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Combined Effect of Mercuric Chloride and Selenium Dioxide on the Growth of the Cyanobacteria, *Anacystis nidulans*

L. H. Lee, B. K. Lustigman, S. R. Murray

Montclair State University, Upper Montclair, NJ 07043, USA

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Anacystis nidulans is a rod-shaped, unicellular cyanobacterium which can be used as indicator species for environmental contamination and ecotoxicology. Cyanobacteria are excellent models for toxicity studies because as photoautotrophs and primary producers deleterious effects on them will have implications for higher organisms in the food chain. Ecosystem health depends on a functional microbial community and adverse effects on microorganisms present a serious problem.

Large quantities of heavy metals are released into the environment through industrial discharges, agricultural run-off and sewage treatment. In minute concentrations, they do not pose a threat and some may act as stimulants to growth (Lee et al.1999). However, high concentrations can be deleterious or even lethal. Microorganisms vary in their tolerance to heavy metals, based on exposure (Zhang and Majidi 1994) and physiological and/or genetic mechanisms (Devars et al. 1998).

Phytoplankton appear to be highly sensitive to mercury displaying greatest sensitivity to this metallic ion. Previous studies using *Anacystis nidulans* have shown that mercury is far more inhibitory than any of the other heavy metals which have been tested (Lee et al. 1992). It is one of the more common toxic contaminants with no known biological function. There are several different forms: elemental, mercuric salts, and organic mercury. The phenomenon of biomagnification of mercury through the aquatic food chain is well known.

Selenium is an essential micronutrient and is needed for the production of certain cellular enzymes and the amino acid, selenocysteine which is directly involved in catalytic reactions. As a nutrient, it acts as an antioxidant by working with glutathione peroxidase to reduce peroxides (Rana and Boora 1992). The toxicity of selenium depends on its chemical form which includes: elemental selenium, selenite, selenate and selenide. Selenium enters the environment through coal-fired power plants, smelting and combustion of fossil fuels (Cassarett and Doull, 1996). In seven species of microalgae tested all demonstrated strong growth inhibition toward selenate and partial growth inhibition to selenite (Faragasova 1994). Selenate can be reduced to elemental selenium by microorganisms, leading to its deposit in the cytoplasm, periplasmic space and on the surface of the cell (Crist et al. 1988).

Correspondence to: L. H. Lee

There are two phases to uptake of heavy metal ions by microorganisms. First, a passive, rapid phase in which the ions bind to the cell wall (Friis and Myers-Keith, 1986). Second, a slower and metabolically dependent uptake into the cytosol (Vymazal, 1990). Both of these processes involve removal of the ions and may produce higher concentrations within the algae (Harris and Ramelow 1990).

Parizek & Ostadolova (1967) first reported an antagonistic effect of selenium on mercury. However, additional studies have provided mixed results of this phenomenon (Belize et al. 2001). This may be due to the species and its environment, or to the experimental conditions used.

The objective of this study was to determine if an antagonistic effect exists between selenium and mercury in *Anacystis nidulans*, and to determine the lethal combinations of the two metals.

MATERIALS AND METHODS

Anacystis nidulans 625 cultures were obtained from Dr. Roy McGowan, New York. They were grown in 100 ml sterile Mauro's Modified Medium (3M) (Kratz and Myers 1955) at pH 7.9. A stock solution of 10^4 mg/L $HgCl_2$ was diluted to achieve final concentrations of 0, 0.1, 0.5, 1, 5, and 10 mg/L. A stock solution of 10^4 mg/L SeO_2 was diluted to achieve final concentrations of 0, 0.1, 0.5, 1, 5, 10 and 20 mg/L. The flasks were then inoculated with 5 ml of *Anacystis nidulans* cells to achieve a concentration of approximately 1 X 10^7 cells/ml. Four cultures were prepared for each combined concentration used. Controls were prepared of untreated *A. nidiulans* in 100 ml of 3M medium and grown under the same conditions.

The cultures were grown under constant fluorescent light at ambient temperature with continuous agitation at 100 rpm, until stationary phase was achieved. Growth of the cultures was determined by two methods: 1. direct count using a Spencer hemocytometer and 2. indirect turbidometric reading using a Beckmann Spectronic 1001 spectrophotometer at 750 nm. Cultures were read every 3-4 days. Cultures were checked for contamination by plating on nutrient agar. The pH was measured at the start and end of the experiment.

Cultures which did not exhibit growth were, at the end of the experiment, centrifuged, washed and resuspended in fresh medium without the heavy metal ions. If the resuspended cultures exhibited growth, these concentrations were determined to be cyanostatic. In those without growth, the concentrations were considered to be cyanocidal.

At the end of the experiment, atomic absorption was used to determine the levels of Hg and Se remaining in the media of 0.5mg/L $HgCl_2$ and 0.5mg/L SeO_2 cultures. Cultures were centrifuged to remove cells and the supernatant retained, allowing for comparison of inoculated and uninoculated samples. Stock standards for calibration for atomic absorption of Hg were prepared by dissolving 0.1354 g

of $HgCl_2$ in 75 ml of distilled water, adding 10 ml HNO_3 and adjusting to 100 ml. Stock standards for selenium were prepared by dissolving 0.1727 g of H_2SO_4 in deionized, distilled water and adjusting to 1000 ml. Atomic absorption for mercury analysis followed USEPA method 245.1 (cold vapor technique) using a Perkin Elmer 2380 Atomic Absorption Spectrophotometer, equipped with cold vapor system. Selenium analysis followed USEPA 270.2 (Graphite furnace technique) using a Perkin Elmer 5100 Atomic Absorption Spectrophotometer (EPA 1979).

One-way ANOVA and Duncan's Multiple range test were used to analyze the data and to determine the differences between the various concentrations (SAS Institute 1995).

RESULTS AND DISCUSSION

Anacystis nidulans is very sensitive to combinations of mercury and selenium. The cells appear to be more sensitive to mercury than to selenium. Previous studies indicated that the inhibitory concentration of SeO_2 was 50 mg/L, while that of 5 mg/L $HgCl_2$ was toxic with mercury (Lee et al 1992, 1999). Cells in all treated cultures were smaller in size than the control.

Figure 1 shows that *A. nidulans* displayed a decrease in overall growth and lower growth rate with 0.5mg/L of mercury and all concentrations of selenium compared to the control. As seen on day 22, it also indicates that the growth response displays three distinct clusters for optical density and cell number: 1) control, 2) 0, 0.1, 0.5, 1.0 mg/L SeO_2 and 3) 5, 10 and 20 mg/L SeO_2. There was a delay in the onset of log phase from 4 days in the control to 10-12 days with concentrations of 0.5mg/L $HgCl_2$ and 0, 0.1, 0.5 and 1 mg/L SeO_2. The levels of growth with selenium and 0.5mg/L $HgCl_2$ were: control >0 > 0.5=1 >0.1 >> 5=10 =20 mg/L SeO_2 as determined by optical density and control >0=0.1=0.5=1 >>5=10=20mg/L SeO_2 for cell number. Concentrations of 5 mg/L SeO_2 and higher completely inhibited the growth of *A. nidulans*.

Figure 2 shows that selenium did not have a significant beneficial effect when the concentration of mercury was 1 mg/L. The overall growth and the growth rate were severely inhibited by concentrations of 1 mg/L $HgCl_2$. The growth response of *A. nidulans* displayed two distinct clusters with optical density: 1) control, 2) 0, 0.1, 0.5, 1, 5, 10 and 20 mg/L SeO_2 and three distinct clusters with cell number: 1) control, 2) 0.1, 0.5, 1, 5 mg/L SeO_2 and 3) 10, 20 mg/L SeO_2. The onset of log phase was delayed to 11-15 days in cultures with 1 mg/L $HgCl_2$ and 0, 0.1, 0.5, 1 and 5mg/L SeO_2. This was more apparent as measured by cell number than optical density. There was no growth in cultures with 10 and 20 mg/L SeO_2. As determined on day 22, levels of growth as determined by optical density were: control>> 0≥ 0.5≥ 1≥ 0.1≥ 5≥ 10= 20 mg/L SeO_2 and for cell number control>>0= 0.5= 1≥ 0.1> 5>>10= 20 mg/L SeO_2. Concentrations of 10 and 20 mg/L SeO_2 with 1 mg/L $HgCl_2$ were cyanocidal.

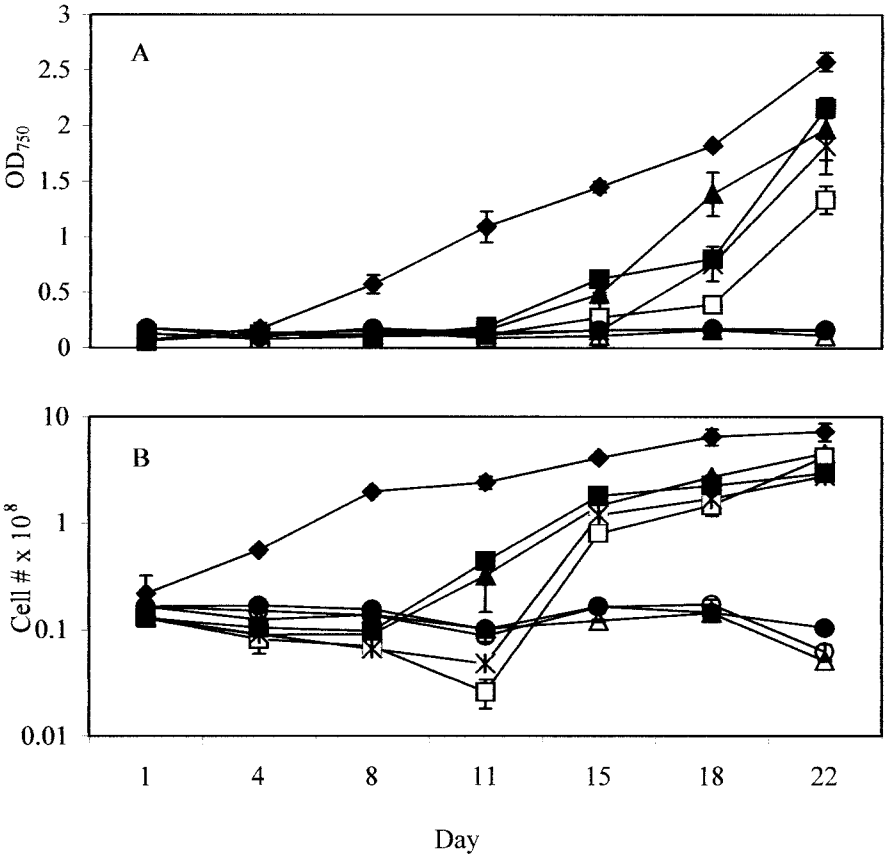

Figure 1. Growth of *Anacystis nidulans* in 100mL of 3M medium containing 0.5mg/L $HgCl_2$ with various concentrations of SeO_2 (0-20 mg/L). Error bars represent standard deviations. Curves were fit by regression analysis. A) Mean optical density with standard deviation. B) Mean cell number with standard deviation. ◆– $0SeO_2/0.5HgCl_2$ ▲– $0SeO_2/0.5HgCl_2$ □– $0.1SeO_2/0.5HgCl_2$ ✳– $0.5SeO_2/0.5HgCl_2$ ■–$1SeO_2/0.5HgCl_2$ ●– $5SeO_2/0.5HgCl_2$ △– $10SeO_2/0.5HgCl_2$ ○– $20SeO_2/0.5HgCl_2$

Figure 3 shows that 5 mg/L $HgCl_2$ alone, or with combinations of SeO_2 ranging from 0.1 to 20 mg/L, completely inhibit the growth of *Anacystis nidulans*. In cultures with 5mg/L $HgCl_2$ and 0.1, 0.5 and 1 mg/L SeO_2, results showed that ½ the cultures recovered in fresh medium, indicating that these levels may be cyanostatic. Concentrations of 5, 10 and 20 mg/L SeO_2 were cyanocidal. Concentrations of 10 mg/$HgCl_2$ completely inhibited the growth of the cells regardless of selenium concentration, and all were cyanocidal (data not shown).

Statistical analysis performed on data from day 18, showed that the control was

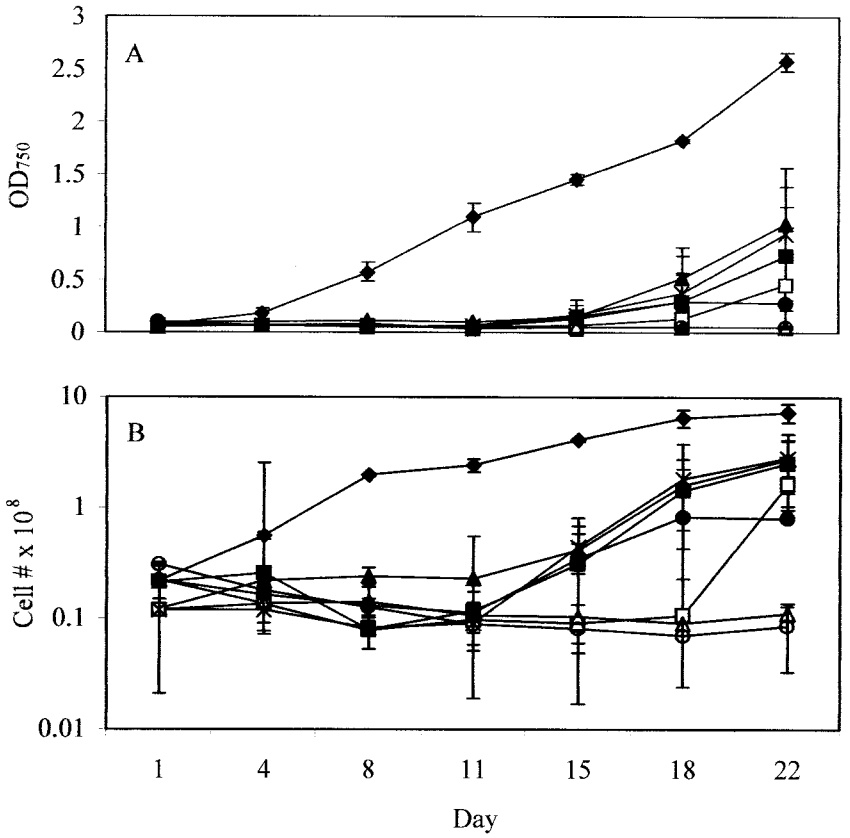

Figure 2. Growth of *Anacystis nidulans* in 100mL of 3M medium containing 1mg/L $HgCl_2$ with various concentrations of SeO_2 (0-20 mg/L). Error bars represent standard deviations. Curves were fit by regression analysis. A) Mean optical density with standard deviation. B) Mean cell number with standard deviation. ◆– $0SeO_2/1HgCl_2$ ▲– $0SeO_2/1HgCl_2$ □– $0.1SeO_2/1HgCl_2$ ✳– $0.5SeO_2/1HgCl_2$ ■– $1SeO_2/1HgCl_2$ ●– $5SeO_2/1HgCl_2$ △– $10SeO_2/1HgCl_2$ ○– $20SeO_2/1HgCl_2$

significantly different from all other concentrations. Although 0.5 and 1 mg/L SeO_2 had a slight effect in reducing the toxicity of $HgCl_2$ at concentrations up to 1mg/L $HgCl_2$, as determined on day 15-18, this was not statistically significant.

The Cold Vapor Atomic Absorption (Table 1) obtained from uninoculated control and two inoculated samples, shows that in three trials, 4.61% of the mercury remained in the inoculated flasks compared with the control. While the cells were not present for this analysis, during growth, *A. nidulans* may have removed Hg to produce a volatile product. Reduction of Hg (II) to elemental Hg is a mechanism

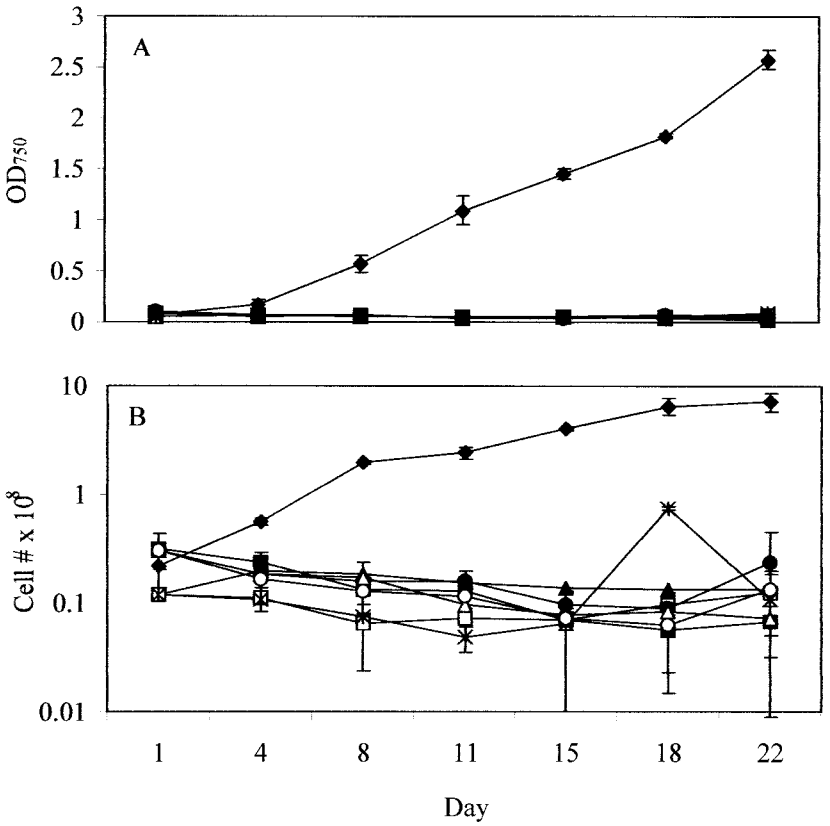

Figure 3. Growth of *Anacystis nidulans* in 100mL of 3M medium containing 5mg/L $HgCl_2$ with various concentrations of SeO_2 (0-20 mg/L). Error bars represent standard deviations. Curves were fit by regression analysis. A) Mean optical density with standard deviation. B) Mean cell number with standard deviation. ◆– $0SeO_2/1HgCl_2$ ▲– $0SeO_2/5HgCl_2$ □– $0.1SeO_2/5HgCl_2$ ✳– $0.5SeO_2/5HgCl_2$ ■– $1SeO_2/5HgCl_2$ ●– $5SeO_2/5HgCl_2$ △– $10SeO_2/5HgCl_2$ ○– $20SeO_2/5HgCl_2$

of resistance found in bacteria, and may have been used by *Anacystis.* Graphite Furnace Atomic Absorption Spectrophotometer data shows that approximately 82.77% of the selenium still remained in the media of the inoculated flasks. This indicates that the cells are much less permeable to selenium since most of the ion remained in the medium.

Table 2 shows that cultures with growth had a final average pH of 8.781 to 9.834. Those without growth had an average final pH of 7.199 to 8.055. The pH in the uninoculated flasks remained unchanged. The range of pH values is well within

Table 1. The remaining Hg & Se concentration in cultures of *Anacystis nidulans* compared to uninoculated flasks (mg/L) at day 18 as measured by atomic absorption.

	Conc. Of Hg	Percentage remaining	Conc. Of Se	Percentage remaining
Uninoculated	0.1263	100%	0.370	100%
Trial I Inoculated	0.0065±0.00016	5.18%	0.304±0.038	82.16%
Trial II Inoculated	0.0051±0.00014	4.03%	0.313±0.047	83.38%
Mean of I & II	0.0058	4.61%	0.309	82.77%

Table 2. Changes (± SD) in pH in *Anacytis nidulans* cultures with various SeO_2 (0-20 mg/L) and $HgCl_2$ concentration (0-10 mg/L).

Control (0Hg 0Se)			Day 1 7.9			Day 18 9.597		
Concentration 0.5 Hg			Concentration 1 Hg			Concentration 5 Hg		
Se	Day 1	Day 18	Se	Day 1	Day 18	Se	Day 1	Day 18
0	7.9	9.214±0.235	0	7.9	8.781±0.464	0	7.9	
0.1	7.9	8.911±0.266	0.1	7.9	9.348±0.113	0.1	7.9	7.778±0.140
0.5	7.9	9.336±0.507	0.5	7.9	9.534±0.059	0.5	7.9	7.660±0.091
1	7.9	9.834±0.237	1	7.9	9.732±0.317	1	7.9	7.199±0.014
5	7.9	9.040±0.940	5	7.9	9.413±0.921	5	7.9	7.450±0.317
10	7.9	8.055±0.005	10	7.9	7.667±0.438	10	7.9	7.373±0.134
20	7.9	8.055±0.035	20	7.9	7.477±0.339	20	7.9	7.368±0.107

those tolerated by *A. nidulans*. Previous research has shown that as growth occurs in the cultures, the pH increases within the range of 8-10 (Lee 1991).

Combinations of ions may have a variety of effects on microorganisms acting antagonistically or synergistically. Previous studies on the effect of selenium andmercury performed on a variety of organisms indicate varied results. There are positive correlations between mercury and selenium in the livers of marine mammals according to Koeman et al. (1973), but Belize et al. (2001) showed that levels of mercury were lower in perch collected from lake waters which are located in areas of high selenium concentration. The results of our study show that although low concentrations of selenium reduce the toxicity of mercury, selenium does not produce a significantly beneficial effect on the toxicity of mercury in cyanobacteria. Further study with other microorganisms will need to be performed in order to determine if the combination of selenium with mercury can reduce the toxicity of mercury on simpler organisms. Possible beneficial effect of other combinations of metal ions with mercury should also be investigated.

REFERENCES

Belzile, N, Chen YW, Gunn J (2001) The antagonistic effect of selenium on mercury uptake by freshwater fish. Limnol Oceanog 46: 1814-1818

Cassarett C, Doull J (1996) Casarett and Doull's Toxicology. Curtis D. Klaassen, Ed. McGraw Hill, New York

Crist R, Oberholser k, Schwarzy D, Marzoff J, Ryder D 91988) Interaction of metals and protons with algae. Environ Sci Technol 22:755-760

Devars S, Hernandez R, Moreno-Sanchez R (1998) Enhanced heavy metal tolerance in two strains of photosynthetic *Euglena gracilis* by preexposure to mercury or cadmium. Arch Environ Contam Toxicol 34:128-135

EPA-600/4-79-020 (1979) Methods for chemical analysis of water and wastes p. 245.1(Mercury), p. 270.2 (Selenium) United States Environmental Protection Agency Environmental Monitoring and Support Laboratory, Cincinnati, Oh

Faragasova A (1994) Comparative toxicity of five metals on various biological subjects. Bull Environ Contam Toxicol 53:317-324

Friis N, Myers-Keith P (1986) Biosorption of uranium and lead by *Streptomyces longwoodensis.* Biotechnol Bioeng 28:21-28

Harris P, Ramelow G (1990) Binding of metal ions by particulate biomass derived from *Chlorella vulgaris* and *Scenedesmus quadricauda.* Environ Sci Technol 24:220-228

Koeman JH, Peeters WHM, Koustaal-Hol CHM, Tjioe PS, DeGoeil JJM (1973) Mercury-selenium correlations in marine animals. Nature 245:385-386.

Kratz WA, Meyer J (1955) Nutrition and growth of several blue-green algae. American J Bot 42:282-287

Lee LH, Lustigman B, Chu IY, Jou HL (1991) Effect of aluminum and pH on the growth of *Anacytis nidulans.* Bull Environ Contam Toxicol 46: 720-726

Lee LH, Lustigman B, Schwinge V, Chu IY, Hsu S (1992) Effect of mercury and cadmium on the growth of *Anacystis nidulans.* Bull Environ Contam Toxicol 49:272- 278

Lee, LH, Lustigman B, Murray S, Koepp S (1999) Effect of Selenium on the growth of cyanobacterium *Anacystis nidulans.* Bull Environ Contam Toxicol 62: 591- 599

Parizek J, Ostadalova I (1967) The protective effect of small amounts of selenite in sublimate intoxication. Experientia 23:142-143

Rana SVS, Boora, PR (1992) Antiperoxidative mechanisms offered by selenium against liver injury caused by cadmium and mercury in rat. Bull Environ Contam Toxicol 48:120-124

SAS Institute (1995) IN: JMP Statistics and Graphics Guide Version 3, Cary, North Carolina

Vymazal J (1990) Uptake of lead, chromium, cadmium and cobalt by *Cladophora glomerata.* Bull Environ Contam Toxicol 51:24-29

Zhang W, Majidi V (1994) Monitoring the cellular response of *Stichococcus bacillaris* to exposure to several different metals using In Vivo 31P NMR and other spectroscopic techniques. Environ Sci Technol 28:1577-1581