TECHNICAL NOTE

Effects of arsenate (As^{5+}) on growth and production of glutathione (GSH) and phytochelatins (PCs) in *Chlorella vulgaris*

Ying Jiang[1,2], Huw Jones[1] , Hemda Garelick[1] and Diane Purchase[1]*

Running head: *Chlorella vulgaris* and arsenate accumulation

[1] Department of Natural Sciences, School of Health and Social Sciences, Middlesex University, The Burroughs, London NW4 4BT, UK.

[2] Current address: School of Civil Engineering and the Environment, University of Southampton, Southampton SO17 1BJ, UK.

[2] Current address: School of Civil Engineering and the Environment, University of Southampton, Southampton SO17 1BJ, UK.

* corresponding author

ABSTRACT

The effect of arsenate (As^{5+}) on growth and chlorophyll *a* production in *Chlorella vulgaris*, its removal by *C. vulgaris* and the role of glutathione (GSH) and phytochelatins (PCs) were investigated.

C. vulgaris was tolerant to As^{5+} at up to 200 mg/L and was capable of consistently removing around 70% of the As^{5+} present in growth media over a wide range of exposure concentrations. Spectral analysis revealed that PCs and their arsenic-combined complexes were absent indicating that the high bioaccumulation and tolerance to arsenic observed was not due to intracellular chelation. In contrast, GSH was found in all samples ranging from 0.8 mg/L in the control to 6.5mg/L in media containing 200 mg/L As^{5+} suggesting that GSH plays a more prominent role in the detoxification of As^{5+} in *C. vulgaris* than PC. At concentrations below 100 mg/L cell surface binding and other mechanisms may play the primary role in As^{5+} detoxification, whereas above this concentration As^{5+} begins to accumulate inside the algal cells and activates a number of intracellular cell defence mechanisms, such as increased production of GSH.

The overall findings complement field studies which suggest *C.vulgaris* as an increasingly promising low cost As phytoremediation method for developing countries.

Keywords: *Chlorella vulgaris*, arsenate accumulation, glutathione, phytochelatins

INTRODUCTION

Arsenic (As) is abundant and widespread in the environment. It is a metalloid that exists in many chemical forms, including trivalent As^{3+} and pentavalent As^{5+} forms (Mohan and Pittman 2007). The toxicity of As has been well characterised and it is recognised as a potent human carcinogen (Choong et al. 2007). It is also known that the toxicity of As varies greatly with its speciation. For example, organic forms such as methylarsonic acid (MMA) and arsenosugars are

typically 2-4 orders of magnitude less toxic than inorganic forms. Long term exposure to inorganic As may result in skin, lung, bladder and kidney cancer (Mohan and Pittman 2007; Mandal and Susuki 2002; WHO 2008). Arsenic is found naturally in rocks and sediment, and is a common constituent of non-ferrous ores such as copper, lead, gold and uranium (Lorenzen et al. 1995). Arsenic is released into the environment via natural processes including weathering, biological and geochemical reactions and volcanic deposits (Korte and Fernando 1991) as well as anthropogenic activities such as mining, combustion of fossil fuels, application of arsenic pesticides and wood preservatives (Mohan and Pittman 2007; Choong et al. 2007). The greatest threat to human health derives from its natural occurrence in groundwater which exposes millions to arsenic poisoning via consumption of drinking water from this source. At least twenty countries worldwide including the USA, China, Mexico, Hungary, Japan and New Zealand are known to be at risk with groundwater arsenic contamination (Mohan and Pittman 2007; Choong et al. 2007). Of the at risk countries, Bangladesh and West Bengal in India are the worst affected (Ahamed et al. 2006; Hassan et al. 2003; Chatterjee et al. 1995; Robertson 1989).

There is clearly a need to develop cost effective technologies to remediate As pollution. Given the differences that exist between arsenic species toxicity, methods capable of converting inorganic arsenic to other, less toxic species have been the subject of much investigation. Microorganisms have shown good potential to detoxify As (Munoz and Guieysse 2006; Jong and Parry 2004). Three major types of As biotransformation have been reported: the reduction or oxidation of inorganic As (Zouboulis and Katsoyiannis 2005), methylation and demethylation (Stolz et al. 2006) and chelation to intracellular cysteine-rich polypeptides (Levy et al. 2005). The most important classes of metal-chelating polypeptides are glutathione (GSH) and its derivative forms, phytochelatins (PCs) which contain thiol groups that bind readily with As species (Schmidt et al. 2007). These peptides can be found in microalgae, related eukaryotic photosynthetic organisms, and some fungi (Perales-Velaet et al. 2006) as organometallic

3

complexes. These may be partitioned inside vacuoles to facilitate appropriate control of the cytoplasmic concentration of heavy metal ions (Cobbett and Goldbrough 2002). In an acid-stable mixed As-SH complex, one molecule of PC_2 (with two –SH groups) and one molecule of GSH were involved in intracellular complexation of each As atom in the green alga *Stichococcus bacullaris* (Pawlik-Skowrońska et al. 2004).

Chorella vulgaris is a common single-cell phytoplankton that tolerates a number of heavy metals and metalloids including As (Nacorda et al. 2007; Rehman and Shakoori 2001; Suhendrayatna Ohki et al. 1999) which has already shown great promise in As removal during field trials in the contaminated district of Ron Phibun in Thailand (Jones et al. 2009). The work presented here studies the effects of As^{5+} on *C. vulgaris*, its ability to accumulate As^{5+} and the role of thiol-peptides in detoxification.

MATERIALS AND METHODS

Culture conditions

Chlorella vulgaris was obtained from Algae and Protozoa, SAMS Research Services Ltd, Dunstaffnage Marine Laboratory (UK). The cells were cultured in Bold Basal medium [$NaNO_3$ (0.25 g), $CaCl_2.2H_2O$ (0.025 g), $MgSO_4.7H_2O$ (0.075 g), $K_2HPO_4.3H_2O$ (0.075 g), KH_2PO_4 (0.025 g) and NaCl (0.025 g) in 1 L sterile distilled water], incubated at room temperature (20-25 °C), aerated at 200 cm^3/min and illuminated at 2500 lux for 72h. In order to prevent any adverse interference with As, no chelating agents were added to the medium.

For the exposure experiment, the algal cells were grown in 500 mL Bold Basal medium containing 5, 10, 15, 50, 100 or 200 mg/L As^{5+} (as Na_2HAsO_4, Fisher Chemicals, UK). The range of concentration used was not intended to stimulate the concentration of As^{5+} present in environmental samples, but to elicit a measurable response in As-exposed *C. vulgaris.* The

control contained no added As^{5+}. The cultures were incubated as described above for 7 days in duplicates.

Analytical methods

As^{5+} was detected using hydride vapour generator (Shimadzu, HVG-1) connected to an atomic absorption spectrometer (HG-AAS, Shimadzu AA6300). Continuous air-acetylene flame was used having flow rates for acetylene and air of 2 L/min and 15 L/min respectively. For hydride generator, the pump speed was set to 5-6 mL/min, carrying gas pressure was 0.32MPa. All reagents used were of analytical grade. AAS grade arsenic standard solution (1mL equivalent to 1mg As^{3+}) was used to prepare the standard solution which was then diluted into 4 gradient levels (5, 10, 15 and 20 µg/L), dionized water was used as blank. AAS grade arsenic standard solutions (1000 mh/L; TraceCERT® Sigma) were prepared immediately before measurement. The standard solutions were prepared immediately before measurement. All the samples were converted to As^{3+} before measurement by addition of 2mL 20% potassium iodide and 2mL (35-37%) hydrochloric acid into 20ml of sample solution, then leave for at least 15 minutes for complete reduction.

As GSH and PCs are normally present at low concentrations in phytoplanktons and are very susceptible to oxidation once isolated from the cells, the handling techniques, rapid sample preparation and storage are critical in ensuring reliability of the results. To ensure all the laboratory glassware were free from metal and organic contamination, they were all acid washed using 1M HCl and rinsed three times using deionised water prior to use. GSH and PCs were extracted using a method modified from Kawakami et al. (2006). To promote the denaturation of enzymes and minimise the oxidation by metals of the –SH group of PCs and GSH, HCl and diethylene triamine pentaacetic acid (DTPA) was added to all samples. Oxidised GSH and PCs were then converted to free thiols by addition of dithiothreitol (DTT). GSH standard was prepared in a mixture of 0.2M HCl containing 5mM DTPA and 5mM DTT in 2:3 ratio; and the

5

final pH adjusted to 11. GSH (reduced, 98%, ACROS Organics) was then dissolved in this reagent to achieve a stock solution of 100 mg/L. Blank samples were prepared using the reagent only without GSH. Standard additions were carried out to determine the recovery of GSH using the above extraction method, samples and blank samples (six of each) were spiked with GSH internal standard to calculate percentage recoveries.

A standard calibration curve was prepared at GSH concentrations between 0 and5 mg/L. The duplicated algal culture (500 mL) was harvested as described above. Cell pellets were resuspended in 2 mL of 0.1M HCl containing 5mM DTPA, and disrupted by rapid freezing in liquid nitrogen followed by defrosting in an ultrasonic bath for 1 hour at 0°C. Prior to the addition of 5 mL of 5 mM DTT, the pH of the cell suspension was adjusted to 10 using 0.1 M NaOH. The sample was then centrifuged for a further 10 min at 500 g and the supernatant analysed for GSH and PCs using reverse-phase HPLC-ESI-MS (Shimadzu, LCMS-2010A) fitted with a reverse phase C18 column (Phenomenex, USA). GSH and PCs were eluted using 1% (v/v) formic acid and LC/MS grade methanol at 0.5mL/min at the following concentration gradients: 0.5% increased to 20% over 25 min and 20% decreased to 0.5% over 5 min. For MS analysis, the nebulizer flow was set at 1.5 L/min, the drying gas at 0.12 MPa and 12 L/min, the detector voltage at 1.5 kV and heater block temperature at 250°C. The signals of GSH (m/z = 308), PC_2 (m/z = 540), PC_3 (m/z = 772), As^{3+}-$(GS)_3$ (m/z = 994), As^{3+}-$(PC_2)_2$ (m/z = 1151) and As^{3+}-PC_3 (m/z = 844) were monitored.

Determination of As^{5+} toxicity

The determination of As^{5+} toxicity was based on changes in cell density and in chlorophyll a content. Cell density was measured using a cell counting chamber (hemacytometer). For each of the experimental group, the initial cell density was 2.5 x 10^5 cell/mL and chlorophyll a level at 2.14 mg/L. Chlorophyll a content was extracted using 90% acetone and determined using a

trichromatic method (EPA-US, 1991). Briefly, a cell suspension (20 mL) was filtered using 25 mm glass fibre filter paper (Whatman FG/C). The filter paper was treated with 10 mL acetone and saturated magnesium carbonate (1 g $MgCO_3$ in 100 mL distilled water) mixture (9:1 v/v) and boiled for 2 min. The extract was separated by centrifugation at 500 g for 10 min and then topped up to 10 mL by with the acetone magnesium carbonate mixture. The optical density of the extract was read at 750nm, 664 nm, 647 nm, 630 nm to calculate the chlorophyll content.

As^{5+} biosorption

The removal of As^{5+} by *C. vulgaris* was measured through its depletion in the growth medium. Algal samples were harvested by centrifuging at 6000 g for 15 min. As^{5+} present in the supernatant was reduced to As^{3+} by treating with 0.4% (w/v) $NaBH_4$ solution. To ensure complete reduction of As^{5+}, 2 mL of 20% (w/v) KI and 2 mL 2M HCl was added to 20 mL of the supernatant and allowed to stand for 15 min at room temperature prior to analysis.

Statistical Analysis

All data analysis in this study was performed using Minitab$^{®}$ 15 statistical software. Assumptions of underlying parametric distributions were tested using the Anderson-Darling normality test. In this paper all data analysed satisfied this assumption. Thus, two sample t-tests and Pearson's correlation were undertaken as appropriate.

RESULTS

As^{5+} toxic effect on cell growth and chlorophyll *a* content

The mean cell counts in the exposed and the control samples did not vary significantly, control and the exposed cultures all having cell counts within the same order of magnitude (2 x 10^7 cell/mL; Table 1). There was no significant difference between the exposed cell counts and the control. The levels of chlorophyll *a* in the exposed cells were lower than those in the control, although the reductions were not statistically significant. A moderate/strong inverse correlation

between chlorophyll *a* production and As^{5+} present in the medium was found. (Pearson correlation [r] = -0.758; p = 0.045).

As^{5+} biosorption and GSH and PCs analysis

A calibration curve for GSH quantification in HPLC-ESI-MS showed a strong regression correlation (r^2 = 0.989) using the procedures listed in the methodology section. Recovery experiment using known concentration of GSH standard achieved an average 88 ± 8 % recovery using the procedures described above. The concentrations of As^{5+} detected in the control and test culture media are presented in Table 1. The lowest (68.6%) and highest (79.7 %) removal efficiency by the algal culture was found to be in media containing 50 and 15 mg/L As^{5+} respectively. A very strong direct correlation (r = 0.991; p<0.001) between the concentration of As^{5+} present in the medium and the amount of As^{5+} removed was observed (Figure 1).

MS spectral analysis of a sample exposed to 5mg/L As^{5+} is presented in Figure 2. PCs were not detected in either the control or the exposed cultures, whereas GSH was found in all samples. Similar patterns were also observed in other samples. The level of GSH in the control was 1.00 ± 0.14 mg/L and in samples exposed up to 50 mg/L As^{5+} a slight increase in GSH level was observed (Table 1). A more substantial increase was recorded in samples exposed to 100 and 200 mg/L As^{5+} where the GSH level was 3.49 ± 0.15 and 6.51 ± 0.53 mg/L respectively. A strong direct correlation between GSH production and concentration of As^{5+} removed was observed (r = 0.969; p < 0.001).

DISCUSSION

This study found *C. vulgaris* to be tolerant to 200 mg/L As^{5+} as the cell density and chloropyhyll *a* content were not significantly affected at this concentration. These findings were in agreement with those of Murray et al. (2003) where *C. vulgaris* was exposed to <0.1, 10, 100 and 1000 mg/L As^{5+} over 5 days. In another study, Goessler et al. (1997) showed that the cell

densities of *C. bohm* and *C. kessleri* were enhanced by 40% in the presence of 2000 mg/L As^{5+} compared to the As^{5+} free control, but similar stimulation was not observed in this study. It is noteworthy that although the reduction of chlorophyll *a* in the exposed samples was not statistically significantly different from the As^{5+} free control, there was an inverse correlation between the level of As^{5+} present in the medium and the chlorophyll produced. It is likely that the presence of phosphate in the growth medium mitigated any toxic effect of As^{5+}. Arsenic is transported through cell membranes into the cell through the phosphate channel (phosphate inorganic transport [Pit] and phosphate specific transport [Pst] systems (Levy et al. 2005)). The high concentration of phosphate in the medium solution (about 5 g/L) may initially compete successfully with As^{5+} resulting in low levels of intracellular As^{5+}. However, as As^{5+} concentration increases, it may out- compete phosphate causing an increased in intracellular As^{5+}. Karadjova et al. (2008) also showed that increases in phosphate content in culture media up to 1.3 mg/L significantly decreased the toxicity of arsenate and arsenite in *Chlorella salina*.

Between the range of 1-200 mg As/L *C. vulgaris* was able to remove between 69 to 79 % of As^{5+} present in the medium irrespective of the initial As^{5+} concentration. This suggests that a defence mechanism in *C. vulgaris* may be triggered at concentrations as low as 5 mg/L. The removal efficiency of As^{5+} *by C. vulgaris* in this study also suggests that it is related to the initial As^{5+} concentration present in the medium. It has been shown that As^{5+} can be removed by mechanisms such as surface binding or intracellular chelation by GSH or PC in a number of green algae (Pawlik-Skowrońska et al. 2004; Morelli et al. 2005; Kobayashi et al. 2006). In this study only GSH was observed in both the control and exposed samples and its level increased significantly with increased concentration of As^{5+} (after the concentration of As^{5+} had reached a certain level). In contrast, no PC was detected in any of the exposed samples, being below the detection limit of the HPLC-ESI-MS method of approximately 0.2 µmol/L. It appears that GSH plays a more prominent role in the detoxification of As^{5+} in *C. vulgaris* than PC.As PC and GSH

degrade readily in the presence of oxygen (Kawakami et al. 2006; El-Zohri et al. 2005), it is possible that very low levels of PC was produced, and despite the care taken during the extraction process, they auto-oxidised to a level below the detection limit. Nevertheless, this still supports the hypothesis that GSH plays a more noticeable role in the remediation of As in *C. vulgaris*. The extraction protocol proposed by Simmons et al. (2009) where samples are treated under an inert gas environment will significantly reduce the loss of PC and should be employed in future studies.

It is noted that 100 mg/L As^{5+} appeared to be the trigger value in the production of GSH in *C. vulgaris* in this study, as there are no significant changes of GSH levels in the cells below this concentration, and significant increases were observed at or above 100 mg/L. At concentrations below 100 mg/L other metalloid-binding mechanisms and the presence of phosphate in the medium may play the primary role in reducing As^{5+} toxicity. Above this critical concentration, As^{5+} may be accumulating inside the algal cells and causing the activation of a number of intracellular cell defence mechanisms, such as increased production of GSH. However, the GSH concentration observed in this study was between 0.8 – 6.5 mg/L (or 2.7 - 21 µmol); and the concentrations of As^{5+} taken up by the cells ranged from 3.5 -155.2 mg/L (or 19.3 to 596 µmol). Assuming 1 mol of arsenic (As^{5+}) reacts with at least1 mol of GSH (Raab et al. 2004), the expected concentration of GSH to chelate 20-596 µmol As^{5+} would be significantly more than the concentration observed in this study. Therefore, it would be possible to surmise that forming intracellular thiol complex is not the major detoxification mechanism in *C. vulgaris* when exposed to As^{5+}.

It is noted that the concentrations used in this study were above those expected in drinking water samples; however, they are comparable to those found in mining wastewater (Garelick et al. 2009). In environmental samples where As level were below those tested in this study, other mechanisms may play an important role in detoxification by *C. vulgaris*. Mechanisms such as

cell surface binding, bio-reduction of As^{5+} to As^{3+} and subsequent methylation may play a significant role in removing As^{5+} from the growth medium (Levy et al. 2005; Hellweger et al. 2003). As^{5+} can be reduced to As^{3+} which can be rapidly expelled possibly via arsenic anion pump comprised of three polypeptide : ArsA, ArsB and AsrC (Levy et al. 2005; Ji and Silver, 1995; Nies and Silver, 1995; Rensing, Ghosh and Rosen, 1999; Hellweger, 2003). This is also supported by the observations that no As^{3+}-(GS)$_3$ was found in any sample (Figure 2).

Anion efflux is a defence mechanism against arsenic toxicity observed in another microalgae *Monoraphidium arcuatum* (Levy et al. 2005). As this study only measured the level of As^{5+}, it will be useful to differentiate the arsenic species present in the medium at the conclusion of the experiment in future studies to verify the involvement of biotransformation of As^{5+} to As^{3+} in *C. vulgaris*. It will also be interesting to ascertain the involvement of any organic As species which indicates positive methylation.

This work along with ongoing studies will contribute to a deeper understanding of the roles of GSH and PCs in As detoxification. Further studies should be carried out to confirm the trigger value for GSH production, based on the current study, the range would be between 50 and 100 mg/L. It is speculated that given the apparent greater prominence of GSH (and likely low/negligible involvement of PCs) in the detoxification mechanism, that targeting of enhanced GSH production (even in the absence of PCs production) via genetic modification or strain selection of the species may ultimately lead to enhancement/optimisation of the detoxification of As by *C. vulgaris.*

CONCLUSION

C. vulgaris was found to tolerate 200 mg/L As^{5+} and was capable in removing up to 70% of the As^{5+} present in the growth medium. In this study, the presence of As^{5+} above 100 mg/L appears to trigger significant production of GSH. The absence of PCs and their arsenic combined

11

complexes indicate the high bioaccumulation and tolerance to arsenic is not due to intracellular chelation. This paper further supports practical field experience that the application of *C. vulgaris* is a promising low cost As phytoremediation method for developing countries.

ACKNOWLEDGEMENT

REFERENCES

Ahamed, S., Sengupta, M. K., Mukherjee, A., Hossain, M. A., Das, B., Nayak, B., Pal, A., Mukhejee, S. C., Pati, S., Dutta, R. N., Chattejee, G., Mukhejee, A., Srivastava, R., and Chakraborti, D. 2006. Arsenic groundwater contamination and its health effects in the state of Uttar Pradesh (UP) in upper and middle Ganga plain, India: a severe danger. *Sci. Total Environ.* **370,** 310-322.

Chatterjee, A., Das, D., Mandal, B. K., Chowdhury, T. R., Samanta, G., and Chakarborti, D. 1995. Arsenic in groundwater in six districts of West Bengal, India: the biggest arsenic calamity in the world. Part 1. Arsenic species in drinking water and urine of the affected people. *Analyst* **120,** 643-656.

Choong, T. S. Y., Chuah, T. G., Robiah, Y., Koay, G., and Azni, I. 2007. Arsenic toxicity, health hazards and removal techniques from water: an overview. *Desal.* **217,** 139-166.

Cobbett, C,. and Goldbrough, P. 2002. Phytochelatin and metallothioneins: Roles in heavy metal detoxification and homeostasis. *Ann. Rev. Plant Biol.* **53,** 159-182.

EPA-US. 1991. *Chlorophyll – spectrophotometric*. EPA, Madison. ESS Methods 150.1.

Garelick, H., Jones, H., Dybowska, A. and Valsami-Jones, E. 2009. Arsenic pollution sources. In *Rev. Environ. Contam. Toxicol.* **197**, 17-60.

Goessler, W., Lintschinger, J., Szakova, J., Mader, P., Kopecky, J., and Doucha, J. 1997.
Chlorella sp and arsenic compounds: An attempt to prepare an algal reference material for
arsenic compounds. *Appl. Organmet. Chem.* **11**, 57-66.

Grill, E., Loffler, S., Winnacker, E-L., and Zenk, M. H. 1989. Phytochelatins, the heavy-metal-
binding peptides of plants, are synthesized from glutathione by a specific γ-
glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). *Proc. Natl. Acad. Sci.
USA.* **86,** 6838–6842.

Hassan, M. M, Atkins, P. J., and Dunn C. E. 2003. The spatial pattern of risk from arsenic
poisoning: A Bangladesh case study. *J. Env. Sci. Heal. Part A.* **38**, 1-24.

Hellweger, F. L., Farley, K. J., Lall, U., and Di Toro, D. M. 2003. Greedy algae reduce arsenate.
Liminol. Oceano. **48,** 2275-2288.Ji, G. Y., and Silver, S. 1995. Bacterial resistance
mechanisms for heavy metals of environmental concern. *J. Ind. Microbiol.* **14**, 61-75.

Jones, H., Visoottiviseth, P., Bux, M. K. Földényi, R., Kováts, N., Borbély, G., and Galbács, Z.
2009. Case Reports: Arsenic Pollution in Thailand, Bangladesh and Hungary. In: *Reviews
of Environmental Contamination and Toxicology. Arsenic Pollution and Remediation: An
International Perspective.* Volume 197, pp. 163-187 (Garelick, H. and Jones, H. Eds).
Springer, New York.

Jong, T., and Parry, D. L. 2004. Adsorption of Pb(II), Cu(II), Cd(II), Zn(II), Ni(II), Fe(II), and
As(V) on bacterially produced metal sulphides. *J. Colloid Inter. Sci.* **275,** 61-71.

Karadjova, I B., Slaveykova, V. I., and Tsalev, D. L. 2008. The biouptake and toxicity of arsenic
species on the green mciroalga *Chorella salina* in seawater. *Aqu. Toxicol.* **87,** 264-271.

Kawakami, S. K., Gledhill, M., and Achterberg, E. P. 2006. Determination of phytochelatins and
glutathione in phytoplankton from natural waters using HPLC with fluorescence detection.
Trac-Trends Anal. Chem. **25**, 133-142.

Kobayashi, I., Fujiwara, S., Saegusa, H., Matsumoto, H., and Tsuzuki, M. 2006. Relief of arsenate toxicity by Cd-stimulated phytochelatin synthesis in the green alga *Chlamydomonas reinhardtii*. *Mar. Biotechnol.* **8**, 94-101.

Korte, N. E., and Fernando, Q. 1991. A review of arsenic (III) in groundwater. *Cri. Rev. Environ. Control* **21**, 1-39.

Levy, J. L., Stauber, J. L, Adams, M. S., Maher, W. A., Kirby, J. K., and Jolley, D. E. 2005. Toxicity, biotransformation, and mode of action of arsenic in two freshwater microalgae (*Chlorella* sp and *Monoraphidium arcuatum*). *Environ. Toxicol. Chem.* **24**, 2630-2639.

Lorenzen, L., van Deventer, J. S. J., and Landi, W. M. 1995. Factors affecting the mechanism of the adsorption of arsenic species pm activated carbon. *Min. Eng.* **8**, 557-569.

Mandal, B. K., and Suzuki, K. T. 2002. Arsenic round the world: a review. *Talanta* **58,** 201-235.

Mohan, D., and Pittman, C. U. 2007. Arsenic removal from water/wastewater using adsorbents - a critical review. *J. Hazard. Mat.* **142,** 1-53.

Morelli, E., Mascherpa, M., and Scarano, G. 2005. Biosynthesis of phytochelatins and arsenic accumulation in the marine microalga *Phaeodactylum tricornutum* in response to arsenate exposure. *Biomet.* **18**, 587-593.

Munoz, R., and Guieysse, B. 2006. Algal-bacterial processes for the treatment of hazardous contaminants: A review. *Water Res.* **40**, 2799-2815.

Murray, L. A., Raab, A., Marr, I. L., and Feldmann, J. 2003. Biotransformation of arsenate to arsenosugars by *Chlorella vulgaris*. *Appl. Organomet. Chem.* **9**, 669-674.

Nacorda, J. O., Martinez-Goss, M. R., Torreta, N. K., and Merca, F. E. 2007. Metal resistance and removal by two strains of the green alga, *Chlorella vulgaris* Beijerinck, isolated from Laguna de Bay, Philippines. *J. Appl. Phycol.* **19**, 701-710.

Nies, D. H., and Silver, S. 1995. Ion efflux systems involved in bacterial metal resistances. *J. Ind. Microbiol.* **14,** 186-199.

Pawlik-Skowrońska, B., Pirszel, J., Kalinowska, R., and Skowroński, T. 2004. Arsenic availability, toxicity and direct role of GSH and phytochelatins in As detoxification in the green alga *Stichococcus bacillaris*. *Aqua. Toxicol.* **70**, 201-212.

Perales-Vela, H. Y., Peña-Castro, J. M., and Cañizares-Villanueva, R. O. 2006. Heavy metal detoxification in eukaryotic microalgae. *Chemosphere* **64**, 1-10.

Raab, A., Feldmann, J., and Meharg, A. A. 2004. The nature of arsenic-phytochelatin complexes in *Holcus lanatus* and *Pteris cretica*. *Plant Physiol.* **134**, 1113-1122.

Rehman, A., and Shakoori, A. R. 2001. Heavy metal resistance *Chlorella* spp., isolated from tannery effluents, and their role in remediation of hexavalent chromium in industrial waste water. *Bull. Environ. Contam. Toxicol*. **66**, 542-547.

Rensing, C., Ghosh, M., and Rosen, B. 1999. Families of soft-metal-ion-transporting ATPases. *J Bacteriol.* **181,** 5891-5897.

Robertson, F. N. 1989. Arsenic in ground water under oxidizing conditions, south-west United States. *Environ. Geochem. Health* **11**, 171-176.

Schmidt, A. C., Koppelt, J., Neustadt, M., and Otto, M. 2007. Mass spectrometric evidence for different complexes of peptides and proteins with arsenic(III), arsenic(V), copper(II), and zinc(II) species. *Rapid Commun. Mass.* **Sp 21,** 153-163.

Simmons, D. B. D., Hayward, A. R., Hutchinson, T. C. and Emery, R. J. N. 2009. Identification and quantification of glutathione and phytochelatins from Chlorella vulgaris by RP-HPLC ESI-MS/MS and oxygen-free extraction. *Anal. Bioanal. Chem*. **395**, 809-817.

Stolz, J. E., Basu, P., Santini, J. M., and Oremland, R. S. 2006. Arsenic and selenium in microbial metabolism. *Ann. Rev. Microbiol.* **60**, 107-130.

Suhendrayatna Ohki, A., Kuroiwa, T., and Maeda, S. (1999) Arsenic compounds in the freshwater green microalga *Chlorella vulgaris* after exposure to arsenite. *Appl. Organomet. Chem.* **13,** 127-133.

WHO (World Health Organisation). 2008. *Guidelines for Drinking-water Quality*. Volume 1 (3rd ed) World Health Organisation, Geneva

Zouboulis, A. I., and Katsoyiannis, I. A. 2005. Recent advances in the bioremediation of arsenic-contaminated groundwaters. *Environ. Int.* **31**, 213-219.

Table 1 Mean cell numbers (x 10^7/mL) and chlorophyll *a* content (mg/L) of *Chlorella vulgaris* culture (± standard deviation) together with GSH levels (mg/L ± standard deviation), arsenic concentrations and removal %.

Initial As^{5+} concentration in growth medium (mg/L)	Cell number (x 10^7 cell/mL)	Chlorophyll *a* content (mg/L)	As^{5+} concentration in growth medium after 7 days (mg/L)	Average As^{5+} removed (mg/L)	As removal (%)	GSH concentration (mg/L)
0 (control)	2.64 ± 0.36	7.20 ± 0.50	0.00 ± 0.00	0	0.00	1.00 ± 0.14
5	2.48 ± 0.07	7.55 ± 0.09	1.45 ± 0.06	3.55	70.89	1.55 ± 0.16
10	2.65 ± 0.13	7.15 ± 0.34	2.25 ± 0.01	7.75	77.50	1.88 ± 0.23
15	2.38 ± 0.23	7.40 ± 0.08	3.09 ± 0.06	11.91	79.73	0.83 ± 0.06
50	2.62 ± 0.08	6.85 ± 0.12	15.70 ± 0.10	34.3	68.60	1.42 ± 0.07
100	2.63 ± 0.03	6.87 ± 0.22	29.91 ± 0.28	70.09	70.09	3.49 ± 0.15
200	2.78 ± 0.09	6.78 ± 0.20	44.76 ± 0.64	155.24	77.62	6.51 ± 0.53

Fig. 1 Linear relationship between the concentration of As removed and the level of As present in the medium. The algal culture was grown in 500 mL Bold Basal medium containing 5, 10, 15, 50, 100 or 200 mg/L As^{5+} at room temperature (20-25 °C), aerated at 200 cm^3/min and illuminated at 2500 lux for 72h. The concentration of As removed is listed ± standard deviation.
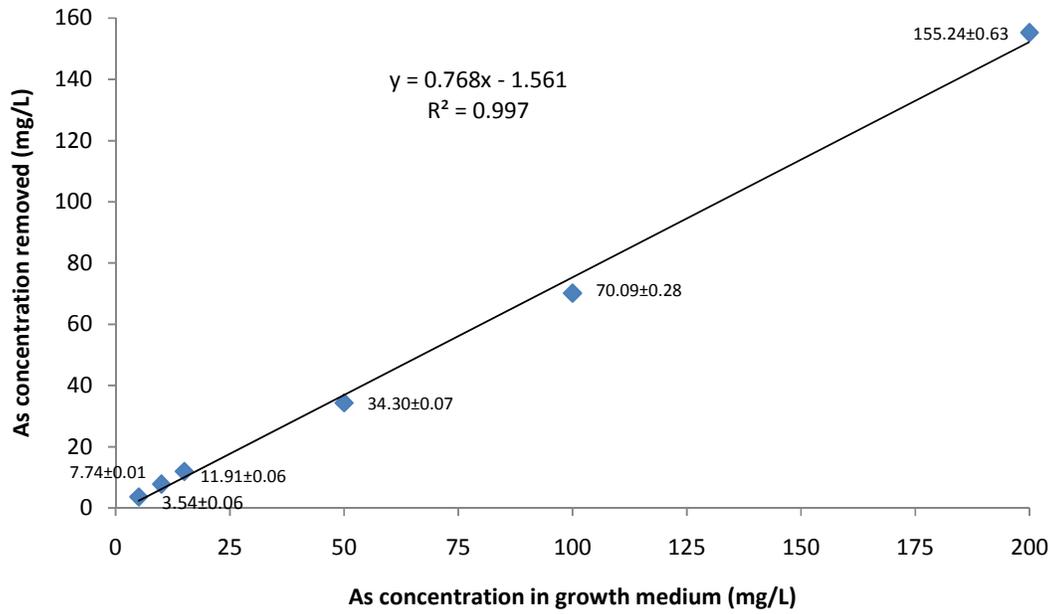
Fig. 2 Total ion counts of cell extracts exposed to 5mg/L As^{5+} in SIM (Select Ion Monitor) mode. GSH (m/z=308), PC$_2$ (m/z=540), PC$_3$ (m/z=772), As^{3+}-(GS)$_3$ (m/z=994), As^{3+}-(PC$_2$)$_2$ (m/z=1151) and As^{3+}-PC$_3$ (m/z=844) ions have been monitored; the only significant signal that can be observed is that of glutathione (GSH).