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Toxicity, biotransformation, and mode of action of arsenic in two freshwater microalgae (Chlorella sp. and Monoraphidium arcuatum)

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Abstract

The toxicity of As(V) and As(III) to two axenic tropical 15 freshwater microalgae, Chlorella sp. and Monoraphidium arcuatum, was determined using 72-h growth rate inhibition bioassays. Both organisms were tolerant to As(III) (72-h IC50, concentration to cause 50% inhibition of growth, of 25 and 15 mg As(III)/L, respectively). Chlorella sp. was also tolerant to As(V) with no effect on growth rate over 72 h at concentrations up to 0.8 mg/L (72-h IC50 of 25 mg As(V)/L). M. arcuatum was more sensitive to As(V) (72-h IC50 of 0.25 mg As(V)/L). An increase in phosphate in the growth medium (0.15 to 1.5 mg PO_4^{3-} /L) decreased toxicity, i.e. the 72-h IC50 value for M. arcuatum increased from 0.25 mg As(V)/L to 4.5 mg As(V)/L, while extracellular As and intracellular As decreased, indicating competition between arsenate and phosphate for cellular uptake. Both microalgae reduced As(V) to As(III) in the cell, with further biological transformation to methylated species (monomethyl arsonic acid and dimethyl arsinic acid) and phosphate arsenoriboside. Less than 0.01% of added As(V) was incorporated into algal cells, suggesting that bioaccumulation and subsequent methylation was not the primary mechanism of detoxification. When exposed to As(V) both species reduced As(V) to As(III), however only M. arcuatum excreted As(III) into solution. Intracellular arsenic reduction may be coupled to thiol oxidation in both species. Arsenic toxicity was most likely due to arsenite accumulation in the cell, when the ability to excrete and/or methylate arsenite was overwhelmed at high arsenic concentrations. Arsenite may bind to intracellular thiols, such as glutathione, potentially disrupting the ratio of reduced to oxidised glutathione and consequently inhibiting cell division.

Keywords

Toxicity, biotransformation, mode, action, arsenic, two, freshwater, microalgae, Chlorella, Monoraphidium, arcuatum, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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TOXICITY, BIOTRANSFORMATION AND MODE OF ACTION OF ARSENIC IN TWO FRESHWATER MICROALGAE (*CHLORELLA* SP. AND *MONORAPHIDIUM ARCUATUM*)

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Abstract- The toxicity of As(V) and As(III) to two axenic tropical freshwater microalgae, *Chlorella* sp. and *Monoraphidium arcuatum*, was determined using 72-h growth rate inhibition bioassays. Both organisms were tolerant to As(III) (72-h IC50, concentration to cause 50% inhibition of growth, of 25 and 15 mg As(III)/L, respectively). *Chlorella* sp. was also tolerant to As(V) with no effect on growth rate over 72 h at concentrations up to 0.8 mg/L (72-h IC50 of 25 mg As(V)/L). *M. arcuatum* was more sensitive to As(V) (72-h IC50 of 0.25 mg As(V)/L). An increase in phosphate in the growth medium (0.15 to 1.5 mg PO_4^{3-} /L) decreased toxicity, i.e. the 72-h IC50 value for *M. arcuatum* increased from 0.25 mg As(V)/L to 4.5 mg As(V)/L, while extracellular As and intracellular As decreased, indicating competition between arsenate and phosphate for cellular uptake. Both microalgae reduced As(V) to As(III) in the cell, with further biological transformation to methylated species (monomethyl arsonic acid and dimethyl arsinic acid) and phosphate arsenoriboside. Less than 0.01% of added As(V) was incorporated into algal cells, suggesting that bioaccumulation and subsequent methylation was not the primary mechanism of detoxification. When exposed to As(V) both species reduced As(V) to As(III), however only *M. arcuatum* excreted As(III) into solution. Intracellular arsenic reduction may be coupled to thiol oxidation in both species. Arsenic toxicity was most likely due to arsenite accumulation in the cell, when the ability to excrete and/or methylate arsenite was overwhelmed at high arsenic concentrations. Arsenite may bind to intracellular thiols, such as glutathione, potentially disrupting the ratio of reduced to oxidised glutathione and consequently inhibiting cell division.

Keywords- Arsenic Algae Toxicity Biotransformation Phosphate

INTRODUCTION

Arsenic is a widespread contaminant in the environment. Anthropogenic sources, together with natural sources, have led to extensive leaching of arsenic into surface, ground and drinking waters [1]. Arsenic concentrations in freshwaters range from <1 to 10 µg/L [2], with up to 5000 µg/L reported in contaminated groundwaters [3].

Most studies investigating arsenic biotransformation have focussed on marine environments [4], due to the formation of arsenoribosides and arsenobetaine in marine invertebrates and macroalgae [5]. Arsenoribosides, believed to be the precursors of arsenobetaine in marine invertebrates, have long been found in marine macroalgae [6], however, their presence in freshwater microalgae has only recently been elucidated [7].

Arsenic biotransformation and cycling in freshwater systems has thus far received little attention, and little is known about the role of freshwater algae. Algae are an important component of freshwater aquatic environments and could potentially remediate arsenic-contaminated waters in wetlands through adsorption and biotransformation of inorganic arsenic. Microalgae in particular, have been shown to accumulate arsenic(V), with bioconcentration factors ranging from 200 – 4000 [8,9]. However, more information about the responses of freshwater algae to arsenic is required if they are to be used in remediation.

Literature data on the toxicity of arsenic to freshwater microalgae are limited to chlorophytes and cyanophytes. Reported IC50 values (concentration to cause a 50% inhibition of growth) range over five orders of magnitude, from 0.048 to 202 mg/L [10,11,12], and are generally well above environmental concentrations of arsenic. The wide variability in sensitivity to arsenic is likely due to biotic factors such as species type, differing uptake/exclusion pathways, detoxification mechanisms and prior-exposure, as well as abiotic factors such as arsenic species, phosphate concentrations, pH and exposure time.

While arsenic is toxic to microalgae at high concentrations, particularly at low ambient phosphate concentrations, few studies have examined the mode of toxic action of arsenic in freshwater microalgae. Most of our information on the mode of toxic action of arsenic comes from studies with terrestrial plants or microorganisms such as bacteria and yeasts [13,14]. A recent review of arsenic toxicity in terrestrial plants [13] showed that arsenic toxicity to biota may be due to: (i) interference in phosphate metabolism, leading to phosphate depletion or inhibition of adenosine triphosphate (ATP); (ii) oxidative stress due to the generation of reactive oxygen species; and/or (iii) binding of arsenite to intracellular thiols (sulfhydryl groups) of enzymes and tissue proteins, such as glutathione.

Aquatic and terrestrial biota have developed several strategies to detoxify metals and metalloids such as arsenic. These include: (i) exclusion of arsenic from the cell [15]; (ii) reduction of arsenate to arsenite followed by either excretion, or complexation with glutathione and sequestration into vacuoles (e.g *Saccharomyces cerevisiae*, [14]); (iii) production of other metal-binding proteins such as phytochelatins [16]; (iv) methylation to less toxic organic forms, together with excretion [17]. Studies with microalgae have largely focused on methylation as a potential detoxification process [17,18].

The objective of this study was to investigate the toxicity, biotransformation and mode of toxic action of arsenic in two axenic tropical freshwater microalgae, one arsenate-tolerant species (*Chlorella* sp.) and one sensitive species (*Monoraphidium arcuatum*). The mode of toxicity of arsenate and the detoxification processes in these two algae were compared. Biotransformation of arsenic and arsenic speciation in cells was determined by microwave-assisted extraction and high performance liquid chromatography- inductively coupled plasma-mass spectrometry (HPLC-ICP-MS), enabling low detection limits for the quantitation of arsenoribosides.

METHODS

Algal stock cultures

Chlorella sp. 12 and *Monoraphidium arcuatum* (Korš.) Hindák, originally isolated from Lake Aesake, Papua New Guinea, were maintained axenically at CSIRO Energy Technology, Sydney. Cultures were checked microscopically monthly and plated on agar (2% bacto agent, 0.1% pepsin and 0.1% yeast extract) several times over 12 months to ensure the absence of bacteria and other microorganisms. The algae were cultured in 1/5 strength Jaworki's medium [19] and incubated at 27 ± 1ºC on a 12:12 h light/dark cycle (Philips TL 40W cool white fluorescent lighting, 75 µmol photons/m^2/s, Caringbah, NSW, Australia).

Growth rate inhibition bioassays

The toxicity of As(V) and As(III) to *Chlorella* sp. and *M. arcuatum* was determined using 72-h growth rate inhibition bioassays. The test medium used in the bioassays was synthetic softwater (96 mg/L $NaHCO_3$, 60 mg/L $CaSO_4.2H_2O$, 123 mg/L $MgSO_4$ and 4 mg/L KCl) (Ajax and Asia Pacific Specialty Chemicals, Bacto Laboratories, Liverpool, NSW, Australia) with a hardness of 80-90 mg $CaCO_3$/L, an alkalinity of 54 mg $CaCO_3$/L and a pH of 7.6 ± 0.1. The medium was vacuum filtered through an acid-washed 0.45 µm cellulose acetate/nitrate membrane filter (Millipore, Bedford, MA, USA) and stored at 4°C.

A batch method was used to conduct growth rate inhibition tests using 250-mL borosilicate glass Erlenmeyer flasks, coated with Coatasil silanising solution (Ajax Chemicals, Auburn, NSW, Australia) to prevent adsorption of arsenic to the glass. Test flasks and sample storage vessels were soaked in 10% (v/v) nitric acid (BDH) overnight and rinsed thoroughly with high purity Milli-Q deionised water (>18 MΩ/cm, Bedford, MA, USA).

On the initial day of each test arsenate stock solutions, 0.2 and 15 g/L As(V) using $Na_2AsO_4.7H_2O$ (May and Baker, Dagenham, England), or an arsenite stock solution, 15 g/L

As(III) using $NaAsO_2$ (BDH, Poole, England), were prepared in test medium. Test media (70 mL) for control treatments or arsenic solutions were added to the flasks. Each test included five arsenic concentrations and a control, each prepared in triplicate. For *Chlorella* sp., As(III) treatments ranged from 10 to 200 mg/L and As(V) treatments from 0.75 to 60 mg/L. For *M. arcuatum*, As(III) treatments ranged from 5 to 50 mg/L and As(V) treatments from 0.025 to 0.4 mg/L. Nutrients KH_2PO_4 (Ajax) and $NaNO_3$ (Merck, Kilsyth, VIC, Australia) were added to all flasks to give a final concentration of 0.15 mg PO_4^{3-}/L and 15 mg NO_3^-/L (N:P molar ratio of 150:1). Additional toxicity tests with *M. arcuatum*, were carried out at a higher phosphate concentration (15 mg NO_3^-/L; 1.5 mg PO_4^{3-}/L; N:P molar ratio of 15:1) and a lower nitrate concentration (1.5 mg NO_3^-/L; 1.5 mg PO_4^{3-}/L; N:P molar ratio of 150:1).

Prior to inoculation with algae, 20-mL subsamples were taken from each flask, pooled for each treatment, for measurement of initial arsenic concentrations and stored at –18°C. Measured concentrations, not nominal, were used to calculate toxicity test endpoints.

Cells in the exponential growth phase (5-6 days old) were used in bioassays after centrifugation (2500 rpm, 7 minutes) and washing three times with Milli-Q water to remove residual culture medium. Flasks were inoculated with 2-4 $\times$ 10^4 cells/mL, shaken by hand and randomly placed in a growth cabinet (27 $\pm$ 1ºC, 12:12 h light/dark cycle, Philips TL 40W cool white fluorescent lighting, 140 µmol photons/m^2/s). Test flasks were rotated, and shaken twice daily to ensure sufficient gas exchange. The pH was recorded initially and after 72 h.

Cell density was determined daily using a Coulter Multisizer IIE Particle Analyser (70 µm aperture; Coulter Electronics, Luton, UK), with a correction count of background particles. The cell density data from 0 to 72 h were used to calculate the growth rate of treatments by fitting a regression line to a plot of $\log_{10}$ (cell density) versus time (h). The slope of the plot gave the cell division rate (µ) calculated as divisions per day. Growth rates for treated flasks were expressed as a percentage of the control cell division or growth rate.

136

137 *Statistical analysis*

138 The 72-h IC50 was calculated using linear interpolation (ToxCalc Version 5.0.23C,
139 Tidepool Software, San Francisco, CA, USA). The data were tested for normality and
140 homogenous variance, and Dunnett's multiple comparison test was used to determine which
141 treatments differed significantly from the controls (1 tailed, $P \leq 0.05$) to estimate the NOEC
142 (no observable effect concentration) and the LOEC (lowest observable effect concentration).

143

144 *Intracellular and extracellular arsenic determination*

145 The concentration of intracellular and extracellular arsenic after 72-h exposure to
146 As(V) at two different phosphate concentrations (both with 15 mg NO_3^-/L) was determined to
147 investigate the potential competitive uptake of phosphate and arsenate, using a modified
148 method of Franklin et al [20]. All manipulations were carried out in a Class-100 clean room.
149 *M. arcuatum* was incubated for 72-h with initial As(V) concentrations of 125, 250 and
150 1000 µg/L for the low phosphate (0.15 mg/L) tests and concentrations of 250, 1000 and 3000
151 µg As(V)/L for the high phosphate (1.5 mg/L) tests. Both tests included control treatments
152 (no arsenic). For each treatment, nine flasks were prepared, combining three after 72 h to
153 gain sufficient biomass for analysis, with three replicates per treatment. Each replicate was
154 mixed thoroughly and the cell density determined on a Coulter Multisizer IIE Particle
155 Analyser (70 µm aperture). Weighed sub-samples (145 mL) were filtered through a 25-mm
156 glass filtration unit (pre-acid-washed and rinsed with Milli-Q water) using a 0.45 µm GH-
157 polypropylene filter (Pall, Ann Arbor, MI, USA). Approximately 50 mL of the filtrate was
158 collected and frozen until analysis (dissolved As fraction).

Cells were rinsed with 20 mL of arsenic-free, nutrient-free growth medium while in the filtration unit to remove excess dissolved As(V) solution and to prevent overestimation of arsenic bound to the outside of cells. This solution was retained for analysis (rinse fraction).

Following preliminary experiments, two 20-min washes with 0.1 M KH_2PO_4/K_2HPO_4 buffer solution (pH 5.95) (Ajax, Merck)) were used for the optimum removal of extracellular arsenic without obvious efflux of intracellular arsenic. Phase contrast microscopy showed that cells were healthy and intact after these treatments. Cells on the filter paper were transferred to a Teflon tube, using 0.1 M phosphate buffer (final volume of 20 mL). This mixture was shaken for 30 s, allowed to stand for 20 minutes, then re-filtered using a new filter. The filtrate was retained for analysis. The process of cell-washing was repeated. These samples were called the "extracellular" fraction.

Algal cells were returned to the Teflon tube using 25% (v/v) HNO_3 (Merck), made up to a volume of 8 mL and left to digest for 30 minutes. The digest was microwaved at 90W for 5 min, diluted to 20 mL with Milli-Q water to give a final concentration of 10% (v/v) HNO_3, and stored at 4°C ("intracellular" fraction).

The dissolved, rinse and extracellular arsenic fractions were analysed for total arsenic by hydride generation-atomic fluorescence spectrometry (HG-AFS). Because the 10% (v/v) acid matrix interfered with the response from the AFS detector, the intracellular arsenic fraction was analysed for total arsenic by ICP-MS (Perkin Elmer Elan-6000, Australia).

Arsenic speciation bioassays

To determine the inorganic and organic arsenic species in solution and in algal cells following 72-h exposure to As(V), two speciation bioassays were conducted for both *Chlorella* sp. and *M. arcuatum*. The first bioassay consisted of As(V) treatments of one replicate each of 0, 10, 25 and 40 mg/L for *Chlorella* sp. and 0, 0.1, 0.2 and 0.3 mg/L for *M.*

184 *arcuatum*. The second speciation bioassay consisted of three replicates at one concentration
185 of arsenic only; 25 mg As(V)/L for *Chlorella* sp. and 0.2 mg As(V)/L for *M. arcuatum*
186 (approximate 72-h IC50). This set-up was required logistically as each replicate required 15
187 flasks of algae to be combined to gain sufficient biomass for analysis.

188 The cell density of the pooled bioassay solution was determined and the solution was
189 filtered and both the filter paper and filtrate were collected for analysis. For the second
190 speciation bioassay, the cells collected on the filter paper were rinsed with 20 mL of Milli-Q
191 water to prevent overestimation of cellular arsenic due to carryover of dissolved solution.
192 This rinse solution was analysed for total arsenic by ICP-MS. The water samples and cellular
193 samples on the filter paper were frozen immediately following collection and were analysed
194 for arsenic speciation by microwave digestion and HPLC-ICP-MS.

195 For quality assurance purposes, three additional flasks were prepared for each
196 treatment concentration, and incubated for 72 h under the standard test conditions. Two of
197 these were blanks (no algae), used to determine arsenic speciation changes in solution due to
198 either chemical reduction, or the process of collecting the sample fractions. The third flask
199 (inoculated with algae) was used to check the overall arsenic mass balance, to account for all
200 the added arsenic as either in solution, on the cells or adsorbed to the flask. Adsorption to the
201 flask was determined by filling the empty flask with nitric acid (20 mL, 0.2% (v/v), Suprapur,
202 Merck). It was shaken, left to stand for 48 h and then analysed for total arsenic by ICP-MS.
203
204 *Extracellular versus intracellular As(V) reduction*

205 Reduction of As(V) to As(III) by *M. arcuatum* was further investigated to determine
206 whether it occurred intracellularly or extracellularly. Control solutions (no arsenic) and
207 As(V) treatments were inoculated with algae and incubated for 48 h. To test for non-
208 biological reduction of arsenic in solution, an additional control was prepared containing

As(V) but not algae. The solutions containing cells were centrifuged and the supernatant was divided into 6 × 50 mL sub-samples, of which three were spiked with more As(V). Thus there were three replicates of the following (cell free) solutions: (i) exposed to As(V) plus an additional arsenic spike after 48 h; (ii) exposed to As(V) with no arsenic spike; (iii) not exposed to As(V) plus an additional arsenic spike after 48 h and; iv)not exposed to As(V) with no arsenic spike. All flasks were placed in the growth cabinet for a further 24 h. The solutions were then stored at –15°C until analysis of inorganic arsenic species by HG-AFS. This experiment was done three times, twice with algae exposed to and additional spikes of 100 µg As(V)/L and once with algae exposed to 300 µg As(V)/L and a spike of 300 µg As(V)/L.

Effect of As(V) on cellular thiol groups in M. arcuatum and Chlorella sp.

To determine whether As(V) reduction to As(III) was coupled to the oxidation of thiol groups such as glutathione in the cell, cellular sulfhydryl groups (-SH) were determined by spectrophotometry with 2-2'-dithiodipyridine using a modified method of Grassetti and Murray [21], adapted for algal cells by Stauber and Florence [22].

Test solutions were prepared in triplicate at three As(V) concentrations for both *M. arcuatum* (0 (control), 260 and 500 µg As(V)/L) and *Chlorella* sp. (0 (control), 25 and 50 mg As(V)/L) using nutrient concentrations of 0.15 mg PO_4^{3-}/L and 15 mg NO_3^-/L. Solutions were inoculated with a high algal cell biomass (2-3 × 10^5 cells/mL) and incubated for either 24 or 48 h under standard growth conditions.

After the exposure period, treatment flasks were combined and 30 mL (by mass) dispensed into four 50 mL polypropylene centrifuge tubes. Three replicates were exposed to 2-2'-dithiodipyridine, with the last replicate a blank. They were processed as per Stauber and Florence [22], and the absorbance of the samples at 341 nm and 233 nm was measured on a

234 UV-Visible spectrophotometer (Ultrospec IIE, LKB Biochrom, Cambridge, UK). Using a
235 calibration curve generated with freshly prepared 0.001 M reduced glutathione (GSH)
236 solution (Sigma) as a standard, the concentration of free thiols in controls and arsenic-treated
237 cells was calculated. Student t-tests were performed for pairs of As(V) concentrations to
238 determine if differences in the number of thiol groups were significant ($P \leq 0.05$).
239
240 *Arsenic analyses*
241 HG-AFS, ICP-MS and HPLC-ICP-MS were all used to determine concentrations of
242 total arsenic and arsenic species in solution and in algal cells. All calibration standards were
243 prepared fresh on the day of analysis using matrix-matched solutions.
244 Total arsenic and inorganic arsenic speciation in solution were analysed by HG-AFS
245 using a PSA Excalibur system (PS Analytical, Kent, UK). Total arsenic was measured after
246 oxidative digestion of organics to As(V) in 1% $K_2S_2O_8$ in an autoclave for 30 minutes
247 (120°C). Quantitative reduction of As(V) to As(III) was achieved by standing for 30 minutes
248 with 32% HCl, 1.3% KI and 0.25% ascorbic acid. Online delivery of 33% (w/v) HCl and
249 1.5% (w/v) $NaBH_4$ (stabilised in NaOH) converted As(III) to AsH_3 for detection. Total
250 inorganic arsenic was determined by eliminating the persulphate digestion. For As(III)
251 determination, online delivery of 0.3M acetic acid-0.2M sodium acetate and 1.5% (w/v)
252 $NaBH_4$ (stabilised in NaOH) converted As(III) to AsH_3. Samples were in the same acetic
253 acid- sodium acetate matrix. Matrix-matched calibration curves using As(III) and As(V)
254 standards were generated and the total inorganic As and As(III) concentrations calculated
255 directly, and As(V) calculated by difference.
256 Samples requiring determination of total As in an acidic matrix (e.g. As adsorbed to
257 flask walls and intracellular As) were measured by ICP-MS (Perkin Elmer Elan-6000,
258 Australia) following a microwave digestion step [23].

259 Where low to trace concentrations of organic arsenic compounds were of interest in
260 cells, microwave-assisted extraction coupled with HPLC-ICP-MS was primarily used for
261 quantitative speciation analysis. Both anion- and cation-exchange HPLC-ICP-MS was used
262 according to the method thoroughly outlined and validated in prior work of Kirby and Maher
263 [24] and Kirby et al. [25]. Because microalgal cell masses were small (1-3 mg) following
264 freeze-drying (Labconco, Australia), they were extracted without subsampling. Calibration
265 curves were prepared using a mixed standard of sodium arsenite, sodium arsenate
266 heptahydrate, sodium dimethylarsenic (Alltech - Specialists, Australia) and disodium
267 monomethylarsenic (Alltech - Specialists, Australia) in Milli-Q water. Characterisation of
268 arsenosugars was done with standards previously isolated and purified as described in Kirby
269 and Maher, [24]. Standards were run at regular intervals throughout sample analysis.
270
271 **RESULTS**
272 *Toxicity of arsenic to microalgae*
273 The effects of As(III) and As(V) on the growth rates of *Chlorella* sp. and *M. arcuatum*
274 are shown in **Table 1**. Growth rates of controls in the toxicity tests ranged from 1.2-1.8
275 doublings/day for *M. arcuatum* and 1.3-1.7 doublings/day for *Chlorella* sp., except for one
276 *Chlorella* sp. test where the growth rate was only 0.9 doublings/day, possibly due to late
277 inoculation at the end of the light cycle. Measured (initial) concentrations of As(III) and
278 As(V) ranged from 68-72% and 69-109% of nominal concentrations, respectively. The pH
279 increased by a maximum of 0.5 pH unit for all tests except for three individual *M. arcuatum*
280 treatments (0, 50, 100 µg As(V)/L) that increased by up to 1.1 pH units.
281 Algal growth rate decreased as the concentration of arsenic increased. Slight
282 stimulation of algal growth (2-8%) occurred at the lowest arsenic concentrations in some
283 tests. Both species were insensitive to As(III) with 72-h IC50 values of 14.6 and 25.2 mg/L

for *M. arcuatum* and *Chlorella* sp., respectively. Complete growth inhibition (< 5% of controls) was found at 50 mg As(III)/L for both species. The direct impact of adding As(III) to these alga was not further investigated due to lack of inter-species sensitivity differences, As(III) concentrations that caused toxicity were orders of magnitude above expected environmental concentrations of total arsenic in freshwater and because As(V) is the thermodynamically-favoured species in oxidised freshwaters [1, 2].

Data from the three individual As(V) toxicity tests for *Chlorella* sp. and *M. arcuatum* were combined to determine a concentration-response curve for the toxicity of As(V) to each alga (**Fig. 1**). Using non-linear regression, the 72-h IC50 for *Chlorella* sp. was 25.4 mg As/L, with 95% confidence limits (CL) of 25.2 to 25.7 mg As/L. This alga showed similar tolerance to both As(III) and As(V), however, both 72-h IC50 values were several orders of magnitude above expected environmental arsenic concentrations. As(V) was about 100 times more toxic to *M. arcuatum* than *Chlorella* sp., with a 72-h IC50 (95% CL) of 0.254 (0.253-0.255) mg As/L. Significant effects of As(V) on the growth rate of *M. arcuatum* were found at As(V) concentrations as low as 50 µg/L in one test, however, the mean LOEC value from three tests was 81 µg/L, with a NOEC of 39 µg As(V)/L.

The toxic mode of action of As(V) on *M. arcuatum* was of interest due to its greater sensitivity to As(V) compared with *Chlorella* sp. Thus a number of more detailed experiments were conducted, using *M. arcuatum*, to try and elucidate the mechanism of toxicity (see below).

Effect of phosphate on As(V) toxicity to M. arcuatum

When the phosphate was increased to 1.5 mg/L, lowering the N:P ratio in solution from 150:1 to 15:1, As(V) was much less toxic to *M. arcuatum.* The 72-h IC50 was 4.53 mg As(V)/L, compared to the standard bioassay with a 72-h IC50 of 0.254 mg/L (Table 1).

309 The NOEC and LOEC values also increased approximately 20-fold when the phosphate
310 concentration was increased. To establish that this was a result of changing the phosphate
311 concentration and not changing the N:P ratio, a separate test with a lowered nitrate
312 concentration (1.5 mg NO_3^-/L) and a molar N:P ratio of 15:1 was conducted (0.15 mg PO_4^{3-}
313 /L). In this test, the 72-h IC50 (0.183 mg As(V)/L) was only slightly (but significantly,
314 $P<0.05$) lower than the 72-h IC50 from the standard test using 15 mg NO_3^-/L (0.254 mg
315 As(V)/L) (Table 1). This suggests that the ameliorating effect on As(V) toxicity observed in
316 the high phosphate growth bioassay (N:P;15:1), was due to increasing phosphate
317 concentration alone.
318
319 *Effect of phosphate on the concentration of intracellular and extracellular arsenic*
320 The distribution of arsenic in the various algal fractions after 72 h are shown in **Table**
321 **2** for low phosphate (0.15 mg PO_4^{3-}/L) and high phosphate (1.5 mg PO_4^{3-}/L) bioassays. Good
322 recovery of arsenic was obtained (96-103% of the initial arsenic in solution), with most of the
323 arsenic (> 99%) in the dissolved arsenic fraction. Arsenic concentrations in the cellular
324 fractions were low. The concentration of arsenic in all fractions increased with increasing
325 initial arsenic in the media.
326 Extracellular and intracellular concentrations of total arsenic on a per cell basis are
327 shown in **Fig. 2**. Results are expressed this way to overcome substantially lower total cell
328 numbers at higher arsenic concentrations, due to toxic effects on growth. Preliminary
329 experiments showed that arsenic did not substantially alter the size of *M. arcuatum*, i.e.
330 arsenic load did not change as a result of surface area or volume changes. Extracellular and
331 intracellular concentrations of arsenic increased with increasing concentrations of arsenic
332 added to the growth medium. The concentrations of intracellular and extracellular arsenic

were significantly higher ($P \leq 0.05$) when the bioassay was carried out in low phosphate (0.15 mg PO_4^{3-}/L) compared to high phosphate (1.5 mg PO_4^{3-}/L) solutions (Table 2).

Speciation and distribution of arsenic in microalgae

The distribution of arsenic after 72-h exposure to As(V) is shown in **Fig. 3** for both *M. arcuatum* and *Chlorella* sp. Of the As recovered, > 94% was in solution, < 0.01% was associated with the cells and < 1.3% was adsorbed to the flask walls. The amount of total As adsorbed to the flask increased with increasing concentrations of As(V) used in the test medium. Addition of a rinsing step resulted in up to 6.2% of total As being recovered in this fraction, with cellular concentrations of As(V), As(III), DMA and MMA in *Chlorella* sp. decreasing by 2-4 fold. This highlighted that carryover of dissolved arsenic, in the mg/L range, results in overestimation of cellular arsenic.

As(III) was present in test media containing *M. arcuatum* after a 72-h exposure to As(V) (Fig. 3). The percentage of initial As(V) reduced to As(III) decreased from 95% to 22% with increasing initial As(V) concentrations. However, no As(III) was detected in the blanks (no algae), indicating that the presence of As(III) in *M. arcuatum* solutions was due to biological reduction. In a separate As(V) exposure test, inorganic As concentrations were measured at 24-h intervals throughout the 72-h bioassay with *M. arcuatum*. As(V) reduction to As(III) was observed in the initial 24-h period and the reduction continued over time: 41% and 65% of the 260 µg As(V)/L treatment was detected as As(III) at 24 and 48 h, respectively; and 46% and 72% of the 500 µg As(V)/L treatment was detected as As(III) at 24 and 48 h. Between 48 and 72-h, As(V) reduction to As(III) was similar.

In contrast, As(III) was not detected in solution after 72 h in *Chlorella* sp. bioassays (Fig. 3). There was no detectable MMA, DMA, phosphate arsenoriboside or other organic species of arsenic in solution at 72 h for either algae.

358 Five arsenic species were detected in the cells; As(V), As(III), MMA, DMA and
359 phosphate arsenoriboside (P-sug) (**Fig. 4**). No other arsenic species were detected in any of
360 the tests. Cells contained predominantly As(V) followed by As(III). Although cellular
361 concentrations of arsenic species in *Chlorella* (after rinsing) were a maximum of 6-fold
362 higher than arsenic species accumulated by *M. arcuatum, M. arcuatum* had been treated with
363 concentrations of As(V) 100 times lower than *Chlorella*. Thus *M. arcuatum* accumulated
364 more arsenic from solution relative to *Chlorella* sp., and was consequently more sensitive to
365 As(V) than *Chlorella* sp.
366 For *Chlorella* sp., concentrations of cellular As(V) and As(III) generally increased
367 with increasing As(V) concentrations in the growth medium, in contrast to *M. arcuatum*
368 where concentrations of As(V), DMA and phosphate arsenoriboside were at a maximum in
369 the 0.210 mg/L (rinsed cell) treatment (Fig. 4). Trace concentrations of phosphate
370 arsenoriboside were occasionally detected in *Chlorella* cells. Higher amounts of phosphate
371 arsenoriboside were detected in *M. arcuatum* with a mean value of $44.7 \pm 19.6 \times 10^{-18}$ g/cell
372 in the 0.210 mg As/L treatment.
373
374 *Cellular reduction of arsenic(V) to arsenic(III) by M. arcuatum*
375 Preliminary experiments demonstrated that *M.arcuatum* did not inherently produce an
376 exudate that could reduce arsenic.
377 *M. arcuatum* exposed to 0.1 mg/L and 0.3 mg/L As(V) for 48 h reduced the As(V) in
378 solution to As(III) by approximately 41% (0.04 mg As(III)/L) and 11% (0.03 mg As(III)/L),
379 respectively. There was no reduction of As(V) to As(III) in the growth medium in the
380 absence of *M. arcuatum* cells, indicating that arsenic reduction was biologically mediated.
381 After the initial 48-h exposure (control, 0.1 or 0.3 mg/L As(V)), the cells were
382 removed and the supernatant spiked with an additional 0.1 or 0.3 mg As(V)/L for a further 24

383 h. No further reduction of As(V) was observed in this period. This showed that the reduction
384 of As(V) to As(III) only occurred in the presence of cells, i.e. the reduction was not due to an
385 exudate released by the cells, generated in the presence of As(V).
386
387 *Effect of As(V) on cellular thiol groups in M. arcuatum and Chlorella sp.*
388 Oxidation of thiols such as glutathione has previously been shown to be a potential
389 mechanism by which cell division is inhibited by metals in algal cells [19]. The hypothesis
390 was that reduced glutathione (GSH) is oxidised (to GSSG) as As(V) is reduced to As(III).
391 Decreased SH concentrations for As(V) treatments compared to controls (no As(V)) indicated
392 that thiol groups were oxidised. Preliminary experiments with As(V) and glutathione in cell-
393 free solution, showed that As(V) did not oxidise GSH to GSSG in the absence of algal cells.
394 Arsenic toxicity and As(V) reduction to As(III) were similar when both high (3×10^5
395 cells/mL) and low ($2\text{-}4 \times 10^4$ cells/mL) initial cell densities were used.
396 In unexposed controls, *M. arcuatum* contained 8.2 ± 1.9 nmol SH 10^{-6} cells. Thiols
397 significantly decreased as the concentration of arsenic and time of exposure increased,
398 however, results were variable. After a 24-h exposure to 500 µg As(V)/L, thiol
399 concentrations were significantly lower ($P < 0.05$) in two of the three tests (16% and 57% of
400 controls). After a 48-h exposure, thiol concentrations were 15 and 37% of controls in two of
401 the three tests.
402 A similar pattern was observed for *Chlorella* sp., with a mean number of thiols of 7.9
403 $\pm$ 3.3 nmol SH 10^{-6} cells in unexposed controls. After 48-h exposure to 25 mg As(V)/L (the
404 approximate IC50 for *Chlorella* sp.) thiol concentrations were significantly decreased (16-
405 75% of controls). After a 72-h exposure, thiol concentrations were also decreased (55% of
406 control and 14% of control at 25 and 50 mg As(V)/L, respectively). When *Chlorella* sp. was
407 exposed to much lower arsenic concentrations, similar to that of *M. arcuatum* (i.e. 500 µg

As(V)/L) thiol concentrations were not significantly different after 24 h (92% of control) but were significantly lower ($P < 0.05$) after a 48- and 72-h exposure (61 and 68% of controls). The variation observed is these results is likely due to the cells being damaged but not lysed by the addition dithiodipyridine solution and vortexing. Thus, conversion of internal thiols may differ between experiments.

DISCUSSION

Arsenic toxicity

The current results confirm that the toxicity of arsenic to freshwater microalgae depends on the chemical species of arsenic, the algal species and the phosphate concentration in the test medium. Arsenate and arsenite were approximately equally toxic to *Chlorella* sp., with IC50s of 25.4 mg As(V)/L and 25.2 mg As(III)/L). *M. arcuatum* was more sensitive to As(V) (IC50: 0.254 mg As(V)/L) than *Chlorella* sp. and more sensitive to As(V) than As(III) (IC50: 14.6 mg As(III)/L).

The 12-14-d growth inhibition IC50 values for As(V) spanned five orders of magnitude for *Scenedesmus obliquus, Ankistrodesmus falcatus*, *Selenastrum capricornutum*, *Scenedesmus quadricauda* and *Chlamydomonas reinhardtii* (0.048, 0.256, 31, 61, 202 mg/L, respectively) [10,11,12]. Although different test durations and conditions such as photoperiods and phosphate concentrations make comparison difficult, this illustrates that even in a single genus, there are large variations in sensitivity of microalgae to arsenic.

The toxicity of arsenic to freshwater microalgae is also dependent on the chemical species of arsenic added. It has been reported that As(V) is more toxic than As(III) to freshwater algae, while the reverse is true for marine algae and humans [1,26]. In 96-h growth inhibition tests with the freshwater green alga *Selenastrum capricornutum*, IC50 values of 31 and 0.69 mg As/L were found for As(III) and As(V) respectively [27], while the

433 toxicity of arsenic decreased in the order As(V)>As(III)>DMA for natural algal assemblages
434 in an arsenic-contaminated freshwater lake [28]. Contrary to this, it was found that As(III)
435 was more toxic than As(V) to *Chlorella vulgaris* (isolated from arsenic-contaminated
436 freshwaters) with growth increasing with As(V) concentrations up to 2000 mg/L, and growth
437 inhibition at As(III) concentrations > 40 mg/L [9].

438 Our results showed that a ten-fold increase in the phosphate concentration decreased
439 the toxicity of As(V) to *M. arcuatum* by approximately twenty-fold (Table 1). The reduced
440 toxicity of As(V) was a result of higher phosphate concentrations, rather than simply due to
441 changing the N:P ratio (Table 1). The concentration of phosphate in solution significantly
442 affected the amount of arsenic adsorbed to the surface of *M. arcuatum* and the amount of
443 arsenic that was accumulated inside the cell (Table 2). At low phosphate concentrations,
444 intracellular and extracellular arsenic concentrations were high, corresponding to increased
445 growth inhibition, compared to the bioassays carried out at high phosphate concentrations.
446 With an increase in phosphate concentration in the bioassay medium, less arsenic binds to the
447 algal cell, and less arsenic is taken up intracellularly (Fig. 2), supporting the hypothesis that
448 arsenate and phosphate compete for uptake in algal cells. This further supports the study by
449 Maeda et al [29] which showed that the toxic effect of 10 mg As(V)/L to *Chlorella vulgaris*
450 decreased when the phosphate concentration increased from 14 to 14000 mg PO_4^{3-}/L.
451 However, these authors used high arsenic concentrations (1-1000 mg As(V)/L), high cell
452 densities, and an isolate from a contaminated environment. High cell densities decrease the
453 toxic load to cells [20], while there exists the potential for adaption and species sucession in
454 polluted environments. Consequently *Chlorella vulgaris* was very tolerant to As(V) (52%
455 growth inhibition at 5 g As(V)/L) when compared *M. arcuatum* in our study (IC50: 254 µg
456 As(V)/L). Maeda et al. [30] also determined intracellular and extracellular As in *Chlorella*
457 *vulgaris* using only water to remove extracellular arsenic. The amount of arsenic adsorbed to

458 and accumulated inside the cells increased 10-fold with each 10-fold increase in the
459 concentration of arsenic in the test medium. Similar trends were observed in *M. arcuatum* in
460 our current study, using a phosphate buffer to desorb As from the algal cell surface.

461 While phosphate has been shown to affect arsenate uptake into *M. arcuatum*, it is not
462 known if arsenate reduces phosphate uptake into the alga, thereby contributing to inhibitory
463 effects of arsenate on algal growth. However, because phosphate is an essential nutrient,
464 competition between arsenate and phosphate for cellular uptake is likely to be one mode of
465 toxic action in microalgae. Increases in arsenic have been shown to decrease phosphate
466 uptake in five freshwater algae, *Anabaena variabilis*, *Chlamydomonas reinhardtii*,
467 *Cryptomonas erosa*, *Melosira granulata* and *Ochromonas vallesiaca* [31]. In contrast, it was
468 found that phosphate uptake in *Synechococcus leopoliensis,* a cyanophyte, was not affected by
469 arsenate even when the concentration of arsenate was fifty times that of phosphate, possibly
470 because this species had a highly specific phosphate transport system [32].
471
472 *Arsenic accumulation and biotransformation*
473 Accumulation of arsenic by freshwater microalgae typically increased with increasing
474 arsenate concentrations in the test medium (Fig. 4), similar to other studies [18,29,32].
475 Maeda et al. [9] also showed that accumulation of As only occurred in live *C. vulgaris* cells,
476 suggesting an active uptake mechanism.
477 In our studies, As(V) was the main arsenic species in cells, followed by 1-6% as
478 As(III). Maeda et al. [33] also found that >95% of arsenic was accumulated by freshwater
479 algae as inorganic species. They found that dimethylated arsenic was the major methylated
480 arsenic compound detected. However, while both *Chlorella* sp. and *M. arcuatum* in our study
481 methylated As(V) to MMA, DMA and phosphate arsenoriboside (Fig. 4), these products were
482 present only in low concentrations in the cells and were not detectable in solution.

In our study 0-12% of cellular arsenic occurred as the phosphate arsenoriboside. Arsenoribosides have only recently been positively identified in one freshwater alga, *Chlorella vulgaris*, with comparison to the retention time of arsenoriboside standards. Glycerol, phosphate and sulfonate arsenoribosides were detected, with phosphate arsenoriboside occurring in the highest concentration of 0.2-5% of accumulated arsenic [7]. Agar plating was not carried out, but no significant differences occurred between cultures treated with and without antibiotics. Arsenoribosides have also been identified in *Nostoc flagelliforme,* a terrestrial cyanobacterium [34]. It is possible that the arsenoribosides detected were produced by bacteria in the cultures rather than the microalgae themselves, but our study with bacteria-free algal cultures confirms that microalgae exposed to low arsenic concentrations can produce trace arsenoribosides, but it does not appear to be a major detoxification pathway.

Bioassays with exponentially growing cells showed that when *M. arcuatum* (but not *Chlorella* sp.) was exposed to As(V), As(III) was excreted into solution. Hellweger et al. [35] found that As(III) excretion into solution was more likely during the exponential phase of growth. Algae in the stationary phase of growth (phosphate limited) were more likely to methylate arsenic to more complex organic arsenic compounds, which are then excreted. Similarly, it was found that when *Chlorella vulgaris* accumulated inorganic, mono- and di-methylated arsenic over 20 days, it excreted inorganic As together with trimethylated arsenic species after 4 days and dimethylated arsenic species after 14 days [18]. Trivalent methylarsenic species have also been detected in the growth medium of the green alga *Closterium aciculare* [36] but were not detected in our current study using HPLC-ICP-MS. It is possible that, if methylation of arsenic occurs to a greater extent in stationary phase cells, then larger concentrations of arsenoribosides may have been detected if stationary phase rather than exponentially growing algae had been used in our experiments.

As(V) reduction to As(III) occurs intracellularly (or in the cell membrane) in *M. arcuatum* and the As(III) is then excreted into the test medium. Similar trends have also been found in bacteria and yeasts [14], with As(V) reduced to As(III) via an arsenate reductase and then removed from the cytosol by either a secondary carrier, using energy from an existing ion gradient, or in a complex with a second protein via an ATP-coupled pump.

Our study supports the biotransformation model of Cullen et al. [17,37] in which arsenate is taken up by algal cells using a phosphate transport system, reduced to As(III) in the cell by thiols and/or dithiols and then excreted into the growth medium, probably by an active transport system. At longer exposure times, As(III) may be methylated to MMA, then to DMA and trimethylated arsenic species, which then diffuses into the growth medium.

In the marine microalga *Nitzchia closterium*, toxicity of copper was shown to be a cytosolic reaction between copper and GSH [22]. The cellular ratio of GSH:GSSG, critical to mitotic cell division, was lowered. We hypothesised that in *M. arcuatum*, reduction of As(V) to As(III) may be coupled with oxidation of GSH, ultimately resulting in inhibitory effects on cell division. If this was the case, total thiol concentrations in the cells should be reduced in the presence of arsenate, at concentrations that are inhibitory to algal growth. Thiol cell concentrations were lower in *M. arcuatum* at high concentrations of As(V) (0.5 mg As(V)/L) at 24 and 48-h compared to controls, but variability in the results suggest improvements must be made to this technique for freshwater algae before strong conclusions can be made (as using marine algae, e.g. [22], osmotic shock effectively lyses the cell). Excretion of As(III) may not keep pace with arsenic reduction, leading to accumulation of As(III) in the cells. As(III) is known to bind strongly to thiols in plants and animals [13]. As(III) appears to only be toxic once accumulated inside cells, as As(III) in the medium was not toxic to either *M. arcuatum* or *Chlorella* sp.

In *Chlorella* sp., thiol oxidation was also observed at As(V) concentrations that inhibit cell division (25 mg As(V)/L). This indicates that As(V) reduction may be coupled to thiol oxidation, but the alga lacks the arsenite transporter to excrete As(III) into the medium. It is possible that *Chlorella* sp. is able to detoxify arsenite inside the cell by sequestering it into subcellular compartments much like the yeast *Saccharomyces cerevisiae* complexes As(III) with glutathione, transferring the product from the cytosol into vacuoles via a specific transporter [14].

In freshwater environments, arsenic is unlikely to be toxic to *M. arcuatum*, except in highly contaminated surface and groundwaters containing >50 µg As/L. In such environments, it is likely that As(V) is taken up by algal cells due to its similarity to phosphate, and is quickly reduced to As(III). Toxicity is most likely due to the presence of As(III) in the cell, when the ability to excrete or sequester As(III) is overwhelmed and the As(III) subsequently binds to intracellular thiols, inhibiting cell division. The disruption of phosphate metabolism by incorporation of As(V) into phosphorylated compounds, vital to the cycling of ATP, may also contribute to arsenic toxicity.

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Table 1. 72-h toxicity of As(III) and As(V) to *Chlorella* sp. and *Monoraphidium arcuatum*, under different nutrient conditions

	$[NO_3^-]$ (mg/L)	$[PO_4^{3-}]$ (mg/L)	N:P (molar)	72-h IC50[a] (mg/L)	LOEC[b] (mg/L)
As(III)					
Chlorella sp.	15	0.15	150:1	25.2 (23.3-29.2)[c]	-[d]
M. arcuatum	15	0.15	150:1	14.6 (11.7-17.7)[c]	3.75[c]
As(V)					
Chlorella sp.	15	0.15	150:1	25.4 (25.2-25.7)	1.93
M. arcuatum	15	0.15	150:1	0.254 (0.253-0.255)	0.081
M. arcuatum- high PO_4^{3-}	15	1.5	15:1	4.53 (4.02-4.83) [c]	1.91 [c]
M. arcuatum- low NO_3^-	1.5	0.15	15:1	0.183 (0.170-0.192) [c]	0.054 [c]

[a] IC50: concentration of As which inhibits growth rate by 50%, calculated from a concentration response curve developed from 3 separate growth inhibition toxicity tests, unless otherwise indicated. Brackets indicate 95% confidence limits

[b] LOEC: lowest-observable-effect concentration, calculated as the geometric mean of three LOECs from three separate tests, unless otherwise indicated

[c] Results are calculated from a single growth inhibition toxicity test

[d] LOEC > IC50 therefore not reported

Table 2. Mean arsenic distribution in *M. arcuatum* fractions after 72-h exposure to varying As(V) and PO_4^{3-} concentrations [a]

$[PO_4^{3-}]$ (mg/L)	Initial nominal [As] (µg/L)	Initial measured [As] (µg/L)[b]	% Recovery[c]	Dissolved As (µg/L)	As in rinse [d] (µg/L)	Extracellular As ($\times 10^{-18}$ g/cell)[e,f]	Intracellular As ($\times 10^{-18}$ g/cell)[f]
0.15	0 (control)	< 0.5	-	0 ± 0	0.0 ± 0.0	140 ± 15	ND[g]
	125	123	102 ± 1	125 ± 2	0.7 ± 0.0	1100 ± 270	1200 ± 390
	250	236	103 ± 0	244 ± 1	1.4 ± 0.2	1400 ± 120	2400 ± 380
	1000	1000	98 ± 2	985 ± 19	5.7 ± 0.3	3900 ± 410	2600 ± 110
1.5	0 (control)	< 0.5	-	0 ± 0	0.0 ± 0.0	15 ± 34	12 ± 21
	250	247	96 ± 1	237 ± 2	1.4 ± 0.4	40 ± 74	180 ± 22
	1000	935	101 ± 2	945 ± 22	7.5 ± 2.5	110 ± 50	400 ± 140
	3000	2880	99 ± 2	2840 ± 43	20 ± 1.5	1100 ± 100	1600 ± 330

[a] Mean calculated from 3 replicates, ± one standard deviation (SD) from the mean

[b] No SD indicated for initial measured arsenic as it was calculated from 3 pooled sub-samples

[c] % Recovery = total As/measured initial As concentrations × 100

[d] Rinse was performed to prevent overestimation of extracellular As due to carryover from dissolved fraction.

[e] Extracellular As is the combination of both phosphate washes

[f] Extracellular blank (phosphate buffer) was 0.6 (± 0.2) µg As/L and intracellular blank (acid matrix) was <0.1 µg As/L

[g] ND not detected

Fig. 1. Effect of As(V) on 72-h growth rate of *Monoraphidium arcuatum* (◆) and *Chlorella* sp. 12 (□). Concentration-response curves were based on combined data from three toxicity tests. Error bars represent one standard deviation of three replicates.

Fig. 2. Intracellular and extracellular arsenic concentrations in *M. arcuatum* when bioassays were carried out with varying As(V) concentrations (0-3000 µg As(V)/L) at low and high phosphate concentrations (0.15 and 1.5 mg PO_4^{3-}/L). Note that the legend is based on nominal concentrations of As, measured initial concentrations are given in Table 2 and vary slightly between low and high phosphate tests.

Fig. 3. Mass balance of arsenic species in solution after 72 hours of growth of (a) *Monoraphidium arcuatum* and (b) *Chlorella* sp. 12. As(V) and As(III) in solution, total arsenic (TAs) in the cells and adsorbed to the flask walls were measured for all test treatments. * This column in each figure is the average from 3 separate bioassays run with 0.210 and 26.4 mg As(V)/L for *M. arcuatum* and *Chlorella* sp. respectively; these tests incorporated a rinsing step of the algal cells to investigate the As carryover from solution to cells in the subsequent analysis, and thus a rinse fraction is shown only for these test treatments.

Fig. 4(a) Concentration of As species in *Chlorella* sp. 12 after 72-h exposure to 8.80-39.6 mg As(V)/L. Values indicated for 26.4 mg As(V)/L were the result of triplicate speciation bioassays which incorporated a rinsing step prior to analysing the cells. Values for control, 8.8 and 39.6 mg As(V)/L were the result of a single speciation bioassay and did not incorporate a rinsing step prior to analysing the algal cells. (b) Concentration of As species in Monoraphidium arcuatum after 72-h exposure to 0.103-0.298 mg As(V)/L. Values indicated for 0.210 mg As(V)/L were the result of triplicate speciation bioassays which incorporated a rinsing step prior to analysing the cells. Values for control, 0.103 and 0.298 mg As(V)/L were the result of a single speciation bioassay and did not incorporate a rinsing step prior to analysing the algal cells. MMA = monomethylarsonic acid; DMA = dimethylarsinic acid; P-sug = phosphate arsenoriboside.

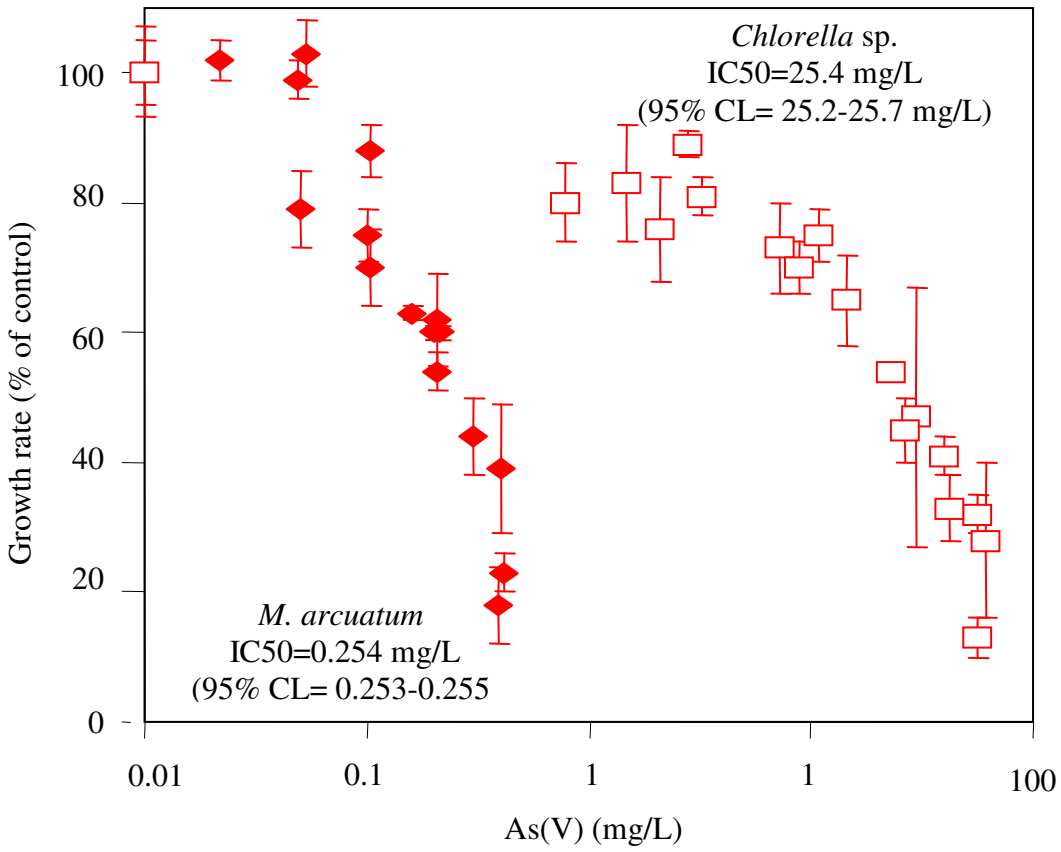

Chlorella sp.
IC50=25.4 mg/L
(95% CL= 25.2-25.7 mg/L)
M. arcuatum
IC50=0.254 mg/L
(95% CL= 0.253-0.255
Growth rate (% of control)
100
80
60
40
20
0
0.01
0.1
1
1
100
As(V) (mg/L)

Fig. 1

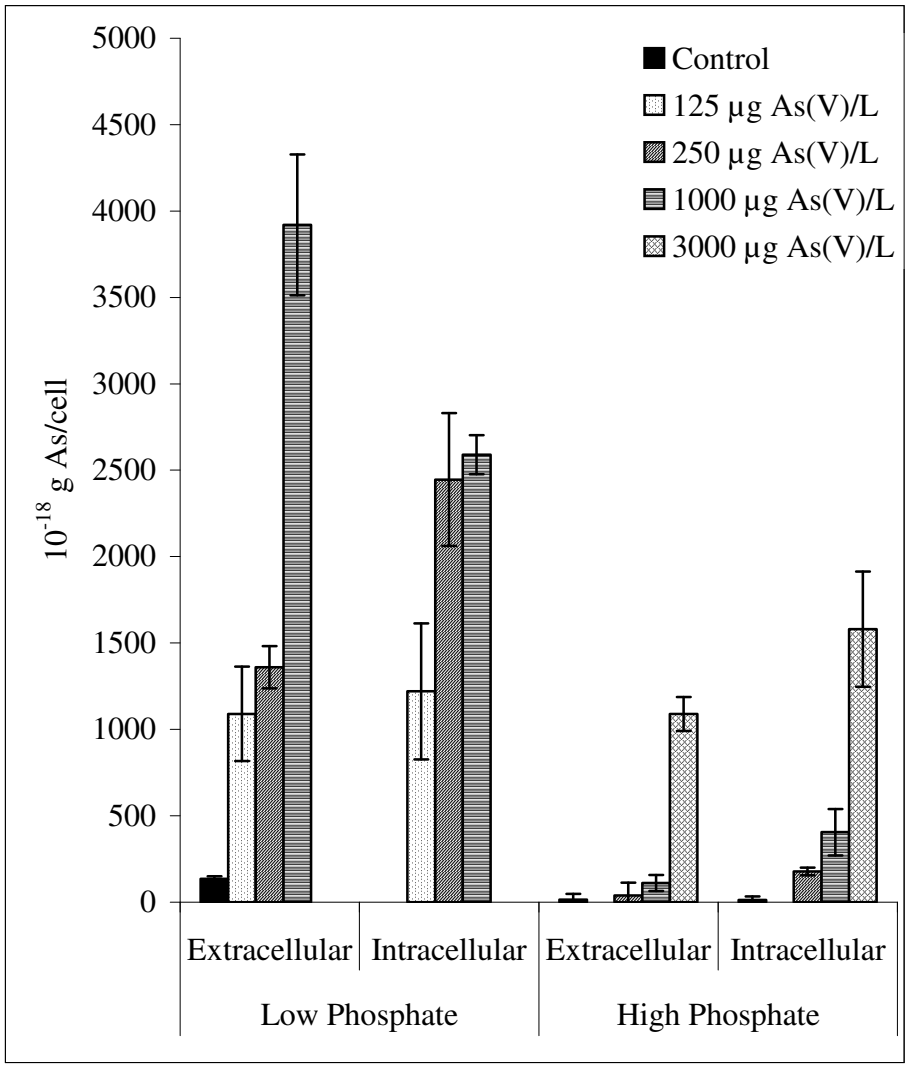

5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
10-18 g As/cell
Control
125 µg As(V)/L
250 µg As(V)/L
1000 µg As(V)/L
3000 µg As(V)/L
Extracellular
Intracellular
Extracellular
Intracellular
Low Phosphate
High Phosphate

Fig. 2

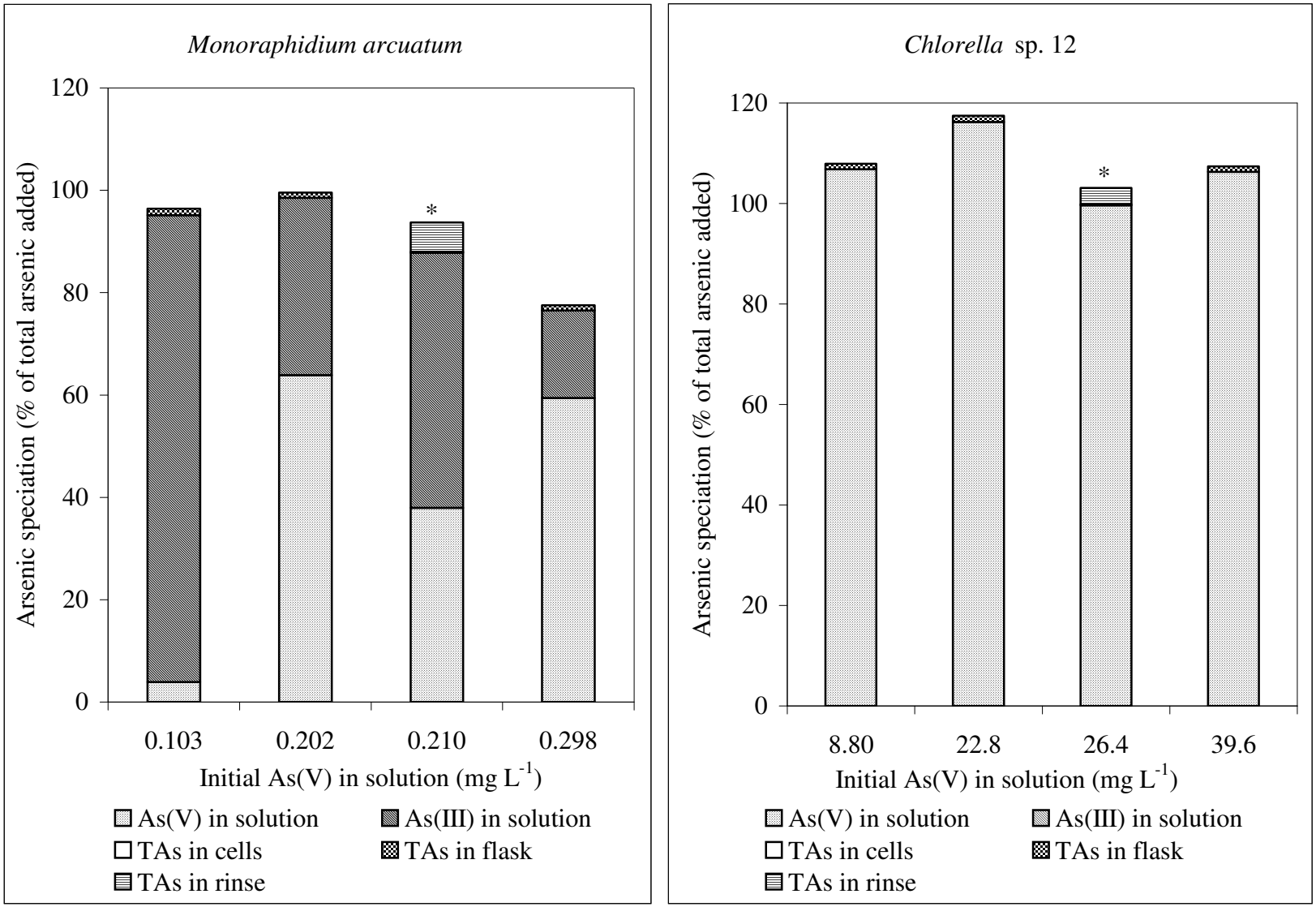
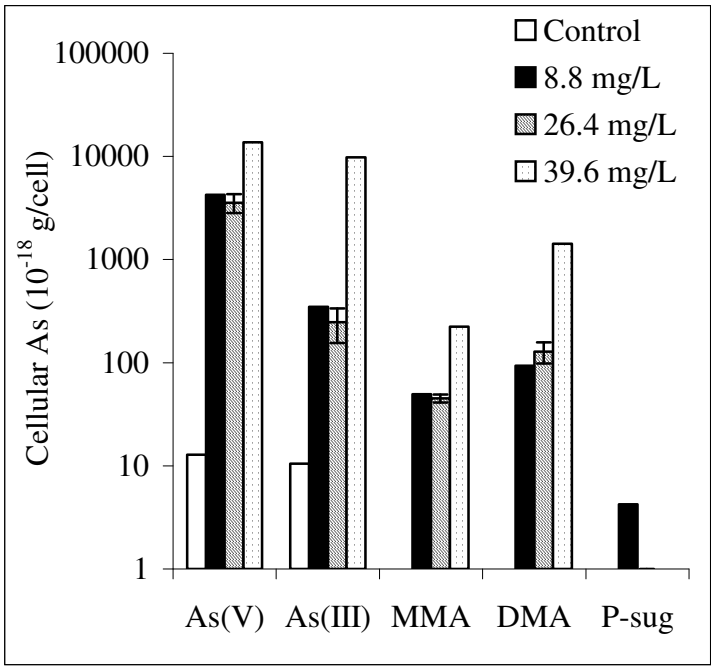

Monoraphidium arcuatum
Arsenic speciation (% of total arsenic added)
120
100
80
60
40
20
0
*
0.103
0.202
0.210
0.298
Initial As(V) in solution (mg L-1)
As(V) in solution
As(III) in solution
TAs in cells
TAs in flask
TAs in rinse
Chlorella sp. 12
Arsenic speciation (% of total arsenic added)
120
100
80
60
40
20
0
*
8.80
22.8
26.4
39.6
Initial As(V) in solution (mg L-1)
As(V) in solution
As(III) in solution
TAs in cells
TAs in flask
TAs in rinse

Fig. 3


Control
8.8 mg/L
26.4 mg/L
39.6 mg/L
100000
10000
1000
100
10
1
Cellular As (10^{-18} g/cell)
As(V)
As(III)
MMA
DMA
P-sug

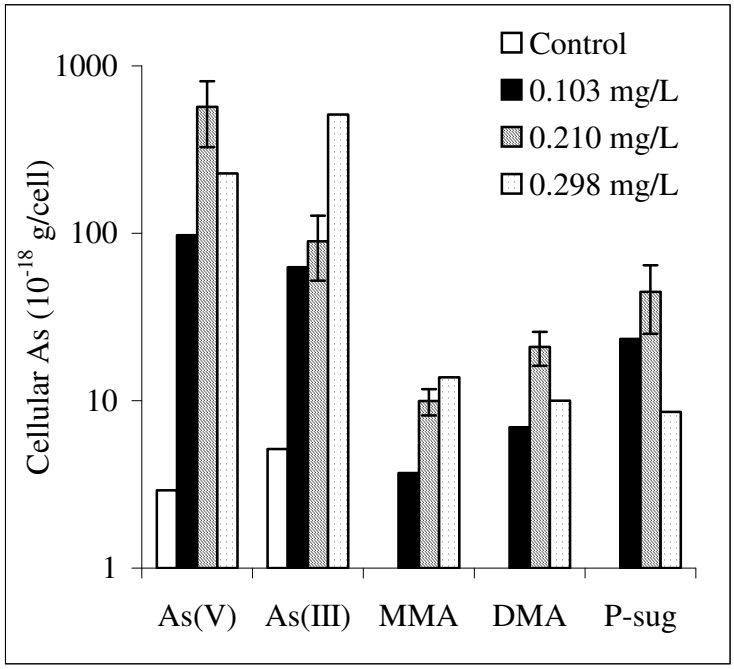

Control
0.103 mg/L
0.210 mg/L
0.298 mg/L
1000
100
10
1
Cellular As (10^{-18} g/cell)
As(V)
As(III)
MMA
DMA
P-sug

Fig. 4.