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The role of phosphorus in the metabolism of arsenate by a freshwater green alga, *Chlorella vulgaris*

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ABSTRACT

A freshwater microalga, *Chlorella vulgaris*, was grown in the presence of varying phosphate concentrations (<10–500 μg/L P) and environmentally realistic concentrations of arsenate (As(V)) (5–50 μg/L As). Arsenic speciation in the culture medium and total cellular arsenic were measured using AEC-ICP-MS and ICP-DRC-MS, respectively, to determine arsenic biotransformation and uptake in the various phosphorus scenarios. At high phosphate concentration in the culture medium, >100 μg/L P, the uptake and biotransformation of As(V) was minimal and dimethylarsonate (DMAs(V)) was the dominant metabolite excreted by *C. vulgaris*, albeit at relatively low concentrations. At common environmental P concentrations, 0–50 μg/L P, the uptake and biotransformation of As(V) increased. At these higher As-uptake levels, arsenite (As(III)) was the predominant metabolite excreted from the cell. The concentrations of As(III) in these low P conditions were much higher than the concentrations of methylated arsenicals observed at the various P concentrations studied. The switchover threshold between the (small) methylation and (large) reduction of As(V) occurred around a cellular As concentration of 1 fg/cell. The observed nearly quantitative conversion of As(V) to As(III) under low phosphate conditions indicates the importance of As(V) bio-reduction at common freshwater P concentrations. These findings on the influence of phosphorus on arsenic uptake, accumulation and excretion are discussed in relation to previously published research. The impact that the two scenarios of As(V) metabolism, As(III) excretion at high As(V)-uptake and methylarsenical excretion at low As(V)-uptake, have on freshwater arsenic speciation is discussed.

Introduction

Elevated arsenic (As) concentrations, whether from natural or anthropogenic origins, are of major concern due to a variety of health effects connected with human exposure to arsenic (Abernathy et al., 1999). The toxic properties of arsenic are dependent on its chemical speciation (Aposhian et al., 2003), and therefore environmental transformations, which affect the speciation of this trace element, are important to study in order to assess this contaminants' risk to humans and the environment.

In fresh surface waters, arsenic is found predominantly as oxidized inorganic arsenate, As(V). The reduced inorganic arsenite, As(III), is routinely found in fresh water environments at higher concentrations than thermodynamic calculation would predict (Cullen and Reimer, 1989). Organic arsenic

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compounds are also found in freshwater environments, generally as minor constituents and are only present in significant concentrations in limited scenarios (Sohrin et al., 1997).

The increased presence of the reduced inorganic and methylated organic forms of As in freshwater environments suggests that factors other than the redox potential determine As speciation. Correlations between biological productivity and As transformations have been made (Chen et al., 2008; Hellweger and Lall, 2004; Howard et al., 1995; Maki et al., 2007; Sohrin et al., 1997; Wang et al., 2014; Wurl et al., 2013), identifying the conversion as a biologically driven process. Algal cells take in As(V) through phosphate transporters due to chemical mimicry (Rosen and Liu, 2009). Arsenic cellular metabolism proceeds with As(V) being reduced to As(III) followed by a, kinetically slower, stepwise methylation through monomethylarsenate, MMAs(V), to DMAs(V) (Challenger et al., 1951). Microorganisms have demonstrated the excretion of both the reduced As(III) form (Knauer and Hemond, 2000) and/or the methylated forms (Hasegawa et al., 2001).

Biotransformation of arsenic has been heavily studied in the marine environment, but comparatively less is known regarding what regulates transformations of arsenic in freshwater environments (Rahman et al., 2012), although some explanations have been suggested. Due to the competitive interaction between arsenate and phosphate, the influence of phosphorus on arsenic speciation under the presence of freshwater algae has been the topic of speculation by many researchers (Baker et al., 1983; Hellweger et al., 2003; Rahman et al., 2012). While researchers have begun to associate arsenic speciation with phosphorus condition, strong empirical data is lacking and divergent results have generated confusion. Studies which have explored arsenic-phosphorus interactions with freshwater algae in laboratory exposures, as well as freshwater observations of arsenic speciation, are summarized in Table 1.

Researchers have demonstrated a higher level of As(V) bio-reduction to As(III) at low phosphorus concentrations, including in work with freshwater green algae (Knauer and Hemond, 2000; Wang et al., 2013) and cyanobacteria (Guo et al., 2011; Markley and Herbert, 2010). Contrastingly, Hellweger et al. (2003) developed a model from arsenic speciation observed in laboratory cultures (Hasegawa et al., 2001) which implicated high phosphorus conditions, and the luxury uptake of phosphorus by algae, as the cause of increased bio-reduction rates of As(V). Meanwhile, methylarsenicals have been connected to highly productive waters (Hasegawa et al., 2009), yet, methylarsenicals can predominate at low phosphorus conditions (Baker et al., 1983; Hasegawa et al., 2001). These incongruities regarding the effect of low or high phosphorus conditions on the bio-production of arsenicals could lead to confusion in the assessment of arsenic speciation in freshwaters.

Phosphorus concentrations in freshwater lakes range from <10 μg/L in oligotrophic waters to >100 μg/L in eutrophic waters (Rast and Holland, 1988); previous research examining arsenic interactions with algae has often used extremely elevated P concentrations, tested only one or two concentrations of P (*e.g.*, a low and a high) and/or has indicated an initial P concentration without tracking the change in, or maintaining, the levels of this nutrient in the exposure. Overall, most research has lacked a quantitative approach to the phosphorus dynamics of the algal system. In addition to elevated P concentration, studies have employed arsenic at concentrations which are orders of magnitude above freshwater levels.

Table 1 – Overview of previous studies in which freshwater algae were exposed to arsenate at varying phosphorus conditions.

Algal species	Stage of algal growth	Phosphorus (μg/L)	As(V) (μg/L)	As speciation	Role of P	Reference
Chlorella sp.[a]	Logarithmic	6 or 6 × 10^3	0.075 to 75	Quick reduction to As(III). Methyl-arsenicals not analyzed.	Increase in bio-reduction under low P concentration	Knauer and Hemond, 2000
C. aciculare[a]	Logarithmic and Stationary	341 or 589	0.75 or 900	DMAs(V) dominant in stat growth while As(III) dominant in log growth.	DMAs(V) production increased with decrease in P:As ratio.	Hasegawa et al., 2001
C. reinhardtii[b] and *S. obliquus*[b]	Logarithmic	−P (limited) +P (enriched)	7.5 to 750	Quick reduction to As(III). Methyl-arsenicals not detected.	Intracellular As:P best determinant of toxicity. Increase in bio-reduction under P-limited condition.	Wang et al., 2013
Chlorella sp.[a] and *M. arcuatum*[a]	Logarithmic	150 or 1.5 × 10^3	100 to 40 × 10^3	As(III) excreted at high (*M. arcuatum*) and low (*Chlorella*) levels. No methylarsenicals excreted after 72 hr.	Increase in P concentration decreased toxicity of As(V).	Levy et al., 2005
Variety	Logarithmic and Stationary	<1–100 (Rast and Holland, 1988)	<1–10 (Terlecka, 2005)	DMAs(V) during stationary growth and As(III) spike associated with logarithmic growth (*i.e.*, algal blooms)	Limiting nutrient which, generally, stimulates growth of freshwater algae. Competes with As for uptake.	Freshwater observations (Hasegawa et al., 2010, Hasegawa et al., 2009; Hellweger and Lall, 2004; Hellweger, 2005; Howard et al., 1995)

[a] Isolated algae;
[b] Laboratory strain.

The drinking water guideline has been set at 10 μg/L in Canada and by the World Health Organization (Health Canada, 2014; WHO, 2011) and concentrations of As, in unpolluted surface waters range from <1 to 10 μg/L (Terlecka, 2005). Thus, as the majority of the work reviewed in Table 1 has employed either elevated concentrations of the elements of interest or a limited number of environmentally relevant scenarios, the data on the interaction between phosphorus and arsenic is incomplete.

The research presented in this study focuses on the forms of arsenic found in a culture of a common freshwater alga exposed to environmentally relevant concentrations of arsenic, with the majority of the exposures initiated with 10 μg As/L, and likewise, environmentally realistic concentrations of phosphorus, well below suggested medium concentrations (Nichols and Bold, 1965). The P concentration range applied in this study is <10–500 μg P/L, and therefore encapsulate levels of starvation, low and elevated P concentrations. Phosphorus concentrations in the medium and cell were monitored throughout the experiment to develop an understanding of the phosphorus dynamics in the culture. The chosen range of P concentration is used to predict arsenic metabolites as a function of nutrient status (*i.e.*, eutrophication). This examination of the dynamics between phosphorus, a freshwater alga and arsenic speciation in a laboratory culture will aid in consolidating previous studies and testing theories postulated in those studies, as well as explaining the speciation dynamics observed in water bodies with risk-relevant concentrations of arsenic.

1. Experimental

1.1. Chemicals

Arsenite (sodium meta-arsenite, J.T. Baker, Phillipsburg, NJ, USA), arsenate (arsenic acid disodium salt heptahydrate, Sigma Aldrich, Oakville, ON, USA), dimethylarsinic acid (cacodylic acid sodium salt trihydrate, Acros, Geel, Belgium) and methylarsonic acid (disodium monomethyl arsenic acid, Chem Service, West Chester, PA, USA) were used for arsenic exposures and as primary arsenic standards. Secondary arsenic standards were used in arsenic analysis: arsenate and arsenite as certified solutions (High Purity Standards, Charleston, SC, USA), dimethylarsinate as dimethylarsinic acid (Sigma Aldrich) and methylarsenate as monosodium acid methane arsonate sesquihydrate (Chem Service, West Chester, PA, USA). Dibasic ammonium phosphate (Sigma Aldrich) was used for exposures and analysis. Sodium hydroxide solution for the ion chromatography eluant was made from 50% (W/W) NaOH purchased from Fisher Scientific (Ottawa, Ontario).

1.2. Culture growth

Chlorella vulgaris, strain UTCC 90, was received from the University of Toronto Culture Collection (now the Canadian Phycological Culture Centre, University of Waterloo). Precautions were taken to ensure that all cultures were free from microbial contamination, including the handling of all experimental solutions under a laminar flow hood, open flame sterilization of the inoculation loop and Erlenmeyer flask tops, ethanol sterilization of the lab bench and autoclaved glassware/media. All culture media, glassware and pipette tips were autoclaved for 25–30 min at 15–20 psi and 121°C. Stock algal cultures were created from single colonies of cells scraped from agar algal plates and were prepared with similar medium conditions as the experimental cultures. Experimental cultures were started through the addition of a 10 mL inoculant, removed from a stock culture in exponential growth, and transferred into 500 mL of fresh culture medium in a 1 L Erlenmeyer flask. Flasks were capped with cotton plugs, placed in an environmental chamber maintained at 25°C, on an 18:6 photoperiod and rotated on a shaker at 110 r/min. Cultures were exposed to arsenic for 7 days during exponential growth.

Cultures began with medium concentrations of 10% Bold's basal medium (Nichols and Bold, 1965) and 10 μg/L of As(V). Single experiments with 10 μg/L As(III) and DMAs(V), respectively, were also performed. Phosphorus in the culture was modified in exposures with As(V), with specific concentrations shown in Table 2. Phosphorus concentrations in the culture medium and cell were determined via spectrophotometry (McKelvie et al., 1995). Nitrogen concentrations were modified to create nitrogen to phosphorus molar ratios of approximately 17, in all exposures and additions. Daily nutrient additions were used to maintain culture medium concentrations, specifically phosphorus, and the addition rates/schedules are presented in Table 2. Exposures to As(III) and DMAs(V) employed additions of 560 μg P/L daily.

Two experiments, indicated as H-1 and H-2 in Tables 2 and 3, were conducted to maintain high [P] (*i.e.*, >150 μg/L). Three experiments were completed maintaining the [P] between 20 and 150 μg/L, indicated as M-1 through M-3 in Tables 2 and 3. Experiments M-2 and M-3 used multiple daily additions, while Experiments L-1 and L-2 utilized only single daily additions of phosphorus, and these latter two experiments simulated low phosphorus conditions (*i.e.*, <20 μg/L). Phosphorus starvation experiments (indicated as S-5, S-10 and S-50 in Tables 2 and 3) were also run to test the effect of this scenario on cellular arsenic transformation. In these experiments the initial concentration of phosphorus, 560 μg/L, was not replenished and after taking up this phosphorus the cells remained without additional phosphorus for the rest of the exposure. All exposures were initiated with 10 μg/L of As(V) except for the P- starvation experiments S-5, S-10 and S-50 which were initiated with 5, 10 and 50 μg/L of As(V), respectively.

All experiments involved the analysis of triplicate exposure cultures and a single (or duplicate) non-arsenic control or blank culture. Chlorophyll *a* (Chl *a*) was extracted from centrifuged algal cell pellets with cold 95% ethanol (ACP Chemicals, Toronto, ON, USA) and determined with a UV/Vis Spectrophotometer (Perkin Elmer, Woodbridge, ON, USA). Centrifuged algal cell pellets were heated in a muffle oven at 60°C for 24 hr to determine dry weight. Cell counts were completed using a Beckman–Coulter Cell Counter (Vi-Cell XR Cell Analyzer; Mississauga, ON, USA). Growth measurements were not determined for the starvation experiments (S-5, S-10 and S-50).

Table 2 – Phosphorus parameters for As(V) exposures.

Experiment	Daily phosphorus (μg/L) [# of additions]	Medium P concentration (μg/L)		Final cellular P concentration (mg/g)	Phosphorus recovery [a]
		Initial	Final		
High-1 [H-1]	455 [once]	1600	1950	23.5	98
High-2 [H-2]	400 [once]	600	540	25.3	90
Medium-1[M-1]	110 [once]	100	41 ± 10	14.0 ± 1.3	107
Medium-2 [M-2]	110 [four times 27.5] [b]	25	28 ± 8	12.1 ± 0.7	106
Medium-3 [M-3]	106 [twice 53] [c]	50	24 ± 1	11.1 ± 0.1	94
Low-1 [L-1]	53 [once]	50	10 ± 2	6.6 ± 0.1	102
Low-2 [L-2]	40 [once]	30	<10	6.3 ± 0.3	103
Starvation-5 [S-5] [d]	0 [none]	560	<10	nd	nd
Starvation-10 [S-10] [d]	0 [none]	560	<10	nd	nd
Starvation-50 [S-50] [d]	0 [none]	560	<10	nd	nd

Final medium and cellular P concentration are presented as the average of triplicate culture samples, ±SD (n = 3). Initial medium P concentration presented as nominal concentration. Triplicate cultures were not performed for experiments H-1 and H-2.

[a] Sum of cellular and medium phosphorus divided by total phosphorus added.

[b] 4 additions of 27.5 μg/L [schedule: 0700 hr, 1200 hr, 1800 hr and 2330 hr];

[c] 2 additions of 53 μg/L [schedule: 0800 hr and 1700 hr];

[d] S-5, S-10, S-50 contained 5, 10 and 50 μg/L As (V), respectively, while all other experiments contained 10 μg/L As (V).

nd: not determined/analyzed.

1.3. *Determination of arsenic compounds and total arsenic*

Culture samples were centrifuged at 4000 r/min for 20 min and the supernatant was used for the determination of arsenic speciation in the medium. Speciation was determined using anion exchange chromatography coupled to inductively coupled plasma mass spectrometry (AEC-ICP-MS). Separation and detection parameters were similar to those used previously for the analysis of inorganic and methylated As (Wallschläger and London, 2008). A standard chromatogram from this speciation analysis can be found in Appendix A Fig. S1.

Total cellular arsenic was determined through the analysis of cell pellets created from centrifugation. Algal cell pellets were digested with concentrated nitric acid (Fisher Scientific) on a hot plate set to 350°F for 2 hr. Samples were diluted to 2% nitric acid and total As concentrations were determined using ICP-MS (Elan DRCII, Perkin Elmer) in dynamic reaction cell mode with oxygen, O_2, as a reaction gas.

Quality assurance/quality control procedures used to ensure accuracy included: blanks, standard reference materials, secondary standards, spikes, duplicates, as well as continuing calibration verification and blanks. AEC-ICP-MS detection limits for

Table 3 – Arsenic speciation in culture medium, cellular arsenic concentrations, arsenic recovery and total arsenic uptake after 7 day exposure.

Experiment	P-daily (μg/L)	As(V) (μg/L)	As(III) (μg/L)	DMAs(V) (μg/L)	MMAs(V) (μg/L)	Other species-OS (μg/L) [a]	Cellular As (μg/g d.w.) [b]	Recovery [c]
H-1	455	8.78	<0.02	**0.06**	<0.01	<0.01	nd	nd
H-2	400	9.40	<0.02	**0.1**	<0.01	<0.01	nd	nd
M-1	110	9.11 ± 0.79	0.09 ± 0.04	**0.25 ± 0.07**	0.04 ± 0.02	0.13 ± 0.04	14.8 ± 0.6 [d]	104
M-2	110	8.59 ± 0.46	0.15 ± 0.05	**0.38 ± 0.04**	0.08 ± 0.03	0.20 ± 0.03	18.3 ± 1.4	104
M-3	106	4.58 ± 0.73	**3.54 ± 0.70**	0.29 ± 0.01	0.06 ± 0.01	0.13 ± 0.01	17.3 ± 0.7	95
L-1	53	1.95 ± 1.13	**7.72 ± 3.12**	0.34 ± 0.04	0.06 ± 0.01	0.18 ± 0.04	18.9 ± 0.0	112
L-2	40	0.90 ± 0.89	**7.29 ± 1.54**	0.31 ± 0.02	0.08 ± 0.01	0.18 ± 0.02	19.2 ± 1.1	96
S-5 [e]	0	0.30	**3.23**	0.35	0.17	nd	nd	nd
S-10 [e]	0	1.04	**6.65**	0.62	0.39	nd	nd	nd
S-50 [e]	0	8.50	**43.82**	0.67	1.38	nd	nd	nd

Dominant produced metabolite in bold. As concentration reported as the average of triplicate cultures and triplicate samples, ±SD (n = 9). Triplicate cultures not performed for experiments H-1, H-2, S-5, S-10 and S-50.

[a] Other species. Arsenic concentration in dead volume. This concentration was quantified using peak area correlations from the closest eluting arsenic species, DMAs(V);

[b] Celullar As. Arsenic determined in cell pellets created after centrifugation;

[c] Sum of culture medium and cellular arsenic divided by initial arsenic (×100%);

[d] Significant difference between Experiment M-1 and M-2/ M-3/ L-1/L-2 ($p < 0.05$);

[e] S-5, S-10, S-50 contained 5, 10 and 50 μg/L As(V), respectively, while all other Experiments contained 10 μg/L As(V).

nd — not determined/analyzed; DMAs: dimethylarsonate.

As(III), As(V), MMAs(V) and DMAs(V) were estimated at 0.020, 0.075, 0.010 and 0.010 μg/L respectively. The certified reference material used was TMDA-61 (Environment Canada, Burlington, ON), and had an average recovery of 31.6 ± 6.4 μg/L (sum of all As species) and 33.8 ± 5.8 μg/L (total arsenic), using AEC-ICP-MS and ICP-DRC-MS analysis, respectively (certified value: 33.5 μg/L).

1.4. Statistics

Statistical analyses were performed using STATISCA 7. Two-sample t-tests were completed to test for differences in cell counts, chlorophyll *a* and dry weight. A one way analysis of variance (ANOVA) was used to determine differences in cellular arsenic concentrations amongst all phosphorus trials. This was followed by Bonferroni post-hoc analyses to distinguish differences between specific phosphorus trials. Simple regression analysis was used to determine the correlation between phosphorus and arsenic accumulation/uptake.

2. Results

2.1. Growth and phosphorus dynamics

The toxicity of As(V) to *C. vulgaris* was analyzed by comparing parameters of arsenic free controls with 10 μg/L As(V) exposures. No significant toxicity was observed when comparing Chl *a*, dry weight and cell counts of the controls with any of the As(V) exposures (Appendix A Table S1).

Although the maintenance of the phosphorus concentrations was completed daily, and in some cases multiple times daily, the concentration of this nutrient in the culture medium displayed large fluctuations, as is to be expected in an algal culture in exponential growth (Appendix A Fig. S5). Comparison of algal phosphorus uptake and the phosphorus added to the culture indicated that the algae were in phosphorus limited conditions (Appendix A Figs. S2–S3). In addition, the uptake of phosphorus by the algal cells did not significantly deviate from a Michaelis–Menten predictions, as has been shown previously for phosphorus-limited cells (Currie and Kalff, 1984), and indicated that no luxury uptake was observed (Appendix A Fig. S4). Phosphorus mass balance results, including tracking of medium and cellular phosphorus, final concentrations at termination of the 7-day exposure, as well as correlations regarding phosphorus-growth and Michaelis–Menten models can be found in Table 2 and in the Appendix A Figs. S2–S5.

2.2. Arsenic uptake

Arsenic concentrations in the *C. vulgaris* cells in experiments with medium and low phosphorus conditions showed only marginal changes, with concentrations ranging from 14.8 to 19.2 μg As/g (d.w.) (Table 3). Experiment M-1, was the only culture within these five experiments which had a significantly different (lower) cellular arsenic concentration ($p < 0.05$, ANOVA), despite the fact that daily phosphorus additions varied by a factor of three between these experiments. The lack of significant difference in cellular arsenic concentrations between M-2, M-3, L-1 and L-2 could lead to the inaccurate conclusion that phosphorus does not play a major role in arsenic uptake. However, cellular arsenic only represents the arsenic which is associated with the cell after 7 days (*i.e.*, assimilated).

Using total arsenic uptake, defined as the sum of cellular arsenic and the biologically-produced arsenic metabolites in the culture medium, a definitive relationship between arsenate and phosphate is established. A strong inverse correlation between total arsenate uptake (sum of cellular arsenic and produced arsenic metabolites) and phosphorus uptake was demonstrated across all cultures (Fig. 1).

Within cultures of *C. vulgaris* exposed to 10 μg/L As(V) with high P concentration (*i.e.*, >150 μg/L), a low level of As-uptake was observed, exhibited through lowered accumulation and biotransformation in experiments H-1 and H-2 (Table 3), likely due to competitive inhibition of As(V)-uptake by phosphate. When medium P concentration was lowered (<150 μg P/L), competition from phosphorus decreased and total As uptake was shown to increase, demonstrated by a slight increase in arsenic accumulation and a large increase in metabolites present in the culture medium of experiments M-1/2/3 through L-1/2 (Table 3).

2.3. Arsenic metabolism and excretion

Small amounts of DMAs(V) in the culture medium were observed in As(V) exposures at high phosphorus conditions in experiments H-1 and H-2 (Table 3). As medium P concentration was lowered, in experiments M-1 and M-2, algae converted As(V) to multiple species. In addition to DMAs(V), the main metabolite found in the culture medium, As(III) and MMAs(V) were also excreted from algal cells. Some arsenic eluted in the dead volume of the anion exchange chromatograph in these exposures, representing one or more other excreted metabolite(s), indicated as other species (OS) in Table 3. Possible identities of the neutral or positively charged unknown form(s) of arsenic found in exposures M-1 through L-2 could include trimethyl arsine oxide (Baker et al., 1983), or an arsenosugar found to elute in the dead volume under similar chromatographic conditions (Price et al., 2013).

Appearance of biologically-produced arsenic species at higher medium P concentration was small in comparison to the exposure concentration of 10 μg/L and the vast majority

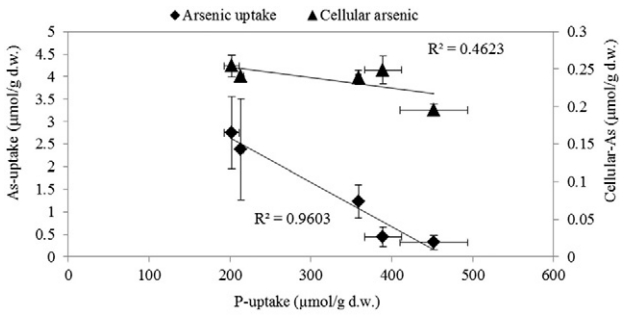

Fig. 1 – Role of P-uptake in cellular arsenic and As-uptake. Arsenic uptake determined by sum of cellular arsenic and the bio-produced arsenic metabolites in the culture medium (from M-1/2/3 and L-1/2 (*n* = 5)). Error bars represent ± SD (*n* = 3).

of aqueous arsenic remained as As(V) (80%–90%). As the biotransformation of As(V) increased at the lower medium P concentration, specifically in experiments M-3, L-1 and L-2, As(III) found in the culture medium began to increase to levels much higher than those of the methylated arsenicals in these experiments, as well as those seen in the high P concentration. This trend continued in the experiments under which the algal cells were phosphorus starved (S-5, S-10 and S-50). In these experiments, the culture mediums did not contain any available phosphorus after 7 days of algal growth and an increase in As(V) exposure concentration led to a correlated increase in As(III) in the culture medium.

MMAs(V) and OS concentrations in the culture medium of experiments H-1 through L-2 were approximately one-third and two-thirds the concentration of DMAs(V), respectively (Table 3). In fact, increases in MMAs(V) and OS correlated with increases in DMAs(V) in all these non-starvation experiments, with R^2 of 0.71 and 0.98 respectively (Appendix A Fig. S7). The concentration of MMAs(V) was higher than that of DMAs(V) in only one experiment, experiment S-50 which had the highest As(V)-uptake of any experiment. Increasing concentrations of As(III) were found at half the concentration of the increasing DMAs(V) (R^2 = 0.99), in Experiments H-1 through M-2 (Appendix A Fig. S7), prior to the stark increase in As(III) prevalence in the low phosphorus experiments (M-3, L-1 and L-2).

Using total As uptake (cellular + metabolites) and culture cell counts, a value of 1 fg As/cell was estimated as an approximate threshold or border value between the two predominant scenarios of As transformation, biomethylation and bioreduction. This was estimated as the average of total cellular uptake values for experiments M-2 and M-3, the experiments between which the dominant metabolite in the culture medium changed from DMAs(V) to As(III) (Table 4). Below 1 fg As/cell the dominant process by the algae is the excretion of the methylated species and cellular assimilation (Fig. 3a–b), but above this level of uptake, the dissimilatory reduction of As(V) to As(III) becomes the dominant process (Fig. 3c).

2.4. Exposures to other arsenicals

Cellular As concentration was determined as 8.0 ± 0.5 μg/g after *C. vulgaris* cultures were exposed to 10 μg/L As(III) for 7 days (average of triplicate cultures ± SD, n = 3). Lower cellular arsenic was observed in algal cultures exposed to 10 μg/L DMAs(V) for 7 days, with cellular As concentration of 2.4 ± 0.4 μg/g (triplicate ± SD, n = 3), accounting for <2% of the initial DMAs(V).

The As(III) introduced at a concentration of 10 μg/L was converted to As(V) by *C. vulgaris*, at higher levels than algal free cultures, demonstrating bio-oxidation (Fig. 4). Over 50% of the 10 μg/L of As(III) introduced to the culture was transformed by the algae to As(V). Although substantial quantities of cellular arsenic were observed in As(III) exposures, no methylarsenicals were observed in the culture medium after the 7 days.

The other major species found, DMAs(V), when introduced into cultures of *C. vulgaris* at a concentration of 10 μg/L, did not produce any other arsenic species in the culture medium after the 7 days, suggesting a lack of demethylation or further methylation of DMAs(V) by *C. vulgaris*.

3. Discussion

Cellular As concentrations determined in arsenate exposures were in agreement with previous cellular concentrations found in As(V) exposures involving freshwater algae by other researchers (Hasegawa et al., 2001; Levy et al., 2005; Murray et al., 2003). Cellular As concentrations were lower after exposure to either As(III) or DMAs(V) than after exposure to As(V), which agrees with previous work completed at arsenic exposure concentrations orders of magnitude higher than those used in our study (Maeda et al., 1985; Pawlik-Skowrońska et al., 2004).

Cellular arsenic (*i.e.*, assimilated arsenic) after As(V) exposure did not mirror the increasing As uptake (*i.e.*, the cellular + produced metabolites) and became a minor component in the fate of the arsenic (Table 4) in the exposures containing <100 μg P/L. In fact, the regression analysis comparing cellular arsenic between exposures M-1 through L-2 was not statistically significant (p = 0.2, Fig. 1). This disconnection between cellular arsenic and phosphorus may be a source of some of the confusion regarding the role of phosphorus in arsenic bioaccumulation and biotransformation. Some previous studies have concluded that phosphate did not have an effect on arsenate uptake (*e.g.*, Budd and Craig, 1981), but did not measure arsenicals in the culture medium to quantify the total amount of arsenic which had experienced an uptake-conversion-excretion process and therefore may have confused As accumulation with uptake. Similarly, researchers working with marine algae demonstrated

Table 4 – Fate of arsenic in *C. vulgaris* cells.

Experiment	DMAs(V) excreted (%) [a]	As(III) excreted (%) [a]	Cellular As (%) [a]	Total As-uptake — (fg/cell) [b]	Major produced metabolite	Dominant cellular process
M-1	19	7	62	0.35	DMAs(V)	Accumulation
M-2	20	8	55	0.63	DMAs(V)	Accumulation
M-3	6	71	19	1.40	As(III)	Excretion
L-1	4	83	11	7.70	As(III)	Excretion
L-2	4	84	9	7.70	As(III)	Excretion

[a] Percent of total As-uptake;
[b] Calculated as sum of DMAs(V), As(III), cellular and MMAs(V) + OS (not shown) per cell of algae.

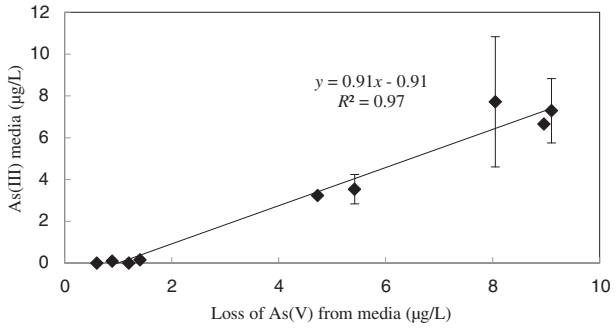

Fig. 2 – Loss of arsenate from culture medium and presence of arsenite after 7 days in low phosphorus media (Exp M-1 to L-2, S-5 and S-10).

that phosphorus did not play a role in arsenic accumulation and therefore concluded that it also did not play a role in arsenic uptake (Foster et al., 2008). They then found contradictory evidence when they further explored the changes in arsenic speciation at various phosphorus levels with the same algae (Duncan et al., 2013). It is therefore important that experiments quantify total As uptake by determining possible excreted metabolites in addition to the arsenic associated with the cell, to garner a better understanding of the quantity of arsenic that has actually been taken in by the cell, as well as the role of the water quality/chemistry on this uptake.

The correlation between phosphorus and arsenic uptake ($p = 0.0032$) is a strong quantitative depiction of the competitive interaction between phosphate and As(V) for uptake by freshwater alga. Other researchers have suggested this competition for uptake by freshwater alga (Baker et al., 1983; Levy et al., 2005; Maeda et al., 1985), but have not provided a quantitative analysis of this effect over a range of relevant phosphate concentrations.

The presence of methylated metabolites in the culture medium can provide insight into arsenic biomethylation in varying environmental conditions. DMAs(V) in the culture medium, and lack of other detectable species, at high P concentration suggests that DMAs(V) is the primary excreted arsenical by algae at low As-uptake levels. The production of methylarsenicals demonstrated here at high [P] contradicts the conclusion made by some researchers that low P concentration is essential in the production of methylarsenicals (Baker et al., 1983; Hasegawa et al., 2001). Nevertheless, the methylated arsenicals increased logarithmically with decrease in medium P concentration, similar to results reported by Hasegawa et al. (2001), but plateaued below 10% of the original total arsenic (*e.g.*, a maximum DMAs(V) concentration of 0.6 µg/L was observed in all As(V) experiments).

The low concentrations of the methylated arsenicals observed in As(V) exposures match the low concentrations generally found in freshwaters (Terlecka, 2005). When methylarsenicals are occasionally detected in freshwater systems they are often found at their highest concentrations during the stationary growth of algae (Hellweger et al., 2003). The connection can therefore be made between the predominant excretion of methylarsenicals and the low uptake of As(V) by algae, caused either by stationary growth (Goessler et al., 1997; Maeda et al., 1985) or elevated competition from phosphorus, as shown in the present study.

Varying levels of methylation observed in previous laboratory cultures may be partially due to the changes in the activity of arsenic methyltransferases between different species of algae, as suggested by the work of Qin et al. (2009). More work is needed to determine a limit of arsenic methylation in other algal species, as was done here for *C. vulgaris*, to delineate

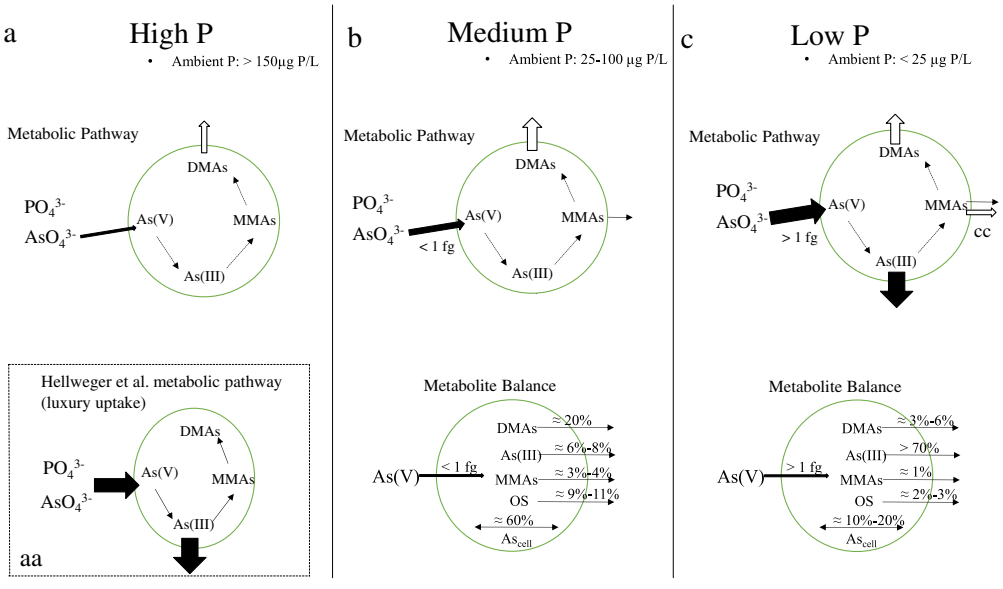

Fig. 3 – Arsenic metabolism pathways in *C. vulgaris* under different phosphate exposure regimes 3a: High ambient P concentration — DMAs excretion 3aa: Hellweger et al. (2003) production of As(III). 3b: Medium P concentration — assimilated arsenic and excreted DMAs 3c: Low P concentration—As (III) as the dominant excreted product. 3cm³: Exp S-50 — MMAs as dominant methylated excreted metabolite.

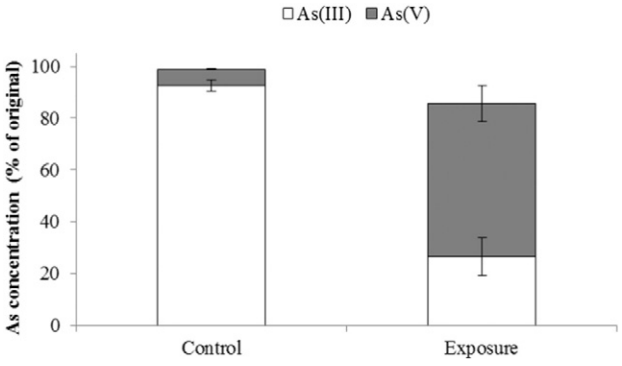

Fig. 4 – Arsenic Speciation of 10 μg/L As(III) after 7 days in the presence and absence of *C. vulgaris* (560 μg/L of daily phosphorus).

the methylation range for freshwater algae. This may help to discern some of the discrepancies observed in freshwater arsenic biotransformation work (Table 1).

Although methylarsenicals were produced, algal reduction of As(V) to As(III) was more significant and highlighted by a strong relationship ($p < 0.001$) between the loss of As(V) and the presence of As(III) in the culture medium (Fig. 2). This relationship indicated that >90% of the metabolized As(V) was present as As(III) in experiments of low P concentration. Similarly, the algal experiments of Geiszinger et al. (2001), Knauer and Hemond (2000) and Wang et al. (2013) observed an increase in reduction rates as arsenic uptake increased, due to higher As concentration and/or lower P concentration in their cultures.

Bio-reduction observed here may be of major importance for the speciation of arsenic in arsenic-contaminated, biologically-productive fresh waters. Correspondingly, the occurrence of As(III) has been associated with the growth of algal communities in freshwater bodies (Hellweger et al., 2003). Our results show a strong competition between As(V) and phosphorus uptake and, therefore, the majority of As(V) uptake by freshwater algal cells may occur towards the end of logarithmic growth, when nutrient (phosphorus) concentrations have been depleted and competition lowered. This may explain the lag between observation of logarithmic growth and that of an As(III) peak (Hellweger, 2005).

Our work suggests that at very high As-uptake rates the methylation pathway may also become overburdened after the first step of methylation, causing MMAs(V) to be the foremost excreted methylated arsenical. Research on the metabolism of arsenic by humans has revealed that an elevated concentration of MMAs(V) in comparison to DMAs(V) in the urine implies poor elimination of arsenic (Vahter, 2002), but this has not been described previously in freshwater algal exposures.

Testing with a cyanobacterium, *Microcystis aeruginosa*, at comparatively elevated concentrations of arsenate (75 and 750 μg/L) and four phosphate situations (deprived, limited, rich and excess) by Guo et al. (2011) determined two arsenic speciation scenarios similar to those observed in the present study with *C. vulgaris*. Both As(III) and DMAs(V) were produced under the phosphate-deprived and phosphate-excess conditions conducted by Guo et al. (2011), with the deprived condition potentially mirroring our low and starved phosphorus algal cultures and the excess condition potentially introducing the luxury uptake described by Hellweger et al. (2003). Meanwhile the cyanobacterium in the Guo et al. (2011) study produced DMAs(V) alone when exposed to the phosphate-limited and phosphorus-rich conditions. Although their phosphate concentrations were higher than those employed in this study, the observation of DMAs(V) alone in their limited and rich phosphorus conditions, mirrors the predominance of this methylarsenical in the medium and high phosphorus conditions of this study.

While a strong connection between As speciation and P concentration was found in the present study, the results did not further define the role of algal growth rate and luxury P-uptake, both of which have been linked to arsenic speciation (Hasegawa et al., 2001; Hellweger et al., 2003). The algae in this study were always in logarithmic growth, yet scenarios with either arsenite or methylarsenicals as the predominant metabolite were found. Therefore, a direct connection between the rate of growth (either logarithmic and/or stationary) and arsenic speciation should only be made with caution. As no algal cultures here were found to exhibit luxury uptake of phosphorus, which was suggested to be the cause of the occurrence of As(III) in freshwaters (Hellweger et al., 2003), the importance of this mechanism for arsenic speciation could not be verified. Although, in situations where luxury uptake of phosphorus is possible, this phenomenon could increase the uptake of As(V), which would indeed lead to an increase in As(III) production, based on our findings.

These results demonstrate that it may be possible to further use the strong link between ambient phosphorus concentration and arsenic speciation as a tool in the predictions of the freshwater arsenic biogeochemical cycle. For example, a qualitative estimation of a switch between the two scenarios of arsenic speciation was identified in our work at an average medium phosphorus concentration of 20 to 40 μg P/L (Table 2). This is a concentration of phosphorus which corresponds with the trophic change generally observed between mesotrophic systems (*i.e.*, moderate algal growth) and eutrophic systems (*i.e.*, large algal growth). Interestingly, the work by the Hasegawa group (Hasegawa et al., 2010, Hasegawa et al., 2009; Rahman et al., 2012) with freshwater bodies in Japan has linked arsenic speciation to differences between the mesotrophic and eutrophic classification. Furthermore, Wurl et al. (2013) discovered a strong quantitative relationship between As(III) and decreasing phosphorus concentrations in a marine environment.

The interactions observed in this study between phosphorus, arsenic and algae are not only important for primary producers and the surrounding water, but may be important for higher trophic level organisms. A study with freshwater cladocerans demonstrated the role of phosphorus condition of algae on the fate of arsenic in these organisms (Miao et al., 2012). Meanwhile, a study completed with mosquitofish, found that the presence of algae in arsenic exposures increased the adverse effects of arsenic to fish (Magellan et al., 2014). These authors suggested that arsenic bioremediation programs with algae should only be carried out with careful consideration, and although no As speciation

measurements were conducted, the excretion of As(III) by their algae may have been the culprit for the increased adverse effects observed. Using algae in bio-remediation programs is currently widely suggested (Bahar et al., 2016; Levy et al., 2005; Murray et al., 2003; Wang et al., 2013; Yin et al., 2012).

While the presence of As(III) in freshwater systems is of concern, the presence of this reduced arsenic compound is typically only observed for a short time period (or spike) (Hellweger, 2005). The quick oxidation of As(III) by the cell in As(III)-algae exposures completed in this study offers an explanation for this observation. Due to the lower concentration of arsenic associated with the cells in As(III) exposures and the lack of methylated metabolites observed in these exposures, it is possible that the As(III) bio-oxidation by *C. vulgaris* is an extracellular process, as previously suggested by the work of Qin et al. (2009). Therefore, after As(V) is bio-reduced to As(III) and excreted from the cell, algal cells may be involved in the re-oxidation of As(III), in addition to non-biological oxidation. Alternatively, freshwater systems with high As(III) concentrations and high biological activity could therefore see similar biotransformation as As(V)-dominated systems, due to the bio-oxidation of As(III) to As(V) and subsequent uptake of As(V) by microorganisms. While the bio-oxidation observed in this work was significant, it was less than the level of bio-reduction observed in the As(V) spiked exposures at environmental P concentration, indicating that As reduction is the net effect of the As-algae interaction.

Our work did not study the role that nutrient levels, such as phosphorus, have on uptake/biotransformation of As(III) and DMAs(V), but this could be a focus of future research. Recent research has indicated that nutrients may also play a significant role on As(III) biogeochemical transformations (Wang et al., 2014).

4. Conclusions

Arsenic uptake by *C. vulgaris* was more strongly connected to phosphorus condition than arsenic accumulation, potentially indicating a cause of confusion in previous research and highlighting the importance of quantifying both cellular arsenic and arsenic speciation in the culture media. Contrasting speciation was related to phosphorus concentration (and therefore cellular uptake), with the excretion of As(III) being the biotransformation pathway of the most importance in cultures with environmental concentrations of phosphorus. Based on findings of this study, we encourage the use of phosphorus condition and microorganism growth in freshwater arsenic assessments, while advising caution in the use of algae in the remediation of arsenic.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2016.10.002.

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