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**Supplementary material**

# Influence of culture regime on arsenic cycling by the marine phytoplankton *Dunaliella tertiolecta* and *Thalassiosira pseudonana*

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## Culture regimes

The species *Dunaliella tertiolecta* and *Thalassiosira pseudonana* were chosen for this study as they are both easily culturable; readily available; represent two of the major groups of phytoplankton – green algae (*Dunaliella*), diatoms (*Thalassiosira*) which have major physiological differences[1–4] and in addition these species, particularly *D. tertiolecta* have been used in previous arsenic cycling research.[5–9]

Batch and continuous *D. tertiolecta* cultures were exposed to $As^V$ at a concentration of ~2 μg $L^{-1}$ and were cultured using seawater f/2 nutrient media,[10] both of which have been used in recent studies.[5,6] In addition, a continuous *D. tertiolecta* culture was also created and supplied with seawater f/2 nutrient media[10] containing no additional $As^V$ (Table 1 in the main manuscript), with the arsenic present in this culture (~0.3 μg $L^{-1}$) originating from the autoclaved seawater used as the base of the f/2 nutrient media. This culture was used to determine if differences in arsenic cycling processes performed by *D. tertiolecta* under batch and continuous culture regimes could be attributed to the constant supply of $As^V$. Selected batch *D. tertiolecta* cultures were also heat treated via autoclaving (Table 1) to create a batch culture comprised purely dead cell material to assess the effects of dead cell tissue accumulation on observed arsenic cycling processes within cultures.

Batch and continuous *T. pseudonana* cultures were also both exposed to the same nutrient media (seawater f/2[10]) as *D. tertiolecta*. *T. pseudonana* cultures, however, were exposed to $As^V$ at a concentration of 5 μg $L^{-1}$ as arsenic uptake and accumulation is typically lower in marine diatoms (e.g. *Phaeodactylum tricornutum*)[6] than in *D. tertiolecta* and thus higher arsenic exposures were required to ensure arsenic concentrations were detectable analytically (Table 1). In addition, a second *T. pseudonana* continuous culture was constantly exposed to 5 μg $L^{-1}$ $As^V$ in sterile seawater medium containing no additional nutrients (Table 1). This was used to assess the importance of constant nutrient supply on the observed arsenic cycling processes performed by *T. pseudonana*.

**Total arsenic and arsenic species analysis**

*Reagents and standards*

Nitric acid ($HNO_3$; Aristar, BDH, West Chester, PA, USA) was used for the digestion of *D. tertiolecta* and *T. pseudonana* tissue for total arsenic determination. Ammonium dihydrogen phosphate (Suprapur, Merck, Darmstadt, Germany) and pyridine (Extra Pure, Merck) were used in the preparation of high performance liquid chromatography (HPLC) mobile phases. Formic acid (Extra Pure, Fluka, Munich, Germany) and ammonia solution (>99.9 %, Aldrich, Munich, Germany) were used for the adjustment of mobile-phase pH. Methanol (HiPerSolv, BDH), acetone, (HPLC, BDH), chloroform (AR, BDH) and deionised water (18.2 mΩ, Millipore, Billerica, MA, USA) were used for the extraction of arsenic species.

Stock standard solutions (1000 mg $L^{-1}$) were made by dissolving the appropriate arsenic standard in 0.01 M HCl–deionised water. Arsenous acid ($As^{III}$) and arsenic acid ($As^{V}$) were from AJAX Laboratory Chemicals, methylarsonic acid (MA) and dimethylarsinic acid (DMA) were from ChemService. Arsenobetaine bromide (AB) was synthesised by the methods of Cannon et al.,[11] arsenocholine iodide (AC) was synthesised by the methods of Minhas et al.[12] Trimethylarsine oxide (TMAO) was synthesised by the methods of Merijanian and Zingaro,[13] tetramethylarsonium ion (TETRA) by the methods of Momplaisir et al.,[14] dimethylarsinoylethanol (DMAE) and dimethylarsinoylacetate (DMAA) by the methods of Edmonds et al.[15] and Francesconi et al.[16] Glycerol arsenoriboside (Gly-ribose), sulfonate arsenoriboside ($SO_3$-ribose), phosphate arsenoriboside ($PO_4$-ribose) and sulfate arsenoriboside ($OSO_3$-ribose) were obtained from a previously characterised Fucus extract.[17] Thio-Gly-arsenoriboside, thio-$PO_4$-ribose, thio-$SO_3$-ribose, thio-$OSO_3$-ribose and thio-DMAE were synthesised by bubbling $H_2S$ (Aldrich, 99.5 %) through an extract of previously characterised *Fucus*[17] or synthetic standard for ~30 s to 1 min with a ~100 % conversion rate. Thio-DMA was synthesised by the methods of Raml et al.[18]

**Determination of cellular total arsenic and phosphorus concentrations**

*D. tertiolecta* and *T. pseudonana* tissue were digested for total arsenic and phosphorus concentrations using a microwave digestion technique, outlined by Baldwin et al.[19] with modifications. Approximately 0.07 g of homogenised lyophilised samples were accurately weighed and recorded into 7-mL Teflon polytetrafluroacetate digestion vessels (A.I. Scientific, Melbourne) and 1 mL of concentrated nitric acid added (Aristar, BDH). Digestion vessels with sample and acid were left in the fume cupboard for ~1 h before digestion. Microwave digestion (MDS 81D, CEM, Indian Trail, NC, USA) with a program cycle of 2 min, 600 W, 2 min 0 W, 45 min 450 W was used for each set of samples with certified reference materials and blanks. Samples were allowed to cool after digestion for ~60 min then diluted to 10 mL with deionised water in 10-mL polyethylene vials. Total arsenic and phosphorus concentrations in

samples were analysed using a Perkin Elmer (Waltham, MA, USA) DRC-e Inductive Coupled Plasma-Mass Spectrometer (ICP-MS) with an AS-90 autosampler (Waltham, USA). Internal standards were added on-line to compensate for any acid effects and instrument drift.[20] The potential interference to arsenic (*m*/*z* 75) from $^{40}Ar^{35}Cl^{+}$ was determined by measuring chloride at *m*/*z* 35, $^{35}Cl^{16}O^{+}$ at *m*/*z* 51, $^{35}Cl^{17}O^{+}$ at *m*/*z* 52 and $^{40}ArCl^{+}$ at *m*/*z* 77. Selenium was monitored at *m*/*z* 82 as a cross check for $^{40}Ar^{37}Cl^{+}$. Other elements were corrected for interferences as outline in Maher et al. [20]Certified reference material, *Ulva lactuca* L. (CRM 279, Community Bureau of Reference, Brussels, Belgium), *Sargasso* (NIES 9, National institute for environmental studies, Environment Agency of Japan) and citrus leaves (NBS 1572, National Bureau of Standards) was routinely used for quality assurance, and was analysed in the same manner as *D. tertiolecta* and *T. pseudonana* tissue for determination of total arsenic and phosphorus concentrations. Measured values for total arsenic in *U. lactuca* ($2.6 \pm 0.7$ μg $g^{-1}$) were in agreement than certified values ($3.09 \pm 0.20$ μg $g^{-1}$) ($n = 13$), as were values for total arsenic in *Sargasso* (measured, $110 \pm 21$ μg $g^{-1}$; certified, $115 \pm 9$ μg $g^{-1}$) ($n = 14$). Total phosphorus in citrus leaves ($0.151 \pm 0.004$ μg $g^{-1}$) were also in agreement with the certified value ($0.13 \pm 0.2$ μg $g^{-1}$) ($n = 3$).

**Total phosphorus and nitrogen analysis in culture media**

Total phosphorus (TP) and total nitrogen (TN) concentrations in culture media from *D. tertiolecta* and *T. pseudonana* cultures was undertaken after an alkaline peroxodisulfate digestion as outlined by Maher et al.[21]

All alkaline peroxodisulfate digestions were carried out in 50-mL screw-capped polycarbonate tubes (Laboratory Supply, Australia). The stock alkaline peroxodisulfate digestion reagent contained 0.27-M potassium peroxodisulfate (Ajax-Univar, Australia) and 0.24 M of sodium hydroxide (BDH-AnalaR, Australia) in deionised water. For digestions, 2 mL of digestion reagent was added to 10 mL of sample. Using this ratio of digestion reagent to sample volume, i.e. 1 : 5 (v/v) the final concentrations of potassium peroxodisulfate and sodium hydroxide in solution before heating were 0.045 and 0.04 M. Samples were digested using a MDS-2000 microwave oven (630 W) (CEM, Matthews, USA) programmed at 95 °C for 40 min.

TP and TN was measured using a Lachat flow injection analyser, Phosphorus as orthophosphate was determined by the formation of a reduced phosphoantimonylmolybdenum complex with ammonium molybdate, potassium antimony tartrate and ascorbic acid and measurement at 880 nm. Nitrogen as nitrite was determined after cadmium column reduction by the formation of a red–purple azo dye by reaction with sulphanilamide coupled to *N*-1-napthylethylene dihydrogen chloride and measurement at 520 nm.

**Fractionation of arsenic**

*Lipid extraction*

Approximately 0.1 g of homogenised lyophilised *D. tertiolecta* or *T. pseudonana* tissue was weighed into a 50-mL polyethylene vial. The extraction process as carried out by Folch et al.[22] allows separation of lipid and water-soluble phases, with analysis of total arsenic for each phase used to determine the amount in each phase. To 0.1 g sample, 5 mL of chloroform/methanol (2 : 1, v/v) were added, vortexed for 30 s to assist mixing and placed on a rotary wheel for 4 h. Samples were then centrifuged for 10 min at 4500*g* at room temperature (20–21 °C) to separate sample and supernatant. The supernatants were pipetted into 50-mL polypropylene vials. This procedure was repeated, and the supernatant was combined with the first extraction. To the supernatants, 4 mL of deionised water were added to assist in separation of the lipid and water-soluble phases, and left to stand overnight. The lipid and water-soluble phases were separated and placed in 10-mL centrifuge tubes and evaporated to dryness using a RVC 2–18 rotary vacuum concentrator (60 °C) (Christ, Osterode am Harz, Germany). Once dry, samples were stored in the freezer (–18 °C) until required for analysis. Prior to measurement of total arsenic and arsenic species in extracts, samples were re-suspended in 2 mL of 1 % v/v $HNO_3$.

*Water extraction*

The residues from the chloroform/methanol/water extractions were lyophilised (~24 h). To residues, 2 mL of hot water were added and placed in a hot water bath (100 °C) for 1 h to remove any water-soluble arsenic species remaining after the previous extraction. The extracts were centrifuged at 4500*g* for 10 min at room temperature (20–21 °C), and the supernatants were transferred into 10-mL polyethylene tubes and combined with the water soluble phase from the lipid extraction. The combined supernatants were evaporated to dryness using a RVC 2–18 rotary vacuum concentrator (Christ, Osterode am Harz, Germany) and stored frozen until required for analysis. Prior to measurement of total arsenic and arsenic species in extracts, the supernatants were made up to 2 mL with deionised water.

*Residue*

The remaining residues were lyophilised (~24 h) and extracted with 2 mL of $HNO_3$ w/w at 95 °C for 1 h.

**Measurement of total arsenic concentrations in fractions**

Total arsenic concentrations in lipid, water-soluble and residue extracts of *D. tertiolecta* and *T. pseudonana* tissue were analysed using Electrothermal Atomic Absorption spectroscopy (ET-AAS; Perkin Elmer; Waltham, USA).[23] Arsenic was measured at a wavelength of 193.7 nm with a slit width of 0.7 nm. Ten μL of sample were injected onto the surface of a pyrolytic graphite coated tube inserted with a pyrolytic graphite L'vov platform. A palladium–magnesium matrix modifier was used for arsenic

analysis.[23] Peak area was used to determine total arsenic concentrations. Detection limits were 0.03 μg $L^{-1}$ for media and 0.06 μg $g^{-1}$ for cells based on ten replicate blank measurements.

**Arsenic species measurement**

All extracts were filtered through a 0.20-μm RC syringe filter (Millipore). Aliquots of 20 or 40 μL were injected onto a high pressure liquid chromatography (HPLC) system consisting of a Perkin Elmer Series 200 mobile phase delivery and auto sampler system (Perkin Elmer). The eluant from HPLC columns was directed by PEEK (polyether-ether-ketone) (internal diameter 0.02 mm) (Supelco, Fluka) capillary tubing into a Ryton cross flow nebuliser of a Perkin Elmer Elan-6000 ICP-MS, which was used to monitor the signal intensity of arsenic at *m*/*z* 75. Potential polyatomic interferences were checked by monitoring for other ions, as described for total arsenic analysis. The column conditions used for separation of arsenic species are outlined in Table S1, with extensive details presented in published literature.[24–26] External calibration curves for quantification of arsenic species were prepared daily by diluting $As^{III}$ for anionic species and AB for cationic species to 0, 0.5, 1, 10, 100 μg $L^{-1}$. Peak area responses ($n$ = 10) relative to AB and $As^{III}$ have been reported previously[27] Purities of arsenic species were determined periodically by HPLC-ICP-MS. The accuracy of arsenic speciation procedure was determined by analysis of the certified reference material DORM-2 (National Research Council, Canada). The concentrations (mean ± s.d.) of AB (16.8 ± 1.6 μg $g^{-1}$) and TETRA (0.2 ± 0.1 μg $g^{-1}$) measured in DORM-2 tissues ($n$ = 7) were similar to certified values (AB, 16.4 ± 1.1 μg $g^{-1}$; TETRA, 0.236 ± 0.001 μg $g^{-1}$). HPLC Column recoveries for all samples are presented in Table S2.

The chromatography software package Total Chrom (Perkin Elmer) was used to quantify arsenic species by peak areas. Arsenic species were identified by spiking with known standards and retention time matching.

 


**Fig. S1.** Photographic representation of the continuous culture set-up used in this study.

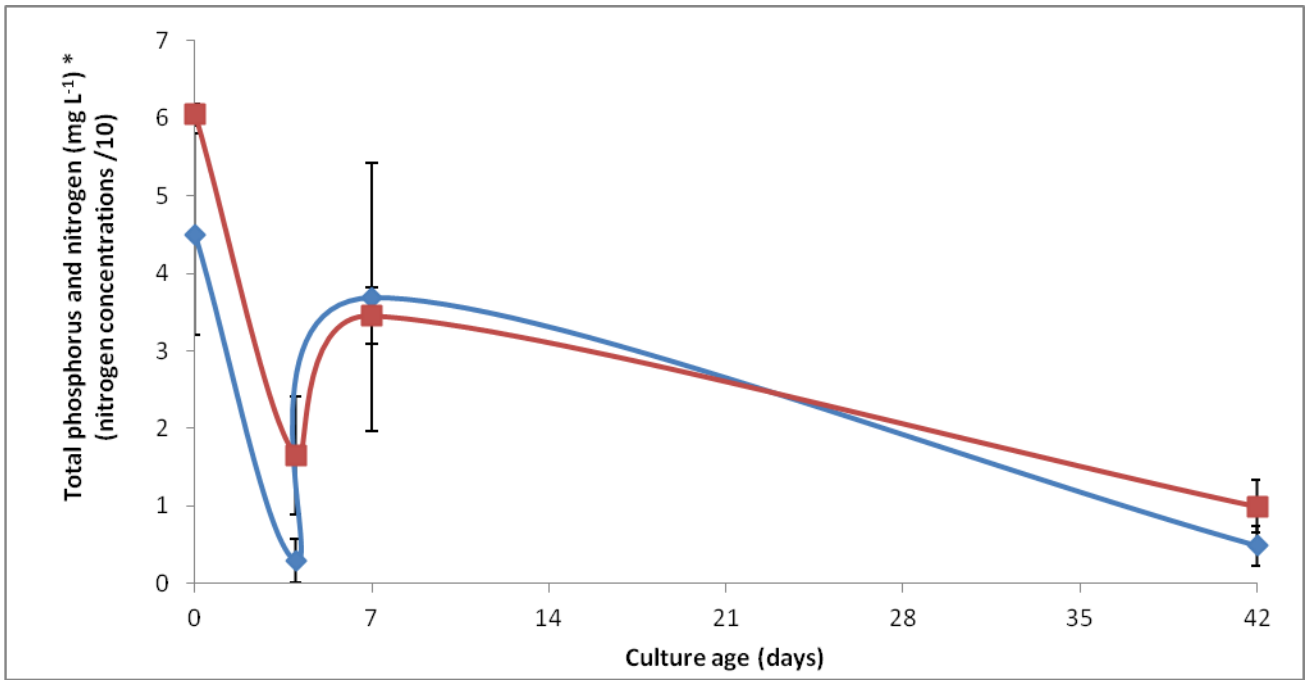


**Fig. S2.** Total phosphorus (blue) and total nitrogen (TN) (red) concentrations in the culture media of batch *D. tertiolecta* cultures. Values are means ± standard deviation ($n$ = 2) (nitrogen concentrations are divided by 10 in order to plot both nutrients on the same axis).

 

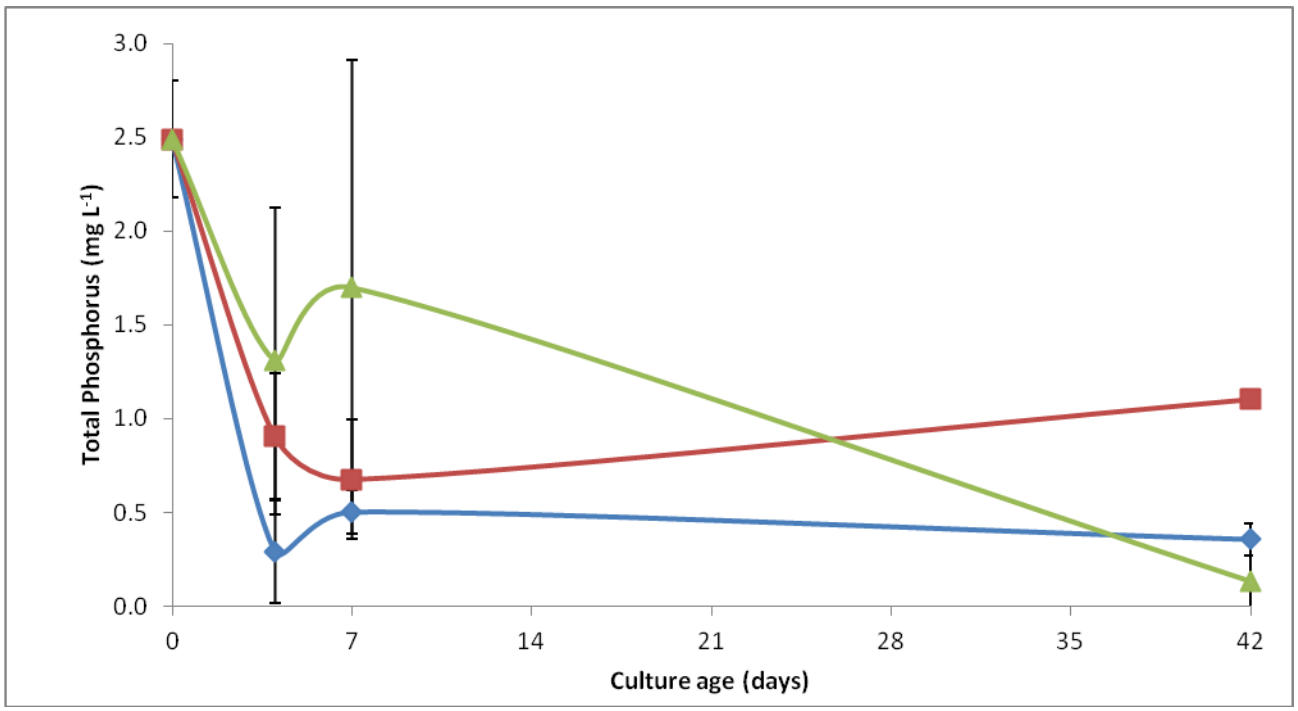


**Fig. S3.** Total phosphorus concentrations in the culture media of *T. pseudonana* when cultured using batch cultures (blue), continuous cultures constantly supplied with f/2 nutrient media and $As^V$ (red) and continuous cultures constantly supplied with $As^V$ and no additional nutrients (green). Values are means ± standard deviation (*n* = 2).

 

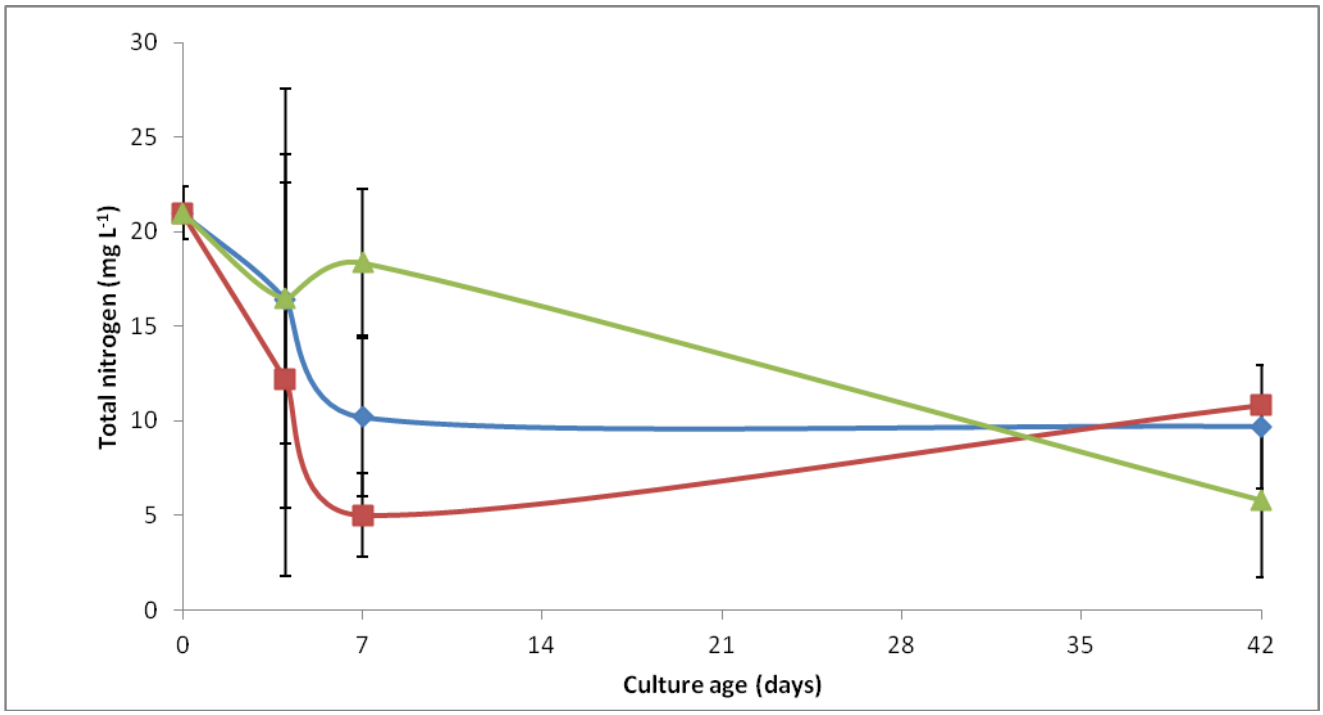


**Fig. S4.** Total nitrogen concentrations in the culture media of *T. pseudonana* when cultured using batch cultures (blue), continuous cultures constantly supplied with f/2 nutrient media and $As^V$ (red) and continuous cultures constantly supplied with $As^V$ and no additional nutrients (green). Values are means ± standard deviation ($n$ = 2).

 

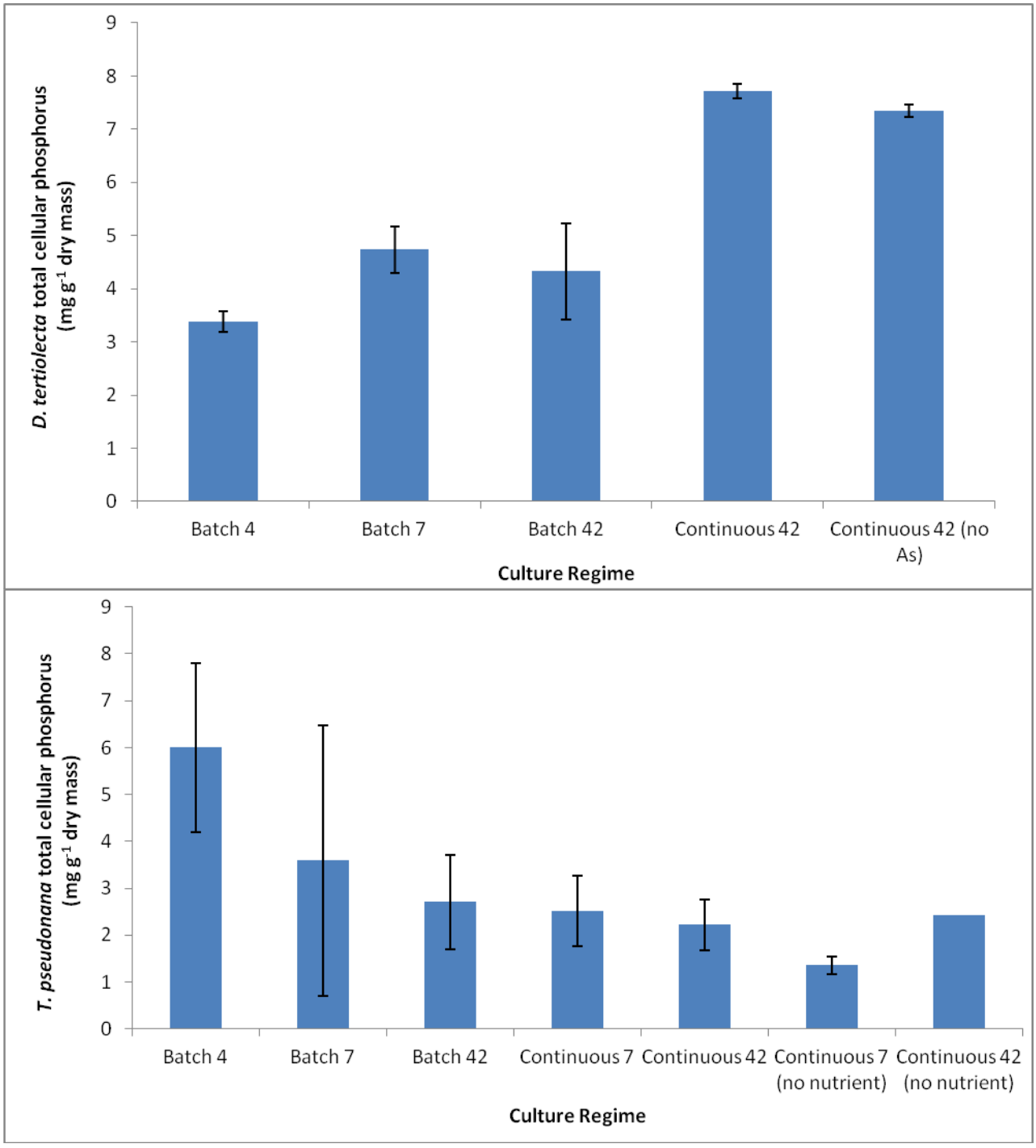


**Fig. S5.** Total phosphorus concentrations (mg $g^{-1}$) in *D. tertiolecta* and *T. pseudonana* tissue grown under batch and continuous culture regimes. Values are means ± standard deviations ($n$ = 2).

 

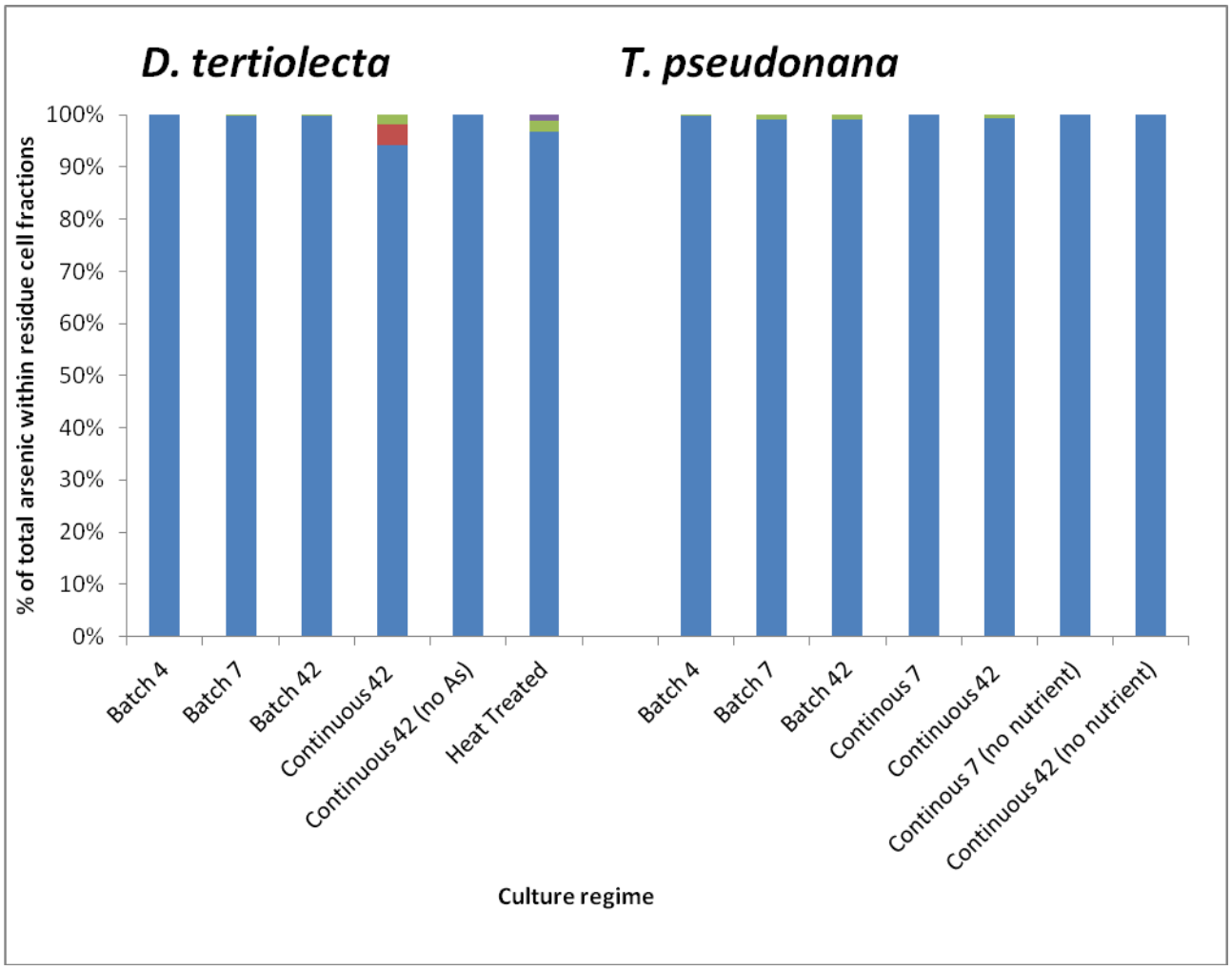


**Fig. S6**. Proportions of individual arsenic species associated with residue cell fractions of *D. tertiolecta* and *T. pseudonana* cultures grown under varying culture regimes. Different colours represent individual arsenic species: blue, inorganic arsenic; red, DMA; green, MA; purple, $PO_4$-riboside. Values are presented as means ($n$ = 2).

**Table S1. HPLC Column conditions for the separation and quantification of arsenic species**

| Column | Hamilton PRP-X100 (PEEK) | Supelocosil LC-SCX | Supelocosil LC-SCX |
|---|---|---|---|
| Size | 150 or 250 mm × 4.6 mm | 250 mm × 4.6 mm | 250 mm × 4.6 mm |
| Particle size | 10 µm | 10 µm | 10 µm |
| Buffer | 20 mM $NH_4H_2PO_4$, 1 % $CH_3OH$ | 20-mM pyridine | 20-mM pyridine |
| pH | 5.6 | 2.6 | 3 |
| Flow rate | 1.5 mL $min^{-1}$ | 1.5 mL $min^{-1}$ | 1.5 mL $min^{-1}$ |
| Temperature | 40 °C | 40 °C | 40 °C |
| Arsenic species | $As^V$, DMA, MA, $PO_4$-, $SO_3$- and $OSO_3$-arsenoribosides | DMAE, glycerol trimethyl arsenoriboside, TETRA, AC and TMAP | AB and OH arsenoriboside |

**Table S2. Column recoveries from lipid-soluble, water-soluble and residue fractions of *D. tertiolecta* and *T. pseudonana* tissue analysed in this study**

*T. pseudonana* continuously cultured with just $As^V$ for 42-days contains no error measurements as one of the two experimental cultures crashed before day 42

| Algae species | Sample | Lipid-soluble fraction (% ± s.d.) | Water-soluble fraction (% ± s.d.) | Residue fraction (% ± s.d.) |
|---|---|---|---|---|
| *D. tertiolecta* | Batch day 4 | 56 ± 2 | 115 ± 4 | 106 ± 9 |
| *D. tertiolecta* | Batch day 7 | 77 ± 2 | 112 ± 2 | 106 ± 5 |
| *D. tertiolecta* | Batch day 42 | 91 ± 9 | 100 ± 4 | 93 ± 23 |
| *D. tertiolecta* | Continuous + $As^V$ + f/2 media | 115 ± 5 | 100 ± 1 | 92 ± 7 |
| *D. tertiolecta* | Continuous + f/2 media | 85 ± 1 | 80 ± 15 | 83 ± 2 |
| *T. pseudonana* | Batch day 4 | 82 ± 2 | 97 ± 11 | 111 ± 13 |
| *T. pseudonana* | Batch day 7 | 77 ± 7 | 90 ± 14 | 101 ± 6 |
| *T. pseudonana* | Batch day 42 | 99 ± 24 | 77 ± 7 | 115 ± 14 |
| *T. pseudonana* | Continuous + $As^V$ + f/2 media day 7 | 82 ± 2 | 81 ± 3 | 97 ± 13 |
| *T. pseudonana* | Continuous + $As^V$ + f/2 media day 42 | 98 ± 6 | 69 ± 5 | 109 ± 8 |
| *T. pseudonana* | Continuous + $As^V$ day 7 | 73 ± 3 | 82 ± 4 | 100 ± 4 |
| *T. pseudonana* | Continuous + $As^V$ day 42 | 79 | 80 | 118 |

 

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