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Cellular quotas and induced toxicity of selenite in the unicellular green alga *Chlamydomonas reinhardtii*

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Abstract. Cellular growth and intracellular concentrations of selenite in the unicellular green alga *Chlamydomonas reinhardtii* showed considerable variability between replicates at ambient concentrations of Se that became toxic to the alga. However, growth inhibition was well correlated to the cellular quota of selenite; the correlation between absorbed selenite and toxicity was better than the simple correlation between ambient selenite and toxicity usually used to express metal toxicity, suggesting selenite toxicity is mainly linked to intracellular accumulation. It is usually expected that effects of contaminants on organisms depend on internal concentrations. These results confirm this hypothesis.

1. INTRODUCTION

Selenium is well known as an essential element in natural systems, both as a nutrient and as a potential toxicant. It is a by-product of certain industrial and agricultural processes and is distributed unevenly in the environment. Se has previously led to several environmental damages [1, 2] and could cause severe problems on a global scale in the future [3, 4]. Se toxicity is mainly linked to its propensity to bioaccumulate within the base of food webs [5]. Micro algae, that transform inorganic Se species into more bioavailable organic species and transfer them to higher organisms, play a crucial role in Se toxicity [1, 6, 7]. Deleterious effects on algae may also induce a decrease of primary production, affecting the whole ecosystem. The aim of our study was to investigate the bioaccumulation and toxicity of selenite (Se (IV)) over a complete algal batch growth cycle. Selenite was chosen because selenium contents in organisms and its toxicity are thought to be mainly linked to the concentration of this form in water. It was shown to be scavenged from water to a greater extent than selenate (Se (VI)) [5, 8, 9]. Only few studies deal with selenium bioaccumulation and toxicity in micro algae. We found one study on the cyanobacterium *Anabaena flos-aquae* [10], and two studies on marine algae [11, 12]. In all three cases, accumulated selenium increased with selenite concentration in the medium. No saturation occurred, even at concentrations reaching $mg.L^{-1}$, which is very high compared to concentrations that can be usually found in the environment. First toxic effects were also observed at those environmentally irrelevant $mg.L^{-1}$ levels. In a previous study on selenite toxicity in *Chlamydomonas reinhardtii*, we showed that the growth rate was not a sensitive parameter, while the maximal cell density decreased with increasing selenite concentrations. Based on maximal cell density, the estimated EC_{50} was 80 µM (95 % IC [64; 98]), the LOEC 50 µM and the NOEC 10 µM [13].

Toxicity of a contaminant may be linked to cell surface interactions (competition for transport sites) or to intracellular accumulation. In the case of selenite, both effects can occur: as an oxyanion, selenite can compete with macronutrients at the membrane surface and hence reduce their availability to algae; it is also known to exercise oxidative stress in the intracellular medium or to substitute to sulphur in sulphur-containing proteins, forming dysfunctional enzymes [14-16]. The aim of this study was to investigate the bioaccumulation of selenite in *Chlamydomonas reinhardtii* and its relationship with toxicity.

2. MATERIAL AND METHODS

2.1 Algal cultures and experimental exposure conditions

Chlamydomonas reinhardtii cultures were obtained from the Culture Collection of Algae and Protozoa (CCAP; Cumbria, United Kingdom; culture 11/32B). Maintenance and experimental culturing were carried out axenically in a modified high salt medium (Table 1) under constant illumination at 100 $\mu mol.m^{-2}.s^{-1}$ and with an ambient temperature of 25°C ± 0.5 °C. The inoculation of algae in Se-contaminated media was carried out with cells from end of exponential growth phase cultures, by addition of the volume needed to obtain an initial density of 2,500 $cells.ml^{-1}$. Triplicate flasks containing exposure media (0, 10, 20, 30, 40 and 50 µM, obtained by addition of aqueous stock solutions of Na_2SeO_3, Sigma Aldrich, Steinheim, Germany) were prepared. Experiments were carried out at a pH of 7, monitored and adjusted daily (KOH, 0.1 M). Water samples were collected and filtered daily (0.2 µm PES filter membrane, Nalgene, USA) to determine major anions / cations and selenite concentrations. The effect of selenite was examined during a four-day exposure period corresponding to the complete algal growth cycle (from inoculation to stationary growth phase). At the end of the exposure (after 96h), 80 ml of cell suspensions per replicate were centrifuged to collect algae (100,000 g, 10 min) for measurement of intracellular selenium concentrations. To remove Se eventually adsorbed to the algal cell surface, algae were rinsed by re-suspension in 80 ml of fresh selenium-free media and collected again by centrifugation (100,000 g, 10 min). Total selenium content in algal pellets was then assessed as indicated in the section 2.3.

2.2 Algal growth measurements and statistical analysis

Cell density was measured daily in each medium using a Coulter Z2 particle counter (1:10 dilution with ISOTON II isotonic solution, using a 100 µm orifice tube; Beckman Coulter, Roissy, France).

We fitted growth curves as described in a previous paper [13]. We used the model proposed by Delignette-Muller [17], that describes the whole growth curve with three relevant parameters: initial population density $N(0)$, maximal cell density $N(\infty)$ (expressed in $cells.ml^{-1}$) and exponential growth rate μ (expressed in d^{-1}). The cell density at time t, $N(t)$, is then given by the equation:

$$N(t) = \frac{N(\infty)}{1 + (\frac{N(\infty)}{N(0)} - 1)\exp(-\mu t)} \quad (1)$$

Non-linear least-squares regressions using the Levenberg-Marquardt algorithm were performed to fit growth in each flask according to equation (1), with $N(0)$ fixed at 2500 $cell.ml^{-1}$, to estimate the two other parameters ($N(\infty)$ and μ).

A Hill model was then fitted to obtain a dose-response relationship between ambient selenite concentrations and maximal cell density. The Hill equation is characterised by two parameters: the Hill number nH and the 50% Effect Concentration EC_{50}, with the probability function written as follows:

$$f(x) = \frac{x^{nH}}{x^{nH} + EC_{50}^{nH}} \quad (2)$$

The Excel macro REGTOX (based on the Marquardt algorithm; [18]) was used to calculate EC_xs. The confidence intervals on the parameters were estimated by a bootstrap non parametric simulation. Finally, an analysis of variance completed by lowest significant difference (LSD) post-hoc comparisons was performed to test for significant differences between maximal cell densities at different contamination levels.

To investigate the relationship between intracellular accumulation and toxicity, estimated maximal cell densities were plotted against cellular quotas (quantity of selenium per cell). Non-parametric Spearman rank correlation coefficients of maximal cell densities against cellular quotas or ambient concentrations were calculated and compared. Tests of their significance were also performed.

The STATISTICA software package was used to perform all statistical analyses (StatSoft, Maisons-Alfort, France).

2.3 Chemical analyses

Anions and cations in water samples were respectively determined by ion chromatography (DIONEX-120, Sunnyvale, CA, USA; detection limits: Cl^-: 50 µg.L^{-1}; NO_3^-, NO_2^-, PO_4^{3-} and SO_4^{2-}: 100 µg.L^{-1}) and inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 4300 DV, Perkin Elmer Norwalk, CP, USA; detection limits: Mg^{2+}: 1 µg.L^{-1}; Na^+ and Ca^{2+}: 5 µg.L^{-1} ; K^+: 10 µg.L^{-1}). Selenite in water samples was analysed directly after filtration by flow-through hydride generation atomic absorption spectrometry, a selenite specific method (FIAS-100 / AAS 4110-ZL Perkin Elmer Shelton, CT USA; detection limit: 0.04 µg.L^{-1}). HCl was added at the time of the analyses to avoid any reduction of Se(VI) (final concentration of 1.2 M HCl).

To determine total selenium in algae, the first step consisted in converting organic selenium into inorganic Se(VI) (digestion with boiling a 1:1 mixture of nitric acid and hydrogen peroxide at 100°C for 90 min). Selenate was then reduced to selenite with boiling hydrochloric acid (final concentration of 4 M HCl) at 90°C for 45 min. Finally, Se(IV) was determined as described previously for aqueous samples.

3. RESULTS AND DISCUSSION

3.1 Toxicity on growth

Means and standard errors of estimated $N(\infty)$ and μ are reported in Table 1. The growth rate did not decrease with increasing selenite concentrations while cell yield decreased markedly, which had already been observed in previous experiments [13]. Estimated benchmark doses based on the relationship between estimated $N(\infty)$ and selenite concentrations and associated 95% confidence intervals at 10, and 50% (i.e. EC_{10} and EC_{50}) based on 5000 bootstrap runs were 2 µM ([0.8; 4.3]), and 14 µM ([10; 18]) respectively. The toxicity we found was higher than expected from previous experiments (estimated EC_{50}: 80 µM [64; 98], [13]). This may be explained by the intergenerational variability, which had already been observed before [19, 20]. Nevertheless, still high concentrations of selenite were necessary to cause significant growth inhibition such that reduction of algal biomass due do selenite is unlikely to occur in the environment.

LSD post-hoc comparisons showed that maximal cell density of each treatment (including controls) against one another was significantly different ($p<0.05$), except when comparing the 30, 40 and 50 µM against one another ($p>0.1$). At those toxic concentrations, it is noticeable that responses are very different from one replicate to the other, as shown by coefficients of variation (Table 1). Important variations in responses of replicates to high contamination pressures are a phenomenon we have also observed in other experiments on selenite, and on uranium (Gilbin, personal communication).

Table 1. Means and standard errors of measured average cell diameters at 96h and estimated μ and $N(\infty)$ when fitting equation (1) to experimental data sets from controls and from each Se treated experimental units. Coefficients of variation associated to $N(\infty)$.

Se conc. (µM)	Control	10	20	30	40	50
μ (d^{-1})	2.23±0.05	2.42±0.19	2.56±0.14	2.48±0.30	2.64±0.00	2.64±0.00
$N(\infty)$ (10^6 cell.ml^{-1})	2.05±0.07	1.25±0.04	0.90±0.04	0.38±0.10	0.42±0.13	0.55±0.10
CV(%)	6	6	7	48	52	32

3.2 Dependence of bioaccumulation on ambient concentration

Dissolved Se(IV) exposure concentrations were stable throughout the experiment with initial measured concentrations (mean ± 1SE , n=3, expressed in µM) of 0, 8.6 (± 0.1), 17.5 (± 0.3), 29.4 (± 0.1), 38.1 (±1.6) and 51 (± 0.2). Concentrations of anions and cations were also stable over time and in good agreement with nominal values (percentage of variation along the experiment: <5%).

Results of intracellular contents of selenite depending on ambient measured concentrations are reported in Fig. 1.

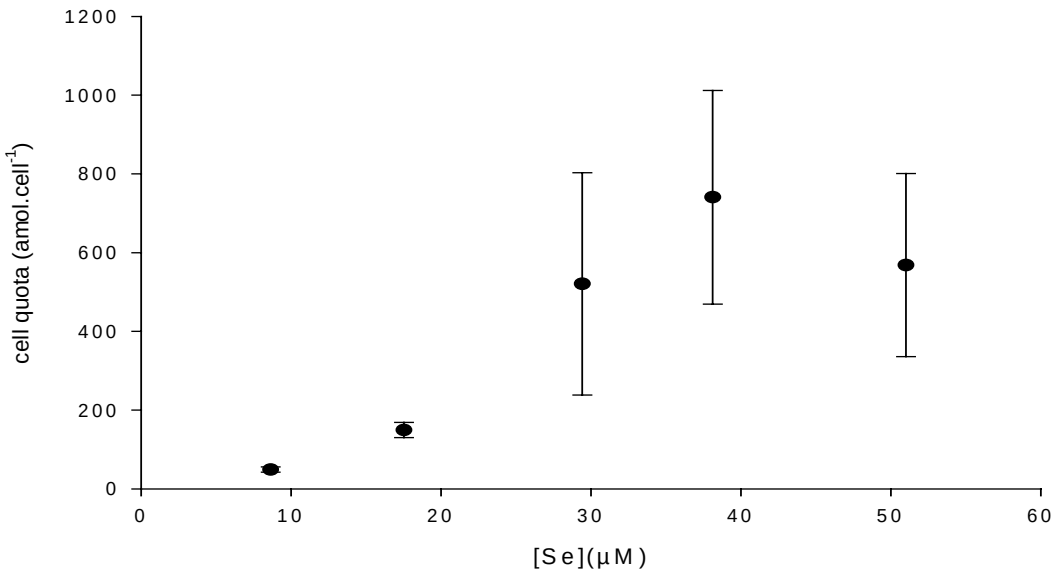

Figure 1. Means and standard errors of intracellular cell contents of selenite after 96h of growth plotted against measured selenite concentrations in media (n=3).

When ambient concentrations reached highly toxic values, we again observed an important variability between replicates: the coefficients of variation corresponding to cellular quotas at 10, 20, 30, 40 and 50 µM were respectively 14, 13, 54, 37 and 41%.

Chlamydomonas cells were able to accumulate large amounts of selenite. The bioaccumulation increased with ambient concentrations. A maximum value seemed to be reached above 20 µM, but the variability was too high to be conclusive. Those results are in agreement with those found by Kiffney (1990), Boisson (1995) and Li (2003), with similar maximal bioaccumulation capacities.

3.3 Link between bioaccumulation and effects

Noting that batches where the toxicity was the highest corresponded to batches where the bioaccumulation was the highest, we plotted estimated maximal densities against cellular quotas (Fig. 2). Algal growth decreased in response to the increase in intracellular Se, as anticipated. The correlation between estimated maximal cell densities and cellular quotas (Spearman, $r=-0.95$, $p<0.05$) was however greatly improved with respect to the correlation between estimated maximal densities and ambient selenite concentrations (Spearman, $r=-0.84$, $p<0.05$). For the 30, 40 and 50 µM treatments where a high variability between replicates appeared, the correlation between estimated

maximal densities and ambient concentrations was no longer significant (Spearman, r=0.18, p>0.05), whereas the correlation between estimated maximal densities and cellular quotas was still high and significant (Spearman, r=-0.77, p<0.05). This suggests that selenite exerts its toxicity through presence in the intracellular medium and is consistent with our previous observations of toxicity linked to ultrastructural damages [13].

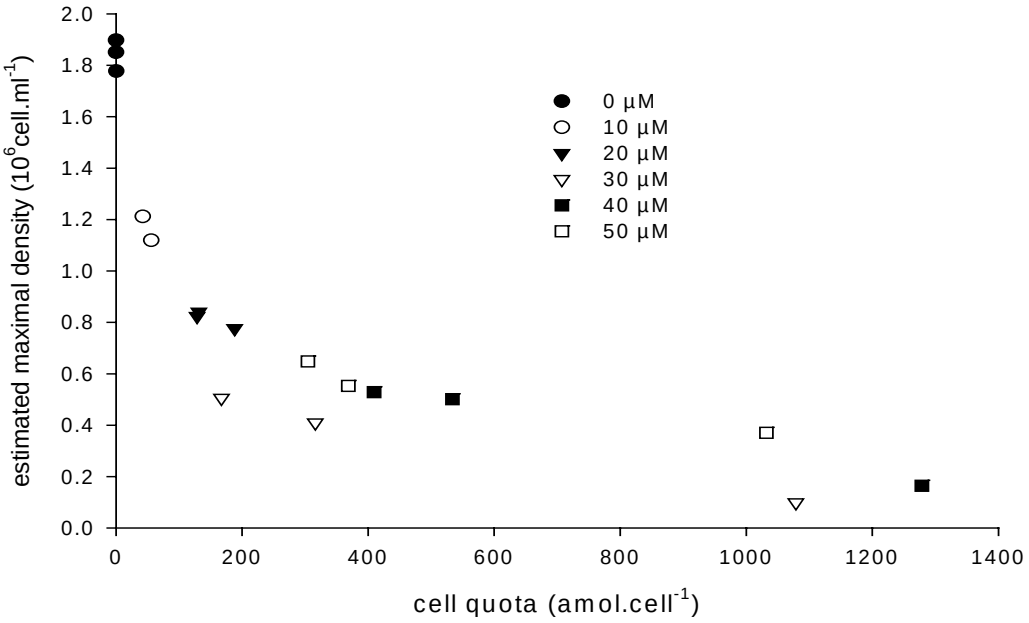

Figure 2. Estimated maximal densities plotted against measured intracellular contents.

4. CONCLUSION

As expected, Se bioaccumulation and toxicity increase with ambient selenite concentrations. However, when ambient Se reaches critical values, large variability in algal response is observed from one replicate to the other. Yet, we showed that intracellular quotas and estimated maximal density are well correlated. Thus, when faced to a high contamination pressure, it seems that in some of the batches algae accumulate a lot and do not grow well, while in others they accumulate less and grow better. The reasons for this variability are not clear. It has been suggested that a physiological adaptation of algae to high selenite concentrations may occur [21, 22]. It may be possible that this adaptation occurs randomly, which could explain the high variability observed.

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References

[1] Fan, T.W.M., et al., *Aquat. Toxicol.* **57** (2002) 65-84.
[2] USEPA, *Report on the Peer Consultation Workshop on Selenium Aquatic Toxicity and Bioaccumulation*. 1998, USEPA.
[3] Hamilton, S.J., *Aquat. Toxicol.* **57** (2002) 85-100.
[4] Lemly, A.D., *Ecotox. Environ. Safe.* **59** (2004) 44-56.
[5] Hamilton, S.J., *Sci.Total. Environ.* **326** (2004) 1-31.
[6] Besser, J.M., et al., *Environ. Toxicol. Chem.* **12** (1993) 57-72.
[7] Zhang, G.H., et al., *Marine Environ. Res.* **30** (1990) 179-190.
[8] Vandermeulen, J.H. and A. Foda, *Mar. Biol.* **98** (1988) 115-123.
[9] Wrench, J.J., *Nature* **299** (1982) 431-433.
[10] Kiffney, P. and A. Knight, *Arch. Environ. Contam. Toxicol.* **19** (1990) 488-494.
[11] Boisson, F., et al., *Arch. Environ. Contam. Toxicol.* **28** (1995) 487-493.
[12] Li, Z.Y., et al., *Bioresource Technol.* **89** (2003) 171-176.
[13] Morlon, H., et al., *Aquat. Toxicol.* (submitted).
[14] Brown, T.A. and A. Shrift, *Biol. Rev.* **57** (1982b) 59-84.
[15] Stadtman, T.C., *Annu. Rev. Biochem.* **49** (1980) 93-110.
[16] Pilon, M., et al., *Plant Physiol.* **131** (2003) 1250-1257.
[17] Delignette-Muller, M.L., *Méthodes de prédiction des aptitudes de croissance des populations de micro-organismes*, in *Laboratoire de Biométrie-Génétique et Biologie des Populations*. 1995, Université Claude Bernard LYONI: Lyon.
[18] Vindimian, E., *REGTOX: macro Excel^{TM} for dose-response modelling*. 2003.
[19] Dutel, L., *Bioaccumulation de l'uranium chez une algue verte unicellulaire: Adsorption et Absorption*. 2000-2001, IPSN. p. 35.
[20] Fortin, C. and P.G.C. Campbell, *Environ. Toxicol. Chem.* **19** (11) (2000) 2769-2778.
[21] Bennet, W.N., *Wat. Res.* **22** (7) (1988) 939-942.
[22] Wrench, J.J., *Mar. Biol.* **49** (1978) 231-236.