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ARTICLE TYPE

Antagonistic interaction of selenomethionine enantiomers on methylmercury toxicity in the microalgae *Chlorella sorokiniana*

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The protective effect of selenium against mercury toxicity is well known especially between selenomethionine and methylmercury and it has been studied in several living organisms, but there is not any information about the interaction of these species in *Chlorella*. Moreover, the investigation about which chiral form of selenomethionine effectively acts against the toxic effects of methylmercury has not been carried out previously. In the present work, two control cultures and two cultures of *C. sorokiniana* grown in standard medium with D,L-SeMet, L-SeMet or D-SeMet. At a certain time after the experiment stated up $MeHg^+$ was added to the cultures with D,L-SeMet, L-SeMet, D-SeMet and to one of the controls. The results show that both SeMet enantiomers counteract the toxicity of $MeHg^+$, because they markedly increases the total contents of chlorophyll, carotenoids, as well as the dry weight and light dependent oxygen production, with respect to the culture non pre-treated with SeMet and only exposed to $MeHg^+$. Also, the levels of $MeHg^+$ measured in cells are lower in the cultures pre-treated with SeMet indicating that the input of $MeHg^+$ into the cells is hardly carried out in the presence of SeMet, or enhances the release of $MeHg^+$. On the other hand, L-SeMet is directly involved in the detoxification of $MeHg^+$, but D-SeMet might be involved only indirectly, since it was neither identified in the medium nor in *C. sorokiniana* after supplementation with this enantiomer, but it might be transformed into SeMeSec and L-SeMet. Moreover, SeMeSec is almost totally released from the cells after 72 hours. None mercury-selenium complex was detected but, since the summation of the different species identified accounts only for 77% of total selenium and mercury measured directly after the sample digestion, it is possible that some of them could be in the form of an undetected Se-Hg complex. This hypothesis can also be supported by the decrease of inorganic selenium along the experiment. Present paper reports new data about the relationship between ability of detoxification of methylmercury and selenomethionine enantiomers through the study of the metabolic intermediates by means of speciation analysis.

Introduction

The protective effect of selenium on mercury toxicity has been issued in several papers[1-6] as well as the antagonist action of selenium against carcinogenic and/or neurotoxic chemicals.[7-9] Simultaneous injection of sodium selenite with mercury chloride (at different sites to avoid precipitation) completely protects rats from the characteristic histological changes associated with mercury chloride toxicity.[10] Further studies have demonstrated this protective effect of selenium in mice,[11] pigs,[12] chicken,[13] fishes[2] and rabbits.[14] The chemical basis of the antagonism between inorganic mercury and selenium in mammals has been established showing that gluthatione (GSH) molecules are attached to the surface of the mercury selenide core and are involved in the binding of the mercury-selenium-sulfur species to selenoprotein P, because selenols and selenides are much more reactive toward mercuric mercury than thiols.[15]

The protective effect of selenium on mercury toxicity differs between species[16] and also with the molar ratios.[17] Formation of a biologically inert 1:1 mercury-selenium complex inside the cells has been suggested to explain this interaction, which has been stated in studies with marine mammals[18] and humans exposed to high levels of inorganic mercury.[19] It has also been established that biological selenium (selenium extracted from liver of rats supplemented with selenite or seleno-dl-selenomethionine) has higher ability to form the mercury-selenium complex when it is incorporated *in vivo* to rats than selenomethionine (SeMet) and this later more than selenite.[20] On the other hand, mercury ions can react with cysteine and selenocysteine through thiols (-SH) and selenols (-SeH). Thus, mercury can be incorporated to

proteins, prosthetic groups of enzymes and peptides. Mercury ions can also react with selenides (Se^{2-}), and hydrogen selenide to form complexes together with glutathione which can be finally bound to selenoprotein P.[21] Numerous studies have been carried out in relation with the protective influence of selenocompounds against methylmercury ($MeHg^+$) toxicity, in different animals such as zebrafish[22], rats[23], diatoms and green mussel[24], and mice[25], especially selenomethionine (SeMet).

Although SeMet has been claimed as the Se-specie most implicated in the detoxification of methylmercury, the interaction of these species in chlorophyll-containing organisms, and in particular in the microalgae *Chlorella* has not been studied until now. Even more important, the potential antagonistic action of chiral forms of SeMet against the toxicity of mercury has not been considered yet, which is important since the bioavailability of D- and L-aminoacids present in food can be different because of the stereoselectivity of organisms. In mice and rats, studies indicate similar bioavailability of both enantiomers[26-27] and that there are few differences in Se-retention in different organs depending on the dose of D-SeMet or L-SeMet, suggesting that rats effectively converts the former into the later in vivo[28], but such that results may not be generalized to man. In humans, the bioavailability of D-methionine is considered almost equal to that of L-methionine,[29] but excretion of D-SeMet in human after ingestion of D,L-SeMet is much higher than L-SeMet, presumably because D-form is not efficiently metabolized.[30-31] In addition, dietary L-SeMet and D-SeMet supplementation could involve antioxidant capability and selenium deposition in serum and tissues and reduce drip loss of breast muscle in broilers compared with sodium selenite. Besides, L-SeMet is more effective than D-SeMet in improving antioxidant status in broilers.[32]

Exposure experiments with selenite have been carried out in unicellular marine algae namely, *Dunaliella primolecta*, *Porhyridium cruetum*, and *Chlorella sp*.[33] Selenium was found to be present in several biochemical components, including proteins, amino acids (*Dunaliella primolecta* and *Chlorella sp*) and perhaps lipids (*Porhyridium cruetum*) and soluble carbohydrates (*Dunaliella primolecta* and *Chlorella sp*). In *C. sorokiniana* three major amino acids were found in the protein hydrolysates: selenocysteic acid, Se-methyl selenocysteine (SeMeSec) and Se-methylselenocysteine selenoxide. On the other hand, the prevailing fraction of selenium analogues in the free amino acids and in the proteins of *C. sorokiniana* is a mixture containing selenocysteine (SeCys), selenocystine ($SeCys_2$) and selenomethionine. The results suggest that the seleno-aminoacid pattern of algae exposed to sub-lethal selenium concentrations depends on the stage of the algae growth. It has also been established that when exposed to sub-lethal, but higher than trace concentrations of Se, the algal cells tend to substitute selenium for part of their sulphur. Thus, under overloading conditions, selenium appears to use the sulphur enzymatic system, while under normal levels, selenium specific enzyme systems seem to be in operation, at least in bacterial systems.[34] In addition, when *Chlorella* grows under low levels of sulphur in the presence of selenite, the main metabolite is SeMet.[35]

On the other hand, mercuric chloride and methylmercury chloride were found to be extremely toxic to the freshwater flagellate *Poterioochromonas malhamensi* and methylmercury disturbs cytokinesis and mitosis of the algae leading to the formation of giant multinucleated cells, polypoid nuclei and numerous alterations in the nuclear and nucleolar structures.[36]

In this paper, the microalgae *Chlorella sorokiniana* has been exposed to $MeHg^+$ and both enantiomers of SeMet to deep insight into the interactions between them in this photosynthetic organism belonging to a genus very important for atmospheric oxygen production in both marine and freshwater systems. The fast growing of the colonies makes them suitable for exposure experiments. For this purpose, we used an analytical method previously published that allows the simultaneous separation of several selenium- and mercury-containing species by HPLC-ICP-MS using a column-switching system.[37-38] Some new data about the interaction of mercury and selenium in *Chlorella sorokiniana* are reported, especially in connection with the different antagonistic action of the chiral forms of SeMet against $MeHg^+$ toxicity.

Materials and methods

Instrumentation

The instrumentation for the chromatographic separation and detection of mercury- and selenium-species has been previously described and published elsewhere.[37-38] Briefly, two HPLC columns were connected using a column switching system comprising two Rheodyne valves model 7725i (Bellefonte, PA. USA) and two columns: Phenomenex Luna C18 (250 mm x 4.60 mm, 5 µm, 100 Å pore size) and Astec Chirobiotic T column (250 mm x 4.6 mm, 5 µm). The injection volume was 100 µl. Elemental detection was performed using an ICP-MS Agilent 7500ce (USA) equipped with a Micromist nebulizer. The column outlet was directly connected to the ICP-MS nebulizer system by a PEEK tubing.

An ultrasonic probe (HD 2200, Bandelin electronic, GmbH & Co. Kg, Berlín, Germany) with a MS 72 tip was used for the extraction of the species. A centrifuge model Sigma Laborzentrifugen 4-10 (Osterode, Germany) was used to accelerate the phase separation process in the extraction of the compounds.

The total chlorophyll and carotenoids concentrations were calculated from the absorbance data obtained with a UV/vis spectrometer (Ultrospec 3100 pro, Biochromm Ltd). The photosynthetic activity was determined using a Clark electrode (Hansatech, UK). A Neubauer camera and an optical microscope Olympus model CX41 was used to measure the number of cells. Cells were grown in graduated bottle (5 L) of borosilicate glass Pyrex® using a volume of 4.5 L of culture.

The mineralization of samples to determine the total content of selenium and mercury was carried out with a microwave accelerated reaction system model MARS (CEM Corporation, Mattews, Carolina del Norte, USA) using MARSXpress vessels.

Standard solutions and reagents

All reagents used were of highest purity. The following reagents were purchased from Sigma-Aldrich (Steinheim, Germany): KH_2PO_4, Na_2HPO_4 x $2H_2O$, $MgSO_4$ x $7H_2O$, $CaCl_2$ x $2H_2O$, KNO_3, EDTA , Na_2EDTA x $2H_2O$, H_3BO_3, $MnCl_2$ x $4H_2O$, $ZnSO_4$ x $7H_2O$, $CuSO_4$ x $5H_2O$, $NaHCO_3$, 2-mercaptoethanol

98%, D,L-selenomethionine (D,L-SeMet), L-selenomethionine (L-SeMet), Se-methylselenocysteine (SeMeSec), selenocystine ($SeCys_2$) and Na_2SeO_3 were purchased from Sigma-Aldrich (Steinheim, Germany). Na_2SeO_4 and tetraethylammonium chloride (TEAC) were obtained from Fluka (Buchs, Switzerland). Methylmercury, inorganic mercury, ammonium acetate, sodium hydroxide, nitric and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Methanol was purchased from Romil Pure Chemistry (Cambridge, UK).

Aqueous stock standard solutions of 1,000 mg Se L^{-1} were prepared from D,L-SeMet and L-SeMet in ultrapure water, SeMeSec and $SeCys_2$ in 3% (v/v) HCl solution, sodium selenate (Na_2SeO_4) in 2% (v/v) HNO_3 solution, and sodium selenite (Na_2SeO_3) in 2% (v/v) HCl solution. Methylmercury stock standard solution was prepared at 1,000 mg Hg L^{-1} by dissolving methylmercury chloride into 2% (v/v) HNO_3 solution. Inorganic mercury stock standard solution was prepared (1,000 mg Hg L^{-1}) by dissolving mercury chloride into 10% (v/v) HNO_3 solution.

Working solutions were prepared daily by stepwise dilution of their stock solutions with high-purity de-ionized water. Ultrapure water (18 MΩ cm) was obtained from a Milli-Q water-purification system and was used throughout (Millipore, UK). All the standard solutions were stored at 4ºC in darkness until the analysis.

The mobile phases were prepared daily as follows: 0.075% tetraethylammonium chloride at pH 4.5 water solution (mobile phase A) and 5% (v/v) methanol-water solution containing 60 mmol L^{-1} ammonium acetate and 0.1% (v/v) 2-mercaptoethanol (mobile phase B). The pH of mobile phase A was adjusted with HCl solution (Merck, Darmstadt, Germany). Protease type XIV was supplied by Sigma–Aldrich (Steinheim, Germany).

D-SeMet was isolated from the commercial racemic mixture using the chiral column previously cited. The mobile phase used was ultrapure water (18 MΩ cm) at a flow-rate 1 ml min^{-1}. The eluate was continuously monitorized using a UV detector at 254 nm, and the D-SeMet fraction was isolated and freeze-dried. The purity of the D-SeMet standard was 99% (HPLC).

A tuning solution containing Li, Y, Tl and Ce (1 mg L^{-1} each) was purchased from Agilent Technologies (USA).

The reference material SELM-1 (selenium enriched yeast, 1381.43 ± 63.21 mg kg^{-1} of SeMet) was obtained from the National Research Council of Canada.

Preparation of *Chlorella sorokiniana* cultures

Microalgae *C. sorokiniana* CCAP 211/8K was obtained from the UTEX culture collection. The culture was in exponential grown phase and was maintained in modified M-8 medium with 160 µmol of photons m^{-2} s^{-1} (continuous illumination) at 30 ºC. The medium contains the following reagents (mol L^{-1}): KH_2PO_4, $5.4x10^{-3}$; Na_2HPO_4 x $2H_2O$, $1.5x10^{-3}$, $MgSO_4$ x $7H_2O$, $1.6x10^{-3}$; $CaCl_2$ x $2H_2O$, $0.9x10^{-4}$; KNO_3, $30x10^{-3}$; EDTA $0.3x10^{-3}$, Na_2EDTA x $2H_2O$, $0.1x10^{-3}$; H_3BO_3, $1.0x10^{-6}$; $MnCl_2$ x 4 H_2O, $0.7x10^{-4}$; $ZnSO_4$ x $7H_2O$, $0.1x10^{-4}$, $CuSO_4$ x $5H_2O$, $0.7x10^{-5}$; $NaHCO_3$, $5x10^{-3}$. The pH was adjusted at 6.7 using NaOH. The sterilization of the medium was carried out during 20 min at 120 ºC at a pressure of 1 atm. The medium was constantly bubbled with 5% (v/v) CO_2 in order to provide a carbon source, homogenize cells and reduce light shielding effect. Cultures were also examined under the microscope to evaluate cell viability and whether the increase in cell optical density was purely due to growth of microalgae or bacterial contaminants.

Exposure experiments with *Chlorella sorokiniana*

Exposure experiments were carried out during three weeks. When the cultures were exposed to both species, the concentration of SeMet was added in equimolar ratio to $MeHg^+$. Seven different cultures were prepared: (i) control (non-exposed culture), *Experiment A (Exp-control)*, (ii) culture exposed to 400 µg L^{-1} $MeHg^+$ (as Hg) at the 7th day, *Experiment B (Exp-7Hg)*, (iii) culture exposed to 787 µg L^{-1} of D,L-SeMet (as Se) at the beginning of the experiment and 400 µg L^{-1} of $MeHg^+$ (as Hg) at the 7th day, *Experiment C (Exp-1DLSe+7Hg)*, (iv) culture exposed to 787 µg L^{-1} of L-SeMet (as Se) at the beginning of the experiment and 400 µg L^{-1} $MeHg^+$ (as Hg) at the 7th day *Experiment D (Exp-1LSe+7Hg)*, (v) culture exposed to 787 µg L^{-1} of the D-SeMet (as Se) at the beginning of the experiment and 400 µg L^{-1} $MeHg^+$ (as Hg) at the 7th day, *Experiment E, (Exp-1DSe+7Hg)*, (vi) culture exposed to 787 µg L^{-1} of the D,L-SeMet (as Se) and 400 µg L^{-1} $MeHg^+$ (as Hg) at the beginning of the experiment, *Experiment F, (Exp-1DLSe+1Hg)*, (vii) culture exposed to 787 µg L^{-1} of L-SeMet (as Se) and 400 µg L^{-1} MeHg (as Hg) at the beginning of the experiment, *Experiment G, (Exp-1LSe+1Hg)* and (viii) culture exposed to 787 µg L^{-1} of D-SeMet (as Se) and 400 µg L^{-1} MeHg (as Hg) at the beginning of the experiment, *Experiment H, (Exp-1DSe+1Hg)*.

On the other hand, the medium cultures were exposed to both species without the algae, to be determined whether any speciation or chirality changes occur in the SeMet without involving the metabolism of Chlorella: (ix) medium culture exposed to 787 µg L^{-1} of D-SeMet (as Se) at the beginning of the experiment and 400 µg L^{-1} $MeHg^+$ (as Hg) at the 7th day without the algae, *Experiment I, (Exp-Medium-1DSe+7Hg)*, (x) medium culture exposed to 787 µg L^{-1} of L-SeMet (as Se) at the beginning of the experiment and 400 µg L^{-1} $MeHg^+$ (as Hg) at the 7th day without the algae, *Experiment J, (Exp-Medium-1LSe+7Hg)*, (xi) medium culture exposed to 787 µg L^{-1} of D,L-SeMet (as Se) at the beginning of the experiment and 400 µg L^{-1} $MeHg^+$ (as Hg) at the 7th day without the algae, *Experiment K, (Exp-Medium-1DLSe+7Hg)*. The speciation of mercury and selenium in the cultures was performed every day.

Measurement of the biochemical parameters in the cultures

The dry mass of cultures was calculated using 0.45 µm filters (cellulose acetate). The filters were cleaned with Milli-Q water and dried at 80 ºC during 24 h in an oven. Then, 5 ml of the culture was filtered and dried. The dry mass was calculated by weight difference.

The total content of chlorophyll and carotenoids was determined using the modified Arnon´s method.[39] 1 ml of the culture was centrifuged during 5 minutes at 4400 rpm. The supernatant was discarded and 4 ml of methanol added to the residue for chlorophyll extraction shaking during 20 seconds. The extract was incubated for 30 minutes at 60 ºC and then during 10 minutes at 0 ºC. After that, the extract was centrifuged during 5 minutes at 4400 rpm obtaining a white pellet. The UV/vis absorbance was measured at 665, 653 and 470 nm and the total content of chlorophyll and carotenoids calculated using the equations proposed by H. Lichtenthaler.[39]

The photosynthetic activity was measured with a Clark electrode. The number of cells was determined using an optical microscope and a Neubauer camera after the addition of 50 µl of ethanol per mL.

The biological activity used to test cell viability was photosynthetic activity. For photosynthetic activity determinations, 1 mL of microalgae cell culture was placed in a Clark-type electrode to measure O_2 evolution. The electrode was equipped with a stir bar, a pressure corrector and a temperature sensor. It was placed into a photosynthetic cylindrical chamber with an outer jacket for thermostated water, all made of plexiglass, with a 15 mm inside diameter and a capacity of 10 ml. Meassurements were made at 25 ºC under saturating white light (1500 $\mu Em^{-2}s^{-1}$) or darkness (endogenous respiration).

Determination of total mercury and selenium

For the determination of total mercury and selenium in the culture medium, the supernatant obtained from cultures centrifugation was filtrated through 0.45 µm and an aliquot of 100 mg was digested with 200 µL of H_2O_2 and 600 µL of HNO_3 (65% w/v). The samples were digested from room temperature to 160ºC in 15 minutes and hold for 40 minutes at this last temperature.

For the determination of total mercury and selenium in *C. sorokiniana*, the pellet obtained from the centrifugation of the culture was lyophilized and an amount of 100 mg was accurately weighted and submitted to acid digestion with 200 µL of H_2O_2 and 600 µL of HNO_3 (65% w/v). The samples were digested from room temperature to 160ºC in 15 minutes and hold for 40 minutes at this last temperature. The final solutions were filtered through 0.45 µm PVDF filters. The elements were measured by ICP-MS (the operative experimental conditions for this equipment are listed in Table 1).

Extraction of mercury and selenium species

The cultures of *C. sorokiniana* were centrifuged to separate the pellet from the medium. The medium of the culture was filtrated using 0.45 µm filters (PVDF) and then directly injected into the HPLC-ICP-MS. The pellet was frozen with liquid nitrogen to break the wall cells and then lyophilized. An amount of 0.040 g was weighted in a centrifuge tube and 0.02 g of Protease XIV and 5 mL of 2-mercaptoethanol 0.1% (v/v) at pH 4.3 were added. The extraction was carried out with the assistance of an ultrasonic probe at 25% power during 2 minutes. After extraction, the extract was centrifuged during 5 minutes at 6000 rpm and the supernatant collected. The procedure was repeated with 5 mL of 2-mercaptoethanol 0.1% (v/v) at pH 4.3. Finally the supernatants were pooled, filtered through 0.45 µm and injected into the HPLC-ICP-MS.

Determination of mercury and selenium species

The instrumental approach for the determination of mercury and selenium species has been previously described and published elsewhere.[37-38] Optimal operation conditions for ICP-MS detection are similar to those for the determination of total mercury and selenium (Table 1). ^{77}Se, ^{80}Se, ^{82}Se ^{198}Hg and ^{202}Hg were monitored, but only isotopes ^{80}Se and ^{202}Hg were used for quantification in this work. A solution containing Li, Y, Tl and Ce (1 µg L^{-1} each) prepared in the mobile phase A was used to tune the ICP-MS for sensitivity, resolution, percentage of oxides and doubly charged ions. The procedure for the chromatographic separation is summarized in Table 2. Chromatographic performance was checked regularly by measuring control standards to ensure enough separation between species and sensitivity of the method after the analysis of a considerable number of samples. Hydrogen at a flow-rate of 4 ml min^{-1} was used as reaction gas to avoid polyatomic interferences in the plasma (such as $^{80}Ar_2$, $^{32}S^{16}O$, $^{10}Ca_2$, and $^{40}Ar^{40}Ca$).

Results and discussion

Quality control of the analytical methods

Recovery experiments were performed in fortified samples at three different concentration levels (10, 200 and 600 mg kg^{-1} in the pellet, and 10, 200 and 600 µg L^{-1} in the culture medium) and the digestion method was applied. The recoveries were always higher than 86 % and 91% for selenium and mercury, respectively. The Relative Standard Deviation (%RSD) of the sample response factor was calculated for three separate samples. The results ranged from 3 to 15 % for the analytes, which were considered acceptable.

In addition, recovery experiments of individual species were performed in fortified samples at three different levels (10, 200 and 600 mg kg^{-1} in the pellet). For SeMet the reference certified material SELM-1 (selenium enriched yeast) was used. The recoveries were always higher than 95% for all the analytes and interconversion of the species was not observed. The Relative Standard Deviation (%RSD) of the sample response factor was calculated for three separate samples. The results ranged from 8 to 11 % for the analytes that were considered acceptable. The limits of detection of the species were 1 ng of the corresponding element, except for SeMeSec and Hg^{2+}, which were 2 and 0.3 ng, respectively (corresponds to 0.02 and 0.003 mg kg^{-1} for SeMeSec and Hg^{2+}, respectively and 0.01 mg kg^{-1} for other species).

Determination of the sub-lethal dose of MeHg+ in cultures of *Chlorella sorokiniana*

C. sorokiniana cells were exposed to different concentrations of $MeHg^+$ to determine the sub-lethal concentration of this specie. For this purpose, several cultures of *C. sorokiniana* were prepared with different concentrations of $MeHg^+$ ranging from 50 to 1000 µg L^{-1} and compared with a control. At the end of the experiment, the sub-lethal concentration of $MeHg^+$ was estimated to be 400 µg L^{-1} (as Hg) since the growth decreases but the culture remain viable (data not shown). The chlorophyll content decreases from 67.78 µg mL^{-1} in the control to 0.4 µg mL^{-1} in the culture exposed to 400 µg/L of $MeHg^+$ (after 72 hours). Then, this critical concentration of $MeHg^+$ that limit the colony viability was selected for further experiments to check the chemical mechanism between chiral forms of SeMet and $MeHg^+$.

Protective effect of SeMet against mercury ($MeHg^+$) in *C. sorokiniana*

The microalgae cultures (*Experiments A-H*) were monitored considering several physiological parameters, namely dry weight of biomass, light dependent oxygen production (LDOP) and

pigment content which reveal culture viability status. The referred parameters were determined in the cultures both before and upon $MeHg^+$ addition, so that protective effect, if produced, may be detected on the roughly constant profile of cell viability parameters.

The cultures of *C. sorokiniana* exposed to both mercury and selenium species simultaneously at the beginning of the experiment (*Experiment F/Exp-1DLSe+1Hg, Experiment G/Exp-1LSe+1Hg and Experiment H/Exp-1DSe+1Hg)* present toxic effects in 24 hours, which induces difficulty in microalgae grow and final collapse of the colony (data not shown).

After $MeHg^+$ was added at the 7th day, (*Experiments C-E*) the physiological parameters of colony exhibit better results in cultures preincubated with SeMet than colony only exposed to $MeHg^+$ (*ExperimentB/Exp-7Hg)*. In this way, the results obtained show that the cultures pre-treated with SeMet (*ExperimentC/Exp-1DLSe+7Hg), ExperimentD/Exp-1LSe+7Hg*) and *(ExperimentE/Exp-1DSe+7Hg)* markedly increases the total contents of chlorophyll (Figure 1A) and carotenoids (data not shown) as well as the dry weight (data not shown) and LDOP (Figure 1B) with respect to the culture non pre-treated with SeMet and only exposed to $MeHg^+$ (*ExperimentB/Exp-7Hg*). On the other hand, the decrease of colony viability after 16 days from the beginning of the experiment (Fig. 1) might be attributed to culture aging. In addition, levels of $MeHg^+$ measured in cells are lower in the cultures pre-treated with SeMet (*ExperimentC/Exp-1DLSe+7Hg, ExperimentD/Exp-1LSe+7Hg and ExperimentE/Exp-1DSe+7Hg*) (Fig. 2). These results might suggest that the input of $MeHg^+$ into the cells is hardly carried out in the presence of SeMet, or enhances the release of $MeHg^+$ as stated by several authors in other organisms.[40] Since $MeHg^+$ is not detected in the culture media after 24 h of exposure, indicating that it is readily absorbed, the reason might be the enhancement of the growth of cultures exposed to SeMet that represent an increase in the number of cells and therefore lower levels of $MeHg^+$ or the conversion of $MeHg^+$ into an insoluble undetected Se-Hg complex.

Furthermore, the evolution of biochemical parameters in those cultures exposed to D,L-SeMet (50/50 mixture of the two enantiomers), L-SeMet and D-SeMet were similar (*Experiment C/Exp-1DLSe+7Hg, Experiment D/Exp-1LSe+7Hg* and *Experiment E/Exp-1DSe+7Hg*) respectively), which led as to suggest that both chiral forms are directly or indirectly involved in the interaction with $MeHg^+$.

Evolution of mercury and selenium species in culture medium

First of all, D-, L- and D,L-SeMet were added to the culture medium without the microalgae (Experiments I-K) to check the occurrence of possible interconversions between chiral forms. The results show that all the species and concentrations remain inalterable in the absence of microalgae.

It has been checked that mercury concentration is under the detection limits in the culture media during the exposure experiment; therefore, $MeHg^+$ has to be easily taken by *C. sorokiniana* due to its ability to cross the plasmatic membrane. On the other hand, total selenium concentration decreases daily during the first six days of exposure (Figure 3A) and correlatively this element increases in the cell in the same period of time, decreasing slowly along the time (Figure 3B). The selenium species identified and quantified in the culture media through the experiment were: $SeCys_2$ (Figure 4A), SeMeSec (Figure 4C), L-SeMet (Figure 4E) and D-SeMet (Figure 4G), which was only detected after 24 h of exposure (1st day), at 119 µg L^{-1} in the culture medium.

Speciation in *Chlorella sorokiniana*

The total concentration of Se in *C. sorokiniana* is collected in figure 3B. Selenium in cells is mainly distributed among several species: [$SeCys_2$ (Figure 4B), SeMeSec (Figure 4D) and L-SeMet (Figure 4F)]. A continuous decrease of $SeCys_2$ and SeMeSec is shown in cells, especially SeMeSec during the first 72h (Fig. 4D) with a correlative increase in the culture medium (Fig. 4C). SeMeSec was present in these samples at 47 mg kg^{-1} at first day and below 3 mg kg^{-1} after day 15. These results suggest that Se-MeSeCys is released from cells. In particular, this compound concentration increases more markedly when either D,L-SeMet or D-SeMet are added to culture medium (*Experiment C/Exp-1DLSe-+7Hg* and *Experiment E/Exp-1DSe-+7Hg*, figure 4C) than that exposed to L-SeMet (*Experiment D/Exp-1LSe-+7Hg*), disappearing correlatively from the cells (figure 4D). This fact can be related with the metabolic transformation of D-SeMet into SeMeSec which has been reported in the literature[41] and that can be supported by the absence of the former in the cells in all the experiments and in the medium after the first 48 hours. Then, if D-SeMet disappears from the medium and cells, it cannot be directly involved in detoxification of $MeHg^+$, but as suggested previously, it should be involved almost indirectly since the evolution of biochemical parameters in those cultures exposed to D,L-SeMet (50/50 mixture of the two enantiomers), L-SeMet and D-SeMet were similar. We can also suggest that D-SeMet might also be transformed into L-SeMet (Figure 4F), because the latter is present in the culture exposed to D-SeMet (*Experiment E/Exp-1DSe+7Hg*). On the other hand, L-SeMet concentration increases until the third day in *C. sorokiniana* cells while the opposite behavior is observed in the culture medium.

In relation to inorganic selenium species, selenate was found below 4 mg kg^{-1} in *C. sorokiniana* and the concentration decreases daily. The presence of selenite was also identified in *C. sorokiniana* at concentration below 2 mg kg^{-1}, decreasing daily.

The total selenium and mercury calculated as the summation of the different species identified, in culture medium and *Chlorella*, were up to 77% of total selenium and mercury measured directly after the sample digestion. This fact together with the decrease of selenite along the experiment may indicate that an inorganic complex of selenium and mercury is formed.[20]

Mass balance of mercury and selenium

The relation between the absolute quantities of mercury and selenium species in a pellet (8.000 g) collected at the 8th day and the added quantity to the medium at the beginning of the experiment (SeMet) and at the 7th day ($MeHg^+$) was calculated.

At the 8th day, in the culture exposed to $MeHg^+$ and D,L-SeMet, 82±15 % (n=5) of selenium initially added to the culture was present in the biomass: 94.8 % as L-SeMet and the remaining quantity distributed as $SeCys_2$ (2.4%), SeMeSec (2.7 %) and selenate (0.1 %). The fraction that remains in the medium is present in the culture medium as $SeCys_2$ and SeMeSec (Figures 4A and 4C). In the case of methylmercury, it easily penetrates

into the cell and 95% was measured in the biomass (8.000 g) at the 8th day (the remaining quantity corresponds to inorganic mercury measured in cells).

Conclusions

The results obtained in this to work shows that both SeMet enantiomers counteract the toxicity of $MeHg^+$, because they enhance biochemical parameters measured in Chlorella, with respect to the culture non pre-treated with SeMet and only exposed to $MeHg^+$. Also, the levels of $MeHg^+$ measured in cells are lower in the cultures pre-treated with SeMet indicating that the input of $MeHg^+$ into the cells is hardly carried out in the presence of SeMet, or enhances the release of $MeHg^+$. However, D-SeMet is not directly involved in the detoxification mechanism of $MeHg^+$ since it was neither identified in the medium nor in *C. sorokiniana* after supplementation with this enantiomer. The increase observed in SeMeSec is higher in cells exposed to D,L- and D- than in ones exposed to L-SeMet that might be explained by the conversion of D-SeMet in that specie. D-SeMet might also be transformed into L-SeMet, because this later is present in the culture exposed to D-SeMet. It has not been found any compound containing both, mercury and selenium, but, since the summation of the different species identified accounts only for 77% of total selenium and mercury measured directly after sample digestion, it is possible that some of them could be in the form of an undetected Se-Hg complex. This hypothesis can also be supported by the decrease of inorganic selenium along the experiment.

The use of ICP-MS is essential for multispeciation purposes as it allows obtaining multielement profiling in real samples in a single chromatographic run and for this reason, it constitutes a reliable technique with high throughput. The possibility of screening both elements in a given chromatographic peak from the same sample is very useful since there are no errors associated to multiple measurements.

Further studies will be focused in the study of the protective effect of L- and D-SeMet against $MeHg^+$ in mammals.

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FIGURE LEGENDS

FIGURE 1. Total chlorophyll (A) and LDOP (B) in *C. sorokiniana* cells. Purple line: Control (*Experiment A, Exp-Control)*; orange line: Culture exposed to $MeHg^+$ at the 7th day (*Experiment B, Exp-7Hg)*; red line: culture exposed to $MeHg^+$ and pretreated with D,L-SeMet (*Experiment C, Exp-1DLSe+7Hg),* green line: culture exposed to $MeHg^+$ and pretreated with L-SeMet (*Experiment D, Exp-1LSe+7Hg)* and blue: culture exposed to $MeHg^+$ and pretreated with D-SeMet (*Experiment E, Exp-1DSe+7Hg).*

FIGURE 2. $MeHg^+$ (mg Kg^{-1}) in *C. sorokiniana cells*. Orange line: Culture exposed to $MeHg^+$ at the 7th day (*Experiment B, Exp-7Hg)*; red line: culture exposed to $MeHg^+$ and pretreated with D,L-SeMet

(*Experiment C, Exp-1DLSe+7Hg)*, green line: culture exposed to $MeHg^+$ and pretreated with L-SeMet (*Experiment D, Exp-1LSe+7Hg)* and blue line: culture exposed to $MeHg^+$ and pretreated with D-SeMet (*Experiment E, Exp-1DSeMet+7Hg*).

FIGURE 3. Total Selenium in the culture medium (μg L^{-1}) (A) and in *C. sorokiniana* cells (mg kg^{-1}) (B). Red line: culture exposed to $MeHg^+$ and pretreated with D,L-SeMet (*Experiment C, Exp-1DLSe+7Hg)*, green line: culture exposed to $MeHg^+$ and pretreated with L-SeMet (*Experiment D, Exp-1LSe+7Hg)* and blue line: culture exposed to $MeHg^+$ and pretreated with D-SeMet (*Experiment E, Exp-1DSe+7Hg).*

FIGURE 4. $SeCys_2$ in the culture medium (μg L^{-1}) (A), $SeCys_2$ in *C. sorokiniana* cells (mg kg^{-1}) (B), SeMeSec in the culture medium (μg L^{-1}), SeMeSec in *C. sorokiniana* cells (mg kg^{-1}) (D), L-SeMet in the culture (μg L^{-1}) (E), L-SeMet in *C. sorokiniana* cells (mg kg^{-1}) (F) and D-SeMet in the culture medium (μg L^{-1}) (G). Red line: culture exposed to $MeHg^+$ and pretreated with D,L-SeMet (*Experiment C, Exp-1DLSe+7Hg)*, green line: culture exposed to $MeHg^+$ and pretreated with L-SeMet (*Experiment D, Exp-1LSe+7Hg)* and blue line: culture exposed to $MeHg^+$ and pretreated with D-SeMet (*Experiment E, Exp-1DSe+7Hg).*

Table 1. Operating conditions for ICP-MS.

ICP-MS CONDITIONS	
RF power	1500 W
Plasma gas flow rate	15 L min-^{1}
Auxiliary gas flow rate	1.0 L min^{-1}
Carrier gas flow rate	0.91 L min^{-1}
Reaction gas (H_2)	4 mL min^{-1}
Makeup gas flow rate	0.12 L min^{-1}
Sampling depth	7 mm
Sampling and skimmer cones	Platinum
Dwell Time	0.3 s per isotope
Isotopes monitored	^{77}Se, ^{80}Se, ^{82}Se, ^{198}Hg, ^{202}Hg

Table 2. Chromatographic method conditions

Time (minutes)	Columns	Mobile phase
0-5	RP	A
5-6.7	RP + Chiral	A
6.7-9	RP	A
9-13	RP + Chiral	A
13-25	RP	B

RP = Phenomenex Luna C18 column, 300 mm × 3.90 mm, 10 μm
Chiral = Astec Chirobiotic T column, 250 mm × 4.6 mm, 5 μm
Mobile phase A = 0.075% tetraethylammonium chloride, pH 4.5
Mobile phase B = 0.1% 2-mercaptoethanol, 0.06M Ammonium acetate, 5% Methanol.