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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