

# Rapid microalgal metabolism of selenate to volatile dimethylselenide

P. M. NEUMANN<sup>1</sup>, M. P. DE SOUZA<sup>2</sup>, I. J. PICKERING<sup>3</sup> & N. TERRY<sup>2</sup>

<sup>1</sup>Division of Agricultural Engineering, Faculty of Civil and Environmental Engineering, Technion Israel Institute of Technology, Haifa 32000, Israel, <sup>2</sup>Department of Plant and Microbial Biology, 111 Koshland Hall, University of California, Berkeley, CA 94720–3102, USA and <sup>3</sup>Stanford Synchrotron Radiation Laboratory, Stanford Linear Accelerator Center, MS 69, 2575 Sand Hill Road, Menlo Park, CA 94025–7015, USA

## ABSTRACT

**An axenically cultured isolate of single-celled freshwater microalgae (*Chlorella* sp.) metabolized toxic selenate to volatile dimethylselenide at exceptionally high rates when transferred from mineral-nutrient solution to water for 24 h. The Se-volatilization rates were orders of magnitude higher than those similarly measured for wetland macroalgae and higher plants. Ninety percent of 20  $\mu$ M selenate supplied to the microalgae incubated without nutrients was removed through accumulation and volatilization. Additions of 1 mM sulphate but not nitrate, inhibited Se accumulation and volatilization so that only 1.8% of the supplied selenate was removed. The microalgae cultured in nutrient solution without sulphate showed increased <sup>35</sup>S-sulphate-transporter activity. Selenium K-edge X-ray absorption spectroscopy of selenate-treated microalgae cultured with or without mineral nutrients, showed that 87% of the selenate accumulated during 24 h was reductively metabolized to intermediate organic compounds such as selenomethionine and selenocystine. This is in complete contrast to higher plants that show very limited reduction of selenate. It appears that high rates of Se accumulation and volatilization by the sulphate-deprived microalgae resulted from reduced competition with chemically analogous sulphate ions for selenate uptake via up-regulated sulphate/selenate transporters and rapid reductive metabolism of selenate. Hyper-volatilization of selenate by microalgal cells may provide a novel detoxification response.**

**Key-words:** detoxification; dimethylselenide; hyper-volatilization; microalgae; selenium accumulation; speciation; sulphate-transporter; wetlands; X-ray-absorption-spectroscopy.

## INTRODUCTION

Agricultural drainage waters resulting from the irrigation of selenium (Se)-rich soils and also industrial wastewater generated during the processing of coal or oil may contain

inorganic selenium salts that act as toxic environmental contaminants. Higher plants are able to accumulate Se in their tissues and can convert some to dimethylselenide (DMSe), a volatile gas which is released from the tissues and is relatively non-toxic to the environment (Wilber 1980; Terry *et al.* 1992; Terry & Zayed 1994; Pilon-Smits *et al.* 1999a). There has therefore been much interest in utilizing wetlands containing such plants for the bio-remediation of Se-contaminated waters (Bañuelos & Meek 1990; Frankenberger & Benson 1994; Zayed *et al.* 1999; Terry *et al.* 2000). However, in most plants studied to date, unmodified selenate is accumulated and only a very small percentage is slowly metabolized to volatile DMSe (Zayed, Lytle & Terry 1998; Pilon-Smits *et al.* 1999a; de Souza *et al.* 2000).

The possible contribution of bacteria and microalgae to Se bioremediation in wetlands has also been studied. Production of DMSe from inorganic Se has been shown for bacteria (Frankenberger & Karlson 1994) and for a euryhaline *Chlorella* species which was isolated from hypersaline Se-contaminated evaporation ponds (Fan, Lane & Higashi 1997). In addition, Oyamada, Takahashi & Ishikazi (1991) showed that three different species of freshwater microalgae were capable of metabolizing Se to DMSe. Although marine macroalgae have been shown to volatilize sulphate (Gage *et al.* 1997), volatilization of chemically analogous selenate by macroalgae does not appear to have been shown. Moreover, there have been no previous attempts to determine comparative Se bioremediation potential, namely absolute rates of DMSe production, in fresh water microalgae, macroalgae and higher plants assayed under similar conditions.

Rates of Se volatilization may be affected by environmental factors. For example, it is known that mineral-nutrient deprivation and particularly sulphate deprivation, can lead to increased activity of the enzymes involved in sulphate uptake and the initial stages of sulphate reduction by plant cells (Biedlingmaier & Schmidt 1989; Zayed & Terry 1992, 1994; Takahashi *et al.* 1997; Leustek & Saito 1999; Takahashi *et al.* 2000). Similarly, sulphate deprivation can increase uptake and volatilization of the chemically analogous selenate by higher plants (Zayed & Terry 1992, 1994). This suggests that selenate accumulation and metabolism may occur via the pathways of sulphate metabolism. Effects of mineral nutrient deprivation in general and sulphate

Correspondence: Peter M. Neumann. Fax: 972 48221529; e-mail: agpetern@tx.technion.ac.il

deprivation in particular, on uptake, intermediate metabolism and volatilization of Se by fresh water micro- or macroalgae do not appear to have been investigated.

We report here on investigations of Se metabolism by an axenically cultured microalgal isolate (apparent *Chlorella* species). The main aims were: (1) to determine whether the microalgae could metabolize selenate, selenite or L-selenomethionine to volatile dimethylselenide; (2) to determine the effects of mineral nutrient supply and particularly sulphate, on Se accumulation, intermediate metabolism and volatilization by the microalgae; and (3) to compare rates of Se volatilization determined for the microalgae with those in similarly treated macroalgae and higher plants.

## MATERIALS AND METHODS

Effluent collected from a constructed wetland (Allegheny Power Services, Springdale, PA, USA), which was cultured with aerated Hoagland solution in the greenhouse for several weeks, turned green as a result of microalgal growth. Preliminary data (not shown) indicated that the mixed culture was able to produce volatile Se when incubated with selenate. Microalgal colonies were produced by plating aliquots of the culture onto sterile 1.5% agar plates containing 0.5× Hoagland nutrient solution, placed in a growth chamber with a 24 h photoperiod, and maintained at 25 °C with light at 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . After repeated subculturing, uniform algal colonies were obtained without microbial contamination. A single algal colony was then removed from an agar plate and axenically grown in 3 L of aerated 0.5× Hoagland solution. Axenic algal cultures were maintained in a growth chamber and subcultured at weekly intervals. A microscopic examination by Dr Paul Silva of the UC Berkeley Herbarium revealed green, uniform, non-motile, single-cells of 4–5  $\mu\text{m}$  diameter, each with a single chloroplast suggesting that this was a *Chlorella* sp. Algal cultures were centrifuged at 6000 *g* for 6 min and the resultant algal precipitate was rinsed with distilled water. The algae were then suspended in distilled water or nutrient solution with the indicated selenium compound.

Two types of macroalgae, *Enteromorpha* and *Chara* (musk grass) were also investigated with respect to their

comparative Se volatilization rates. The *Enteromorpha* were taken from the same APS constructed wetland as the microalga. They were harvested and transported to the laboratory for analysis. The *Chara*, originally from cell 2 at the Tulare Lake Drainage District wetland in Corcoran California, were harvested from a wetland microcosm maintained in a UC Berkeley greenhouse. Both macroalgae were identified by Dr Paul Silva at the UCB Herbarium. In each case the algae were rinsed several times in distilled water and appropriate solutions were added to weighed aliquots prior to assay.

## Se uptake and volatilization

Algal suspensions (200 mL) containing Se compounds, were introduced into Pyrex-glass gas-washing bottles. Antibiotics (60 mg L<sup>-1</sup> ampicillin and 100 mg L<sup>-1</sup> penicillin G) were routinely added to the algal solutions in order to minimize any build up of microbial contaminants during the 24 h assay. Experiments were conducted with selenium compounds at concentrations of 20  $\mu\text{M}$  (1580  $\mu\text{g Se L}^{-1}$ ) to allow comparison with several previous investigations of Se volatilization rates in higher plants in which similar concentrations of Se compounds were used (Table 1). Moreover 20  $\mu\text{M}$  Se represents the upper end of Se concentrations likely to be encountered in wetlands and did not have visible toxic effects during the 24 h incubations used for the rate assays. During the assay of volatilization rates each gas wash bottle was connected via short lengths of Tygon tubing to a second wash bottle. This contained 200 mL of alkaline peroxide solution (3.84 g NaOH, 480 mL 30% H<sub>2</sub>O<sub>2</sub>, 1920 mL H<sub>2</sub>O) in which any volatile selenium compounds given off by the algae in the first wash bottle were trapped and oxidized. A vacuum line was attached to the trap wash bottle via a pressure regulator so that a stream of 0.22  $\mu\text{m}$  filtered air was forced through both wash bottles at a rate of 1.5 L min<sup>-1</sup>. Control experiments in which three gas traps were used in series showed that the first trap retained 96 ± 1% (*n* = 3) of the volatile selenium released by nutrient-deprived microalgal cultures over 24 h. The set up was maintained in a temperature controlled (22–24 °C) sun-lit glasshouse with a 16 h light period maintained by supplementary lighting. At indicated

**Table 1.** Reported rates of volatilization of selenate by higher plants

Organism	Selenate conc. ( $\mu\text{M}$ )	Se volatilization rate ( $\mu\text{g Se g}^{-1} \text{DW d}^{-1}$ )	Reference
<i>Brassica juncea</i>	20	0.46	<sup>a</sup> de Souza <i>et al.</i> (1998)
<i>Brassica juncea</i>	20	0.32	<sup>b</sup> de Souza <i>et al.</i> (2000)
<i>Salicornia</i>	15–50	0.5	<sup>c</sup> Z.Lin <i>et al.</i> unpublished.
Six crop species	20	1 to 38	<sup>d</sup> Terry & Zayed (1994)
15 crop species	20	0.2–2.4	<sup>e</sup> Terry <i>et al.</i> (1992)
20 aquatic species	20	0 to 1	<sup>f</sup> Pilon-Smits <i>et al.</i> (1999a)

<sup>a,b,d,e,f</sup> Assays of selenate volatilization under similar conditions to those used in the microalgal assay, except that the intact plants were pretreated with selenate for 7 d before Se volatilization was measured for 24 h in the presence (b,d,e) or absence (a,f) of mineral nutrients in the root medium. <sup>c</sup> Unpublished data from field assays.

times 5 mL of trap solution was removed for Se sampling. The samples were first heated in a loosely capped boiling tube at 95 °C for 20 min in order to remove the remaining peroxide. The cooled samples were then heated again for 30 min with 5 mL of concentrated HCl in order to reduce the selenium to selenite prior to assay by hydride-generation flame atomic absorption spectroscopy (Varian spectra AA220FS spectrometer; Varian Inc., Mulgrave, Victoria, Australia). The assay was sensitive to Se levels above 1 p.p.b. and was calibrated with Se standards at 2, 5, 10 and 20 p.p.b. to produce a calibration curve by the instrument's (New Rational) program (Varian Inc.) before each set of assays.

At the end of each run 50 to 100 mL aliquots of microalgae were collected by filtration onto weighed glass-fibre filter discs with 0.22 µm pore size, or by two cycles of centrifugation with intermediate washes in distilled water. In order to determine selenium removal by the algae, 2 mL of the clear culture filtrate was sampled, 2 mL of 30% hydrogen peroxide was added to it, and the Se content was determined as described above. The filtered algae were rinsed with distilled water and dried at 55 °C for 48 h prior to dry weight determination. Macroalgae were similarly treated. In some experiments the dry algae were digested by stepwise additions of 70% nitric acid and then peroxide at 95 °C in a modification of US Environmental Protection Agency Protocols 3050 B, 1996, and 7742, 1994. The Se content was then assayed by atomic absorption. Uptake and volatilization data are shown as means (± SE) for parallel assays of three replicate batches of algae. All the chemicals used were of high purity grade. Experiments were repeated one or more times with similar results.

Samples of the volatile selenium compound produced by the algae were analysed by gas chromatography/mass spectrometry as described in de Souza *et al.* (2000). Briefly, the precipitated algae obtained after centrifuging 200 mL of algal culture at 6000 g for 6 min, were rinsed with distilled water, re-suspended with 5 mL of distilled water and transferred to 10 mL serum vials. The samples were closed with Teflon-faced butyl rubber stoppers, crimp-sealed, incubated in a growth chamber for 24 h, and frozen at -20 °C. The gas phase was sampled immediately after thawing and assayed against known standards of DMSe, dimethyldiselenide (DMDSe) and dimethylsulphide (DMS).

To determine the effect of sulphate deprivation on sulphate uptake rates by the microalgae, they were grown in 500 mL of 0.5× Hoagland solution, which was prepared with or without sulphate to obtain the following treatments: (1) no sulphate, no Se; (2) + 1 mM sulphate, no Se; (3) no sulphate + 20 µM selenate; and (4) 1 mM sulphate + 20 µM selenate. These media were dispensed into 1 L flasks, inoculated with a 5% inoculum of microalgae and maintained on a shaker at 150 r.p.m. with continuous light. After 1 month of growth, the cultures were centrifuged at 6000 g, re-suspended in 0.5× Hoagland solution without sulphur, and short-term <sup>35</sup>S-sulphate influx was measured for 5 min as described for yeast cultures (Cherest *et al.* 1997).

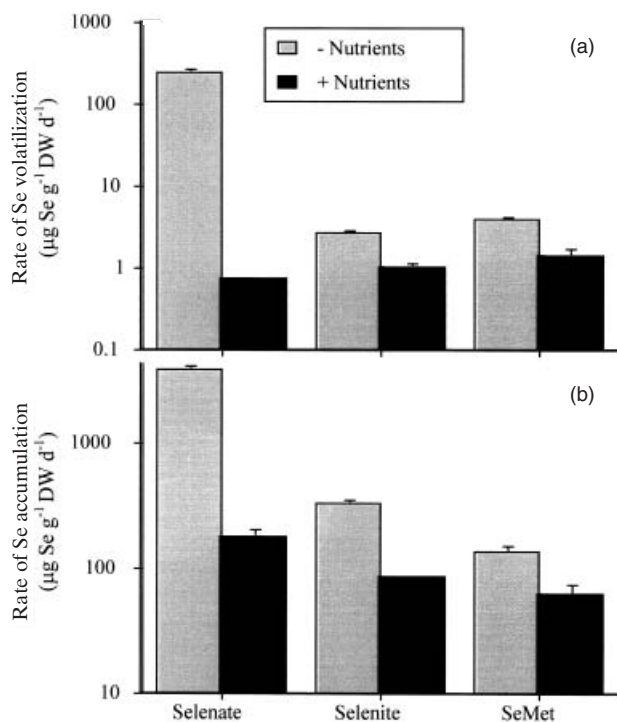
## Cellular metabolism of Se

Microalgal samples for X-ray absorption spectroscopy (XAS) were collected by centrifugation. The algal pellet was suspended in distilled water, centrifuged again, re-suspended in 200 µL 15% glycerin and stored at -80 °C in capped microcentrifuge tubes prior to assay. XAS at the Stanford Synchrotron Radiation Laboratory provided a qualitative in-vivo estimation of the degree to which inorganic selenium supplied to the cells was converted to organic selenium compounds. XAS analyses of all frozen samples were performed on Beam Line 4-3 [Stanford Synchrotron Radiation Laboratory (SSRL), Menlo Park, CA, USA]. A Si(220) double crystal monochromator was used with an upstream vertical aperture of 1 mm, and harmonic rejection was achieved by detuning one crystal by 50%. The electron energy was 3.0 GeV with a current of 50–100 mA. Frozen samples were positioned in a liquid He cryostat at an angle of 45° to the X-ray beam. Selenium K-edge X-ray absorption spectra were collected by monitoring the Se K<sub>α</sub> fluorescence using a Canberra 13-element Ge detector (SSRL), in a series of scans dependent on trace element concentration. Spectra were also collected for dilute reference solutions. All samples were calibrated against a reference of hexagonal Se(0) collected simultaneously with the data in transmission; the first energy-inflection of the reference was assumed to be 12658.0 eV. Data were collected using the program XAS-COLLECT (George 2000) and analysed using the EXAFSPAK suite of programs (<http://ssrl.slac.stanford.edu/exafspak.html>). Quantitative analysis using an edge-fitting method was carried out according to the method of Pickering, Brown & Tokunaga (1995). Statistical analyses were performed using the JMP IN statistical package (SAS Institute, Cary, NC, USA) using analysis of variance procedures.

## RESULTS

### The microalgal production of DMSe from selenate is greatly increased by removing mineral nutrient supply

Production of DMSe by the microalgae was initially assayed during incubation for 24 h in mineral nutrient solution with the addition of 20 µM selenate. The mean Se volatilization rate for three separate experiments was  $1.6 \pm 0.7 \mu\text{g Se g}^{-1} \text{ DW algae d}^{-1}$ . This low rate is similar to those previously found in higher plants supplied with selenate at near equivalent concentrations and conditions (cf. Table 1). However, when the microalgae were centrifuged and re-suspended in distilled water to which selenate was added, the rate of Se accumulation increased 22-fold and the rate of Se volatilization increased 313-fold to  $232 \pm 39 \mu\text{g Se g}^{-1} \text{ DW d}^{-1}$  (Fig. 1; NB the logarithmic scale of the y axis). Increasing external selenate concentrations to 200 µM did not produce higher rates of Se volatilization by the nutrient-deprived microalgae, suggesting that Se was saturating under these conditions (data not shown). Moreover the acceleration of DMSe flux was not particularly



**Figure 1.** Effect of mineral nutrient deprivation on uptake and volatilization of selenate by microalgae. Algae were treated for 24 h with selenate (20  $\mu\text{M}$  or 1.59 p.p.m. Se), selenite (10  $\mu\text{M}$ ), or selenomethionine (20  $\mu\text{M}$ ) with or without mineral nutrient solution. Columns are means of three replicate determinations, vertical bars indicate standard error where large enough to appear. Note that the Y-axis has a logarithmic scale. Increases induced by nutrient deprivation were significant ( $P = 95\%$ ) for all treatments. Algal concentrations were  $137 \pm 9 \text{ mg DW L}^{-1}$  in the plus-nutrient experiment and  $85 \pm 5 \text{ mg DW L}^{-1}$  in the minus-nutrient experiment.

sensitive to cell mass since it was obtained despite the fact that the algal density was greater in the plus nutrient experiment than in the minus nutrient experiment (137 and 85 mg DW  $\text{L}^{-1}$ , respectively). In other experiments, rates of volatilization by the microalgae supplied with selenate in the absence of mineral nutrient solution reached values as high as  $900 \pm 38 \mu\text{g Se g}^{-1} \text{ DW d}^{-1}$ . The mean rate for five separate experiments was  $550 \pm 110 \mu\text{g Se g}^{-1} \text{ DW d}^{-1}$  (equivalent to  $7.0 \pm 1.4 \mu\text{mol Se g}^{-1} \text{ DW d}^{-1}$ ).

The volatile Se compound released by the microalgae was identified using gas chromatography as dimethylselenide (DMSe). DMDS and DMS were not detected (data not shown).

Low rates of Se volatilization also occurred when the microalgae were supplied with either selenite or selenomethionine. However, mineral nutrient deprivation resulted in relatively smaller (two- to three-fold) increases in comparison with the selenate-supplied microalgae (Fig. 1). Thus, withdrawal of mineral nutrients for 24 h appeared to activate greatly enhanced rates of microalgal Se accumulation from selenate and of subsequent Se volatilization to DMSe.

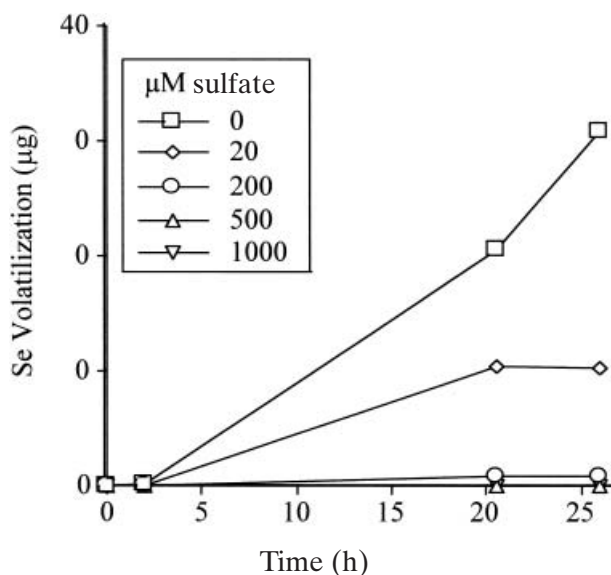
Rates of Se volatilization by two fresh water macroalgae supplied with 20  $\mu\text{M}$  selenate and similarly deprived of

nutrients for 24 h were assayed under identical conditions. The rates were comparatively low:  $0.36 \pm 0.04 \mu\text{g Se g}^{-1} \text{ DW d}^{-1}$  for the *Enteromorpha* and  $3.8 \pm 0.6 \mu\text{g Se g}^{-1} \text{ DW d}^{-1}$  for the *Chara*. Thus, nutrient deprivation in these two macroalgae did not result in rates of volatilization comparable to those given by the microalgae.

### Sulphate alone inhibits Se accumulation and volatilization

The high rates of Se volatilization induced by incubation of the microalgae in 20  $\mu\text{M}$  selenate without mineral nutrient solution for 24 h were effectively inhibited by the specific presence of 1000  $\mu\text{M}$  sulphate alone in the aqueous incubation solution (volatilization rate was reduced from 760 to 0  $\mu\text{g Se g}^{-1} \text{ DW d}^{-1}$ ). This concentration is equivalent to the sulphate concentration in the mineral nutrient solution used to culture the microalgae. In a subsequent experiment the inhibition of Se volatilization by additions of 20, 200, 500 or 1000  $\mu\text{M}$  sulphate were investigated (Fig. 2). Concentrations of sulphate down to 20  $\mu\text{M}$  clearly inhibited volatilization although the degree of inhibition by 20  $\mu\text{M}$  sulphate was comparatively low. The overall rates of Se volatilization for increasing sulphate concentrations (0, 20, 200, 500 and 1000  $\mu\text{M SO}_4$ ) declined from  $900 \pm 30$  to  $344 \pm 8, 22 \pm 7, 0$  and  $0 \mu\text{g Se g}^{-1} \text{ DW d}^{-1}$ , respectively.

In addition to inhibiting Se volatilization, sulphate also inhibited the accumulation of Se by the microalgae. Accumulation was estimated by measuring Se removal from the



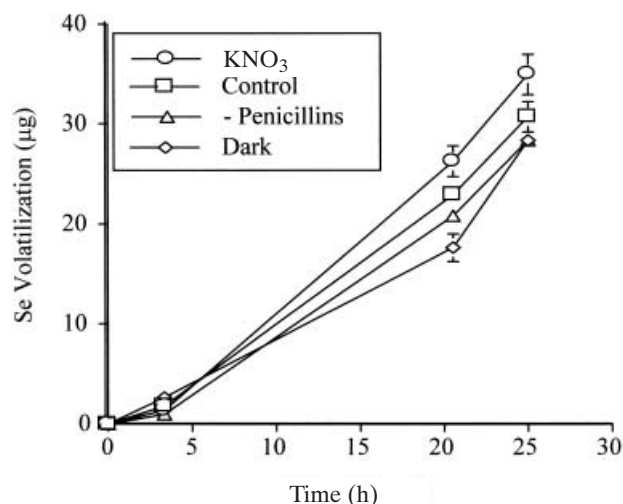
**Figure 2.** Time course of inhibition of selenium volatilization by increasing external concentrations of sulphate. Algae were washed, suspended in distilled water and treated with 20  $\mu\text{M}$  selenate for indicated times with additions of 0, 20, 200, 500 and 1000  $\mu\text{M}$  sulphate. The largest standard error for the three replicates (0.48) is smaller than the symbols. Inhibition of uptake by all sulphate treatments was highly significant ( $P = 95\%$ ). Mean algal concentration was  $149 \pm 10 \text{ mg DW L}^{-1}$ .

incubation medium. Ninety percent of the selenate supplied was removed from the incubation solution by algae treated with  $20\ \mu\text{M}$  selenate and distilled water for 24 h. However, only 1.8% of the selenate was removed by algae incubated with  $20\ \mu\text{M}$  selenate and  $1000\ \mu\text{M}$  sulphate. These findings suggest that a major effect of sulphate was to reduce selenate accumulation, which in turn drastically reduced Se volatilization. Moreover, the presence of sulphate in mineral nutrient solution was probably the cause of the reduced rate of production of volatile DMSe by algae incubated for 24 h with selenate plus mineral nutrient solution. Finally, the fact that additions of sulphate alone inhibited the stimulatory effect of nutrient deprivation on selenate metabolism indicated that the stimulatory effect was not due to any irreversible damage caused by nutrient deprivation.

Selenate is chemically analogous to sulphate (same charge and virtually the same chemical structure) and is likely to be accumulated via sulphate transporters. We therefore determined the specific effect of sulphate deprivation and selenate on  $^{35}\text{S}$ -sulphate transport into the microalgae. The microalgae were grown for 1 month in nutrient media with or without sulphate or selenate as follows: (1) no sulphate, no selenate; (2) 1 mM sulphate, no selenate; (3) no sulphate,  $20\ \mu\text{M}$  selenate; and (4) 1 mM sulphate,  $20\ \mu\text{M}$  selenate. After 1 month they had optical densities (at 680 nm) of 0.196, 0.239, 0.010 and 0.279, respectively. The algae did not grow well in treatment 3 (-S+Se) and the activity of membrane sulphate transporters was therefore measured in the three remaining cultures, after adjusting their optical densities to 0.680 by re-suspension in  $0.5\times$  Hoagland solution without sulphate. After 5 min of treatment with  $^{35}\text{S}$ -sulphate, the -S-Se, +S-Se and +S+Se cultures took up  $133 \pm 30 \times 10^5$ ,  $50 \pm 10 \times 10^5$  and  $23 \pm 17 \times 10^5$  c.p.m.  $^{35}\text{S}\ \text{g}^{-1}\ \text{DW}$ , respectively. Thus, sulphate deprivation (-S-Se) significantly increased (2.6-fold;  $P < 0.05$ ) the activity of membrane sulphate transporters in the microalgal cultures in comparison with the sulphate-supplied algae (+S-Se). Sulphate transporter activity in the sulphate-supplied microalgae (+S-Se culture) was further reduced (two-fold lower) by selenate in the +S+Se culture; however, this difference was not significant ( $P > 0.05$ ).

### Minimal effects of nitrate, bacteria and light level on Se volatilization by selenate-supplied microalgae

The inhibitory effect of mineral nutrient supply on Se volatilization by the microalgae was not a general effect since it could be achieved by additions of sulphate alone. This conclusion is further supported by the fact that additions of 1 mM  $\text{KNO}_3$  alone to nutrient-deprived microalgal cultures did not inhibit Se volatilization (Fig. 3). Moreover, the high rates of Se volatilization by nutrient-deprived microalgae were not attributable to bacterial contamination since the presence or absence of penicillins did not significantly affect Se volatilization. Penicillins inhibit essential processes of cell wall synthesis in bacteria, thus causing osmotic



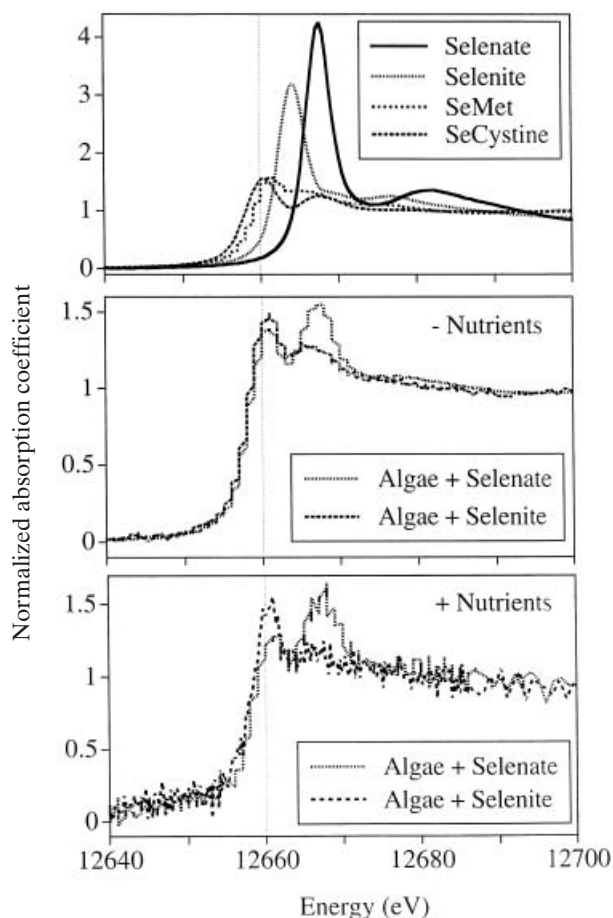
**Figure 3.** Effects of nitrate, dark treatment and assay in absence of penicillin on kinetics of Se-volatilization. For control treatment starved microalgae were incubated in distilled water with  $20\ \mu\text{M}$  selenate. Alternatively, algae were incubated in darkness or with 1 mM  $\text{KNO}_3$  or without routine additions of penicillins (-PEN) for indicated assay times. Vertical bars indicate standard error for three replicates when larger than the symbols. Algal density was  $352 \pm 7\ \text{mg DW L}^{-1}$ .

swelling and death but are thought not to inhibit algal metabolism (Halling-Sorensen 2000). Finally, incubation of algal cultures in darkness resulted in only small changes in volatilization after 20 h so that the effects of nutrient deprivation on Se volatilization are unlikely to be related to any differences in illumination.

### XAS speciation shows that the microalgae effectively reduced accumulated selenate to organic Se in the presence or absence of mineral nutrient supply

Higher plants accumulate selenate mainly in an unchanged form because they are unable to efficiently metabolize it further into reduced metabolites. We were interested in determining to what extent nutrient deprivation might accelerate reductive metabolism, particularly to selenomethionine (SeMet), a known precursor in the formation of DMSe.

X-ray absorption spectra were obtained from microalgae supplied with selenate or selenite in the presence or absence of mineral nutrients. These spectra were used to investigate possible differences in the intermediate metabolism of selenate by the nutrient-supplied and nutrient-deprived microalgae. X-ray absorption spectra of algal samples were compared with spectra from standard solutions of selenate, selenite, selenocystine and SeMet (Fig. 4). These were chosen to be representative of potential Se species present (Pickering *et al.* 1999). Note that due to the similarity in their spectra, SeMet and DMSe cannot be distinguished from one another (Van Fleet-Stalder *et al.* 2000) and SeMet is used as representative of a C-Se-C moiety.



**Figure 4.** Se K near-edge X-ray absorption spectra of selenium accumulated by microalgae. The top graph (a) shows the spectra for aqueous solutions of selenate (highest right hand peak) continuing with selenite, L-selenocystine (selenocystine dimer), and SeMet which were used as Se standards. Middle (b) and bottom graph (c) show spectra for algae after 24 h treatment with selenate ( $20 \mu\text{M}$ ) or selenite ( $10 \mu\text{M}$ )  $\pm$  mineral nutrient solution.

Analysis of the algal spectra (Table 2) showed that Se speciation, namely the relative distribution of Se species was the same in the presence or absence of mineral nutrient supply and within the confidence limits of the fits. In both cases, 87–90% of the selenate taken up by the algae, was converted into more reduced forms (Fig. 4b & c; Table 2).

Treatment	Selenate	Selenite	Selenocystine	SeMet
Selenate + nutrients	13 (2)	NS	48 (10)	39 (10)
Selenate – nutrients	10 (1)	3 (1)	52 (2)	35 (3)
Selenite + nutrients	NS	NS	76 (15)	24 (15)
Selenite – nutrients	NS	5 (1)	64 (3)	31(4)

The percentage contribution of each standard compound to the fit is shown. Values in parentheses are three times the estimated standard deviation and represent the 95% confidence limit in the least squares fit. Treatments of algae + selenate ( $20 \mu\text{M}$ ), and selenite ( $10 \mu\text{M}$ ), with or without mineral nutrient solution for 24 h. NS, component not significant. Selenocystine standard is a stable dimer of selenocystine

Moreover, at least one-third of the accumulated Se was found as an organic Se compound which was modelled as SeMet, a precursor of DMSe. The greatly increased Se volatilization rate measured in the absence of mineral nutrients did not therefore appear to be due to the relief of an inhibition by mineral nutrients of one or more of the biochemical steps involved in the metabolism of accumulated selenate to intermediate organic compounds such as SeMet.

## DISCUSSION

Our results show that a freshwater microalgal isolate of a *Chlorella* species which was axenically cultured in sterile nutrient solution, was capable of slowly producing volatile selenium (DMSe) when supplied with  $20 \mu\text{M}$  selenate, selenite or selenomethionine. This result is consistent with two previous reports showing Se volatilization to DMSe by: (1) a euryhaline *Chlorella* species (Fan *et al.* 1997); (2) freshwater isolates of *Chlorella vulgaris*, *Ankistrodesmus* sp. and *Selenastrum* sp. (Oyamada *et al.* 1991). The comparatively low rates of volatilization shown by our microalgal isolate when supplied with  $20 \mu\text{M}$  selenate and mineral nutrients were similar to low rates previously reported for higher plants from terrestrial and aquatic origins (Table 1). However, the same microalgae were able to produce DMSe from selenate at spectacularly high rates (ranging from 200 to  $900 \mu\text{g Se g}^{-1} \text{ DW algae d}^{-1}$ ) when deprived of mineral nutrients for 24 h. These rates were several orders of magnitude greater than comparable volatilization rates measured by us under identical conditions of nutrient deprivation for two freshwater macroalgae (*Chara* and *Enteromorpha*) and for a large number of terrestrial and aquatic higher plants which, in previous investigations, were also supplied with  $20 \mu\text{M}$  selenate for 24 h in the absence of mineral nutrients (de Souza *et al.* 1998; Pilon-Smits *et al.* 1999a).

Oyamada *et al.* (1991) supplied three different species of microalgae with very high external concentrations of selenate ( $630 \mu\text{M}$ ) together with  $300 \mu\text{M}$  sulphate in the nutrient solution. At this 2 : 1 concentration ratio of selenate to sulphate, selenate may have effectively competed with sulphate for uptake by the microalgae. In all events, graphical data in their report can be used to calculate a DMSe production rate of approximately  $600 \mu\text{g Se g}^{-1} \text{ DW d}^{-1}$  for

**Table 2.** Results of fitting Se K near edge X-ray absorption spectra of microalgae

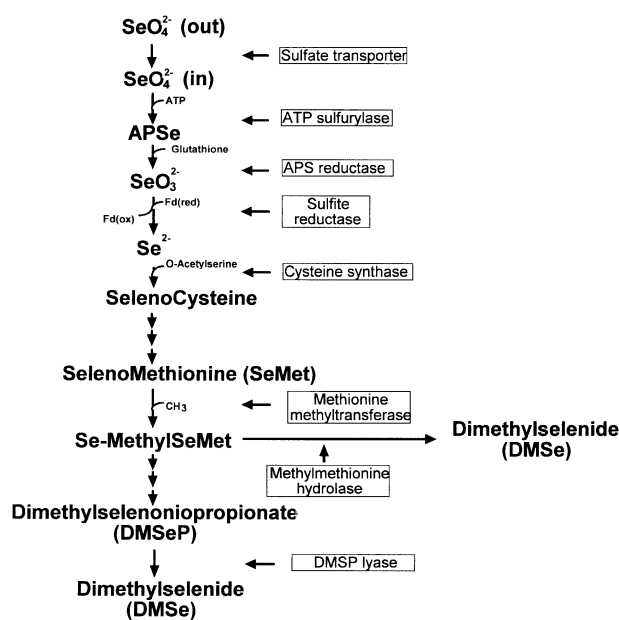
*Chlorella vulgaris* and similar rates for the *Ankistrodesmus* and *Selenastrum* species they assayed. These rates are within the range of volatilization rates shown by our nutrient-deprived *Chlorella* species. Although we clearly cannot answer for all species, it therefore seems that under environmental conditions that favour rapid selenate accumulation, several microalgal species are able to volatilize accumulated selenate at rates far higher than those attained by the wetland macroalgae assayed in this report or by a wide range of higher plants previously assayed. By analogy with heavy metal hyper-accumulation (Whiting, Neumann & Baker 2003), we suggest that the high rates of Se volatilization shown by such microalgae be termed hyper-volatilization.

Our nutrient-deprived microalgae accumulated Se from selenate at much faster rates than from selenite or SeMet. Higher plants also tend to accumulate Se from selenate at faster rates than from selenite or SeMet (Zayed *et al.* 1998) but not to the extent shown here for the microalgae.

The stimulatory effects of withdrawing nutrient solution on Se metabolism by the microalgae were almost certainly attributable to sulphate deprivation alone. Thus, addition of 1 mM sulphate (but not 1 mM KNO<sub>3</sub>) to the nutrient-deprived microalgae, completely reversed the stimulatory effects of nutrient deprivation on selenate accumulation and production of volatile DMSe. Since selenate is chemically analogous to sulphate in charge and structure it is likely to be taken up and further metabolized via the enzymes normally used in sulphate metabolism. Moreover, when the microalgae were grown in nutrient solution without sulphate they showed 2.6-fold higher sulphate transporter activity (<sup>35</sup>S influx) in comparison with the sulphate-supplied microalgae. These findings are consistent with many previous reports indicating that sulphate deprivation can lead to increased activity of the enzymes involved in sulphate uptake and the initial stages of sulphate reduction by plant cells (Biedlingmaier & Schmidt 1989; Takahashi *et al.* 1997; Leustek & Saito 1999; Takahashi *et al.* 2000). In addition, the presence of external sulphate appeared to competitively reduce the uptake of selenate into the root cells of higher plants via sulphate transporters (Zayed & Terry 1992, 1994; Terry *et al.* 2000). Thus, the absence of external sulphate may increase the uptake of selenate into the microalgae by increasing transporter activity and by reducing the competition between selenate and sulphate for sites on the transporter.

Based on the Se assimilation pathway for higher plants and by analogy with the pathways for assimilation of chemically analogous sulphate to DMS by marine algae (Gage *et al.* 1997), we propose that the microalgae assimilated selenate and metabolized it to DMSe by the enzymatic pathway shown in Fig. 5.

In addition to its effects on sulphate transporters, external sulphate has been specifically shown to repress the transcription levels and activity of ATP sulphurylase, the first enzymatic step involved in the reduction of accumulated sulphate, and presumably selenate, in higher plants (Leustek & Saito 1999; Pilon-Smits *et al.* 1999b). Higher



**Figure 5.** Proposed pathway of selenate assimilation and volatilization by the microalgae. In this pathway, selenate is accumulated via sulphate transporters, activated by ATP sulphurylase and reduced. Reduced Se is incorporated into selenocysteine which is then converted to SeMet. SeMet is methylated to methylSeMet which in turn may be converted to DMSeP. Both methylSeMet and DMSeP may be cleaved to produce DMSe.

plants may have intrinsic difficulty in reducing selenate to selenite and then to intermediate organic forms of Se, because ATP sulphurylase is rate-limiting (Pilon-Smits *et al.* 1999b; Terry *et al.* 2000). This suggestion was supported by XAS speciation studies showing that the wild type of *Brassica juncea*, when supplied with 20  $\mu$ M selenate over an 8-day period, accumulated mainly selenate, i.e. further reduction of selenate and subsequent metabolism to organic intermediates was minimal (Zayed *et al.* 1998). However, over-expression of ATP sulphurylase in *Brassica* plants increased the conversion of selenate to SeMet to 70% (Pilon-Smits *et al.* 1999b). Even so, it is important to note that these transgenic plants gave only low rates of Se volatilization to DMSe that were similar to those in the wild-type plants and far below the rates measured here for the microalga (Pilon-Smits *et al.* 1999b; de Souza *et al.* unpublished results). Thus, additional steps later in the pathway may be rate-limiting for Se volatilization by the higher plant. For example, when *Brassica juncea* plants were incubated with DMSeP, a selenonium precursor of DMSe further down the reduction pathway, the rate of volatilization increased from 0.46 to 36.16  $\mu$ g Se g<sup>-1</sup> DW d<sup>-1</sup> (de Souza *et al.* 2000). Even this rate is far lower than rates of Se volatilization shown by the nutrient-deprived microalgae in this report. Assuming that reductive metabolism of selenate takes place in the cytoplasm, it is conceivable that selenate reduction in higher plants is further restricted by Se transport into the large vacuolar compartment for storage.

The finding that ATP sulphurylase is an early rate-limiting step for selenate reduction in higher plants suggested to us that the increased Se volatilization rates in nutrient-deprived microalgae might have been associated with up-regulation of ATP sulphurylase. However, XAS analysis of the microalga showed that 87% of accumulated selenate was readily metabolized to more reduced forms of organic Se (selenocystine- and SeMet-like compounds) after 24 h in the presence or absence of the sulphate added with mineral nutrients (Fig. 4; Table 2). Thus, in complete contrast to higher plants, the activity of ATP sulphurylase did not appear to be rate limiting for selenate reduction by the microalgae. The possibility remains that nutrient deprivation, in addition to accelerating selenate uptake, also accelerated later stages in the pathway from organic selenium intermediates to volatile DMSe (Fig. 5).

Do microalgae derive some advantage from the ability to convert accumulated selenate to volatile DMSe at comparatively high rates? Our studies showed that when the *Chlorella* isolate was cultured for 1 month in nutrient solution without sulphate but with selenate, it did not grow (OD, 0.010). Conversely, microalgae grown without sulphate or selenate grew to an OD of 0.196 over the same period with sulphate for growth probably being scavenged from trace impurities in the nutrient medium. High rates of Se accumulation by the microalgae, whether induced by reduced competition with sulphate for uptake, or high external concentrations (cf. Fan *et al.* 1997), therefore appear to have toxic effects on long-term development. The surface to volume ratio is large in a microscopic single-celled alga so that Se-uptake rates can be relatively high while space available for storage of toxic Se compounds is relatively limited. Higher plants have much larger cells and may be able to transport and safely store some selenate in the vacuoles. However, for single-celled microalgae, a capacity for rapid cellular conversion of accumulated selenate to less toxic DMSe that can be removed by volatilization, could be particularly advantageous.

In conclusion, this report provides for the first time, a comparison of Se volatilization potential in a microalgae, two species of macroalgae and higher plants previously assayed under similar conditions. We show that under conditions of sulphate deprivation which accelerate Se uptake, rates of Se volatilization to DMSe by a freshwater *Chlorella* species (and by three species of freshwater microalgae assayed by others at high external selenate to sulphate ratios) are orders of magnitude higher than rates similarly measured for two macroalgal species, or previously, for a wide range of higher plants with different limitations for selenate storage and metabolism. The possible contribution to Se bio-remediation of microalgae with novel mechanisms of detoxification deserves attention.

## ACKNOWLEDGMENTS

Thanks to Rebekah Grassl for help with Se analyses. This work was supported by the Electric Power Research Institute, Grant Nos. W08021-30 and W04163. The XAS analysis

was performed at SSRL, which is funded by the Department of Energy, Offices of Basic Energy Sciences and Biological and Environmental Research; the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program, and the National Institute of General Medical Sciences.

## REFERENCES

- Bañuelos G.S. & Meek D.W. (1990) Accumulation of selenium in plants grown on selenium-treated soil. *Journal of Environmental Quality* **19**, 772–777.
- Biedlingmaier S. & Schmidt A. (1989) Sulfate transport in normal and S-deprived *Chlorella fusca*. *Zeitschrift für Naturforschung* **44c**, 495–503.
- Cherest H., Davidian J.-C., Thomas D., Benes V., Ansoerge W. & Surdin-Kerjan Y. (1997) Molecular characterization of two high affinity sulfate transporters in *Saccharomyces cerevisiae*. *Genetics* **145**, 627–635.
- Fan T.W.M., Lane A.N. & Higashi R.M. (1997) Selenium biotransformations by a euryhaline microalga isolated from a saline evaporation pond. *Environmental Science and Technology* **31**, 569–576.
- Frankenberger W.T. & Benson S. (1994) *Selenium in the Environment*. p. 456. Marcel Dekker, Inc. New York, USA.
- Frankenberger W.T. & Karlson U. (1994) Microbial volatilization of selenium from soils and sediments. In *Selenium in the Environment* (eds W.T. Frankenberger & S. Benson), pp. 369–387. Marcel Dekker, Inc., New York, USA.
- Gage D.A., Rhodes D., Nolte K.D., Hicks W.A., Leustek T., Cooper A.J.L. & Hanson A.D. (1997) A new route for synthesis of dimethylsulphoniopropionate in marine algae. *Nature* **387**, 891–894.
- George M.J. (2000) XAS-Collect: a computer program for X-ray absorption spectroscopic data acquisition. *Journal of Synchrotron Radiation* **7**, 283–286.
- Halling-Sorensen B. (2000) Algal toxicity of antibacterial agents used in intensive farming. *Chemosphere* **40**, 731–739.
- Leustek T. & Saito K. (1999) Sulfate transport and assimilation in plants. *Plant Physiology* **120**, 637–643.
- Oyamada N., Takahashi G. & Ishikazi M. (1991) Methylation of inorganic selenium compounds by freshwater green algae, *Ankistrodesmus* sp., *Chlorella vulgaris* and *Selenastrum* sp. *Japanese Journal of Toxicology and Environmental Health (Esei Kagaku)* **37**, 83–86.
- Pickering I.J., Brown G.E. Jr & Tokunaga T.K. (1995) Quantitative speciation of selenium in soils using X-ray absorption spectroscopy. *Environmental Science and Technology* **29**, 2456–2459.
- Pickering I.J., George G.N., Van Fleet-Stalder V., Chasteen T.G. & Prince R.C. (1999) X-ray absorption spectroscopy of selenium-containing amino acids. *Journal of Biological Inorganic Chemistry* **4**, 791–794.
- Pilon-Smits E.A.H., de Souza M.P., Hong G., Amini A., Bravo R.C., Payabyab S.T. & Terry N. (1999a) Selenium volatilization and accumulation by twenty aquatic species. *Journal of Environmental Quality* **28**, 1011–1018.
- Pilon-Smits E.A.H., Hwang S., Lytle C.M., Zhu Y., Tai J.C., Bravo R.C., Chen Y., Leustek T. & Terry N. (1999b) Overexpression of ATP sulfurylase in Indian mustard leads to increased selenate uptake, reduction and tolerance. *Plant Physiology* **119**, 123–132.
- de Souza M.P., Lytle C.M., Mulholland M.M., Otte M.L. & Terry N. (2000) Selenium assimilation and volatilization from dimethylselenoniopropionate by Indian mustard. *Plant Physiology* **122**, 1281–1288.
- de Souza M.P., Pilon-Smits E.A.H., Lytle C.M., Hwang S., Tai J.,

- Honma T.S.U., Yeh L. & Terry N. (1998) Rate limiting steps in Se assimilation and volatilization by *Brassica juncea*. *Plant Physiology* **117**, 1487–1494.
- Takahashi H., Watanabe-Takahashi A., Smith F.W., Blake-Kalff M., Hawkesford M.J. & Saito K. (2000) The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in *Arabidopsis thaliana*. *Plant Journal* **23**, 171–182.
- Takahashi H., Yamazaki M., Sasakura N., Watanabe A., Leustek T., De Almeida E.J., Engler G., Van Montagu M. & Saito K. (1997) Regulation of sulfur assimilation in higher plants: a sulfate transporter induced in sulfate-starved roots plays a central role in *Arabidopsis thaliana*. *Proceedings of the National Academy of Science* **94**, 11102–11107.
- Terry N. & Zayed A.M. (1994) Selenium volatilization by plants. In *Selenium in the Environment* (eds W.T. Frankenberger & S. Benson), pp. 343–367. Marcel Dekker, Inc., New York, USA.
- Terry N., Carlson C., Raab T.K. & Zayed A.M. (1992) Rates of selenium volatilization among crop species. *Journal of Environmental Quality* **21**, 341–344.
- Terry N., Zayed A.M., de Souza M.P. & Tarun A.S. (2000) Selenium in higher plants. *Annual Review of Plant Physiological and Plant Molecular Biology* **51**, 401–432.
- Van Fleet-Stalder V., Chasteen T.G., Pickering I.J., George G.N. & Prince R.C. (2000) Fate of selenate and selenite metabolized by *Rhodobacter sphaeroides*. *Applied Environmental Microbiology* **66**, 4849–4853.
- Whiting S.N., Neumann P.M. & Baker A.J.M. (2003) Nickel and zinc hyperaccumulation by *Alyssum murale* and *Thlaspi caerulescens* (Brassicaceae) do not enhance survival and whole-plant growth under drought stress. *Plant, Cell and Environment* **26**, 351–360.
- Wilber C.G. (1980) Toxicology of selenium: a review. *Clinic Toxicology* **17**, 171–230.
- Zayed A. & Terry N. (1992) Selenium volatilization in broccoli as influenced by sulfate supply. *Journal of Plant Physiology* **140**, 646–652.
- Zayed A. & Terry N. (1994) Selenium volatilization in roots and shoots: effects of shoot removal and sulfate level. *Journal of Plant Physiology* **143**, 8–14.
- Zayed A., Lytle C.M. & Terry N. (1998) Accumulation and volatilization of different chemical species of selenium by plants. *Planta* **206**, 284–292.
- Zayed A.M., Pilon-Smits E.A.H., de Souza M.P., Lin Z.Q. & Terry N. (1999) Remediation of selenium-polluted soils and waters by phytovolatilization. In *Phytoremediation of Metal-Contaminated Water and Soils* (eds N. Terry & G. Bañuelos), pp. 61–83. CRC Press, Boca Raton, FL, USA.

Received 25 November 2002; accepted for publication 16 December 2002