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

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ABSTRACT

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 ($Na_2SeO_4$) 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 ($H_2O_2$) 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 macroalga, 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, $Se^{3+}$ (selenite) and $Se^{6+}$ (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 Skulberg, 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,

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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 (selenate and selenite) 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 be also 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 selenate (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 (Loughnane 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 $\mu mol\ m^{-2}\ s^{-1}$ 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 selenate ($Na_2SeO_4$, 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 selenate addition was undetectable, being below the limit determined via ICP-AES. The wide range of selenate concentrations was useful to determine the relationship between physiological and ultrastructural changes with increasing selenate 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 (99%, v/v) as described by Zarcinas et al. (1987). Inductively coupled plasma atomic emission spectroscopy (ICP-AES, Spectrum CirosCCD, 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 selenate 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 $Na_2CO_3$/1 mM $NaHCO_3$ in $H_2O$, 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 μ. Then the samples were analyzed via HPLC using the same procedure described above. To check the consistency of ambient selenate concentrations during experiments, culture medium samples were analyzed by HPLC as reported for sulfate. Sulfate contents in seaweeds and in seawaters, as well as selenate 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 664 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 Inskeep 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 ($H_2O_2$) in *Ulva* sp. was measured using dichlorofluorescein diacetate ($H_2$DCHF-DA, Molecular Probes, Leiden, The Netherlands) as fluorescent dye. Once inside cells, $H_2$DCHF-DA can be oxidized into highly fluorescent 2′,7′-dichlorofluorescein (DCF) by intracellular $H_2O_2$ 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 Ultratturrax T8 (IKA). The homogenates were sonicated for 10 s at 4 °C, centrifuged at 13,400 × *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 $K_2PO_4/K_2HPO_4$ (pH 8.6), 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 $H_2O_2$ ($\varepsilon = -0.0436\ mM^{-1}\ cm^{-1}$) in 50 mM phosphate buffer ($KH_2PO_4/Na_2HPO_4$, 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 $H_2O_2$ 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, w/v). The extracts were placed in an ice bath for 1 h and centrifuged at 3000 × *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% $Na_2CO_3$ 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

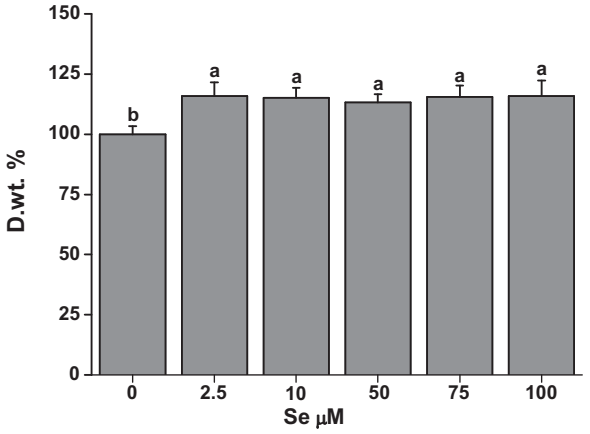


**Fig. 1.** Effect of different selenate concentrations on dry weight (d.wt.) of *Ulva* sp. thalli. Values are reported as percent of control, which was set at 100%. Different letters above bars indicate significant differences between treatments ($P < 0.05$, ±SD).

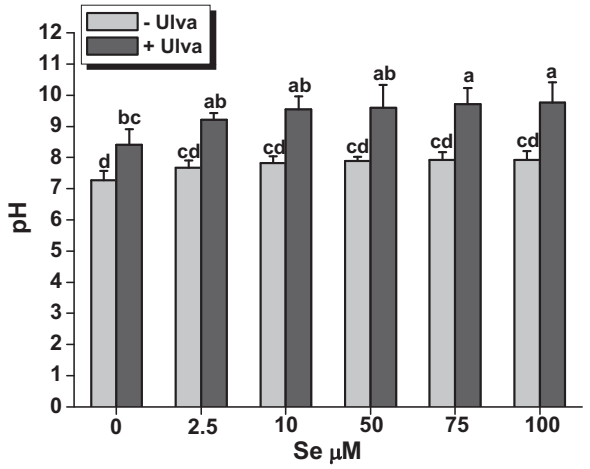


**Fig. 2.** Values of pH in control seawater and Se-added seawater either used for *Ulva* sp. cultivation (*+Ulva*) or let inside the flasks without algae (*−Ulva*). The values reported were measured after 10 days since the beginning of the experiment. At time 0, the pH of seawater was 7.2. Different letters above bars indicate significant differences between treatments ($P < 0.05$, ±SD).

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. thallus 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 1**
Concentration of selenium (Se), sulfur (S) and sulfate ($SO_4^{2-}$) in the seawater used either as the culture medium of *Ulva* sp. thalli (*+Ulva*) or let inside the flasks without thalli (*−Ulva*). The measurements were performed at the beginning of the experiment ($t = 0$), and at the end ($t = 10$ days). In each column, values are the mean of five replicates. Different letters indicate significant differences among treatments ($P < 0.05$, ±SD) and among conditions: $t = 0$, $t = 10$ days (*−Ulva*) $t = 10$ days (*+Ulva*). The statistical analysis was performed independently for Se, S and $SO_4^{2-}$.

| Se (μM) | Se ($mg L^{-1}$) | | | S ($mg L^{-1}$) | | | $SO_4^{2-}$ ($mg L^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | $t = 0$ | $t = 10$ days(*−Ulva*) | $t = 10$ days(*+Ulva*) | $t = 0$ | $t = 10$ days(*−Ulva*) | $t = 10$ days(*+Ulva*) | $t = 0$ | $t = 10$ days (*−Ulva*) | $t = 10$ days (*+Ulva*) |
| 0 | <0.005 | <0.005 | <0.005 | 986.04 ± 32.44a | 956.22 ± 36.12a | 963.08 ± 36.87a | 2956.72 ± 96.10a | 2954.21 ± 154.33a | 2841.52 ± 68.83a |
| 2.5 | 0.197 ± 0.012h | 0.193 ± 0.017h | 0.150 ± 0.010i | 973.07 ± 21.38a | 913.07 ± 67.85a | 997.14 ± 43.89a | 2919.09 ± 91.12a | 2881.75 ± 123.52a | 2990.22 ± 17.46a |
| 10 | 0.768 ± 0.011f | 0.767 ± 0.019f | 0.635 ± 0.015g | 971.43 ± 24.61a | 936.66 ± 50.01a | 967.19 ± 22.55a | 2914.25 ± 91.07a | 2993.54 ± 121.11a | 2900.09 ± 173.78a |
| 50 | 3.910 ± 0.212d | 3.789 ± 0.305d | 3.278 ± 0.021e | 922.54 ± 27.32a | 947.18 ± 27.32a | 937.25 ± 42.67a | 2781.84 ± 86.93a | 2849.64 ± 173.44a | 2801.59 ± 100.35a |
| 75 | 6.228 ± 0.178b | 6.175 ± 0.114b | 4.671 ± 0.097c | 978.32 ± 32.66a | 965.63 ± 41.08a | 937.43 ± 40.73a | 2833.55 ± 69.79a | 2797.35 ± 143.56a | 2580.16 ± 407.87a |
| 100 | 7.842 ± 0.303a | 7.672 ± 0.224a | 6.175 ± 0.127b | 954.11 ± 31.98a | 939.89 ± 28.88a | 896.77 ± 78.54a | 2912.74 ± 67.87a | 2931.88 ± 166.21a | 2971.72 ± 112.32a |

**Table 2**
Concentration of selenium (Se), sulfur (S) and sulfate ($SO_4^{2-}$) 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).

| Se (μM) | Se (mg $kg^{-1}$ d.wt.) | S (g $kg^{-1}$ d.wt.) | $SO_4^{2-}$ (mg $kg^{-1}$ d.wt.) |
|---|---|---|---|
| 0 | <0.005f | 22.952 ± 1.213a | 91.901 ± 16.753b |
| 2.5 | 0.620 ± 0.026e | 23.073 ± 0.432a | 78.007 ± 11.671b |
| 10 | 1.677 ± 0.044d | 22.668 ± 0.879a | 119.601 ± 42.975b |
| 50 | 13.273 ± 0.243c | 22.236 ± 0.453a | 114.660 ± 22.662b |
| 75 | 21.320 ± 0.379b | 21.941 ± 0.612a | 185.967 ± 46.732a |
| 100 | 30.752 ± 0.468a | 22.750 ± 1.133a | 181.326 ± 22.352a |

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 ($y = 0.293x$, $R^2 = 0.992$) (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 $O_2$ evolution

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

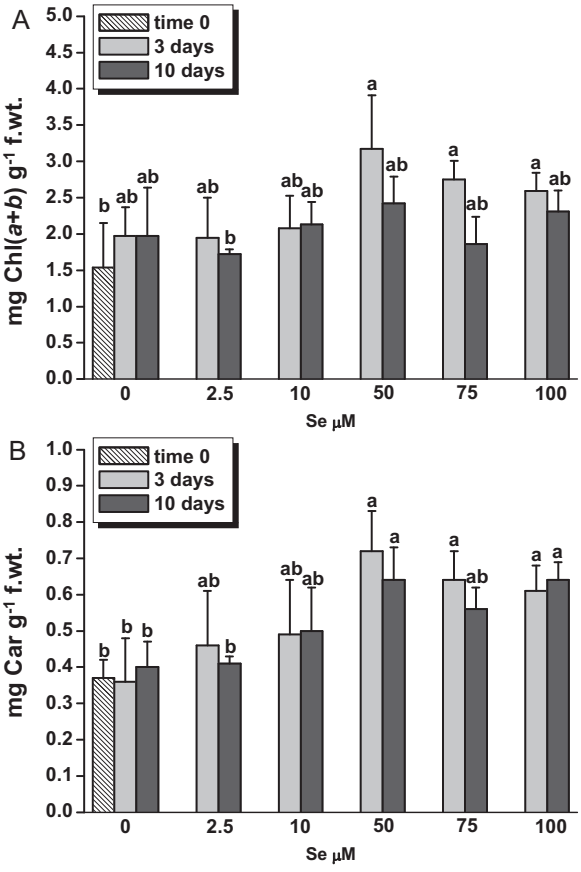


**Fig. 3.** Effect of different selenate concentrations on the level of chlorophyll (A) and Carotenoids (B). The measurements were performed after 3 and 10 days of *Ulva* sp. cultivation. Different letters indicate significant differences between treatments ($P < 0.05$, ±SD). Values are expressed as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

**Table 3**
Photosynthetic oxygen evolution by *Ulva* sp. measured using as suspension medium the seawater culture medium (pH > 8.5) or fresh seawater (pH = 7.2). $O_2$ measurement was performed in thalli after a 10 day-cultivation period either 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).

| Se (μM) | μmol $O_2$ $mg^{-1}$ Chl $h^{-1}$ | |
|---|---|---|
| | Culture medium | Fresh seawater |
| 0 | 13.3 ± 1.20a | 13.30 ± 0.87a |
| 2.5 | 4.76 ± 0.93bc | 15.02 ± 1.22a |
| 10 | 5.91 ± 1.02bc | 14.61 ± 1.57a |
| 50 | 3.73 ± 0.66b | 15.38 ± 1.32a |
| 75 | 5.22 ± 0.97bc | 14.54 ± 0.96a |
| 100 | 7.33 ± 1.98c | 15.84 ± 1.46a |

The content of chlorophyll (*a*+*b*) in thalli cultivated for 10 days in the presence of selenate did not change significantly compared to thalli of the minus Se condition, although at the 3rd day an increase of these pigments was observed at the highest selenate concentrations (Fig. 3A). On the contrary, the level of carotenoids significantly increased in the thalli exposed to selenate both at 3 and 10 days of exposure, especially when Se was supplied at high doses ranging from 50 to 100 μM (Fig. 3B).

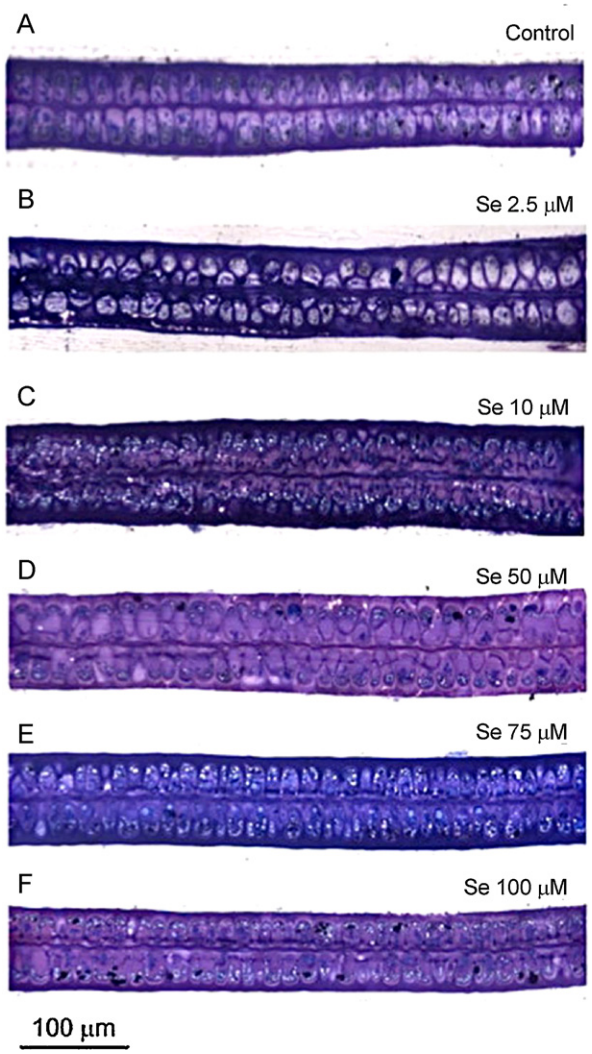


**Fig. 4.** Light microscopy photographs of control (A) and 10 days-Se-treated (B–F) thalli of *Ulva* sp.

The oxygraphic analysis demonstrated lower photosynthetic $O_2$ 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 $O_2$ 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 $O_2$ 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 selenate 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).

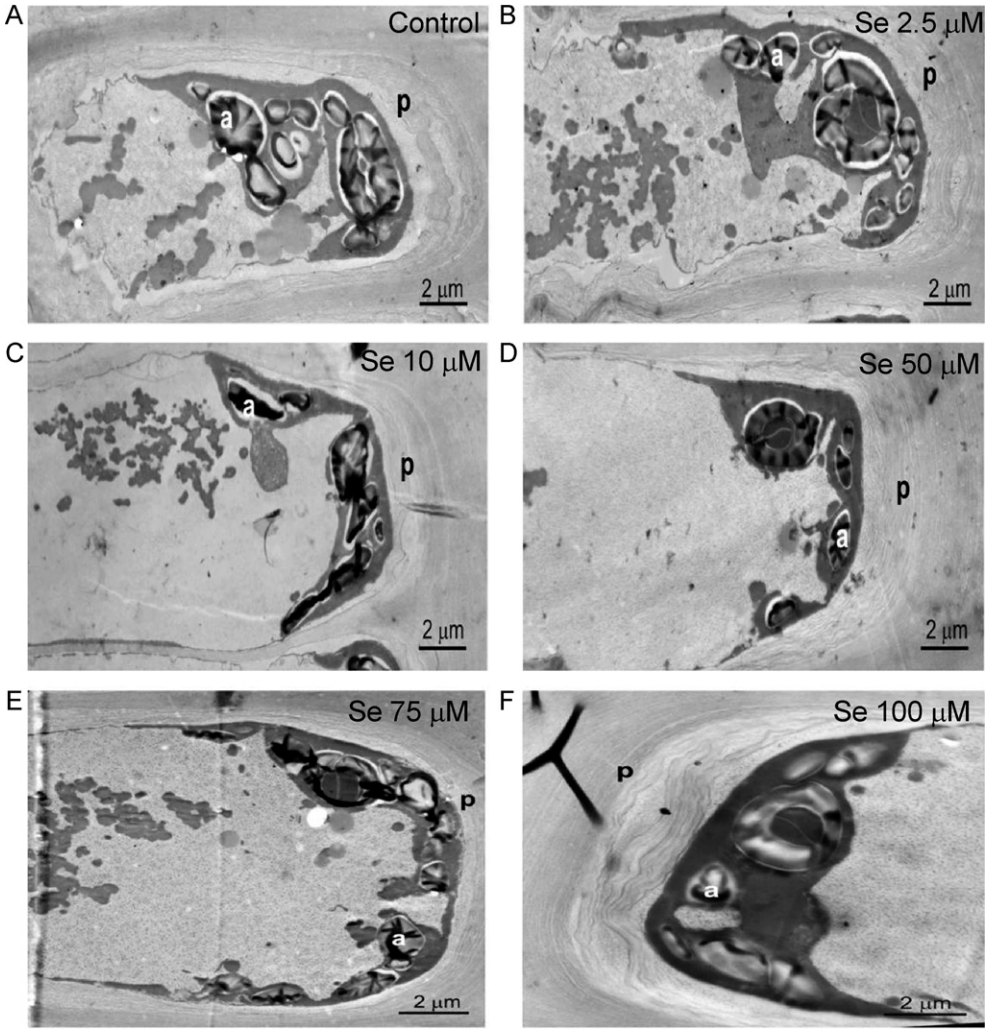


**Fig. 5.** Transmission electron microscope details of cells of control (A) and 10 days-Se-treated (B–F) thalli of *Ulva* sp. Note the chloroplasts with starch in the stroma. No evident alterations of ultrastructure are visible in cells.

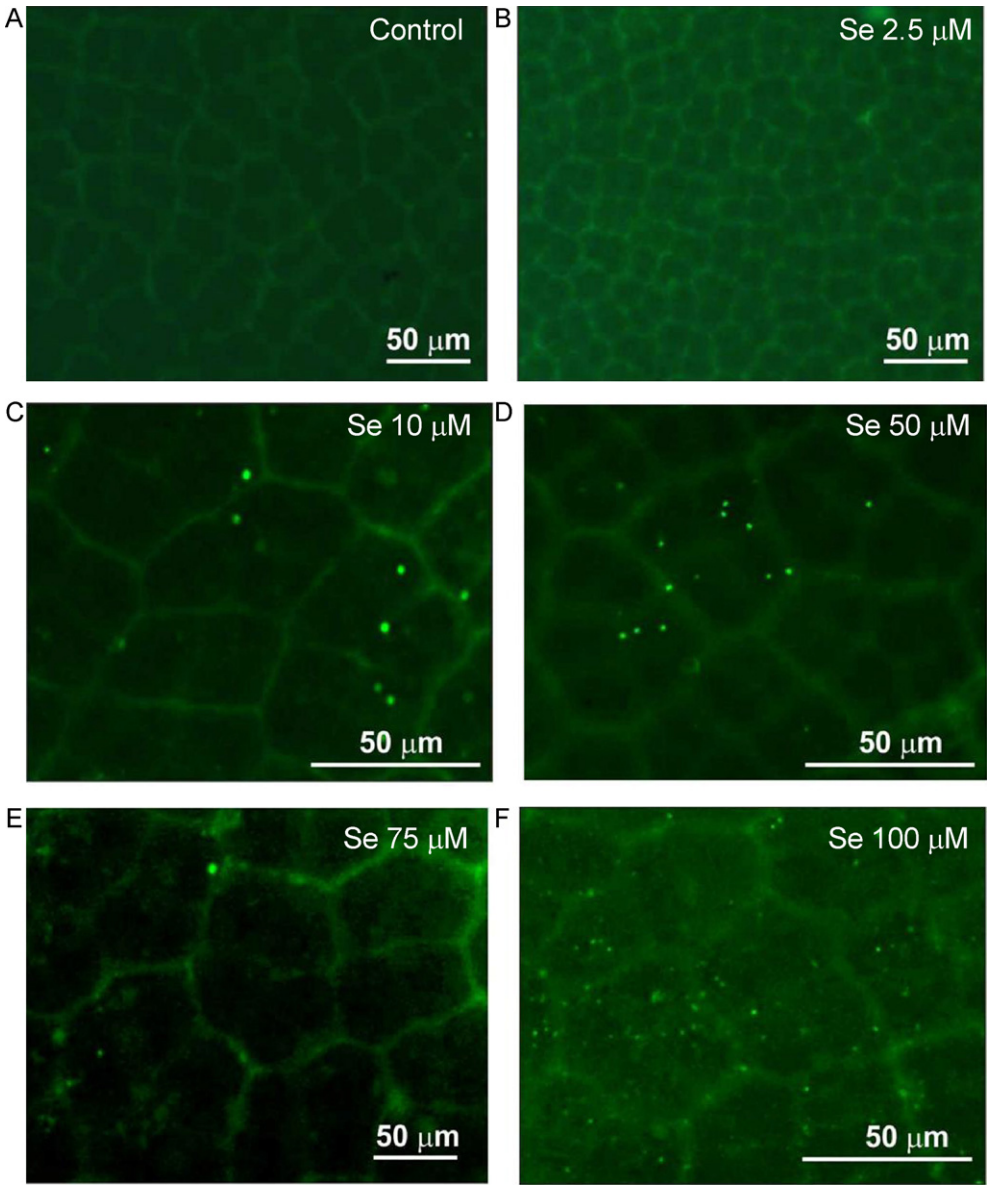


**Fig. 6.** Detection of $H_2O_2$ production with DCFH-DA of control (A) and Se-treated (B–F) thalli of *Ulva* sp. Note the green fluorescence signal in thalli of *Ulva* sp. cultivated with Se. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 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

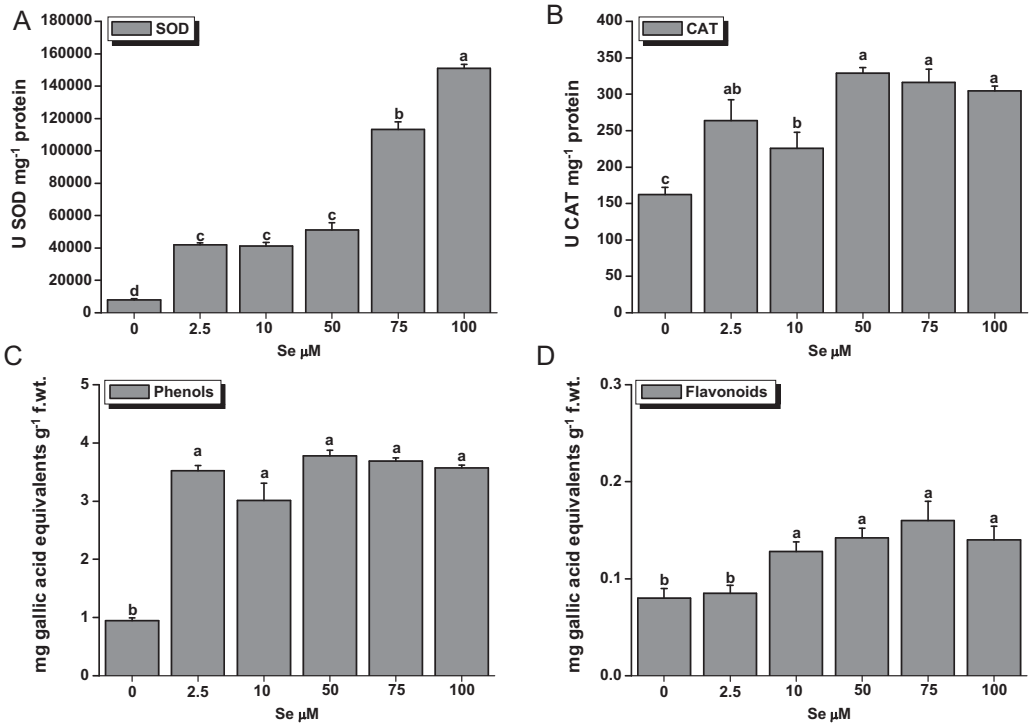


**Fig. 7.** Effect of different selenate concentrations on superoxide dismutase (SOD, A) and catalase (CAT, B) activities, and on the content of phenols (C) and flavonoids (D). The measurements were performed in thalli of *Ulva* sp. after a 10 day-cultivation period. Different letters indicate significant differences between treatments ($P < 0.05$, ±SD). The enzyme activity is expressed as U enzyme $mg^{-1}$ protein, the content of phenols and flavonoids as mg gallic acid equivalents $g^{-1}$ fresh weight.

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; Shrift, 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 microalga *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* $CO_2$-users the inhibition of the capacity to absorb $CO_2$ 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 $CO_2$ concentration in the medium and by lowering the affinity of the algae to free $CO_2$ (Azov, 1982). The progressive alkalinization of the culture medium is often observed when algae perform photosynthetic $CO_2$ fixation in closed $CO_2$ systems (Granum and Myklestad, 2002). Under such conditions, in fact, the inorganic carbon equilibrium in the surrounding medium is shifted towards the formation of $HCO_3^-$, 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 $O_2$ 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 ($H_2O_2$) formation in the algal cells. $H_2O_2$ 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 $H_2O_2$, 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 $H_2O_2$, and CAT further converts $H_2O_2$ 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


Abdel-Hamid, M., Skulberg, O.M., 2006. Effect of selenium on the growth of some selected green and blue-green algae. Lakes and Reservoir Management 1, 205–211.

Aebi, H., 1984. Catalase in vitro. Methods in Enzymology 105, 121–126.

Arnaldos, T.L., Muñoz, R., Ferrer, M.A., Calderón, A.A., 2001. Changes in phenol content during strawberry (*Fragaria ananassa*, cv. Chandler) callus culture. Physiologia Plantarum 113, 315–322.

Araie, H., Shiraiwa, Y., 2009. Selenium utilization strategy by microalgae. Molecules 14 (12), 4880–4891.

Axelsson, L., Mercado, J.M., Figuero, F.L., 2000. Utilization of $HCO^{3-}$ at high pH by the brown macroalga *Laminaria saccharina*. European Journal of Phycology 35, 53–59.

Azov, Y., 1982. Effect of pH on inorganic carbon uptake in algal cultures. Applied and Environmental Microbiology 43 (6), 1300–1306.

Bradford, M., 1976. Rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Chapman, P.M., Adams, W.J., Brooks, M.L., Delos, C.G., Luoma, S.N., Maher, W.A., Ohlendorf, H.M., Presser, T.S., Shaw, D.P., 2010. Ecological Assessment of Selenium in the Aquatic Environment. CRC Press, Boca Raton, FL.

Crapo, J.D., Mccord, J.M., Fridovich, I., 1978. Preparation and assay of superoxide dismutases. Methods in Enzymology 53, 382–393.

Dalla Vecchia, F., Moro, I., Marzocchi, M., 2007. Some effects of cadmium on *Ulva laetevirens* Areschoug collected in Lagoon Venice. Algological Studies 125, 1–15.

Dalla Vecchia, F., Marzocchi, M., Maistro, S., Moro, I., 2012. Morpho-physiological effects of cadmium on two Ulva species. Algological Studies 138, 13–25.

Dazhi, W., Zhaodi, C., Shaojing, L., Yahui, G., 2003. Toxicity and accumulation of selenite in four microalgae. Chinese Journal of Oceanology and Limnology 21, 280–285.

De Alcantara, S., Lopes, C.C., Wagener, K., 1998. Controlled introduction of selenium into Chlorella cells. Indian Journal of Experimental Biology 36, 1286–1288.

Duan, X.J., Zhang, W.W., Li, X.M., Wang, B.G., 2006. Evaluation of antioxidant property of extract and fractions obtained from a red alga. Polysiphonia urceolata. Food Chem. 95, 37–43.

Fan, T.W.-M., The, S.J., Hinton, D.E., Higashi, R.M., 2002. Selenium biotransformations into proteinaceous forms by foodweb organisms of selenium-laden drainage waters in California. Aquatic Toxicology 57, 65–84.

Fassel, V.A., 1978. Quantitative elemental analyses by plasma emission spectroscopy. Science 202, 183–191.

Fini, A., Brunetti, C., Di Ferdinando, M., Ferrini, F., Tattini, M., 2011. Stress-induced flavonoid biosynthesis and the antioxidant machinery of plants. Plant Signaling and Behavior 6 (5), 709–711.

Fleurence, J., 1999. Seaweed proteins: biochemical, nutritional aspects and potential uses. Trends in Food Science and Technology 10, 25–28.

Fournier, E., Adam-Guillermin, C., Potin-Gautier, M., Pannier, F., 2010. Selenate bioaccumulation and toxicity in *Chlamydomonas reinhardtii*: influence of ambient sulphate ion concentration. Aquatic Toxicology 97, 51–57.

Freeman, J.L., Tamaoki, M., Stushnoff, C., Quinn, C.F., Cappa, J.J., Devonshire, J., Fakra, S.C., Marcus, M.A., McGrath, S.P., Van Hoewyk, D., Pilon-Smits, E.A.H., 2010. Molecular mechanisms of selenium tolerance and hyperaccumulation in *Stanleya pinnata*. Plant Physiology 153 (4), 1630–1652.

Fu, L-H., Wang, X-F., Eyal, Y., She, Y-M., Donald, L.J., Standing, K.G., Ben-Hayim, G., 2002. A selenoprotein in the plant kingdom. Journal of Biological Chemistry 227, 25983–25991.

Geoffroy, L., Gilbin, R., Simon, O., Floriani, M., Adam, C., Pradines, C., Cournac, L., Garnier-Laplace, J., 2007. Effect of selenate on growth and photosynthesis of *Chlamydomonas reinhardtii*. Aquatic Toxicology 83 (2), 149–158.

Gomes-Junior, R.A., Gratao, P.L., Gaziola, S.A., Mazzafera, P., Lea, P.J., Azevedo, R.A., 2007. Selenium-induced oxidative stress in coffee cell suspension cultures. Functional Plant Biology 34 (5), 449–456.

Grant, K., Carey, N.M., Mendoza, M., Schulze, J., Pilon, M., Pilon-Smits, E.A.H., Van Hoewyk, D., 2011. Adenosine 5′-phosphosulfate reductase (APR2) mutation in *Arabidopsis* implicates glutathione deficiency in selenate toxicity. Biochemical Journal 438, 325–335.

Granum, E., Myklestad, S.M., 2002. A photobioreactor with pH control: demonstration by growth of the marine diatom *Skeletonema costatum*. Journal of Plankton Research 24 (6), 557–563.

Harrison, P.J., Yu, P.W., Thompson, P.A., Price, N.M., Phillips, D.J., 1988. Survey of selenium requirements in marine phytoplankton. Marine Ecology Progress Series 47, 89–96.

Hartikainen, H., 2005. Biogeochemistry of selenium and its impact on food chain quality and human health. Journal of Trace Elements in Medicine and Biology 18, 309–318.

Havaux, M., 1998. Carotenoids as membrane stabilizers in chloroplasts. Trends in Plant Science 3 (4), 147–151.

Inskeep, W.P., Bloom, P.R., 1985. Extinction coefficients of chlorophyll-*a* and chlorophyll-*b* in *N*,*N*-dimethylformamide and 90-percent acetone. Plant Physiology 77 (2), 483–485.

Ishii, W.P., Yamagishi, T.Y., Murata, Y., 1977. One method for measuring photosynthesis and respiration of leaf slices with an oxygen electrode. Japanese Journal of Crop Science 46, 53–57.
Kuda, T., Tsunekawa, M., Hishi, T., Araki, Y., 2005. Antioxidant properties of dried kayamo-nori, a brown alga Scytosiphon lomentaria (Scytosiphonales, Phaeophyceae). Food Chem. 89, 617–622.
Lee, W.Y., Wang, W.X., 2001. Metal accumulation in the green macroalga *Ulva fasciata*: effects of nitrate, ammonium and phosphate. Science of the Total Environment 278 (1–3), 11–22.
Loughnane, C.J., McIvor, L.M., Rindi, F., Stengal, D.B., Guiry, M.D., 2008. Morphology, rbcL phylogeny and distribution of distromatic *Ulva* (Ulvophyceae, Chlorophyta) in Ireland and southern Britain. Phycologia 47, 416–429.
Mazid, M., Khan, T.A., Mohammad, F., 2011. Role of secondary metabolites in defense mechanisms of plants. Biology and Medicine 3 (2), 232–249.
Michalak, A., 2006. Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Polish Journal of Environmental Studies 15 (4), 523–530.
Moran, R., Porath, D., 1980. Chlorophyll determination in intact tissue using *N*,*N*-dimethylformamide. Plant Physiology 77, 483–485.
Morlon, H., Fortin, C., Adam, C., Garnier-Laplace, J., 2006. Selenite transport and its inhibition in the unicellular green alga *Chlamydomonas reinhardtii*. Environmental Toxicology and Chemistry 25, 1408–1417.
Neumann, P.M., de Souza, M.P., Pickering, I.J., Terry, N., 2003. Rapid microalgal metabolism of selenate to volatile dimethylselenite. Plant, Cell and Environment 26, 897–905.
Norusis, M.J., 1993. SPSS for Windows: base system user's guide, release 6.0. SPSS Inc., Chicago.
Novoselov, S., Rao, M., Onoshko, N., Zhi, H., Kryukov, G., Xiang, Y., Weeks, D., Hatfield, D., Gladyshev, V., 2002. Selenoproteins and selenocysteine insertion system in the model plant cell system, *Chlamydomonas reinhardtii*. EMBO Journal 21, 3681–3693.
Pastierova, J., Kramarova, Z., Molnarova, M., Fargasova, A., 2009. Comparison of the sensitivity of four freshwater macroalgae to selenate and selenite. Fresenius Environmental Bulletin 18 (11), 2029–2033.
Pelah, D., Cohen, E., 2005. Cellular response of *Chlorella zofingensis* to exogenous selenium. Plant Growth Regulation 45, 225–232.
Pilon-Smits, E.A.H., Hwang, S.B., Lytle, C.M., Zhu, Y.L., Tai, J.C., Bravo, R.C., Leustek, T., Terry, N., 1999. Overexpression of ATP sulfurylase in *Brassica juncea* leads to increased selenate uptake, reduction and tolerance. Plant Physiology 119, 123–132.
Pilon-Smits, E.A.H., LeDuc, D.L., 2009. Phytoremediation of selenium using transgenic plants. Current Opinion in Biotechnology 20 (2), 207–212.
Pilon-Smits, E.A.H., Quinn, C.F., Tapken, W., Malagoli, M., Schiavon, M., 2009. Physiological functions of beneficial elements. Current Opinion in Plant Biology 12, 267–274.
Plant, J.A., Kinniburgh, D.G., Smedley, P.L., Fordyce, F.M., Klinck, B.A., 2004. Arsenic and selenium. In: Holland, H.D., Turekian, K.K. (Eds.), Treatise on Geochemistry, vol. 9, Environ Geochem. Elsevier, Amsterdam, pp. 17–66.
Posmyk, M.M., Kontek, R., Janas, K.M., 2009. Antioxidant enzymes activity and phenolic compounds content in red cabbage seedlings exposed to copper stress. Ecotoxicology and Environmental Safety 72 (2), 596–602.
Pyrzynska, K., 1998. Speciation of selenium compounds. Analytical Sciences 14, 479–483.
Rascio, N., Mariani, P., Tommasini, E., Bodner, M., Larcher, W., 1991. Photosynthetic strategies in leaves and stems of *Egeria densa*. Planta 185, 297–303.
Reunova, Y.A., Aizdaicher, N.A., Khristoforova, N.K., Reunova, A.A., 2007. Effects of selenium on growth and ultrastructure of the marine unicellular alga *Dunaliella salina* (Chlorophyta). Russian Journal of Marine Biology 33 (2), 125–132.
Riedel, G.F., Sanders, G.J., 1996. The influence of pH and media composition on the uptake of inorganic selenium by *Chlamydomonas reinhardtii*. Environmental Toxicology and Chemistry 15, 1577–1583.
Ross, C., Van Alstyne, K.L., 2007. Intraspecific variation in stress-induced hydrogen peroxide scavenging by the ulvoid macroalga *Ulva lactuca*. Journal of Phycology 43 (3), 466–474.
Schiavon, M., Pilon-Smits, E.A.H., Wirtz, M., Hell, R., Malagoli, M., 2008. Interactions between chromium and sulfur metabolism in *Brassica juncea*. Journal of Environmental Quality 37, 1536–1545.
Schiavon, M., Pittarello, M., Pilon-Smits, E.A.H., Wirtz, M., Hell, R., Malagoli, M., 2012. Selenate and molybdate alter sulfate transport and assimilation in *Brassica juncea* L. Czern: implications for phytoremediation. Environmental and Experimental Botany 75, 41–51.
Simmons, D.B.D., Emery, R.J.N., 2011. Phytochelatin induction by selenate in *Chlorella vulgaris* and regulation of effect by sulfate levels. Environmental Toxicology and Chemistry 30 (2), 469–476.
Shrift, A., 1954. Sulfur–selenium antagonism. Antimetabolite action of selenate on the growth of *Chlorella vulgaris*. American Journal of Botany 41, 223–230.
Tamaoki, M., Freeman, J.L., Pilon-Smits, E.A.H., 2008. Cooperative ethylene and jasmonic acid signaling regulates selenite resistance in *Arabidopsis*. Plant Physiology 146 (3), 1219–1230.
Terry, N., Zayed, A.M., de Souza, M.P., Tarun, A.S., 2000. Selenium in higher plants. Annual Review of Plant Physiology and Plant Molecular Biology 51, 401–432.
Tuzen, M., Sari, A., 2010. Biosorption of selenium from aqueous solution by green algae (*Cladophora hutchinsiae*) biomass: equilibrium, thermodynamic and kinetic studies. Chemical Engineering Journal 158, 200–206.
Umisová, D., Vìtovà, M., Douskovà, I., Bisovà, K., Hlavovà, M., Cizkovà, M., Machàt, J., Doucha, J., Zachleder, V., 2009. Bioaccumulation and toxicity of selenium compounds in the green alga *Scenedesmus quadricauda*. BMC Plant Biology 9, 58–74.
Vítová, M., Bišová, K., Hlavová, M., Zachleder, V., Rucki, M., Čížková, M., 2011. Glutathione peroxidase activity in the selenium-treated alga *Scenedesmus quadricauda*. Aquatic Toxicology 102, 87–94.
Wellburn, A.R., 1993. The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. Journal of Plant Physiology 144, 307–313.
Wheeler, A.E., Zingaro, R.A., Irgolic, K., 1982. The effects of selenate, selenite and sulphate on the growth of six unicellular marine algae. Journal of Experimental Marine Biology and Ecology 57, 181–194.
Williams, M.J., Ogle, R.S., Knight, A.W., Burau, R.G., 1994. Effects of sulfate on selenate uptake and toxicity in the green alga *Selenastrum capricornutum*. Archives of Environmental Contamination and Toxicology 27 (4), 449–453.
Young, A.J., 1991. The photoprotective role of carotenoids in higher plants. Physiologia Plantarum 83 (4), 702–708.
Zarcinas, B.A., Cartwright, B., Spouncer, L.R., 1987. Nitric acid digestion and multi-element analysis of plant material by inductively coupled plasma spectrometry. Communications in Soil Science and Plant Analysis 18, 131–146.
Zhu, Y-G., Pilon-Smits, E.A.H., Zhao, F-J., Williams, P.N., Meharg, A.A., 2009. Selenium in higher plants: understanding mechanisms for biofortification and phytoremediation. Trends in Plant Science 14 (8), 436–442.
Zhang, Q., Yu, P., Li, Z., Zhang, H., Xu, Z., Li, P., 2003. Antioxidant activities of sulfated polysaccharide fractions from Porphyra haitanesis. J. Appl. Phycol. 15, 305–310.