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## **Effects of arsenate (As<sup>5+</sup>) on growth and production of glutathione (GSH) and phytochelatins (PCs) in *Chlorella vulgaris***

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### **ABSTRACT**

The effect of arsenate (As<sup>5+</sup>) 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<sup>5+</sup> at up to 200 mg/L and was capable of consistently removing around 70% of the As<sup>5+</sup> 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<sup>5+</sup> suggesting that GSH plays a more prominent role in the detoxification of As<sup>5+</sup> in *C. vulgaris* than PC. At concentrations below 100 mg/L cell surface binding and other mechanisms may play the primary role in As<sup>5+</sup> detoxification, whereas above this concentration As<sup>5+</sup> 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<sup>3+</sup> and pentavalent As<sup>5+</sup> 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

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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, Deventer and Landi, 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, Atkins and Dunn, 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 Guiyesse, 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-Vela, Peña-Castro and Cañizares-Villanueva, 2006) as organometallic 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<sub>2</sub> (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).

*Chlorella 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, Kuroiwa and Maeda, 1999) and 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<sup>5+</sup> on *C. vulgaris*, its ability to accumulate As<sup>5+</sup> 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<sub>3</sub> (0.25 g), CaCl<sub>2</sub>.2H<sub>2</sub>O (0.025 g), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.075 g), K<sub>2</sub>HPO<sub>4</sub>.3H<sub>2</sub>O (0.075 g), KH<sub>2</sub>PO<sub>4</sub> (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<sup>3</sup>/min and illuminated at 2500 lux for 72h. Trace metals and chelating agent was not added in the medium to prevent adverse interferences and absorption of As.

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<sup>5+</sup> (as Na<sub>2</sub>HAsO<sub>4</sub>, Fisher Chemicals, UK), no As precipitation was observed in any of the solution. The range of concentration used was not intended to stimulate the concentration of As<sup>5+</sup> present in environmental samples, but to elicit a measurable response in As-exposed *C. vulgaris*. The control contained no added As<sup>5+</sup>. The cultures were incubated as described above for 7 days in duplicates.

### Analytical methods

As GSH and PCs are normally present at low concentrations in phytoplankton 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 deionized 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 minimize the oxidation by metals of the –SH group of PCs and GSH, HCl, and diethylene triamine pentaacetic acid (DTPA) was added to all samples. Oxidized GSH and PCs were then converted to free thiols by addition of dithiothreitol (DTT). GSH standard was prepared in a mixture of 0.2 M HCl containing 5 mM DTPA and 5 mM DTT in 2:3 ratio; and the 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 and 5 mg/L. The duplicated algal culture (500 mL) was harvested as described above. Cell pellets were resuspended in 2 mL of 0.1 M HCl containing 5 mM 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 0.1M NaOH. The sample was then centrifuged for a further 10 min at 500 g and the supernatant analyzed 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 0.5 mL/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 GSH (m/z = 308), PC<sub>2</sub> (m/z = 540), PC<sub>3</sub> (m/z = 772), As<sup>3+</sup>-(GS)<sub>3</sub> (m/z = 994), As<sup>3+</sup>-(PC<sub>2</sub>)<sub>2</sub> (m/z = 1151) and As<sup>3+</sup>-PC<sub>3</sub> (m/z = 844) were monitored.

### **Determination of As<sup>5+</sup> toxicity**

The determination of As<sup>5+</sup> 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  $2.5 \times 10^5$  cell/mL and chlorophyll *a* level 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<sub>3</sub> 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<sup>5+</sup> biosorption**

The removal of As<sup>5+</sup> 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<sup>5+</sup> present in the supernatant was reduced to As<sup>3+</sup> by treating with 0.4% (w/v) NaBH<sub>4</sub> solution. To ensure complete reduction of As<sup>5+</sup>, 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<sup>5+</sup> toxic effect on cell growth and chlorophyll *a* content**

The mean cell counts in the exposed and the control samples did not vary significantly (two sample t-test,  $p>0.05$ ), the control and the exposed cultures all having cell counts within the same order of magnitude ( $2 \times 10^7$  cell/mL; Table 1). The levels of chlorophyll *a* in the exposed cells were lower than those in the control, although the reductions were not statistically significant (two sample t-test,  $p>0.05$ ). A moderate/strong inverse correlation between chlorophyll *a* production and As<sup>5+</sup> present in the medium was found. (Pearson correlation [ $r$ ] = -0.758;  $p$  = 0.045).

### As<sup>5+</sup> biosorption and GSH and PCs analysis

A calibration curve for GSH quantification in HPLC-ESI-MS showed a strong relationship ( $r^2 = 0.989$ ) using the procedures listed in the methodology section. The concentrations of As<sup>5+</sup> 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<sup>5+</sup> respectively. A very strong direct correlation ( $r = 0.991$ ;  $p<0.001$ ) between the concentration of As<sup>5+</sup> present in the medium and the amount of As<sup>5+</sup> removed was observed (Figure 1).

MS spectral analysis of a sample exposed to 5mg/L As<sup>5+</sup> 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 mg/L and in samples exposed up to 50 mg/L As<sup>5+</sup> a slight increase in GSH level was observed. A more substantial increase was recorded in samples exposed to 100 and 200 mg/L As<sup>5+</sup> where the GSH level was 3.49 and 6.51 mg/L respectively. Pearson correlation analysis demonstrated a strong direct correlation between GSH production and concentration of initial As<sup>5+</sup> concentration ( $r = 0.964$ ;  $p < 0.001$ ), as well as concentration of As<sup>5+</sup> being removed ( $r = 0.969$ ;  $p < 0.001$ ).

## DISCUSSION

This study found *C. vulgaris* to be tolerant to 200 mg/L As<sup>5+</sup> as the cell density and chlorophyll *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<sup>5+</sup> 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<sup>5+</sup> compared to the As<sup>5+</sup> 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<sup>5+</sup> free control, there was an inverse correlation between the level of As<sup>5+</sup> 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<sup>5+</sup>. 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<sup>5+</sup> resulting in low levels of intracellular As<sup>5+</sup>. However, as As<sup>5+</sup> concentration increases, it may out-compete phosphate causing an increased in intracellular As<sup>5+</sup>. Karadjova, Slaveykova and Tsalev (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<sup>5+</sup> present in the medium irrespective of the initial As<sup>5+</sup> 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<sup>5+</sup> by *C. vulgaris* in this study also suggests that it is related to the initial As<sup>5+</sup> concentration present in the medium. It has been shown that As<sup>5+</sup> can be removed by mechanisms such as surface binding or intracellular chelation by GSH or PC in a number of green algae (Pawlak-Skowrońska *et al.*, 2004; Morelli, Mascherpa and Scarano, 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<sup>5+</sup> (after the concentration of As<sup>5+</sup> 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  $\mu$ mol/L. It appears that GSH plays a more prominent role in the detoxification of As<sup>5+</sup> in *C. vulgaris* than PC. The synthesis of PC from GSH is catalysed by PC synthase (Grill *et al.*, 1989), a constitutive enzyme (a type of enzyme continuously produced in the cell without external induction to trigger its production, as opposed to an

adaptive enzyme) with no apparent gene regulatory activity (Perales-Vela, Peña-Castro and Cañizares-Villanueva, 2006). Deficiency in the PC synthase gene may account for the absence of PCs even though GSH was found in the exposed cells. Although beyond the scope of this study, further investigation into the genetic makeup of *C. vulgaris* may shed light on this apparent paradox.

It is noted that 100 mg/L As<sup>5+</sup> appeared to be the trigger value in the production of GSH in *C. vulgaris* 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<sup>5+</sup> toxicity. Above this critical concentration, As<sup>5+</sup> 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 mmol); and the concentrations of As<sup>5+</sup> taken up by the cells ranged from 3.5 -155.2 mg/L (or 19.3 to 596 mmol). Assuming 1 mol of arsenic (As<sup>5+</sup>) reacts with at least 1 mol of GSH (Raab *et al.*, 2004), the expected concentration of GSH to chelate 20-596 mmol As<sup>5+</sup> 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<sup>5+</sup>.

Mechanisms such as cell surface binding, bio-reduction of As<sup>5+</sup> to As<sup>3+</sup> and subsequent methylation may play a significant role in removing As<sup>5+</sup> from the growth medium (Levy *et al.*, 2005; Hellweger *et al.*, 2003). As<sup>5+</sup> can be reduced to As<sup>3+</sup> 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<sup>3+</sup>-(GS)<sub>3</sub> 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<sup>5+</sup>, 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<sup>5+</sup> to As<sup>3+</sup> 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. 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<sup>5+</sup> and was capable in removing up to 70% of the As<sup>5+</sup> present in the growth medium. The presence As<sup>5+</sup> above 100 mg/L appears to trigger significant production of GSH. The absence of PCs and their arsenic combined 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.

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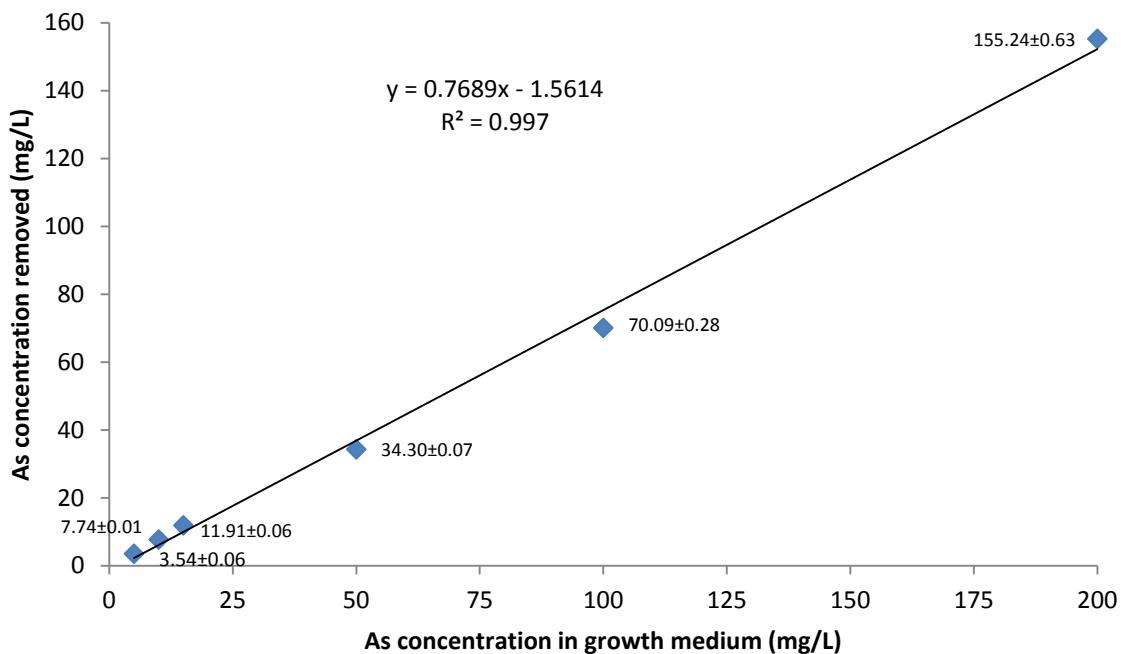
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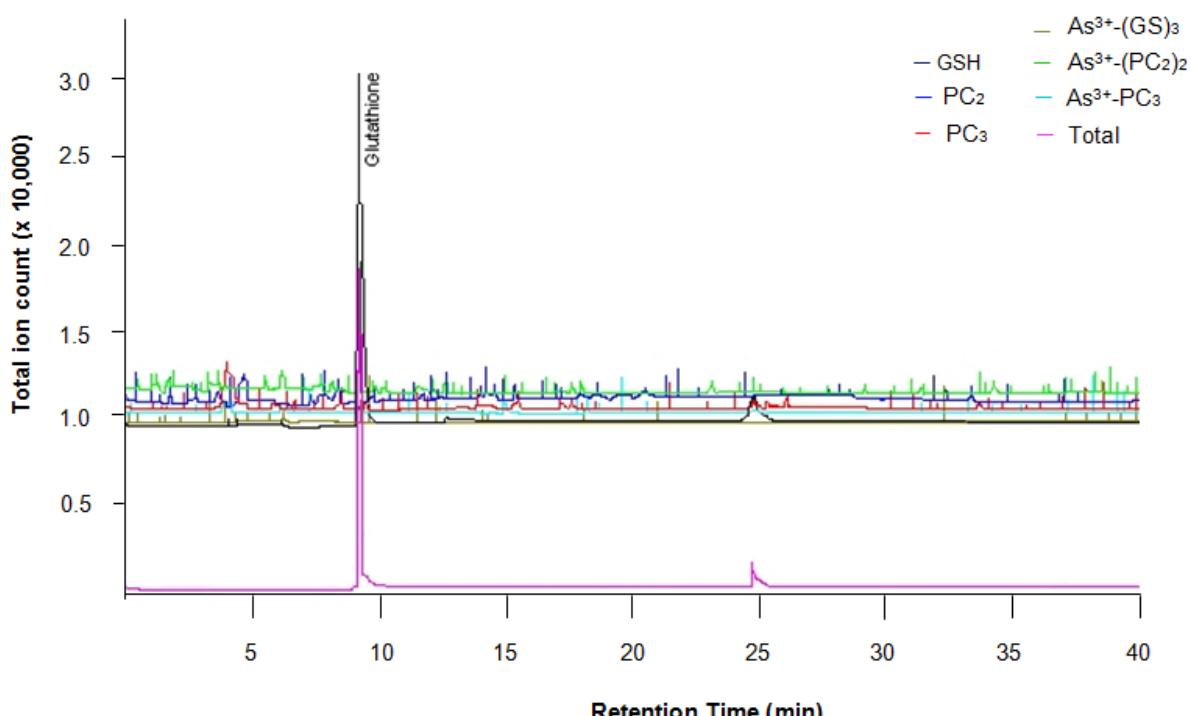
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**Fig. 1** Linear relationship between the concentration of As removed and the level of As present in the medium. The algal culture was grown in 500 mL Bold Basal medium containing 5, 10, 15, 50, 100 or 200 mg/L As<sup>5+</sup> at room temperature (20-25 °C), aerated at 200 cm<sup>3</sup>/min and illuminated at 2500 lux for 72h. The concentration of As removed is listed  $\pm$  standard deviation.



**Fig. 2** Total ion counts of cell extracts exposed to 5mg/L As<sup>5+</sup> in SIM (Select Ion Monitor) mode. GSH (m/z=308), PC<sub>2</sub> (m/z=540), PC<sub>3</sub> (m/z=772), As<sup>3+</sup>-(GS)<sub>3</sub> (m/z=994), As<sup>3+</sup>-(PC<sub>2</sub>)<sub>2</sub> (m/z=1151) and As<sup>3+</sup>-PC<sub>3</sub> (m/z=844) ions have been monitored; the only significant signal that can be observed is that of glutathione (GSH).

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

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