \times g for 30 min at 4 °C, and the supernatant (SN) was collected for enzyme activity measurement.

Total SOD activity was measured in SN with the xanthine oxidase/cytochrome c method according to Crapo et al. (1978). The reaction mixture contained 10 µL SN, 46.5 µM K\(_2\)PO\(_4\)/K\(_2\)HPO\(_4\) (pH 8.8), 0.1 mM EDTA, 195 µM hypoxanthine, 16 µM cytochrome c, and 2.5 µU xanthine oxidase. The cytochrome c reduction by superoxide anion generated by the xanthine oxidase/hypoxanthine reaction was detected at 550 nm at room temperature for 30 s. Enzyme activity was expressed as U SOD mg\(^{-1}\) protein, one unit of SOD being defined as the amount of sample that causes 50% inhibition in the assay conditions.

CAT activity assay was measured according to the method of Aebi (1984). Decreases in absorbance of a solution composed of 10 µL of SN, 50 mM H\(_2\)O\(_2\) (c = –0.0436 mM cm\(^{-1}\)) in 50 mM phosphate buffer (KH\(_2\)PO\(_4\)/Na\(_2\)HPO\(_4\), pH 7.8), were continuously recorded at 240 nm at 10 s intervals for 1 min. Results were expressed as U CAT mg\(^{-1}\) proteins, one unit of CAT being defined as the amount of enzyme that catalyzes the dismutation of 1 µmol of H\(_2\)O\(_2\) in 1 min at 25 °C. For both SOD and CAT assays, the total protein concentration in SN was determined via the Bradford method (1976) using bovine serum albumin (BSA) as standard.

2.8. Extraction and measurement of soluble phenols and flavonoids

Soluble phenolic acids were extracted from control and Se-treated thalli disks of Ulva sp. by crushing them (1 g) in a mortar in the presence of pure methanol (1:1, v/w). The extracts were placed in an ice bath for 1 h and centrifuged at 3000 \times g for 40 min at 4 °C. The supernatants were stored at –20 °C until use. Total phenols were measured according to Arnaldos et al. (2001). One mL of 2% Na\(_2\)CO\(_3\) and 75 µL of Folin–Ciocalteau reagent (Sigma–Aldrich) were added to 100 µL of phenolic extract. After 15 min incubation at 25 °C in the dark, the absorbance at 725 nm was measured. Gallic acid was used as a standard. Flavonoids were extracted from thalli (1 g) in 50 mL of acidified methanol solution. The extracts were kept at 4 °C for 16 h before measuring the absorbance at 300 nm. Phenols and flavonoids were expressed as gallic acid equivalents g\(^{-1}\) fresh weight.

2.9. Statistical analysis

A one-way analysis of variance (one-way ANOVA) was applied to the data. Statistical analysis was performed using SPSS 10.0 (Norusis, 1993). All probabilities were two-tailed. Data were checked for normality and homogeneity of variance (Levene test) and are presented as mean ± SD of five replicates. Differences between means were evaluated for significance at P < 0.05 by using the Duncan’s multiple range test (DMRT). Statistically significant differences at P < 0.05 were indicated by different letters reported in tables and figures. Similar letters in tables and figures indicate no significant differences between mean values.

3. Results

3.1. Impact of Se on algal growth and culture medium pH

The effect of selenate on Ulva sp. growth was evaluated in terms of dry weight production relative to the control treatment (0 Se) at 10 days (Fig. 1). The application of selenate to Ulva sp. increased the dry weight of thalli (roughly 15% more than the control). Interestingly, this increase was the same at all external selenate concentrations.

The pH value of the seawater in the flasks before the addition of selenate and Ulva sp. thalli was 7.2. After 10 days, the pH of the seawater supplemented with selenate without the thalli, was slightly higher (7.5–7.6) than the pH of control seawater (Fig. 2). When Ulva thalli were cultivated in the flasks, the pH values were lower than 8 in the first 6 days, with no difference between treatments (data not shown). A remarkable increase of the seawater pH (from 8.46
at 0 Se to 9.71 at 100 μM Se) was observed at the end of the experiment (10 days). It is noteworthy that the pH measured at 10 days in seawater where Ulva sp. thalli were grown was not significantly different between control (0 Se) and 2.5, 10, 50 μM Se.

3.2. Determination of Se, S and sulfate in Ulva sp. thalli, and quantification of Se, S, sulfate and selenate in seawater

The analysis of nutrient concentrations in the seawater was used to assess the potential impact and fate of Se in algae. The level of Se and S in the seawater culture medium was measured at the beginning of the experiment and after 10 days of Ulva sp. thalli cultivation (Table 1). The concentration of total Se was additionally measured in the seawater contained in the flasks where Ulva thalli were not cultivated. This measurement was performed in order to evaluate whether microorganisms potentially growing in the seawater contributed to the removal of Se after 10 days. As reported in Table 1, the concentration of total Se did not change in seawater after 10 days exposure, and results obtained via HPLC confirmed that ambient Se remained all in the selenate form during the experimental period if Ulva sp. thalli were not cultivated in the flasks (data not shown).
Table 2
Concentration of selenium (Se), sulfur (S) and sulfate (SO₄²⁻) in thalli of Ulva sp. The measurements were performed in thalli after a 10 day-cultivation period in the absence (control) or in the presence of varying doses of selenate. In each column, values are the mean of five replicates and different letters indicate significant differences between treatments (P<0.05, ±SD).

<table>
<thead>
<tr>
<th>Se (µM)</th>
<th>Se (mg kg⁻¹ d.wt.)</th>
<th>S (g kg⁻¹ d.wt.)</th>
<th>SO₄²⁻ (mg kg⁻¹ d.wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;0.005f</td>
<td>22.952 ± 1.213a</td>
<td>91.901 ± 16.753b</td>
</tr>
<tr>
<td>2.5</td>
<td>0.620 ± 0.026e</td>
<td>23.073 ± 0.432a</td>
<td>78.007 ± 11.671b</td>
</tr>
<tr>
<td>10</td>
<td>1.677 ± 0.044d</td>
<td>22.668 ± 0.879a</td>
<td>119.601 ± 42.975b</td>
</tr>
<tr>
<td>50</td>
<td>13.273 ± 0.243c</td>
<td>22.236 ± 0.453a</td>
<td>114.660 ± 22.662b</td>
</tr>
<tr>
<td>75</td>
<td>21.320 ± 0.379b</td>
<td>21.941 ± 0.612a</td>
<td>185.967 ± 46.732a</td>
</tr>
<tr>
<td>100</td>
<td>30.752 ± 0.468a</td>
<td>22.750 ± 1.133a</td>
<td>181.326 ± 22.352a</td>
</tr>
</tbody>
</table>

Sulfur in seawater was present almost exclusively in the form of sulfate (Table 1). Both total S and sulfate concentration were constant throughout the experimental period and values were similar in the Se-treatment and in the control, regardless the presence of thalli (Table 1).

The accumulation of Se in thalli of Ulva sp. linearly correlated with the culture medium concentration of selenate (y=0.293x, R²=0.992) (Table 2). Selenate apparently did not affect the content of total S in the thalli, but at doses as high as 75 and 100 µM significantly increased the level of sulfate (Table 2).

3.3. Effect of Se on chlorophylls, carotenoids and photosynthetic O₂ evolution

The chlorophyll quantification was performed in Ulva sp. thalli after 3 days and 10 days from the beginning of the experiment.

![Image](Fig. 3. Effect of different selenate concentrations on the level of chlorophyll (A) and Carotenoids (B). The measurements were performed after 3 and 10 days of Ulva sp. cultivation. Different letters indicate significant differences between treatments (P<0.05, ±SD). Values are expressed as mg pigment g⁻¹ fresh weight.)

![Image](Fig. 4. Light microscopy photographs of control (A) and 10 days-Se-treated (B–F) thalli of Ulva sp.)
The oxygraphic analysis demonstrated lower photosynthetic O₂ evolution in thalli cultivated for 10 days with Se than in the control when the seawater contained in the flasks was used as the suspension medium (Table 3). However, if the photosynthetic O₂ emission was measured in Se-treated Ulva sp. thalli suspended in fresh seawater (pH = 7.2), the values of net photosynthesis were higher, although not significantly, than those reported for the controls. A similar increase of photosynthetic O₂ evolution was observed during the first days of Ulva sp. cultivation in the presence of Se (data not shown).

Interestingly, the net photosynthesis did not change in thalli cultivated without Se when they were transferred to a fresh seawater medium (Table 3).

3.4. Effects of Se on thallus morphology and ultrastructure

The thallus disks of Ulva sp. cultivated in the presence of selenate concentrations ranging from 2.5 to 50 μM shared a similar thickness and bilayered morphology with those of the control condition (Fig. 4A–F). Only at the highest Se dose (100 μM) was the thickness of Ulva thalli reduced (Fig. 4F). With respect to cell ultrastructure, no significant alteration was observed between control thallus disks and the selenate-treated ones (Fig. 5A–F). Indeed, thallus cells of all experimental conditions showed a normal organization, with a well-visible plastid characterized by the presence of abundant starch and pyrenoid in the stroma.

3.5. Cytochemical analysis and effects of Se on antioxidant enzymes

Ulva sp. thalli treated with selenate above 10 μM showed the production of hydrogen peroxide, as evidenced by the presence of a fluorescent signal in the cytoplasm of cells (Fig. 6). Interestingly, the activity of antioxidant enzymes SOD and CAT increased significantly for all Se treatments (Fig. 7A and B). The highest levels of SOD activity were observed at 75 μM and 100 μM Se (Fig. 7A). The variation pattern of CAT activity also showed an increase relative to the controls (Fig. 7B). Interestingly, the difference in SOD activity between Se-treated and control thalli was higher (10 fold) than that reported for CAT (2 fold).

3.6. Effects of Se on total phenols and flavonoids

The exposure of Ulva sp. thalli to selenite enhanced the level of phenolic compounds (Fig. 7C and D). The total phenol content increased of 3.5 fold even at the lowest Se concentration applied (2.5 μM) (Fig. 7C), while values of flavonoid content were approximately 1.5 fold higher in thalli cultivated with Se concentrations ranging from 10 to 100 μM than in the control (Fig. 7D).

Fig. 5. Transmission electron microscope details of cells of control (A) and 10 days-Se-treated (B–F) thalli of Ulva sp. Note the chloroplasts with starch in the stroma. No evident alterations of ultrastructure are visible in cells.
4. Discussion

Macroalgae may represent important bioindicators of metal pollution in freshwater and marine environments because of their distribution, large size, longevity, presence at pollution sites, and ability to accumulate metals to a satisfactory degree (Lee and Wang, 2001).

The essentiality of Se for macroalgae has not been demonstrated yet, while for many phytoplankton species this element is needed at low doses (Araie and Shiraiwa, 2009). Toxic effects of Se on algae are generally evaluated using phytotoxicity tests based on growth inhibition. In our study, the toxicity of selenate to Ulva sp. was assayed by measuring the effect of a broad range of selenate concentrations on algal growth during a 10 days-exposure period. Interestingly, selenate at concentrations up to 100 μM did not inhibit Ulva sp. growth at all tested doses; rather, this form of Se exerted a positive effect on algal biomass by increasing the dry weight of thalli by 15%.

Despite the advantages of low Se concentrations on growth that have also been reported for a number of land plants (Hartikainen, 2005; Pilon-Smits et al., 2009), inhibition of growth by selenate is frequently observed in microalgae (Fournier et al., 2010; Geoffroy et al., 2007; Pastierova et al., 2009; Reunova et al., 2007). This inhibition is probably because in most of these studies environmentally relevant Se concentrations (up to 400 μM) are used, which exceed the Se requirements for normal growth by microalgae. Moreover, microalgae generally exhibit a stronger capacity of Se bioaccumulation compared to macroalgae, so that Se levels inside cells are often high enough to cause toxicity (Fournier et al., 2010; Geoffroy et al., 2007; Reunova et al., 2007; Umisová et al., 2009). For example, S. quadricauda cells accumulated 3730 mg Se kg⁻¹ dry weight when exposed to 50 mg L⁻¹ Se and cultivated in the presence of 40 mM S (Umisová et al., 2009).

The capacity of Ulva sp. to accumulate Se was likely affected by the high sulfate concentration measured in the culture medium. Indeed, owing to its chemical similarity with selenate, sulfate may...
reduce selenate uptake by algae as a consequence of the competition for transport into cells (Fournier et al., 2010). In support of this hypothesis, there is broad evidence that selenate and sulfate compete for uptake at the level of sulfate transporters in plants and microalgae (Fournier et al., 2010; Neumann et al., 2003; Riedel and Sanders, 1996; Schiavon et al., 2012; Terry et al., 2000; Umisová et al., 2009; Williams et al., 1994). In Chlorella vulgaris and Chlamidomonas reinhardtii, for instance, the uptake of selenate negatively correlated with sulfate concentrations in the growth medium (Fournier et al., 2010; Riedel and Sanders, 1996; Shift, 1954).

Interestingly, the linear relationship between Se content in Ulva sp. biomass and ambient selenate concentration was not altered by the high sulfate concentration in seawater. A positive correlation between Se and selenate was also observed in the microalgae S. quadricauda (Umisová et al., 2009).

Selenate and sulfate are also believed to share the same assimilation pathway in plants and microalgae, which are usually more sensitive to selenate when cultivated under S starvation (Schiavon et al., 2012; Simmons and Emery, 2011; Umisová et al., 2009). Variation of sulfate content in algal biomass may occur when algae absorb selenate, since the two anions are antagonist substrates for the first enzyme of the S assimilatory pathway (Pilon-Smits et al., 1999; Schiavon et al., 2008, 2012). In Ulva sp. sulfate accumulated significantly more when thalli were exposed to high concentrations of selenate (75 and 100 μM), while total S level did not change in response to Se treatments. This may suggest that the uptake of sulfate was not affected by selenate, and increased sulfate accumulation in thalli was the result of the reduced delivery into the S metabolic flux, due to Se interfering with S assimilation in this macroalga.

Selenate apparently did not cause significant alterations of Ulva sp. thallus morphology and ultrastructure. Conversely, previous studies provided evidence of a number of ultrastructural changes induced by selenate in microalgae, and highlighted chloroplasts as the first target of Se cytotoxicity (Geoffroy et al., 2007; Vítová et al., 2011). The lack of chloroplast modifications in Ulva sp. cells was in agreement with the lack of growth inhibition by Se. The Se treatments significantly up-regulated the levels of antioxidant compounds and enzymes. These likely contributed to the selenate resistance by Ulva sp. and enhanced growth, due to their protective effects on membrane integrity. Carotenoids, in particular, are known to protect chloroplast membranes from damage caused by reactive oxygen species (ROS) produced under stress (Young, 1991; Havaux, 1998). In our study, the formation of hydroxide peroxide was revealed in thalli of Ulva sp. exposed to selenate, and the concomitant synthesis of carotenoids was observed.

Despite the absence of chloroplast alterations, the photosynthetic activity of Ulva sp. decreased after 10 days of selenate exposure, as indicated by the oxygraphic analysis. Previous studies have shown in many species of Ulvaceae CO₂-users the inhibition of the capacity to absorb CO₂ when pH increases (Axelsson et al., 2000). At a value as high as 9.5, pH was proven to affect algal photosynthesis both by decreasing free CO₂ concentration in the medium and by lowering the affinity of the algae to free CO₂ (Azov., 1982). The progressive alkalization of the culture medium is often observed when algae perform photosynthetic CO₂ fixation in closed CO₂ systems (Granum and Myklestad, 2002). Under such conditions, in fact, the inorganic carbon equilibrium in the surrounding medium is shifted towards the formation of HCO₃⁻, and the pH increases as a result (Riedel and Sanders, 1996).

In our study, the high pH appeared not to be the only responsible for the net photosynthesis decrease observed in all Se-treated Ulva sp. thalli after 10 days. Indeed, the pH of the seawater used for the cultivation of thalli and enriched with 2.5, 10 or 50 μM Se was not significantly different from that measured in the control medium. Furthermore, the pH in the seawater without Se after 10 days of Ulva sp. cultivation was significantly higher than that reported for fresh
seawater, but no differences in photosynthetic oxygen evolution were evident in thalli.

Selenate treatment may have accounted for the reduction of net photosynthesis in Ulva sp. thalli. In the first days of cultivation, the net photosynthesis values measured in Se-treated thalli were higher than those determined in the controls, as well as after 10 days of Se exposure if thalli were transferred into fresh seawater. The higher photosynthesis rates likely determined the stimulation of algal biomass production during the first days of Ulva sp. cultivation, and reduced the availability of dissolved inorganic carbon in Se-enriched seawater at 10 days with respect to the control medium. The recovery of the capacity to produce photosynthetic O2 by Se-treated thalli once transferred in a fresh culture medium indicated that selenate did not compromise the photosynthetic apparatus of Ulva sp.

Although Se did not injure Ulva sp. growth, morphology and ultrastructure, there was evidence of hydrogen peroxide (H2O2) formation in the algal cells. H2O2 represents the most stable form of ROS and the enhancement of its synthesis suggests that Se was able to induce oxidative stress in Ulva sp. to some extent, as reported for plants (Freeman et al., 2010; Gomes-Junior et al., 2007; Grant et al., 2011; Tamaoki et al., 2008). Despite their role in oxidative stress induction, ROS can sometimes be correlated with Se resistance in plants depending on their tissue level, perhaps acting as signal molecules in the induction of resistance pathways (Tamaoki et al., 2008). On this account, we cannot exclude that ROS production could be useful for acquisition of Se resistance in Ulva sp., as previously reported for Arabidopsis thaliana (Tamaoki et al., 2008).

Concomitant to higher production of H2O2, the activity of antioxidant enzymes, such as SOD and CAT, was significantly stimulated in Ulva sp. These enzymes are involved in the sequential scavenging of ROS, as SOD catalyzes the dismutation of superoxide into oxygen and H2O2, and CAT further converts H2O2 into water and oxygen. In the present study, the two enzymes appeared to undergo differential regulation by Se in Ulva sp., as the enhancement of SOD activity was tightly dependent on Se accumulation, while CAT activity was induced to a similar degree by most of tested selenate doses. Increase of SOD activity was also observed in Chlorella zofingiensis cells exposed to selenite (Pelah and Cohen, 2005), as well as in Ulva lactuca in response to ROS production caused by different Se compounds (Ross and Van Alstyne, 2007). Unfortunately, no information concerning Se effects on CAT activity in algae is available in the literature.

In addition to the stimulation of antioxidant enzyme activity, the level of phenols and flavonoids increased in Ulva sp. thalli cultivated in the presence of selenate. Phenolic compounds are known to play several important protective roles in plants (Mazid et al., 2011; Michalak, 2006). Flavonoids, in particular, display a critical function in plant defence responses accounting for a secondary ROS-scavenging system in plants exposed to severe/prolonged stress conditions (Fini et al., 2011; Posmyk et al., 2009). Our findings indicate that, similarly to plants, Ulva sp. possess a highly efficient antioxidant machinery to keep the level of ROS under a tight control, which involves the activation of both enzymatic and non-enzymatic antioxidants.

5. Conclusion

The results obtained in the present study show that Ulva sp. accumulated Se depending on selenate dose, and Se accumulation in thalli interfered with S metabolism, as indicated by the increase of sulfate:sulfur ratio. The resistance of Ulva sp. to selenate appears to be due to its capacity to keep cellular Se at low levels and to efficiently activate different metabolic pathways that involve enzymatic and non-enzymatic antioxidant compounds functioning in the scavenging of ROS produced upon Se accumulation.

References

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