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Aquatic Toxicology

The mechanisms of detoxification of As(III), dimethylarsinic acid (DMA) and As(V) in the microalga *Chlorella vulgaris*

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Abstract

The response of *Chlorella vulgaris* when challenged by As(III), As(V) and dimethylarsinic acid (DMA) was assessed through experiments on adsorption, efflux and speciation of arsenic (reduction, oxidation, methylation and chelation with glutathione/phytochelatin [GSH/PC]). Our study indicates that at high concentrations of phosphate (1.62 mM of HPO_4^{2-}), upon exposure to As(V), cells are able to shift towards methylation of As(V) rather than PC formation. Treatment with As(V) caused a moderate decrease in intracellular pH and a strong increase in the concentration of free thiols (GSH). Passive surface adsorption was found to be negligible for living cells exposed to DMA and As(V). However, adsorption of As(III) was observed to be an active process in *C. vulgaris*, because it did not show saturation at any of the exposure periods. Chelation of As(III) with GS/PC and to a lesser extent hGS/hPC is a major detoxification mechanism employed by *C. vulgaris* cells when exposed to As(III). The increase of bound As-GS/PC complexes was found to be strongly related to an increase in concentration of As(III) in media. *C. vulgaris* cells did not produce any As-GS/PC complex when exposed to As(V). This may indicate that a reduction step is needed for As(V) complexation with GSH/PC. *C. vulgaris* cells formed DMASV -GS upon exposure to DMA independent of the exposure period. As(III) triggers the formation of arsenic complexes with PC and homophytochelatins (hPC) and their compartmentalisation to vacuoles. A conceptual model was devised to explain the mechanisms involving ABCC1/2 transport. The potential of *C. vulgaris* to bio-remediate arsenic from water appeared to be highly selective and effective without the potential hazard of reducing As(V) to As(III), which is more toxic to humans.

1. Introduction

Chlorella vulgaris (*C. vulgaris*) is a common single-cell green microalga that tolerates a number of heavy metals and metalloids including arsenic (Jones et al., 2008; Wang et al., 2015). The mech- anisms for such tolerance may be via the reduction or oxidation of inorganic arsenic (Zouboulis and Katsoyiannis, 2005), methylation and demethylation (Stolz et al., 2006) and chelation to intracel- lular cysteine-rich polypeptides such as glutathione (GSH) and its homologues (hGSH) (where Gly is substituted by Ala, Ser, Glu and Gln or is absent) (Levy et al., 2005; Nacorda et al., 2007; Rehman and Shakoori, 2001; Suhendrayatna et al., 1999).

Production of phytochelatins (PC_n, (')'-glutamyl-l-cysteinyl)n-glycine, where n = 2-11) and GSH/hGSH derivatives including homophytochelatins (hPC) by *C. vulgaris* has been investigated by several groups but has only been found in experiments with cells challenged with cadmium and selenate (under oxygen-free condi- tions) in which only the presence of GSH, PC_2, PC_3 were reported (Simmons and Emery, 2011; Simmons et al., 2009). All attempts to identify intact metal/loid-GS/PC complexes in *C. vulgaris* cells have failed until the recent application of an enhanced sonication extraction method by Pantoja et al. (2014).

It has been demonstrated that the formation of As-GS/PC alone does not confer tolerance in model plant organisms, but a compart-mentalisation step is required to lower the cytosolic concentration of toxic arsenic as indicated by the contradictory results in which the overexpression of PC has led to hypersensitive mutants (Lee et al., 2003; Li et al., 2004; Wojas et al., 2008) and ABCC (ATP Binding Cassette subfamily C) knock-out gene mutants continued

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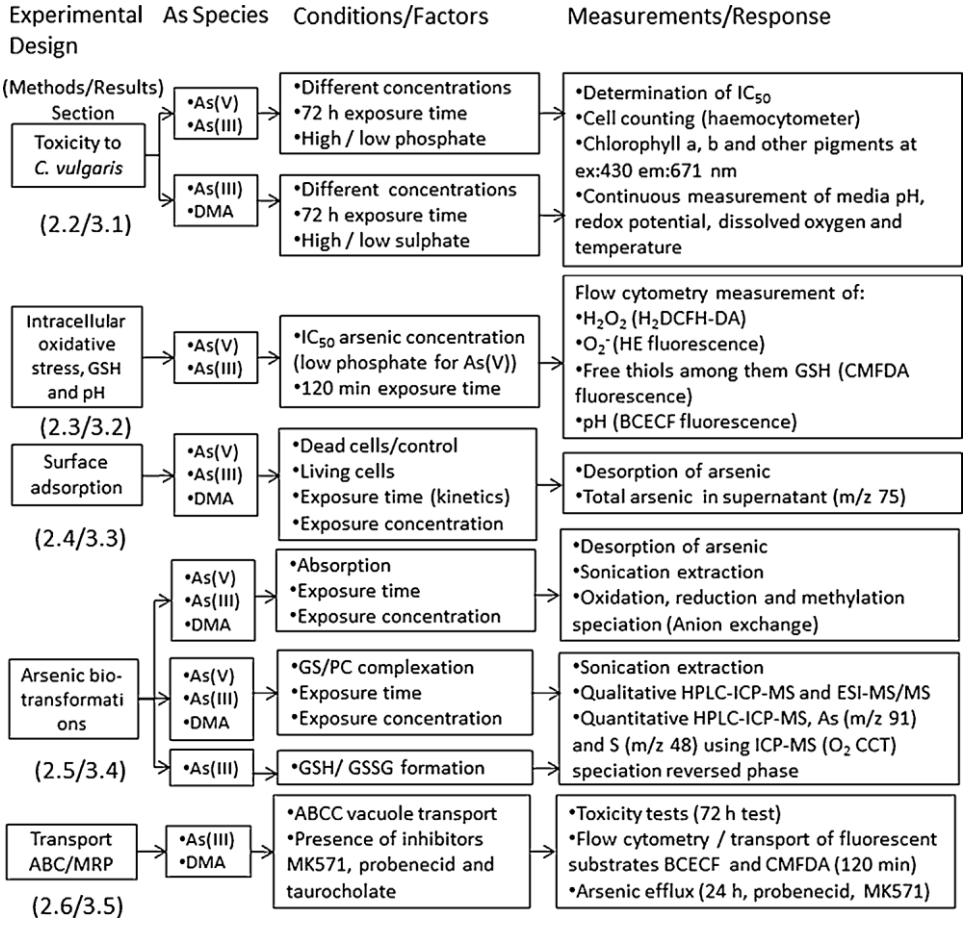

Fig. 1. Overview of the experimental methodology with methods/results sections outlined.

to exhibit PC accumulation into vacuoles (Prévéral et al., 2009; Sooksa-Nguan et al., 2009). The transport mechanism for As-PC complexes to vacuoles has been elucidated in Arabidopsis thaliana where it is mediated by two proteins: AtABCC1 and AtABCC2 (Song et al., 2010). Other studies have demonstrated that As-GS transport is mediated by MRP2, an analogue of ABCC2 in mammalian cells (Kala et al., 2000; Lu et al., 1998). ABCC 1/2 are a subfamily of ABC/MRP (ATP Binding Cassette, Multidrug Resistance Associated Protein) transporters in plants. These proteins are Mg-ATP hydrolysis driven pumps which are situated in cell membranes and membranes of vacuoles (Lu et al., 1998). Specific inhibitors of ABCC1/2 transport can help the investigation of arsenic tolerance in algae by measuring sensitivity of cells with and without their addition; e.g. MK571, taurocholate and probenecid (Long et al., 2011; Lu et al., 1998; Wong et al., 2007).

A number of fluorescent dyes that act as substrates for ABCC1/2 and MRP1/2 in a variety of cells have been used to study such transport (Table S1). To date, most of the ABCC1/2 transporters characterized in plants are localized in the vacuolar membrane and considered to be involved in the intracellular sequestration of xenobiotics (Forestier et al., 2003; Perales-Vela et al., 2006; Swanson et al., 1998). Only certain ABCC1/2 transporters expressed in the plasma membrane might be involved in defence metabolite secretion, and thus their expression is regulated by the concentration of these metabolites (Jasinski et al., 2001).

Recent experiments have demonstrated that at low concentrations of phosphate, different species of microalgae and bacteria are more sensitive to As(V) than As(III), because phosphate reduces some of the toxicity of its 'analogue' arsenate (Karajova et al., 2008; Knauer et al., 1999; Levy et al., 2005; Pawlik-Skowronska et al., 2004; Rubinos et al., 2014; Stolz et al., 2006; Wang et al., 2014) and probably because there is no analogue for As(III) that controls growth rate as phosphate does in the presence of As (V). However, some photosynthetic organisms such as the floating macrophyte Lemna disperm are more sensitive to As(III) than As(V) under low phosphate conditions (Rahman et al., 2014). Thus, it is imperative to measure arsenic toxicity under different concentrations of phosphate and perhaps other nutrients in order not to under or overestimate such toxicity.

Oxidative stress is one of the main toxic effects in plant species that are sensitive to arsenic. These effects are manifested as enzyme inhibition, protein oxidation, lipid peroxidation and DNA and RNA damage (Meharg and Hartley-Whitaker, 2002; Shahid et al., 2014). However, as a redox-inactive stressor, only As(III) can inhibit enzymatic activities as a result of its affinity for −SH groups. In contrast, As(V) does not have such strong affinity for −SH and therefore, strictly speaking, should not be considered an oxidative stressor unless enzymatic reduction takes place (Cuypers et al., 2011; Mishra et al., 2006). In such cases, GSH plays an important role as a reducing agent, a scavenger of reactive oxygen species, substrate of PC formation and a redox buffer. While the redox buffer pairs (e.g. GSH/GSSG, reduced PC/oxidised PC, and reduced Protein/oxidised Protein) can protect cells from oxidative damage (Tsuji et al., 2002), this produces an imbalance in the redox status that may lead to other unwanted effects such as changes in intracellular pH, which is, to our knowledge, an effect that has not been explored to date.

The present investigation aims to elucidate the mechanisms of arsenic interactions in *C. vulgaris* by examining the involvement of media concentration of phosphate and sulphate, oxidative stress, intracellular pH, surface adsorption, GSH and PC and their transport

systems in the detoxification of As(III), dimethylarsinic acid (DMA) and As(V) by *C. vulgaris*. We also propose a conceptual model for the transport of As in *C. vulgaris* that can provide a methodological framework for interpretation tools for future research.

2. Materials and methods

An overview of the methodology used in this study is outlined in Fig. 1.

2.1. Reagents, algal culture and growth conditions

All reagents, growth media and eluents were made with 18.2 MΩcm deionised water from Purite (Thame, UK). Sodium arse- nate dibasic heptahydrate [As(V)], dimethylarsinic acid (DMA), l-glutathione reduced, oxidised glutathione, cerium, uranium, HPLC grade formic acid and HPLC grade methanol were purchased from Sigma Aldrich (Poole, UK); Sodium arsenite [As (III)], gallium, indium, arsenic solutions and nitric acid (trace metal analysis) from Fisher Scientific (Loughborough, UK); cobalt standard from Hopkin & Williams Ltd. (London, UK); lithium nitrate from BDH Chemi- cals (Poole, UK); phytochelatin PC_2 standard was purchased from Cambridge Biosciences (Cambridge, UK).

C. vulgaris was obtained from Algae and Protozoa, SAMS Research Services Ltd., Dunstaffnage Marine Laboratory, UK (CCAP 211/11B). Cells were grown in conical flasks (100 and 250 mL) capped with foam stoppers (Fisher Scientific) containing Bold's medium without EDTA and high phosphate (1.61 mM HPO_4^{2-}) (Perales-Vela et al., 2006). Cells were grown under continuous illumination at 2646 lux (n = 33, SE = 154) and 25 ± 0.5 ° C (LMS Cooled Incubator, Model 300WA). All nutrients were reagent grade if not stated otherwise. In these experiments, we deliberately sought to avoid the potentially confounding variable of light/dark cycle use, consistent with algal test procedures (e.g. OECD, 2011) that use continuous light.

2.2. Tests for arsenic toxicity to *C. vulgaris*

Arsenic [As(III), As(V) and DMA] toxicity to *C. vulgaris* was investigated using 72 h tests to calculate the 50% inhibitory concentration (IC_{50}) following the "Standard guide for conducting static toxicity tests with microalgae" (ASTM, 2004). The concentrations of arsenic used in this study were chosen to elicit a response in *C. vulgaris* cells rather than to reflect natural environment conditions. For measuring cell health, chlorophyll a, b and other pigments were used and extracted using a whole water extraction technique as described by Mayer et al. (1997) using a ratio of sample volume/solvent (1/12 mL). Fluorescence in arbitrary units was measured at 430 nm excitation and 671 nm emission wavelengths using a spectrofluorometer (Shimadzu, RF-1501) and standard 10 mm cuvettes.

Toxicity tests were performed in 100 mL borosilicate conical flasks containing 40 mL of media and 0.03–1.73 mM of arsenic. Cells were supplemented with 0.05% w/v dextrose (Oxoid, bacteriological grade) and buffered with 0.2 M MOPS (Fisher Scientific) solution (pH 7) for the low phosphate experiments. All controls (low phosphate, no arsenic) were buffered with MOPS in the same way to ensure that the response was due to the presence of arsenic rather than the buffer itself. Toxicity was investigated at low (0.003 mM) and intermediate concentration of phosphate (0.137 mM). Toxicity was also investigated in low (7.3 μM) and high (0.3 mM) sulphate conditions (in controls, sulphate salts were replaced by their corresponding chloride salts). Six controls (with no added arsenic) and triplicate experiments for arsenic concentrations were used. Cells growing in the exponential phase (5–6 days old) were used to inoculate controls and arsenic experiments to an initial concentration of 5×10^3 cells mL^{-1}.

Redox potential/dissolved oxygen or pH/dissolved oxygen and temperature were monitored continuously in control media containing an intermediate concentration of arsenic (III and V); cells were not counted for this control.

2.3. Intracellular oxidative stress, GSH and pH

The following reagents were used: Bold's media, N,N-Dimethyl-formamide (DMF, Sigma-Aldrich D4551), dimethyl sulfoxide (DMSO, Sigma-Aldrich D5879), ethanol, H_2DCF-DA (2^t,7^t-dichlorodihydrofluorescein diacetate, Invitrogen D-399), HE (Dihydroethidium, Invitrogen D-1168), BCECF (2^t,7^t-Bis-(2-Carboxyethyl)-5 and 6-Carboxyfluorescein, Acetoxymethyl Ester, Invitrogen B-1170), Menadione (MEN Sigma-Aldrich M57405), H_2O_2 (AnalaR, 30%, 100 vol), N-ethyl-maleimide (NEM Sigma-Aldrich E3876) and CMFDA (5-chloromethylfluorescein diacetate, Invitrogen C-7070). Preparation details are provided in Supplementary material (Materials and methods).

Flow cytometry was used to investigate the response of C. vulgaris to As(III) and As(V) and the influence of intracellular H_2O_2, O_2^-, GSH (low molecular thiols) and pH on toxicity based on the work of Cossarizza et al. (2009). The IC_{50} obtained in the toxicity experiments for As(III) and As(V) were used as the exposure concentration and high and low phosphate respectively for flow cytometry experiments, 0.67 mM As(III) at 1.61 mM HPO_4^{2-} and 0.013 mM As(V) at 0.003 mM HPO_4^{2-} for 120 min.

The analysis was carried out in a FACSCalibur (Becton Dickson) flow cytometer. Channel detection included: forward angle light scatter (FSC), side angle light scatter (SSC), fluorescence channel 1 (FL1, 515–545 nm), channel 2 (FL2, 564–606 nm), and channel 3 (FL3, 670 nm).

Three controls were: stained negative (heat-killed cells, 20 min at 80 ° C), stained cell blanks (no arsenic added, only labelled with dye), cell blanks (no arsenic, no dye) and specific controls (details are provided in supporting information). Cells were assumed killed when fluorescence was below the minimum limit of quantification of number of cells, (7.94 × 102). All dyes were excited by argon-ion laser at 450–490 nm. Fluorescence of H_2DCF-DA and CMFDA were detected through the FL1 filter, HE and BCECF were detected through the FL1 and FL2 filters whereas chlorophyll fluorescence was detected at emission 670 nm, filter FL3.

Samples and controls were incubated for 120 min in triplicates, collecting at least 20,000 events per replicate. Instrument settings are provided in Supplementary material (Materials and methods, Table S2).

2.4. Arsenic adsorption to cell surface

Dry *C. vulgaris* cells (0.2 g) were used as controls for surface adsorption. Cells were transferred to 50 mL screw top polyethylene tubes with a solution containing 15 mL of 0.013 mM of As(V) or As(III). The mixture was shaken (IKA, KS 130 Basic) for 2, 4, 6, and 8 h, then centrifuged for 10 min at 4500 g. The supernatant was analysed for total arsenic.

Living *C. vulgaris* cells were studied for arsenic adsorption. Cells were grown in Bold's medium supplemented with 0.05% w/v dextrose and free of arsenic for 3–5 days. A similar number of cells were taken and exposed to arsenic (As(III), As(V) or DMA) by adding 0.67 mM for different periods (4, 24, 48 and 72 h) and at different arsenic concentrations (0.13, 0.67, 1.33 and 2.67 mM) for a fixed period of 48 h. Only after the exposure period, cells were counted using a whole water extraction technique (see Section 2.2). Briefly, cells were harvested by centrifugation (5 min 3000 g) and washed twice with deionised water. The pellet was then washed for 10 min

with 10 mL of ice-cold desorption solution (1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2$, and 5 mM MES, pH 5.9) (Abedin et al., 2002; Sandau et al., 1996). The supernatant was analysed for arsenic. The pellet was washed one more time with deionised water (arsenic in the final rinse was below the detection limits comparable to calibration blanks), freeze-dried and analysed for total arsenic absorbed (see following section). Experiments were performed in triplicate.

2.5. Arsenic biotransformation

2.5.1. Arsenic extraction

C. vulgaris cells were cultured for 3–5 days in Bold's media free of arsenic. Cells were then exposed to 0–2.67 mM of As(III), As(V) and DMA for 48 h and to 0.67 mM As(III), As(V) or DMA for different time periods (4, 24, 48 and 72 h).

After arsenic exposure, cells were counted and harvested by centrifugation (5 min, 3000 g) washed with deionised water twice and transferred to 15 mL centrifuge tubes. Cells were then washed with desorption solution for 10 min. The supernatant was discarded and the pellet was used for two types of extraction (total absorbed arsenic and speciation). For total absorbed arsenic, cells were digested using the EPA 3051A (2007) method. Briefly, cells were transferred to Teflon® tubes, 3 mL of concentrated nitric acid, 3 mL of deionised water were added and the mixture was digested in a MARS XPRESS microwave digestion system (temperature rise to 175 ± 5 °C in approximately 5.5 ± 0.25 min and retained at 175 ± 5 °C for the 10 min digestion period). The digested sample was transferred and weighed in 15 mL centrifuge tubes.

For speciation analysis, the method of Pantoja et al. (2014) was used. In short, cells were extracted with 2 mL of 1% formic acid; the mixture was sonicated for 30 s using a Minidelta 8935 generator (FFR ultrasonics, 500 W, 35 kHz) fitted with a 3 mm titanium micro-tip. The micro-tip was rinsed with methanol, and then sonicated in 1% formic acid for 10 s between extractions to avoid cross-contamination.

2.5.2. Total arsenic quantitation

Total arsenic was quantified using an ICP–MS (X series II, Thermo Scientific, UK) in CCT (Collision Cell Technology) mode with He/H as collision cell gas using 20 μg L^{-1} gallium as internal standard. The instrument was tuned daily using 10 μg L^{-1} indium, cerium, cobalt, uranium and lithium solution and manufacturer software (Plasmalab, Thermo Scientific).

2.5.3. Speciation analysis (Anion exchange)

Speciation conditions for inorganic and methylated arsenic species were are as follows. Separation was done on a Hamilton PRP-X100 (10 μm, 250 × 4.1 mm) column. Isocratic elution was used with 6.6 mM $NH_4H_2PO_4$ (Sigma 17842) and 6.6 mM NH_4NO_3 (Sigma 256064) buffer (pH = 6.3, NH_4OH). The flow rate was 1 mL min^{-1} based on the method of Liu et al. (2010). Instrument conditions were the same as for total arsenic quantitation.

2.5.4. Speciation analysis (reversed phase)

Samples were analysed immediately after extraction. Separation was done in a Discovery C_{18} column (15 × 2.1 mm) and pre-column Discovery C_{18}. Experimental/instrumental conditions are reported elsewhere (Bluemlein et al., 2009). A gradient elution was used with 0.1% formic acid in water (A) and 99.9% HPLC grade methanol with 0.1% formic acid (B). Elution profile was: 0–20 min linear increase 0–20% B, 20–30 min 20% B, 30–32 min 20–0% B and 32–40 min 0% B with a flow rate of 0.2 mL min^{-1}.

Parameters were adjusted for O_2 CCT in ICP-MS according to the manufacturer's instructions: O_2 cell gas flow 0.6–1.45 mL min^{-1}, hexapole bias −9 V, quadrupole bias −14 V, focus voltage −2 V, D2 voltage −100 V. A post column make up flow was achieved with a tee connector and 0.9 mL min^{-1} indium (20 μg L^{-1} in 2% HNO_3) as internal standard. Injection volume was 50 μL, monitored masses were: As, m/z 91 ($[^{75}As^{16}O+]$), S, m/z 48 ($[^{32}S^{16}O+]$) and In, m/z 115. For arsenic, different solutions of DMA were used as standards and l-cysteine for the quantitation of sulphur. GSH and GSSG were separated and quantified using the same conditions at m/z 48.

Method quantitation limits (MQL) and detection limits (MDL) were calculated by multiplying respectively 10 and 3.14 times the standard deviation signal (at the retention time of the eluting species) of 7 consecutive blank samples (USEPA, 1984).

Correction for methanol in the mobile phase was performed according to Amayo et al. (2011): A blank was injected through the same chromatographic conditions. A post column addition of 100 μg L^{-1} DMA and 20 μg L^{-1} indium (as internal standard) was made. The blank was analysed by ICP-MS for arsenic, sulphur and indium at m/z 91 ($[^{75}As^{16}O+]$), m/z 48 ($[^{32}S^{16}O+]$) and m/z 115 (In).

2.6. ABCC1/2 transport experiments

For ABCC1/2 transport, toxicity, flow cytometry and arsenic efflux was investigated. Probenecid (Sigma, P8761), sodium taurocholate (Sigma, 86339) and MK571 (Sigma, M7571) were used as specific inhibitors of ABCC1/2.

2.6.1. Toxicity as a measure of arsenic transport

A 72 h, As(III) (0.67 mM) toxicity test was performed in the presence and absence of inhibitors (Probenecid, 500 μM; sodium taurocholate, 50 μM and MK571, 25 μM). Here, the percentage of growth with respect to controls (only arsenic added) was compared.

2.6.2. Flow cytometry to measure arsenic transport

Flow cytometry was used to investigate the fate of ABCC1/2 substrates, BCECF (3.5 μM) and CMFDA (2.5 μM). Transport was evaluated in the presence of inhibitors of ABCC1/2, probenecid (50 μM) and MK571 (25 μM). Three controls were used: stained negative (heat-killed, 20 min 80 °C), background (no inhibitor, only labelled with dye) and blanks (no inhibitor, no dye). Incubation for all fluorescent substrates was 60 min. Samples were run in triplicates collecting at least 20,000 events for each sample. No arsenic was used in this experiment.

2.6.3. Arsenic efflux

The fate of As-GS/PC complexes inside *C. vulgaris* cells was inves- tigated after exposure to 2 mM As(III) and high phosphate for 24 h using the inhibitors probenecid (500 μM) and MK571 (25 μM). Inhibitors were added 1 h before arsenic exposure. After arsenic exposure, cells were harvested by centrifugation (10 min at 3000 g), washed twice with deionised water then with desorption solution. The pellet was extracted and analysed for total arsenic as previously described.

2.7. Statistical analysis

Statistical analyses were undertaken using Minitab® v15. Correlation coefficients were calculated using a simple regression, least squares method, analyses of difference using two-sample t-tests and one-way analysis of variance (ANOVA). Normality and equal variance assumptions were tested in all experiments. General Linear Model (GLM) was used to perform univariate analysis of variance (Tukey-Kramer method).

For flow cytometry analysis, Kolmogorov-Smirnov (K-S) statistics were calculated using BD CellQuest software (Young, 1977) generating the K-S test statistics (D-value) ranging between 0 and 1,

with higher values indicating a greater difference between distributions. Given the large number of individual observations generated during flow cytometry experiments, very small difference in distribution will generate a significant p-value and so it is the size effect as indicated by the D-value that is focus of interpretation of distribution differences. D values were categorised in the following way: Strong ($D > 0.30$), moderate ($0.20 < D < 0.30$), weak ($0.15 < D < 0.20$) and negligible ($D < 0.15$) difference (Lebedeva et al., 2011).

3. Results

3.1. Arsenic toxicity in *C. vulgaris*

Physico-chemical background parameters of dissolved oxygen, pH, redox potential and temperature were monitored for controls at 0.6 mM As(III) and 0.001 mM As(V) (Supplementary material Figs. S1A and S1B) and were deemed satisfactorily stable.

Inhibition concentration of As(III) to *C. vulgaris* at 0.137 mM phosphate was $IC_{50} = 0.74$ mM (Fig. S2). Cells growing at 0.003 mM phosphate had a very similar inhibition concentration $IC_{50} = 0.86$ mM (Fig. S3). In contrast, cells growing at 0.137 mM phosphate did not show inhibition by As(V) at the concentrations tested whereas at 0.003 mM phosphate, cell growth was inhibited at a concentration of $IC_{50} = 0.014$ mM (Fig. S4).

Growth inhibition for cells in DMA was only about 25% at the highest concentration of arsenic (0.67 mM). When the concentration of sulphate was lowered to 7.3 μM (0.34% of the concentration used in high sulphur experiments), cells treated with As(III) and DMA grew similarly to cells in high sulphur concentration (Fig. S5) indicating that the amount of sulphur in the media does not play an important role in As(III) and DMA tolerance.

3.2. Induced intracellular oxidative stress, GSH and pH changes

Treatment with As(III) and As(V) had a negligible effect on H_2DCFH-DA [As(III) $D = 0.02$, As(V) $D = 0.07$, Fig. 2A,B and Table S3] and HE [As(III) $D = 0.003$, As(V) $D = 0.1$] fluorescence (Fig2C,D and Table S4). In contrast, treatment with As(V) resulted in a strong increase ($D = 0.56$) in CMFDA fluorescence and therefore is associated with an increase in intracellular GSH (as free thiols) whereas CMFDA fluorescence was not affected by treatment with As(III) ($D = 0.03$), (Fig. 2E,F and Table S5). BCECF fluorescence was not affected by As(III) but a moderate inhibition was observed in the presence of As(V) (Fig. 2G,H and Table S6).

3.3. Arsenic adsorption to cell surface

Poor adsorption of As(III) and As(V) was observed using dried *C. vulgaris* cells (Fig. 3A–B). An apparent negative adsorption com- pared to controls was observed that eventually equilibrates to zero at 8 h for pH 7. For pH 4, we assume that before 8 h, conditions did not reach equilibrium and there is an apparent release of As(V); however longer experiments were not performed due to negligible observed arsenic adsorption.

When living *C. vulgaris* cells were exposed to 0.67 mM arsenic for different periods (4, 24, 48 and 72 h), adsorption of As(V) and DMA showed saturation after 48 h (Fig. 3D). In contrast, adsorption of As(III) was 15 times higher than the other forms of arsenic (Fig. 3C).

When cells were exposed to different concentrations of As(III), As(V) and DMA (0.13, 0.67, 1.33 and 2.67 mM) for 48 h, adsorption increased with arsenic concentration in media for all arsenic forms. However adsorption of As(III) was 17 times higher than the average of both As(V) and DMA (Fig. 3E).

3.4. Arsenic biotransformations

Total arsenic inside cells exposed to 0.67 mM As(III), As(V) and DMA did not change significantly with time (from 4 to 72 h). However, cells absorbed 8 times more As(III) than As(V) or DMA (Fig. 4A). Cells absorbed more arsenic when the concentration of arsenic in media increased for all forms of arsenic. Absorption of As(III) was higher than the other forms, being on average 55 times higher than As(V) and 9 times higher than DMA (Fig. 4B and C).

3.4.1. Speciation analysis (anion exchange)

Speciation of As-GS/PC complexes using strong anion exchange columns has been shown to break the As-S bonds giving only As(III) signals (Raab et al. 2004). Therefore the signal for As(III) in this set of results could come either from As-GS/PC complexes or free As(III).

When cells were exposed to different concentrations of DMA (0.13–2.6 mM) for 48 h and to 0.67 mM over different periods (4–72 h), only DMA and As(V) were detected (Fig. 5A and B). Most arsenic was still in DMA form and only up to 7% was converted to As(V). When cells were exposed to As(V) in the same way, As(III), DMA and As(V) were detected (Fig. 5C and D). On average 41% was still in As(V) form, followed by 30% of DMA and there was a 29% reduction to As(III). After exposure to As(III), only As(III) and trace amounts of As(V) and DMA were observed with the exception for treatments with 0.67 mM for 72 h where 38% of DMA was found (Fig. 5E and F). Across all exposure to As(III), 90% of arsenic was still in As(III) form, and only 5% was oxidised or methylated to DMA.

3.4.2. Speciation analysis (reversed phase)

Qualitative analysis for identification of the eluting molecules has been reported previously (Pantoja et al., 2014). In thus study, the MQL for S at m/z 48 (O_2, CCT) was 20.20 $\mu g\,L^{-1}$ (SE = 2.28, n = 13) and for arsenic at m/z 91 (O_2, CCT) was 0.55 $\mu g\,L^{-1}$ (SE = 0.07, n = 10).

3.4.2.1. Biotransformation after arsenic(V) exposure. Pantoja et al. (2014) reported that exposure to As(V) resulted in signals for GSH, GSSG and oxidised PC_2 but no As bound to GS/PC at any of the concentrations used (0.013–2.67 mM) or exposure period (1, 4, 24, 48, 72 and 96 h). In the present study, the levels of GSH did not appear to alter significantly for the different exposure periods or exposure concentrations (ANOVA-GLM, $p > 0.05$, $n = 3$, Fig. 6, Table S7) but there was strong statistical evidence for a difference ($p = 0.0084$, Fig. 6A, Table S7) in levels of GSSG for the first 4 h after exposure to 0.67 mM As(V). However there seemed to be a recovery in the level of GSSG after 24 h which continued up to 48 h. A moderate positive correlation was found for an increase in the level of GSH + GSSG with respect to treatment time (simple linear least squares, $R^2 = 0.56$, $n = 3$) and a weak positive correlation with respect to concentration of arsenic in media ($R^2 = 0.14$).

3.4.2.2. Biotransformation after arsenic(III) exposure. Strong statistical evidence was observed for a reduction in levels of GSSG with time compared to controls as well as the ratio of GSH/(GSH + GSSG) in cells exposed to As(III) (Fig. 6C and Table S8). However, there was no difference in the concentration of GSH with an increase of As(III) concentration (Fig. 6D and Table S8) with the exception of levels of GSH and GSSG in cells exposed to 1.33 mM compared to 2.67 mM.

It has been demonstrated that cells exposed to As(III) form complexes of arsenic bound to GS/PC as well as newly reported hGS/hPC complexes (Pantoja et al. 2014). In the present study, we report for the first time their quantitation. Some statistical evidence was found for an increase in the amount of As-GS/PC complexes with time (Fig. 7A and Table S9). Strong statistical evidence was found for an increase in the formation of As-GS/PC complexes with an increase in As(III) concentration (Fig. 7B and Table S10).

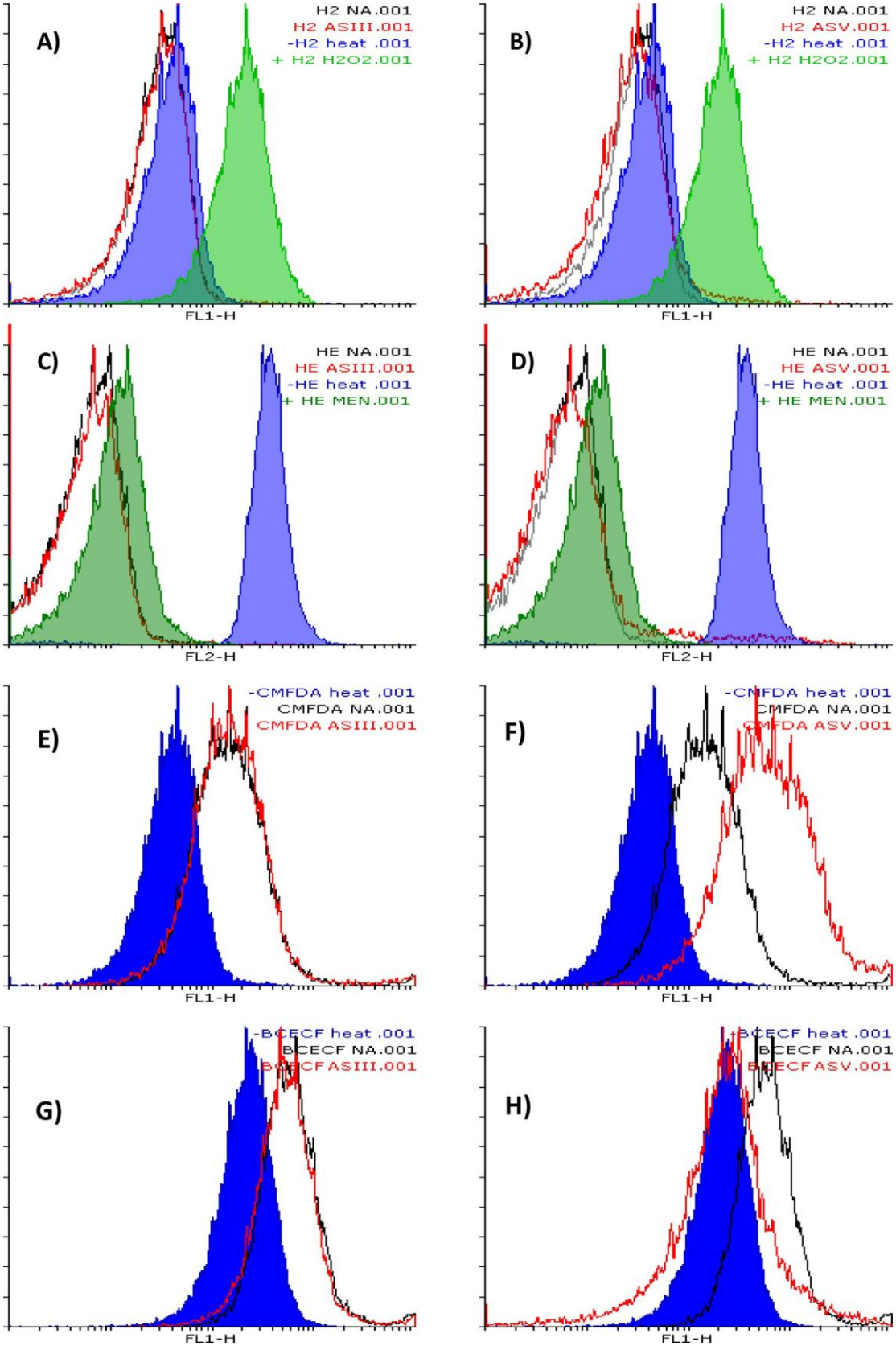

Fig. 2. Flow cytometry histogram distributions of negative controls (heat-killed, blue), non-treated cells (black) as well as As(III) and As(V) treated cells (red). Stain used, treatment, channel: (A) H2DCFH-DA (H2 O2 radical indicator), As(III), FL1; (B) H2DCFH-DA, As(V), FL1; (C) HE (superoxide indicator), As(III), FL2; (D) HE, As(V), FL2; (E) CMFDA (low molecular thiols), As(III), FL1; (F) CMFDA, As(V); (G) BCECF (intracellular pH indicator), As(III), FL1; (H) BCECF, As(V), FL1 measured at 530 ± 15 nm, FL2 measured at 585 ± 20 nm. Treatment time 120 min.

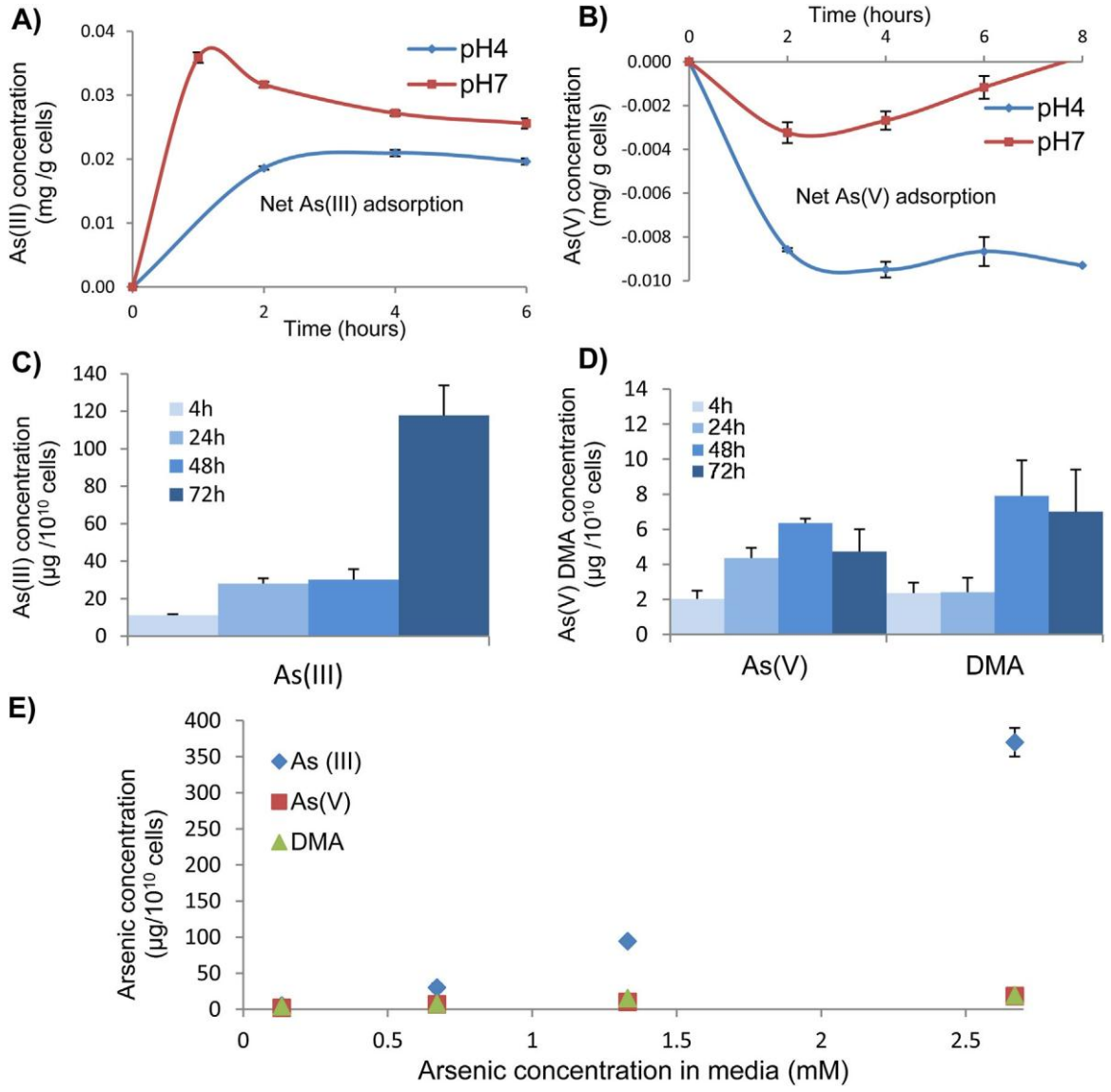

Fig. 3. Kinetics study on arsenic adsorption onto *C. vulgaris* cells, initial concentration 0.013 mM As and 0.2 mg of dry algae, pH adjusted with MOPS (pH 7) and potassium hydrogen phthalate (pH 4). Measured using ICP-MS, (A) dry cells As(III) adsorption; (B) dry cells As(V) adsorption; (C) and (D) surface adsorption of living *C. vulgaris* cells exposed to 0.67 mM As(III), and As(V) and DMA; (E) adsorption curves for living cells exposed to different concentrations of As(III), As(V) and DMA, for 48 h, n = 3, vertical bars denote ± 1 standard error.

3.4.2.3. Biotransformation after DMA exposure. Insufficient evidence was found for a difference in levels of GSH, GSSG or the ratio GSH/(GSH + GSG) at any of the concentrations or exposure periods with the exception of treatment for 72 h compared to controls (Fig. 6E and F and Table S11). Cells exposed to As(III) were able to complex up to 73% of arsenic inside cells. In contrast cells exposed to DMA were only able to form $DMAS^{V}$-GS after 48 h and it accounted only for up to 3.1% of total arsenic inside cells (Fig. 8A and B).

3.5. ABC/MRP transport experiments

3.5.1. Toxicity as a measure of arsenic transport

Growth was not affected by addition of 0.67 mM of As(III) at 1.61 mM HPO_4^{2-}. The presence of inhibitor MK571 itself reduced growth to 47% (two-sample t-test, $p <= 0.001$, $n = 3$) with respect to controls in the absence of arsenic and to 50% ($p = 0.015$) in arsenic's presence. Probenecid alone increased cell growth to 133% ($p <= 0.001$) and in the presence of arsenic, decreased growth significantly to 77% ($p = 0.021$) compared to controls. The difference in growth can be attributed to inhibition of As-GS/PC transport and therefore increasing arsenic's toxicity to cells. The drug sodium tau- rocholate did not have any significant effect ($p > 0.05$) on growth. Summary findings of these initial transport experiments are given in Supplementary Material (Fig. S9). As a result of these obser- vations, the inhibitor probenecid alone was further investigated at different concentrations of probenecid (50, 150, 300, 450 and 600 µM) (Fig. 9). Again, an insignificant growth increase in the sole presence of 0.67 mM As(III) was observed ($p > 0.05$). As previously observed, cells grew significantly more (52% − 60%) in the presence of the inhibitor alone ($p <= 0.002$). In contrast, all cell growth when As(III) was present showed a significant decrease ($p <= 0.01$). This decrease in growth ranged between 60–81% with respect to inhibitor/no As(III). Inhibition of growth in the presence of As(III) was not dependent on concentration of probenecid (50–600 µM).

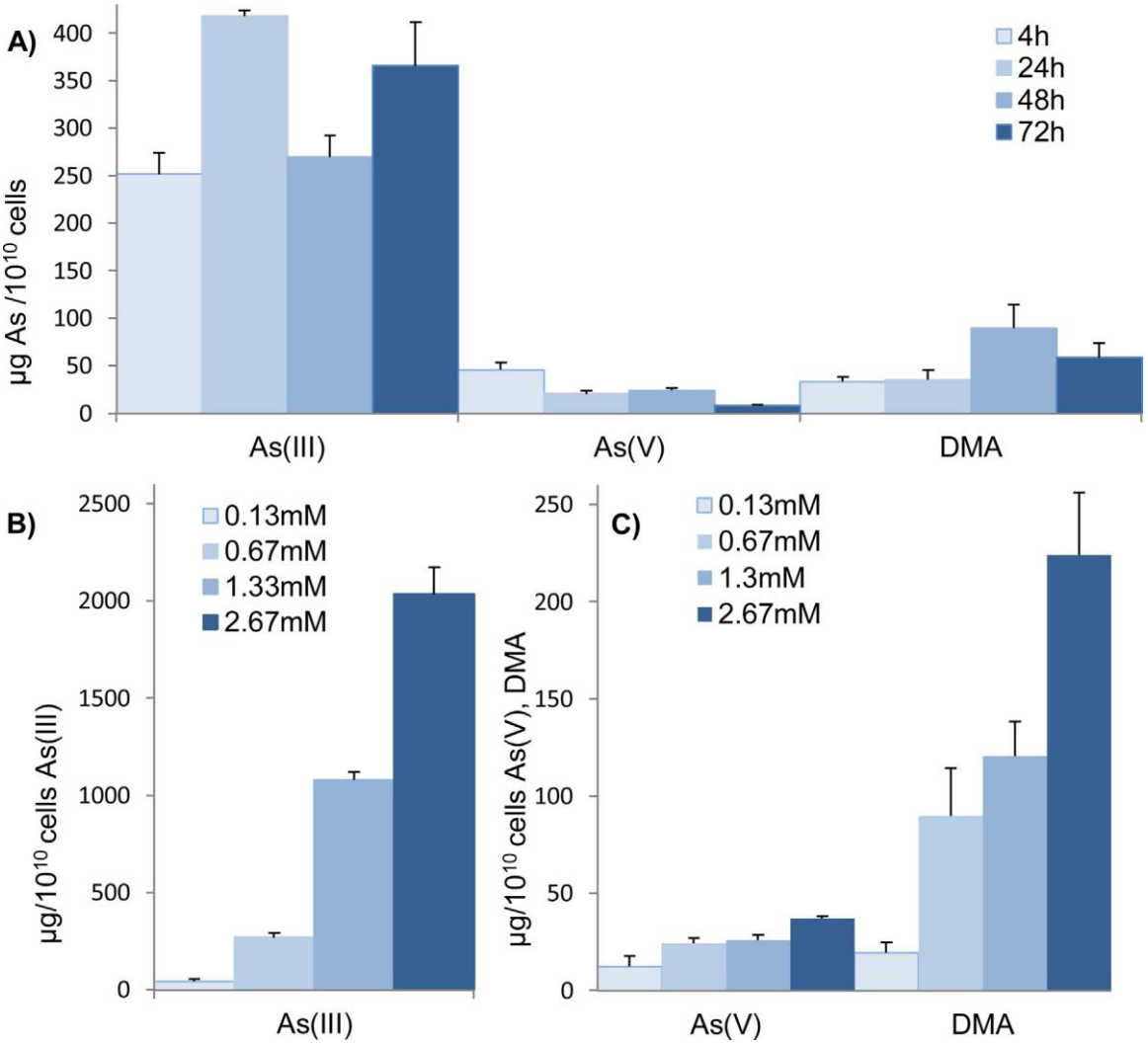

Fig. 4. Total arsenic absorbed in *C. vulgaris* cells exposed to (A) 0.67 mM As(III), As(V) and DMA, over different time intervals; (B) different concentrations of As(III); (C) As(V) and DMA, for 48 h, vertical bars indicate 1 standard error, n = 3.

3.5.2. Flow cytometry to measure arsenic transport

3.5.2.1. Probenecid effect. When negative controls (heat-killed cells) were stained with BCECF, fluorescence was higher than probenecid treated cells and non-treated cells for the two different channels FL1 (D = 0.20) and FL2, (D = 0.38) (Fig. S6A and B and Table S12). When negative control cells were stained with CMFDA, fluorescence was lower than probenecid treated cells and non-treated cells (D = 0.74, Fig. S6E).

Cells treated with probenecid showed weaker fluorescence than non-treated cells when stained with BCECF for the two different channels FL1 (D = 0.18) and FL2 (D = 0.16, S6A and B). In contrast, the difference was negligible when cells were treated with CMFDA (D = 0.08, Fig. S6E).

3.5.2.2. MK571 effect. BCECF fluorescence of negative controls was moderately higher than that of non-treated cells (MK571) (D = 0.25) but lower than MK571 treated cells (Fig. S6C and D). When negative controls were stained with CMFDA, fluorescence was considerably lower than that of non-treated cells (MK571) (D = 0.62) and MK571 treated cells (Fig. S6F).

Cells treated with MK571 (stained with BCECF) exhibited greater fluorescence than non-treated cells and even higher intensity than negative controls for the two different channels FL1 (D = 0.43) and FL2 (D = 0.42) (Fig. S6C and D) which was not the case for probenecid treated cells. When stained with CMFDA, cells exhibited greater fluorescence than non-treated cells (D = 0.28) and negative controls (Fig. S6F). Fluorescence of the two dyes was strongly affected by MK571 (Table S12), whereas only BCECF fluorescence was moderately affected by probenecid and with negligible results for CMFDA fluorescence by probenecid.

3.5.3. Arsenic efflux

There was insufficient evidence for a difference in the amount of arsenic inside treated (probenecid) and control cells (non-treated) (two-sample t-test, $p > 0.05$, $n = 12$). There was, however, strong evidence for a difference in the amount of arsenic inside treated (MK571) and control cells (non-treated, $p = 0.005$, $n = 10$, Fig. 10).

4. Discussion

This study has found that As(V) is at least 50 times more toxic than As(III) to *C. vulgaris* under low phosphate conditions (0.003 mM). One possible explanation is that As(V) can replace phosphate in energy molecules such as arsenylated ATP, ADP and AMP. These molecules are more easily hydrolysed and have lower energy levels than their phosphate analogues (2–3 kcal mol^{-1}

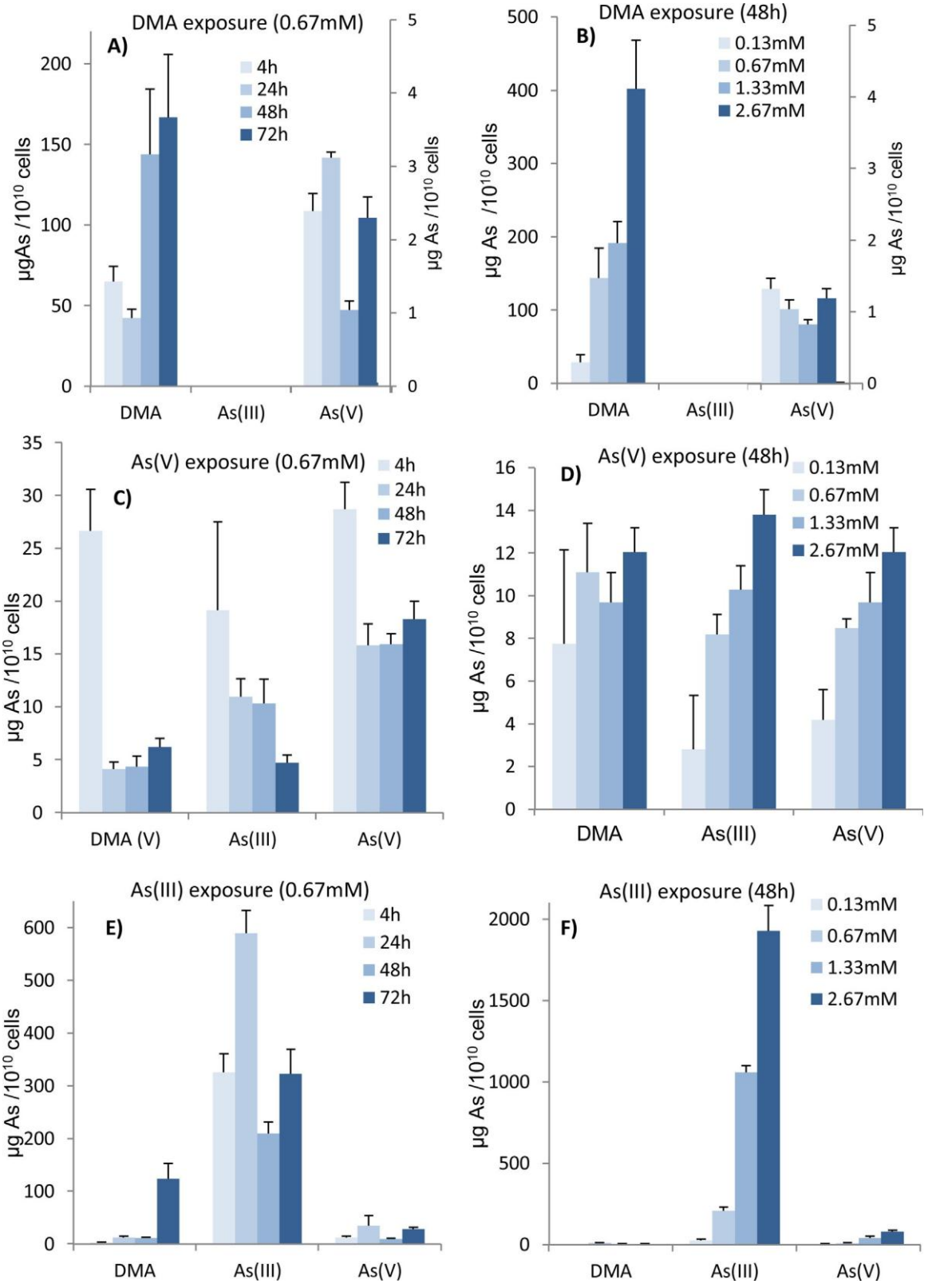

Fig. 5. Speciation of arsenic (Ion exchange) for cells exposed to 0.67 mM at different exposure times (A) DMA, (C) As(V) and (E) As(III); (B) DMA, (D) As(V) and (F) are the experimental results obtained at different As concentrations after incubating the cells for 48 h. Vertical bars denote 1 standard error, n = 3.

lower) therefore rendering algae less likely to grow (Xu et al., 2012). Another disadvantage for cells living in low phosphate and high As(V) environments is that when As(V) binds to ADP, AMP, glucose, ribose and adenine, many enzymes have been found to reduce As(V) in the presence of glutathione. This reduction does not happen with phosphate, since it is more stable and useful at storing energy than As(V) analogues (Gregus et al., 2009; Nemeti et al., 2012, 2010).

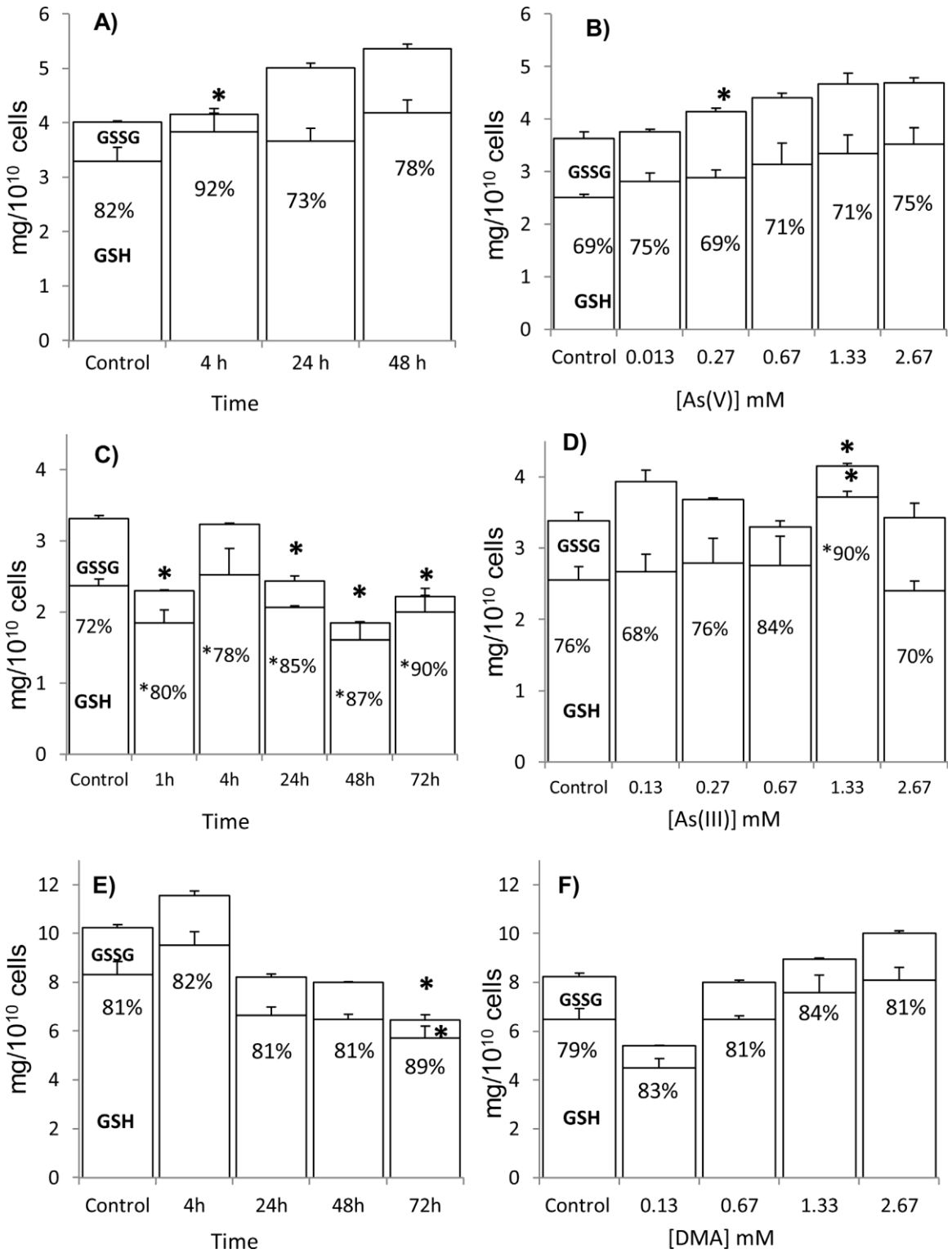

Fig. 6. Concentration of GSH (bottom bars mg/10^{10} cells) and GSSG (top bars mg/10^{10}cells) for *C. vulgaris* exposed to (A) 0.67 mM As(V) at different periods; (B) different As(V) concentrations for 48 h; (C) 0.67 mM of As(III) at different periods; (D) different As(III) concentrations for 48 h; (E) 0.67 mM of DMA at different exposure times and (F) different DMA concentrations for 48 h. Analysed by HPLC-ICP-MS (O_2 , CCT) m/z 48. Percentages indicate the ratio of GSH/(GSH + GSG). * denotes statistical difference using ANOVA-GLM ($p <= 0.05$). Vertical bars denote + 1 standard error, n = 3.

4.1. Arsenic (III) effects in *C. vulgaris*

Passive surface adsorption was found to be negligible for living cells exposed to DMA and As(V) (Fig. 3). However, adsorption of As(III) was observed to be an active process in *C. vulgaris*, because dried cells showed negligible capacity to adsorb As(III) as illustrated in Fig. 3A, whereas living cells did not show saturation at any of the concentrations tested or exposure periods used (Fig. 3B). Since the algal cell surface is a strong electron donor and weak electron acceptor and the iso-electric point of the cell wall is 2.9 (Hadjoudja

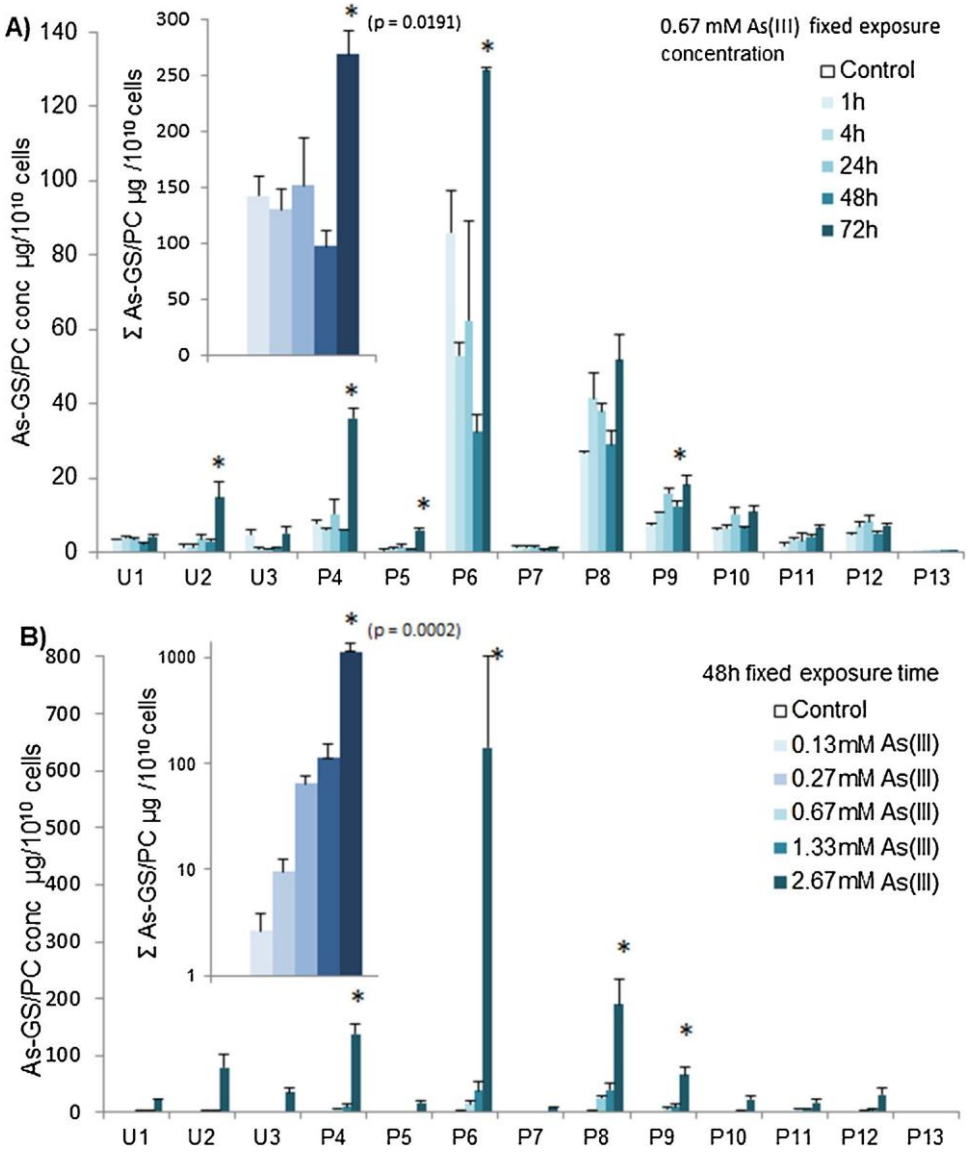

Fig. 7. Concentration of As-GS/PC complexes in *C. vulgaris* cells exposed to (A) 0.67 mM of As(III) for different periods of time and (B) different concentrations of As(III) for 48 h, (inset, total As-GS/PC concentration), analysed by HPLC-ICP-MS (O_2, CCT) m/z 91. * denotes statistical difference using ANOVA-GLM ($p <= 0.05$). Vertical bars denote +1 standard error. Peak identification: **U1-3** Unknowns, **P4** GS-As(III)-PC_2/GS-As(III)-')'-(Glu-Cys)$_2$, **P5** As(III)-')'-(Glu-Cys)$_2$, **P6** GS-As(III)-PC_2, **P7** GS-As(III)-')'-(Glu-Cys)$_2$-Ala, **P8** As(III)-PC_3/MMA(III)-PC_2, **P9** MMA(III)-PC_2, **P10** As(III)-PC_3/As(III)-$(PC_2)_2$, **P11** As(III)-$(PC_2)_2$/As(III)-')'-(Glu-Cys)$_3$-Ala/As(III)-')'-((Glu-Cys)$_2$)$_2$-Ala/MMA(III)-')'-(Glu-Cys)$_2$-Ala, **P12** As(III)-PC_4 and **P13** As(III)-PC_4.

et al., 2010), cells will attract only positively charged molecules efficiently (pH 2.9–14) therefore explaining the weak adsorption of negatively charged As(V). Both As(III) and DMA are predominantly present as uncharged molecules and are not likely to be affected by electrostatic interactions. DMA is not likely to be chelated, so no more active interactions are expected. These facts indicate that the increased adsorption of As(III) is due to a different active mechanism than just physical adsorption (electrostatic attraction and van der Waals forces). We can infer that As(III) adsorption was active because the active formation of PC upon exposure to As(III), the high affinity of As(III) for thiol groups followed by complexation with As(III) and compartmentalization to vacuoles can make the process an active coordination/complexation mechanism for this form of arsenic and thus could also dynamically promote an increase in surface adsorption.

After exposure to As(III), only trace amounts of As(V) and DMA were observed inside the algae, by contrast, other researchers have reported up to 67% oxidation inside microalgae (Wang et al., 2014) and 45% oxidation in cyanobacteria, although the later was measured in growing media (Zhang et al., 2014).

Treatment with As(III) had a negligible effect on the induction of oxidative stress (H_2O_2 free radical and O_2^- super oxide) as well as in the concentration of free thiols (GSH) and intracellular pH. However, strong statistical evidence ($p <= 0.01$) indicated that there was a reduction in levels of oxidised glutathione over time compared to controls as well as in the ratio of GSH/(GSH + GSSG). These results highlight the fact that measurements of the ratio of GSH/(GSH + GSSG) are more meaningful than measurements of only reduced GSH or free thiols and give a more clear indication of redox status and therefore stress and toxicity.

The increase of bound As-GS/PC complexes was found to be strongly related to an increase in concentration of As(III) in media (Fig. 7), with GS-As(III)-PC_2 being the most abundant and also probably the most stable complex. Often methods to measure GSH and related thiols rely on indirect determination with a derivatisation reaction or on conditions in which bound As-GS/PCs are converted

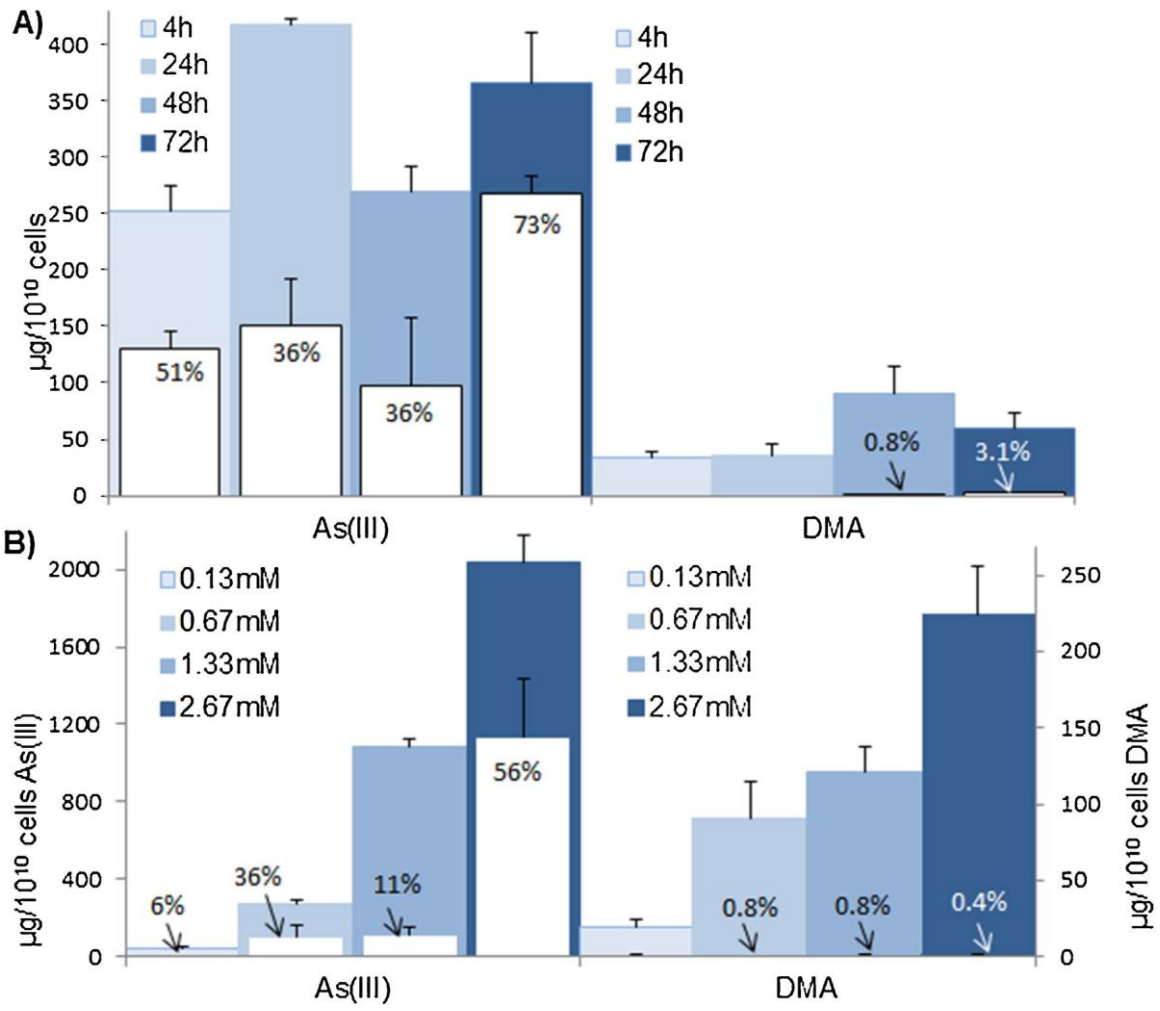

Fig. 8. (A) Kinetic studies for the formation of As-GS/PC complexes in *C. vulgaris* cells exposed to 0.67 mM As(III) and DMA for different periods of time; (B) Concentration effect for cells exposed for 48 h to different concentrations of As(III) and DMA; Coloured bars denote total amount of arsenic, white bars denote the amount of As-GS/PC complexes inside the cells; the amount of As-GS/PC is expressed as percentage of the total arsenic inside cells. Vertical bars denote +1 standard error, n = 3.

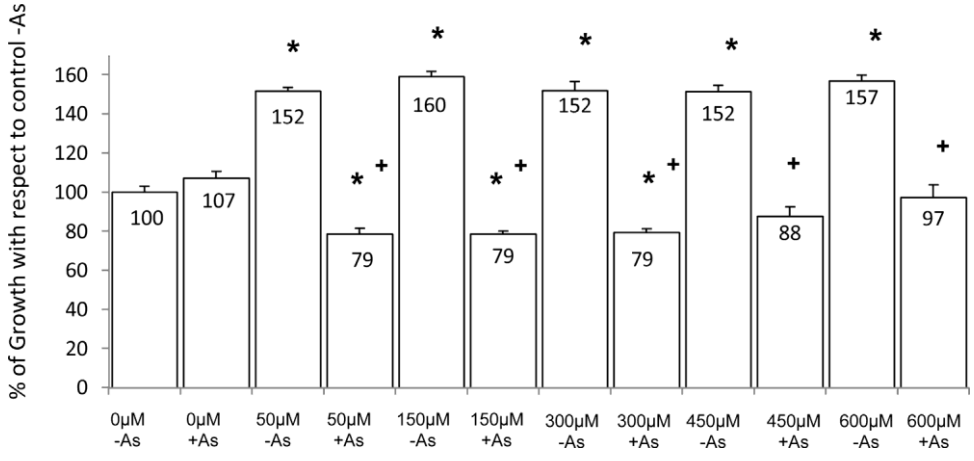

Fig. 9. Toxicity to As(III) (0.67 mM, 72 h test) in the presence/absence of the ABCC1/2 inhibitor probenecid (50, 150, 300, 450 and 600 µM), * denotes statistical difference ($p <= 0.05$) using two-sample t-test with respect to the control. + denotes statistical difference with respect to the absence/presence of arsenic for the same inhibitor. Vertical bars denote +1 standard error, n = 3.

to free thiols by addition of reducing agents. Also, it has been found that other compounds such as coumarins may give false positive signals thus overestimating the amount of PCs and related molecules (Berlich et al., 2002).

It would appear that immediately after As(III) enters cells, GSH binds/chelates with As(III) and triggers the formation of PCs that chelate As(III) in an even stronger way because of the multiple cysteine residues compared to one cysteine in GSH. Inside cells,

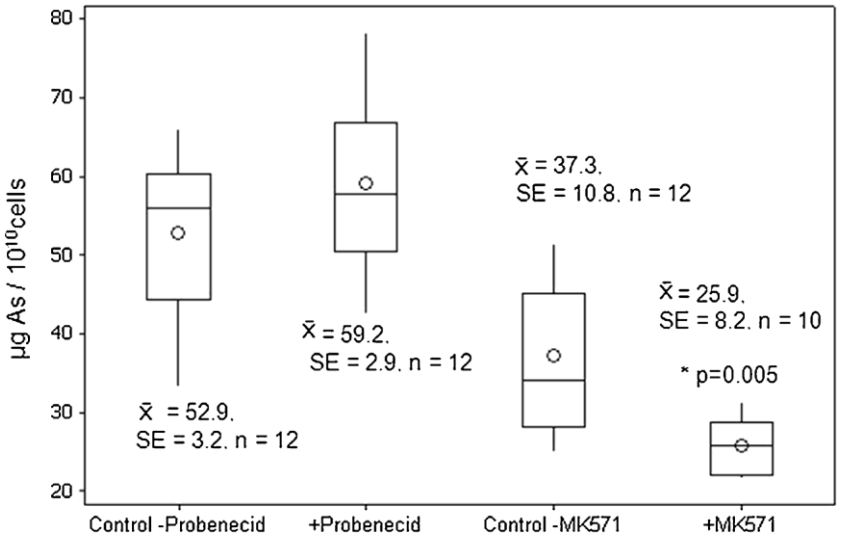

Fig. 10. Box plot of intracellular concentration of arsenic in *C. vulgaris* cells exposed to 2 mM As(III) for 24 h with and without the specific ABCC1/2 inhibitors probenecid (50 µM) and MK571 (25 µM). Circles denote mean values (o).

reduced PCn and oxidised PCn may act as redox buffer systems in the same way as GSH/GSSG, thus the redox pair NADP/NADPH and glutaredoxin may also act as cofactors and reducing enzymes respectively preventing oxidative damage (Hirata et al., 2005). Moreover, phytochelatins have been found to be more effective at preventing oxidative stress than GSH (Tsuji et al., 2002). However, in contrast to GSH, the concentration of PC inside cells depends exclusively on the presence of metal/loids as can be seen in Fig. 7 (Grill et al., 2002; Klapheck 1988). Leguminosae (Klapheck et al., 1995), graminae (Batista et al., 2014; Oven et al., 2002), maize (Chassaigne et al., 2001), horseradish (Kubota et al., 2000) and some species of fungi (Collin-Hansen et al., 2007) have been found to produce hGSH and hPC in addition to GSH/PC (Mounicou et al., 2001), which can give an even greater redox buffering capacity than that observed for *C. vulgaris*.

As previously reported (Pantoja et al., 2014), all arsenic signals eluting after 10 min correspond to As-GS/PC related complexes and therefore can be quantified (Fig. 7). Cells exposed to 0.67 mM As(III) over different periods were able to complex/chelate up to 73% of arsenic and when exposed to different concentrations of As(III) for 48 h, cells were able to complex more than 50% of arsenic, giving a strong indication that chelation of As(III) with GS/PC and to a lesser extent hGS/hPC is a major detoxification mechanism employed by *C. vulgaris* cells when exposed to As(III).

4.2. Arsenic(V) effects in *C. vulgaris*

Treatment with As(V) resulted in a moderate decrease in intracellular pH (D = 0.30) and a strong increase in the concentration of free thiols (GSH) (D = 0.56). This increase was also observed in cells growing in high phosphate where a moderate positive correlation ($R^2 = 0.56$) was found for an increase in levels of GSH + GSSG with respect to treatment period. However, there was a negligible effect on the induction of oxidative stress, H_2O_2 and O_2^- super oxide radicals. It seems that once As(V) enters cells, it is capable of inducing stress which is reflected by an increase of free thiols (GSH) but due to the much lower affinity of GSH to As(V) than to As(III), no chelation or binding is observed. This in turn, allows GSH to scavenge the free radicals immediately formed. Even though this strategy alleviates damaging oxidative stress, reactive As(V) is still able to exert its toxicity by replacement of phosphate in metabolic pathways, interference in enzymatic activity, ATP and energy processes and DNA fragmentation among others (Nemeti et al., 2010; Xu et al., 2012). To our knowledge a change in intracellular pH upon exposure to As(V) has never been reported and its significance is not fully understood. However, this, along with a higher redox capacity given by the increased level of free GSH will certainly have major effects on cell function since most of the cytoplasmic processes are sensitive to the concentration of H^+ ions, mostly involving chemical (ATP) and electrical (NADPH) energy processes, since the two are pH and redox potential dependent respectively (Nobel, 2009).

Our findings indicate that As(V), in presence of high phosphate concentration, is excluded from surface adsorption and intracellular absorption, being 17 and 55-fold lower, respectively, when compared to As(III). It was also found that only 29% of As(V) was reduced to As(III). Furthermore, it has also been reported that the reduction of As(V) mediated by phosphorolitic-arsenolitic enzymes is inhibited by increasing concentrations of phosphate, ATP and glycine (Gregus et al. 2009). These enzymes are involved in the formation of the intermediates, Ribose-1-As(V), Glucose-1-As(V), Glycerate-3-phosphate-1-As(V), Acetyl-As(V), Adenoside-monophosphate-As(V) and Adenosine-diphosphate-As(V) (Gregus et al., 2009; Nemeti et al., 2012, 2010).

It was observed that upon exposure of As(V) to *C. vulgaris*, 41% remained in this form followed by 30% methylation to DMA, and 29% reduction to As(III). Interestingly cells were not capable of chelating these newly formed arsenic species. Since glutathione synthase facilitates the reduction of As(V) by catalysing the arsenolysis of GSH and converting it into the arsenylated product')'-Glu-Cys-As(V) (Nemeti et al., 2012), we hypothesise that high concentrations of phosphate, such as the one used in this experiment, are able to shift the reaction towards methylation once the reduction of As(V) has happened as illustrated in Fig. 11. It is also possible that for As-PC formation, a certain concentration of As(III) needs to be present as "trigger".

For the aforementioned reasons, it is highly unlikely for cells growing under high phosphate to take up high amounts of As(V), to reduce it to As(III) to enable GS/PC complexation.

4.3. DMA effects on *C. vulgaris*

In the present study, formation of $DMAS^V$-GS was found to be unrelated to exposure period or DMA concentration in media. $DMAS^V$-GS has been predicted as the breakdown of a heavier complex molecule, rather than one synthesised via a specific mechanism (Bettencourt et al., 2011). After exposure of DMA, (up to 72 h)

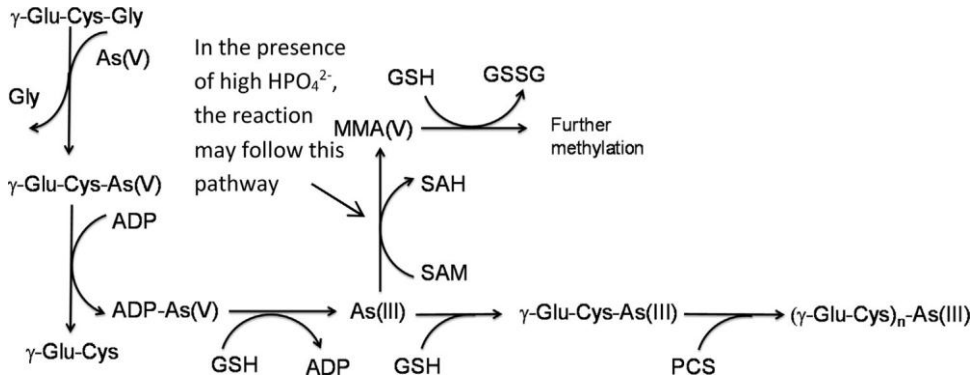

Fig. 11. Arsenic(V) reduction catalysed by glutathione synthase followed by alternative "Challenger" methylation pathway or phytochelatin production. S-adenosyl methio- nine (SAM), S-adenosyl homocysteine (SAH), reduced glutathione (GSH) oxidised glutathione (GSSG), adenosine diphosphate (ADP), phytochelatin synthase (PCS). Developed from Nemeti et al. (2012).

most of the arsenic was still in DMA form, nonetheless, cells were able to demethylate up to 7% of DMA to As(V) but no As(III) was observed. Complex formation ranged from 0.4–0.8% of the total arsenic present.

4.4. Arsenic transport mechanism (ABCC1/2)

Our findings regarding As(III) strongly suggest that *C. vulgaris* possesses not only the mechanism for GSH/PC complexation but also a specific transport system to store such complexes in vacuoles.

Experiments involving transport inhibitors have commonly targeted cells expressing ABCC1/2 proteins in plasma membrane (to export toxicants) whereas in experiments involving vacuole sequestration in plant/algae cells, in addition to passing through the cell membrane, inhibitors have to be directed across the cytosol and to vacuole membranes to be effective. Our results showed that the inhibition of cell growth (72 h) with MK571 (25 μM) was independent of the presence of arsenic (two-sample t-test, $p = 0.841$). The increased inhibition when cells were exposed to MK571 is not rare as ABCC1/2 transporters are ubiquitously expressed in cells and are implicated in many natural processes such as chlorophyll catabolite excretion and folate homologues storage into vacuoles (Kang et al., 2011).

In other studies, 5–50 μM of MK571 and 0.1–2.0 mM of probenecid have been shown to reduce ABCC1/2 like transport in ZF4 zebrafish cells by means of accumulation of Calcein-AM and rhodamine 123 (both substrates of ABCC1/2) in a concentration-dependent manner with very similar inhibition. The two inhibitors showed an increase in toxicity in cells exposed to Cd, Hg and As(III). In contrast, inhibitors did not change the toxicity of As(V) (Long et al., 2011).

Remarkably, probenecid (500 μM) had a significant stimulatory effect on cell growth (133% vs. 100% in control, $p <= 0.001$) but when 0.67 mM of As(III) and probenecid were present, cell growth was reduced (77% vs. 100% in control, $p = 0.021$). Interestingly, other studies have reported reversible inhibition of ATP mediated transport by probenecid after incubation in media without probenecid (Steinberg et al. 1987,1988).

Inhibition of ABCC1/2 transport in eukaryotic algae was reported with the marine diatom Thalassiosira rotula where verapamil, probenecid and MK571 were used as inhibitors using the fluorescent dye Fura 2 acetoxymethylester (AM) as model substrate. Dye loading into the cells was only observed using MK571 at more than 50 μM and not with other inhibitors. The authors concluded that MRP rather than MDR1 or organic anion transport was implicated (Scherer et al., 2008).

As BCECF shows a pH dependent spectral shift, lower fluorescence may indicate that cells treated with probenecid have lower internal pH than non-treated cells. Another possibility, one that has been consistently reported e.g. for Barley aleurone vacuoles and A. thaliana (Forestier et al., 2003; Jiang et al., 2011), is that the dye was effectively transported by non-treated cells via ABCC1/2 transport. In such case, the effect could be effectively reversed by probenecid, reflected in increased fluorescence. However, ABCC1/2 transport would not explain the increase in fluorescence for negative controls (heat killed) that have no active transport systems (Fig. S6). In contrast to BCECF, CMFDA contains a chloromethyl group that reacts with thiols, and does not show a pH dependent spectral shift. Also, once inside the cell, CMFDA is transformed into cell-impermeant molecules. Therefore a greater intensity indicates that the dye is effectively transported by non-treated cells. This strongly suggests that ABCC1/2 transport was in fact reversed by MK571.

Based on the observations in this paper, we propose a conceptual model to explain the mechanisms involving ABCC1/2 transport (Fig. 12). When ABCC1/2 transport is expressed in the plasma membrane with cells having no vacuoles, cells show less fluorescence than cells treated with inhibitor (Fig. 12a). Fluorescence with/without inhibitors will depend on the presence/absence of vacuole like organelles which can/cannot express ABCC1/2 transport. Fluorescence will also depend on the size of vacuole-like organelles (Fig. 12c and d). When ABCC1/2 inhibitor is present, fluorescence would be expected to increase for cases illustrated in Fig. 12a, b and c2. In contrast, lower fluorescence would be expected for cases illustrated in Fig. 12c1 and d.

When cells having vacuoles express ABCC1/2 transport in the plasma membrane but not in the vacuole membrane, the expected behaviour is the same as with cells having no vacuoles (Fig. 12B) (Cordula et al., 2008; Lebedeva et al., 2011; Long et al., 2011).

If cells express ABCC1/2 in the plasma and vacuole membranes, interpretation of results depends on microscopic observations or efflux measurements, because the difference in fluorescence alone depends on the size of vacuoles themselves, as shown in Fig. 12c1 and 2. In Fig. 12c1, higher intensity is expected in non-treated cells than in treated cells (ABCC1/2 inhibited) when vacuoles occupy a high volume of the cell. However, the behaviour is reversed when the vacuoles occupy a small volume, where higher fluorescence is expected in treated cells (ABCC1/2 inhibited) than non-treated cells (Fig. 12c2) (Forestier et al., 2003; Gayet et al., 2006; Swanson et al., 1998).

Some evidence suggests that ABCC1/2 transport is not active in plasma membranes of plants, but is triggered by high concentrations of xenobiotics inside cells. For example, in the case of the plant Nicotiana plumbaginifolia, its excretion of the antifungal sclareolide using ABC transport occurs only after relatively high concentration of the chemical is inside its cells (Jasinski et al., 2001).

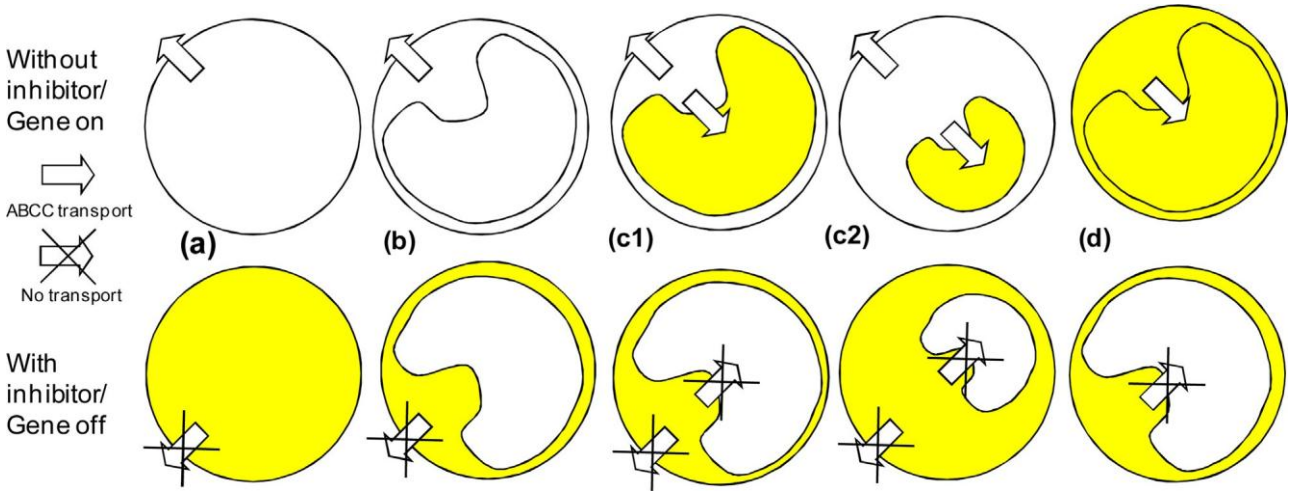

Fig. 12. Schematic illustration of scenarios for ABCC1/2 activity assay in cells after exposure to fluorescent substrates (e.g. Calcein-AM, Fura2, Lucifer Yellow, BCECF, CMFDA) (fluorescence indicated in yellow) with/without specific ABCC1/2 inhibitors (e.g. probenecid, MK571, taurocholate) or on/off gene silenced cells. ABCC1/2 expression in plasma membrane is observed in cases (a, b and c). Presence of vacuole-like organelles is observed in cases (b, c and d).

Apparently contradictory results were found in the present study, where increased fluorescence was observed by treatment with the two inhibitors (60 min). This contrasted with the results for arsenic efflux experiments (24 h) where a decrease in the amount of arsenic inside cells treated with MK571 compared with non-treated cells was observed. In order to correctly interpret these results, further reference to scenarios represented in Fig. 12 should be made. Firstly, the scenario described by Fig. 12a is clearly not feasible because algal cells do have vacuoles. Secondly, the scenario described in Fig. 12b is not likely to be observed as it would not be beneficial for non-motile cells to solely express outer membrane-only transport to eliminate substances in media where the substance can re-enter. Therefore, cases described in Fig. 12c and d, although dependent on the toxic load/concentration of the chemical, are the most probable scenarios. Summarising, Fig. 12c2 scenario would explain the increase in fluorescence observed in treated cells, whereas Fig. 12c1 and d would both explain the decrease in arsenic inside cells. In either case, strong statistical evidence has been found in this study for a difference in the behaviour of toxicity and accumulation of specific ABCC1/2 substrates (As-GS/PC, BCECF and CMFDA) upon exposure to specific inhibitors (probenecid and MK571).

When the vacuolar arsenic-PC transporter (at ABCC1/2) was reported for the first time, vacuoles from model organisms were isolated to subsequently measure transport. Two in vitro strategies were used: transport was measured in vacuoles inhibited by milimolar concentrations of vanadate and vacuoles taken from atABCC1/2 knockout plants. It was found that atABCC1 and atABCC2 were needed to mediate transport of As-PC in addition for cells to be able to produce PCs (Song et al., 2010). However this strategy allows the analysis of only a handful of As-PC complexes due to their labile nature in vitro. More recently it has been found that these two transporters are also needed for cadmium and mercury PC conjugate transport (Park et al., 2012).

5. Conclusions

Surface adsorption was found to be negligible for *C. vulgaris* cells exposed to DMA and As(V). However, adsorption of As(III) was found to be an active process on the surface of *C. vulgaris* cells perhaps enhanced by internal chelation with small thiol rich molecules (GS/PC).

The effects on *C. vulgaris* after exposure to As(V) can be explained in a number of ways depending on the concentration of phosphate in media: At high concentration of phosphate (1.62 mM of HPO_4^{2-}); poor adsorption of As(V) leads to low arsenic availability for intracellular uptake. At low concentrations of phosphate (3.2 μM of HPO_4^{2-}) strong evidence was found for an increase in the level of free thiols (where GSH is the most abundant). A decrease in intracellular pH was also observed and there was insufficient evidence of signals of oxidative stress. *C. vulgaris* cells did not produce any GS/PC complexes when exposed to As(V).

The formation of $DMAS^V$-GS was observed when *C. vulgaris* cells were exposed to DMA. The formation of $DMAS^V$-GS was found not to be related to the concentration of arsenic in the media or to exposure time. This could mean that the formation of $DMAS^V$-GS is part of another detoxification mechanism and that the complex is perhaps a fragment of a bigger molecule.

It was found that As(III) triggers the formation of hGSH/PCs, complexes of As(III) with hGS/PCs and compartmentalisation to vacuoles. *C. vulgaris* cells are able to chelate As(III) in three ways: cells are able to produce As-')'-Glu-Cys, As-Ala-GS and As-GS/PC complexes. No signs of oxidative stress nor changes in intracellular pH were observed as a result of As(III) exposure.

Strong statistical evidence was found in this study for a difference in the behaviour of As(III) toxicity and accumulation of specific ABCC1/2 substrates (As-GS/PC, BCECF and CMFDA) upon exposure to specific inhibitors (probenecid and MK571). The findings also suggest that there is ABCC/MRP related efflux into media also mediated by ABCC/MRP1/2 transport.

Many authors state or assume that oxidative stress is a toxic response of cells when challenged with arsenic. In this study we propose that this is not true for *C. vulgaris*. We present direct evidence that on exposure to As(III) the formation/complexation of GSH, hGSH, PC and hPC and their ensuing compartmentalisation to vacuoles in fact alleviates potential oxidative damage. Further, exposure to As(V) also did not increase signals of oxidative stress but elicited another response—a change in intracellular pH. These combined phenomena which we describe in *C. vulgaris* when challenged by arsenic have, to our knowledge, not been previously discussed.

Our overall observations point to clear future directions in the design of more complete remediation strategies using C. vulgaris since the microalga appeared to have dif-

ferent bioremediation potential depending on the arsenic form. It is better suited to bioremediate As(III) than As(V) from water and to do so over a wide range of phosphate concentrations (3.2 μM–1.62 mM of HPO_4^{2-}). Moreover, C. vulgaris appeared to store As(III) in vacuoles while retaining a high growth rate. Notably, the potential to transform As(V) into more toxic (for human life) As(III) is very low.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.02.020.

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