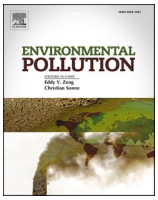
Environmental Pollution 334 (2023) 122176

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol



Arsenic speciation in low-trophic marine food chain – An arsenic exposure study on microalgae (*Diacronema lutheri*) and blue mussels (*Mytilus edulis* L.)☆

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ARTICLE INFO

Keywords:
Biotransformation
Bioaccumulation
HPLC
ICP-MS
Arsenosugars
Feed safety

ABSTRACT

Microalgae and blue mussels are known to accumulate undesirable substances from the environment, including arsenic (As). Microalgae can biotransform inorganic As (iAs) to organoarsenic species, which can be transferred to blue mussels. Knowledge on As uptake, biotransformation, and trophic transfer is important with regards to feed and food safety since As species have varying toxicities. In the current work, experiments were conducted in two parts: (1) exposure of the microalgae *Diacronema lutheri* to 5 and 10 μg/L As(V) in seawater for 4 days, and (2) dietary As exposure where blue mussels (*Mytilus edulis* L.) were fed with *D. lutheri* exposed to 5 and 10 μg/L As (V), or by aquatic exposure to 5 μg/L As(V) in seawater, for a total of 25 days. The results showed that *D. lutheri* can take up As from seawater and transform it to methylated As species and arsenosugars (AsSug). However, exposure to 10 μg/L As(V) resulted in accumulation of iAs in *D. lutheri* and lower production of methylated As species, which may suggest that detoxification mechanisms were overwhelmed. Blue mussels exposed to As via the diet and seawater showed no accumulation of As. Use of linear mixed models revealed that the blue mussels were gradually losing As instead, which may be due to As concentration differences in the mussels' natural environment and the experimental setup. Both *D. lutheri* and blue mussels contained notable proportions of simple methylated As species and AsSug. Arsenobetaine (AB) was not detected in *D. lutheri* but present in minor fraction in mussels. The findings suggest that low-trophic marine organisms mainly contain methylated As species and AsSug. The use of low-trophic marine organisms as feed ingredients requires further studies since AsSug are regarded as potentially toxic, which may introduce new risks to feed and food safety.

1. Introduction

In the marine environment, As exists as different inorganic and organic species. In seawater, the predominant form of As is inorganic As (iAs), with arsenate (As(V)) usually more abundant than arsenite (As (III)) (Neff, 2002). In contrast, the non-toxic arsenobetaine (AB) is the most prevalent As compound in marine animals (Francesconi, 2010). Seaweed usually contains high proportions of arsenosugars (e.g. AsSug OH, AsSug PO_4, AsSug SO_3, AsSug SO_4) (Feldmann and Krupp, 2011; Taylor et al., 2017; Luvonga et al., 2020). Arsenolipids (AsLipids) are the main As compounds in marine fats and oils (Sele et al., 2012), but also present in seaweed (Pétursdóttir et al., 2019), bivalves (Freitas et al., 2020), and phytoplankton (Glabonjat et al., 2021). Clearly, there is a large variation in the occurrence of As species in marine organisms. This has been usually attributed to environmental factors (e.g. salinity, nutrient availability, etc.), the organism's feeding habit or position in the marine food chain, and ability to take up, biotransform, and eliminate As species (Azizur Rahman et al., 2012; Zhang et al., 2022).

Being at the base of the marine food chain, microalgae play a key role in As cycling, acting as the first interface for As transfer by taking up

☆ This paper has been recommended for acceptance by Sarah Harmon.

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https://doi.org/10.1016/j.envpol.2023.122176
Received 27 March 2023; Received in revised form 12 June 2023; Accepted 9 July 2023
Available online 10 July 2023

mostly As(V) from seawater (Duncan et al., 2010). As a detoxification mechanism, As(V) is biotransformed by microalgae to other organic As species through a series of methylation and reduction processes first described by Challenger (1945) (Fig. 1). More complex organic As species can arise following the Challenger pathway, such as the formation of AsSug after adenosylation and glycosidation (Edmonds and Francesconi, 1987). As primary producers, microalgae have the potential to transfer As to primary consumers such as blue mussels (Van Der Spiegel et al., 2013).

Elevated levels of iAs (up to 5.8 mg/kg ww) were reported in blue mussels, which was hypothesized to be due to the microalgae in their diet (Sloth and Julshamn, 2008). In a study by Whaley-Martin et al. (2012), levels of total As (tAs) and iAs in blue mussels were positively correlated with the degree of As contamination in the area, both particle-bound and in the dissolved phase, suggesting that As was taken up by blue mussels from the sediments and water column. Hence, As uptake in blue mussels may occur through the dissolved phase as diffusion via e.g. gills, or through ingestion of particulates (Sloth and Julshamn, 2008; Whaley-Martin et al., 2012; Schöne and Krause, 2016; Beyer et al., 2017). While several studies in blue mussels have investigated the uptake of different As compounds via the dissolved phase, the concentrations of As used (as high as 100 μg/L) were magnitudes higher than what is naturally found in seawater (around 2 μg/L) (Gailer et al., 1995; Francesconi et al., 1999; Clowes and Francesconi, 2004). Moreover, there is limited knowledge on how As species are accumulated and biotransformed by blue mussels via the ingestion of microalgae.

Blue mussels and microalgae are both being considered as future feed ingredients due to their nutritional composition and low-carbon-footprint production (Torres-Tiji et al., 2020; Albrektsen et al., 2022; Tamburini et al., 2022). Knowledge regarding As speciation in microalgae and blue mussels is essential in terms of feed and food safety since As species have varying toxicities (Francesconi and Raber, 2013). Hence, the present work aims to investigate (1) the uptake and biotransformation of iAs in microalgae, (2) the accumulation and biotransformation of As species in blue mussels fed with microalgae exposed to iAs, and (3) the uptake and biotransformation of iAs in blue mussels exposed via seawater. Overall, this study aims to give an insight into As biotransformation at the base of the marine food chain.

2. Materials and methods

2.1. Design of the study

In this study, exposure experiments were divided into two parts (Fig. 2). The first part involved the exposure of microalgae to iAs. Three different experimental cultures were produced: (1) one control culture with no addition of As(V) ('Microalgae Control'), (2) one culture spiked with 5 μg/L ('Microalgae 5'), and (3) one culture spiked with 10 μg/L ('Microalgae 10'). The second part of the experiment comprised of exposure of blue mussels to As through the diet and the dissolved phase. Twelve tanks were randomly assigned to four exposure groups (3 replicate tanks per exposure): the control group (Group A), mussels fed with 'Microalgae 5' (Group B), mussels fed with 'Microalgae 10' (Group C), and mussels exposed to 5 μg/L As(V) in seawater (Group D). Mussels in Groups A and D were fed with 'Microalgae Control'. Group A represents mussels grown under non-exposed conditions. Whereas for Group D, seawater was spiked with a standard solution of As(V) to a nominal concentration of 5 μg/L, simulating an environment with slightly higher

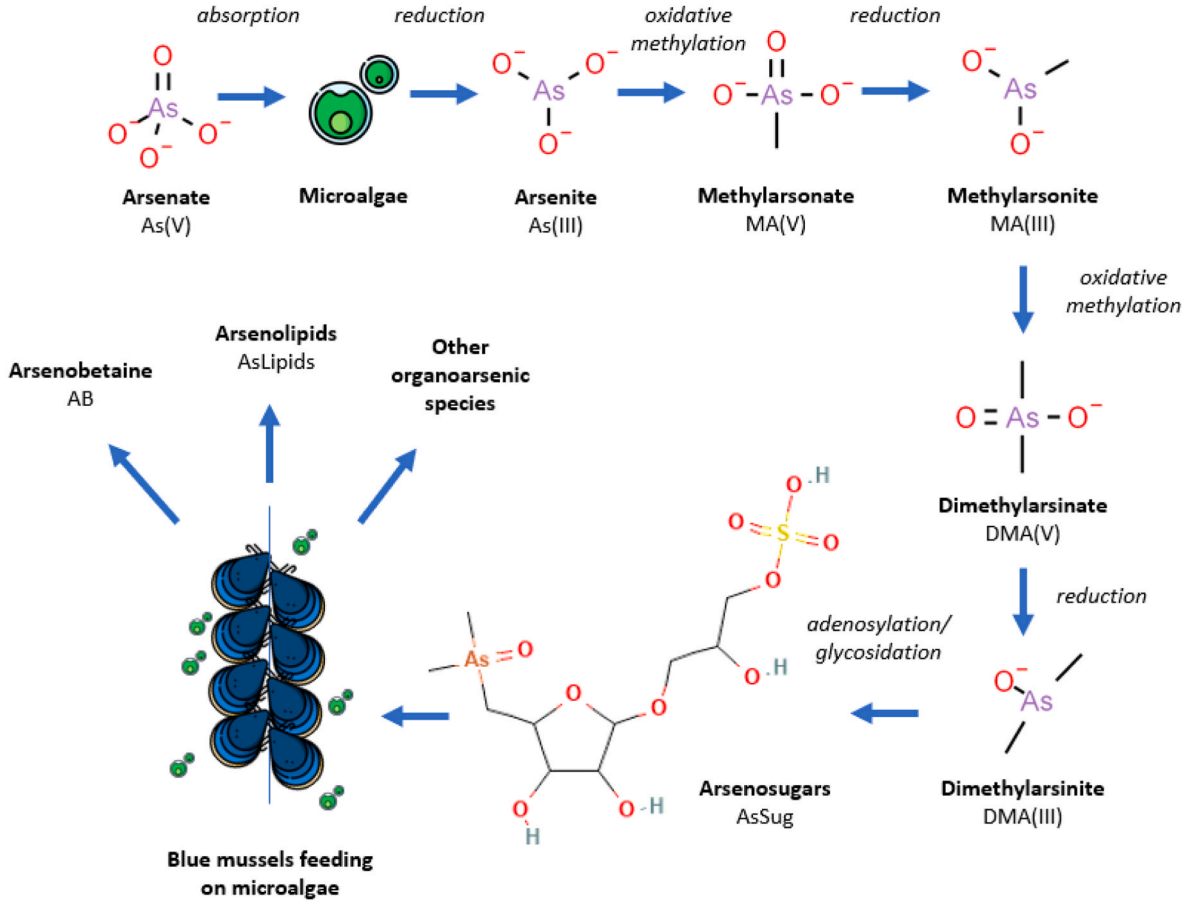

Fig. 1. Pathway for the biotransformation of inorganic arsenic (iAs) to more complex organoarsenic species in microalgae and blue mussels, based on the work of (Challenger, 1945). *Chemical structures retrieved from ChemSpider and PubChem, and icons from Flaticon.* (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

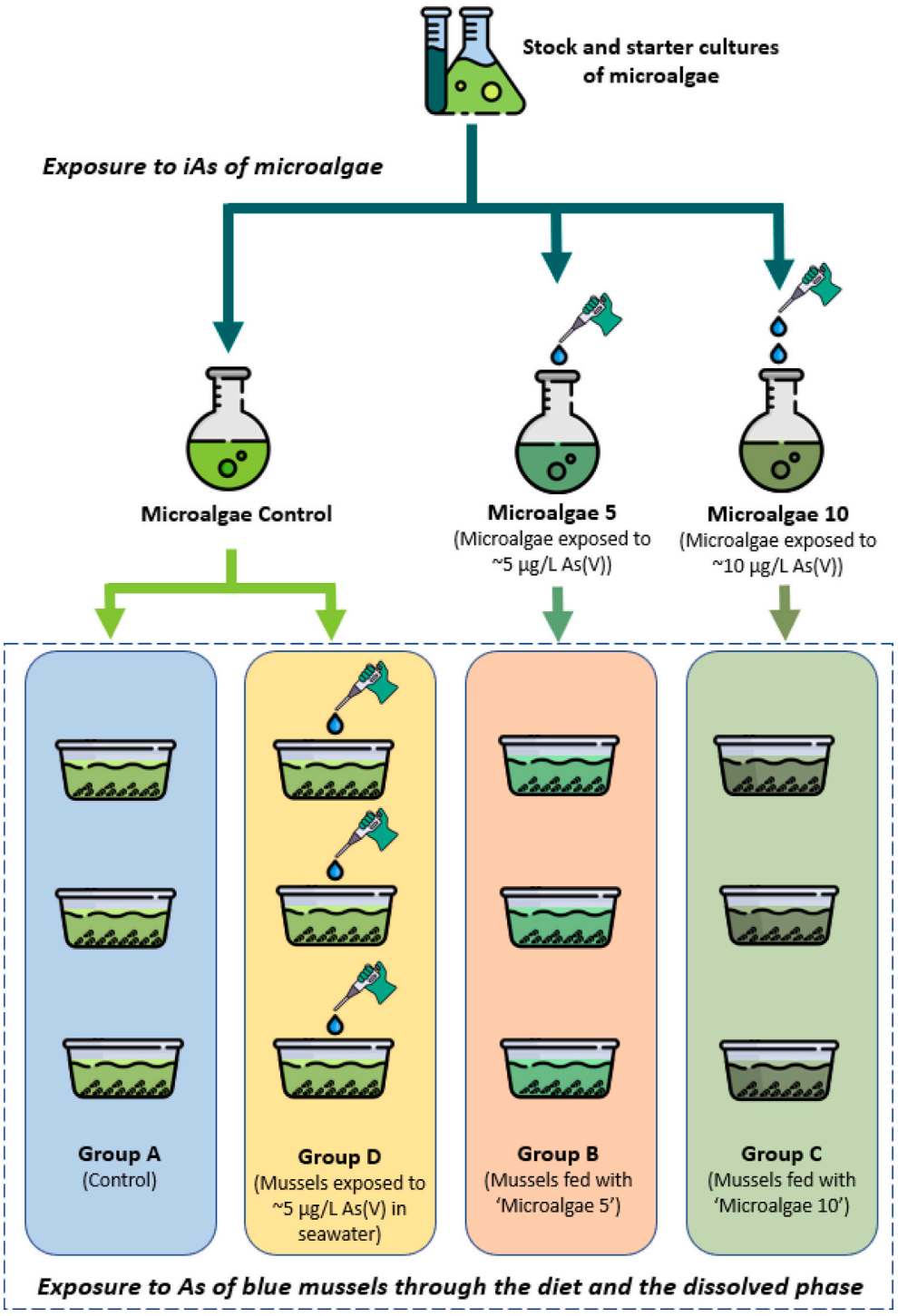

Fig. 2. Overview of the experimental design, divided into exposure of microalgae to inorganic arsenic (iAs) (top) and blue mussels exposed to arsenic (As) through the diet and the dissolved phase (bottom, enclosed in dashed lines). 'Microalgae Control' was cultivated with no addition of As in the culture media, while 'Microalgae 5 and 10' were cultivated with addition of As(V) concentrations of 5 and 10 µg/L in the media, respectively. Blue mussel Groups A and D were fed with 'Microalgae Control', while Groups B and C were fed with 'Microalgae 5 and 10', respectively. *Icons retrieved from Flaticon.* (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

As levels.

2.2. Experimental organisms

2.2.1. Microalgae

Diacronema lutheri (Bendif et al., 2011), formerly known as *Pavlova lutheri*, was the microalgae species chosen for the experiments due to its use as aquaculture feed, usually for bivalves (Ponis et al., 2008). Axenic stock cultures of *D. lutheri* were obtained from the Norwegian Institute for Water Research (NIVA, Oslo, Norway). The strain is part of the Norwegian Culture Collection of Algae (NORCCA), with identification number NIVA-4/92. To produce the starter culture (inoculum), 300 mL of the stock culture was grown in 4-L round-bottom flasks with filtered (2 µm), diluted, sterile seawater (20% ultrapure water) enriched with f/2 medium (Guillard, 1975) to a final volume of 1.8 L. Starter cultures were maintained under continuous light and aeration. The growth curve was monitored by daily measurements of cell density using a Z2 Coulter Particle Count and Size Analyzer (size range: 3.4–8 µm; Beckman Coulter, Brea, CA, USA), by which the stationary phase was noted after five days. Starter cultures were sub-cultured aseptically once a week to maintain the starter culture line.

2.2.2. Blue mussels

Specimens of blue mussels, with shell length ranging from 3.5 to 4.8 cm ($n \approx 1000$), were collected from Hardangerfjord in western Norway between August and September 2020. Mussels were transported to the Institute of Marine Research's (IMR) research station at Austevoll where they were acclimated for two weeks (initial acclimation) in lantern nets submerged at 3 m depth. Thereafter, a total of 672 mussels were cleaned of epiphytes. The mussels were randomly placed into one of 12 tanks (i.e. 56 mussels per tank), containing 40 L of filtered natural seawater pumped from a depth of 160 m. The mussels were acclimated further for two weeks under laboratory conditions (second acclimation), with a 10 h light:14 h dark photoperiod and continuous aeration. During the first days, feeding rates (microalgae cell density and frequency) were examined by testing different cell densities, systematic measuring of particles in the water inside the tanks, and visually inspecting the feces to ensure no formation of pseudo-feces. From these initial tests, it was determined that a microalgae cell density of 6×10^6 cells per L is optimum for feeding the mussels.

2.3. Chemicals and solutions

Solutions of 10 and 40 mg/L were prepared by diluting aliquots of a 1000 mg/L As(V) standard solution (Spectrascan Teknolab, Ski, Norway) using ultrapure water (18.2 MΩ-cm). The f/2 medium was prepared based on the procedure described by Guillard (1975). The following chemicals (purity: ≥97%) were purchased from Sigma-Aldrich (Saint Louis, MO, USA): Sodium phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$), ethylenediaminetetraacetic acid, disodium salt (EDTA-Na_2), iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), copper(II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$), cobalt(II) chloride hexahydrate ($CoCl_2{\cdot}6H_2O$), manganese(II) chloride tetrahydrate ($MnCl_2{\cdot}4H_2O$), sodium molybdate dihydrate ($Na_2MoO_4{\cdot}2H_2O$), Vitamin B12, Vitamin B1, and biotin. Sodium nitrate ($NaNO_3$, reagent grade) was obtained from Merck (Darmstadt, Germany).

2.4. Microalgae exposure experiment

2.4.1. Microalgae production and exposure to iAs

Batches of microalgae were produced twice a week and covered 3–4 days of feeding. First, a sample of the starter culture was checked for cell viability under a microscope and for cell density. Considering the cell density of the starter culture, the required experimental culture volume, and the target initial cell density of 2×10^6 cells per L, the required volume of the starter culture was calculated and transferred aseptically to an appropriate volume of diluted sterile seawater (20% ultrapure water) with f/2 medium in 4-L round-bottom flasks. Three different experimental cultures were produced (Fig. 2). The cultures with spiked concentrations of 5 and 10 μg/L As(V) were prepared by adding appropriate volumes of 10 and 40 mg/L As(V) solutions, respectively. Similar growing conditions as the starter culture were applied (e.g. use of f/2 medium, light regime, aeration, etc.).

After 5–8 days of incubation (corresponding to the stationary growth phase), a sample of experimental culture was checked for cell viability and density. Taking into account the cell density, number of tanks, the volume of seawater in each tank, and the number of times mussels are to be fed, an appropriate volume of the experimental culture was transferred to 50-mL centrifuge tubes. These were then centrifuged for 5 min at 4000 rpm and 15 °C (Centrifuge 5810 R; Eppendorf, Hamburg, Germany). The supernatant was subsequently removed from each tube using a Pasteur pipette. Cells were resuspended in 50 mL of diluted sterile seawater (20% ultrapure water). Cell densities were again measured, which formed the basis for the volume of microalgae suspension to be added to each mussel tank to arrive at a cell concentration of 6×10^6 cells per L.

2.4.2. Microalgae sample preparation

For the microalgae sample preparation, 200–400 mL of each experimental culture was transferred to 50-mL centrifuge tubes. These were then centrifuged followed by removal of supernatant. The precipitates were washed with 5 mL diluted sterile seawater (20% ultrapure water), pooled into individual tubes, and filled with diluted sterile seawater (20% ultrapure water) up to 50 mL. The tubes were subsequently centrifuged, and supernatant was removed. Samples were freeze-dried prior to analysis. Microalgae from the same treatment groups were pooled corresponding to the feeding periods of mussels.

2.5. Blue mussel exposure experiment

2.5.1. Blue mussel exposure to As

Blue mussels were exposed to As either through the diet (*D. lutheri*) or the dissolved phase (seawater). There were four exposure groups with 3 replicate tanks each (Fig. 2). Feeding with microalgae was done manually three times a day. Due to logistic reasons, a feeding interval of 2–3 h was chosen to fit three feeding instances during regular working hours, thereby ensuring a consistent feeding regime throughout the 25 days of the experiment. For each feeding instance, depending on the cell density of the experimental culture and volume of seawater in the tank, a calculated specific volume of microalgae suspension was added to the tanks using an adjustable pipette (5 mL, Eppendorf, Hamburg, Germany) to achieve a cell density of 6×10^6 cells per L. After adding the microalgae suspension, a portable particle counter (PAMAS S4031 GO; PAMAS, Rutesheim, Germany) was used to measure the particles in the tanks. After 2–3 h, particles in the tanks were again measured. The difference between the initial and final counts provide an estimate of the feed intake. The exposure experiment lasted for a total of 25 days. During the exposure period, salinity, temperature, and pH were recorded daily. Check for mortality was also part of the routine. Throughout the experiment, only three mussels died – one each from two different control tanks, and one from a tank fed with 'Microalgae 5'.

2.5.2. Sampling of blue mussels

Mussels ($n = 9$) were collected from each tank before the first feeding of the day at exposure days 0, 2, 6, 12, 18, and 24, corresponding to time points t0, t1, t2, t3, t4, and t5, respectively. Mussels from t0 were collected before As exposure started (i.e. after second/laboratory acclimation). Seawater was replaced every second day and volume was adjusted corresponding to the remaining number of mussels. Throughout the study, a total of 54 mussels were collected per tank.

Blue mussels were cleaned prior to analysis. After allowing the samples to dry, shell length, height, and width were measured using a caliper. Whole body weights were then measured, followed by removal of soft tissue. The empty shells were weighed. After a few minutes of draining with tissue paper, soft tissues were also weighed. Subsequently, soft tissues of the nine mussels collected in each tank per time point were pooled, homogenized in a blender, and freeze-dried (FreeZone 18L Freeze Dryer; Labconco, Kansas, MO, USA). A second homogenization was performed afterwards (GRINDOMIX GM 200; Retsch, Haan, Germany).

2.6. Sampling of seawater

During the start-up of microalgae production, the following samples were collected: filtered natural seawater, seawater with f/2 medium, and seawater with f/2 medium spiked at approximately 5 and 10 μg/L. This was to verify the As contribution from seawater and f/2 medium (background), and to measure the actual exposure concentration. During harvesting of cells, samples of the supernatant (culture medium) were also collected after centrifugation to verify whether As had been taken up by the microalgae.

During laboratory acclimation of the blue mussels, seawater samples (at least 50 mL) were collected in 50-mL polypropylene (PP) tubes.

Similarly, throughout the exposure period, seawater samples were taken from the inlet using 200-mL Teflon bottles, i.e. directly from the tube providing the seawater pumped from a depth of 160 m, every time the seawater in the tanks was changed. For all tanks, seawater samples were collected before blue mussels were sampled (i.e. at t0, t1, t2, t3, t4, t5). For Group D tanks, seawater collection was more frequent: daily, and before and after seawater was changed, to monitor if there were any changes in As concentration during the exposure period and if correct spiking was done. Seawater samples from replicate tanks (same treatment) were pooled and stored at −20 °C until analysis.

2.7. Chemical analysis

2.7.1. Determination of total As

Total As in solid samples was determined after microwave digestion by inductively-coupled plasma mass spectrometry (ICP-MS) as described elsewhere (Julshamn et al., 2007). Briefly, approximately 0.2 g of sample was weighed into quartz digestion vessels followed by addition of 2 mL of concentrated (65%) HNO_3 (purified by sub-boiling distillation; Merck, Darmstadt, Germany). Samples were placed in the UltraWAVE system (Milestone, Sorisole, Italy) and the final clear solution was diluted with ultrapure water to 25 mL. Solutions were analyzed for tAs with iCAP Q ICP-MS (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a helium collision cell and FAST SC 4Q DX autosampler (Elemental Scientific, Omaha, NE, USA). Quantification was carried out using an external calibration curve generated from multielement standard solutions and online addition of germanium as the internal standard (both from Spectrascan Teknolab, Ski, Norway).

2.7.2. Determination of inorganic As

Determination of iAs was carried out by anion-exchange high performance liquid chromatography (HPLC) coupled to ICP-MS (HPLC-ICP-MS) based on EN 16802:2016 (CEN, 2016) and Julshamn et al. (2012). Approximately 0.2 g of sample was weighed into 13-mL PP tubes. Thereafter, 10 mL of 0.1 M HNO_3 in 3% H_2O_2 (30%; Merck, Darmstadt, Germany) was added, followed by vortex mixing (MS 1; IKA, Staufen, Germany). Samples were left to stand overnight. The tubes were then placed in a water bath (OLS200; Grant, Cambridge, UK) for 1 h at 90 °C while shaking at 100 rpm. After allowing to cool, tubes were centrifuged for 10 min at 3800 rpm (Centrifuge 5702; Eppendorf, Hamburg, Germany). The supernatant was filtered using a 5-mL syringe (Henke-Sass Wolf, Tuttlingen, Germany) with disposable filters (0.45-μm PTFE; Sartorius, Göttingen, Germany) into 1-mL PP HPLC vials. Quantification was achieved using a 1260 Infinity HPLC coupled to a 7900 ICP-MS (Agilent Technologies, Santa Clara, CA, USA), equipped with an anion-exchange IonPac AS7 column (2 × 250 mm) and an IonPac AG7 guard column (2 × 50 mm) (both from Dionex, Sunnyvale, CA, USA). Isocratic elution was carried out using 50 mM $(NH_4)_2CO_3$ in 3% CH_3OH (pH 10.3) as mobile phase. An external calibration curve from As(V) standard solutions (Spectrascan Teknolab, Ski, Norway) was used for the quantification of iAs.

2.7.3. Determination of water-soluble As species

Water-soluble As species were determined by anion- and cation-exchange HPLC-ICP-MS as described elsewhere (Tibon et al., 2021). Approximately 0.2 g of sample was weighed into 13-mL PP tubes and 5 mL of extraction solution (CH_3OH:H_2O, 50% v/v) ($CH_3OH \geq 99.97\%$; Merck, Darmstadt, Germany) was added. This was followed by vortex mixing for a few seconds and water bath heating for 30 min (90 °C, shaking speed at 100 rpm). The samples were allowed to cool and then centrifuged at 3800 rpm for 10 min. The supernatant was transferred into a 5-mL syringe and filtered (0.45 μm) into another 13-mL PP tube. An aliquot of the supernatant was transferred into a 1-mL HPLC vial and diluted accordingly with the extraction solution. Analysis was carried out using HPLC-ICP-MS (same setup used for iAs determination) but with two different instrument methods. Cationic As species were separated using a Metrosep C6 column (250 × 4.0 mm, 5 μm; Metrohm, Herisau, Switzerland) by gradient elution with pyridine-based mobile phases. For anionic As species, a PRP-X100 column (250 × 4.6 mm, 5 μm; Hamilton, Reno, NV, USA) was used, and gradient elution was employed with carbonate-based mobile phases. Concentrations were determined based on an external calibration curve from mixed standard solutions of As compounds.

2.7.4. Analysis of seawater samples

Seawater samples were analyzed for tAs using atomic fluorescence (P S Analytical Millennium Excalibur, Kent, UK), after pre-reduction of As (V) to As(III) by 1:1 mixture of seawater with a solution containing 0.5% potassium iodide (KI) and 0.25% ascorbic acid (both Merck pro analysis grade, Darmstadt, Germany) in 7 M hydrochloric acid (HCl, Merck suprapure grade, Darmstadt, Germany) for 30 min at 25 °C. This was followed by hydride generation using in-line mixture of the reduced seawater in a 1.2 M HCl carrier stream with 0.7% sodium tetrahydroborate ($NaBH_4$) dissolved in 0.1M sodium hydroxide (NaOH). The generated gaseous arsine was separated in a gas-liquid separator by an Ar-gas stream and passed through a Perma Pure dryer before being introduced to the fluorescence chamber in a hydrogen-flame, excited with an As hollow cathode lamp (Photron, Victoria, Australia) perpendicular to the measured fluorescence signal (P S Analytical APP017). Quantification was done using an external calibration curve of 0.5–10 μg/L solutions from the Agilent Multi Element Calibration Standard 2A (Agilent, Santa Clara, California, USA).

2.7.5. Quality assurance and control

Certified and in-house reference materials were included in every analysis and results were in good agreement with reference values. Some of the reference materials used were BCR-627 (tuna fish tissue), ERM CE278k (mussel tissue) (both from IRMM, Geel, Belgium), DORM-4 (fish protein), and NASS-6 and CASS-5 (seawater) (all from National Research Council Canada, Ottawa, Ontario, Canada). For each sample, two or three technical replicates were analyzed. Extraction blanks were also included, and one calibration standard was injected periodically throughout the run to monitor possible instrument drifts. Both iAs and tAs methods, including the method for tAs in seawater, are accredited according to ISO/IEC 17025:2017, while the method for water-soluble As species has undergone single-laboratory validation (Tibon et al., 2021). For tAs in seawater, the method is also regularly tested against QUASIMEME proficiency samples (bi-yearly set of three samples) and measurement uncertainty is estimated at < 3%.

2.8. Statistics, equations, and software used

2.8.1. Condition index

Using the biometric parameters obtained, condition indices (CI) were calculated as follows:

$$CI = \left(\frac{SDW}{L \times \frac{W}{H}} \right) \times 1000$$

where *SDW* is the soft tissue dry weight (in g), *L* is the length, *W* is the width, and *H* is the height of the mussel (all in mm) (Lundebye et al., 1997). CIs reflect the mussels' physiological status which may affect clearance, respiration, and other physiological factors affecting the uptake and accumulation of As.

2.8.2. Bioconcentration factor

Bioconcentration factor (BCF) is expressed as the ratio of tAs concentration in the microalgae (μg/kg) to the tAs concentration in the dissolved phase or medium (μg/L) (Huang et al., 2021). Mathematically, this is shown as:

$$BCF = \frac{tAs_{microalgae}\ in\ \mu g/kg}{tAs_{medium} in\ \mu g/L}$$

2.8.3. Linear mixed models

The time series of tAs content in blue mussels was analyzed using a general linear mixed model. The model was used to gain insight into the process of accumulation or release of As over time, and how the treatments, feeding, and mussel condition may have affected this process. In the model, Tank ID was added as random variable to account for the repeated sampling over time in each tank. The model selection was done considering treatment, time, mussel CI, and their interactions as initial covariates. Model selection also aimed to determine the best distribution and link function to be used. Model selection was based on Akaike information criteria (AIC). Model performance and fit were also evaluated by model residuals plots (Zuur et al., 2009). Final model predictions along a combination of covariates were used to further explore the underlying mechanism in As uptake in blue mussels. Statistical analyses were performed using R v4.1.2 (R Core Team, 2020) in RStudio (RStudio Team, 2015), with the packages lme4 (Bates et al., 2015) and lmertest (Kuznetsova et al., 2017).

2.8.4. Software

Microsoft Excel and PowerPoint (Microsoft, Redmond, Washington, USA), OriginPro 2020b (v. 9.7.5.184, OriginLab, Northampton, Massachusetts, USA), and R v4.1.2 (R Core Team, 2020) in conjunction with RStudio (RStudio Team, 2015) were used for statistical analyses and creating figures.

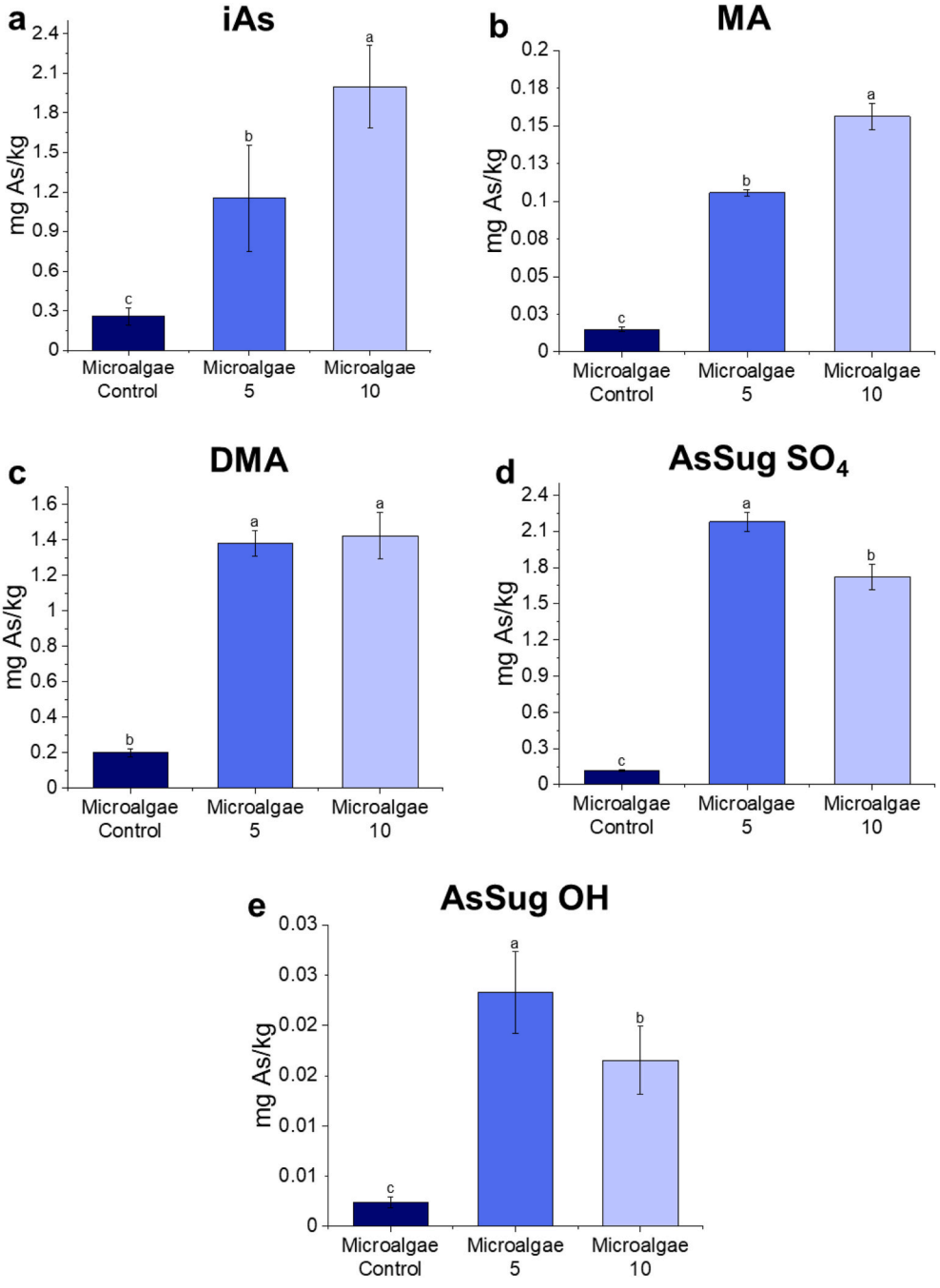

Fig. 3. Concentrations (mg/kg) of (a) inorganic arsenic (iAs), (b) methylarsonate (MA), (c) dimethylarsinate (DMA), (d) sulfate-arsenosugar (AsSug SO_4), and (e) glycerol-arsenosugar (AsSug OH) in *Diacronema lutheri* grown under different treatments. Treatments: 'Microalgae Control' – *D. lutheri* cultured without added As(V), 'Microalgae 5' – *D. lutheri* exposed to 5 μg/L As(V), 'Microalgae 10' – *D. lutheri* exposed to 10 μg/L As (V). Different lower-case letters denote statistically significant difference between exposure groups (Tukey test; $p < 0.05$).

3. Results

3.1. Total As concentrations in media and microalgae

The mixture of seawater with f/2 medium had a background concentration of As of 1.7 μg/L ($n = 1$). The seawater with f/2 medium spiked at 5 and 10 μg/L As(V) showed tAs concentrations of 9.7 μg/L ($n = 1$) and 12.8 μg/L ($n = 1$), respectively. Considering the background As from seawater, the concentrations for seawater with f/2 medium spiked at 5 and 10 μg/L As(V) were acceptable, suggesting that target As exposure concentrations were achieved. For the microalgae samples, 'Microalgae Control' had an average tAs concentration of 1.2 ± 0.1 mg/kg ($n = 5$), while 'Microalgae 5' had 6.6 ± 0.4 mg/kg ($n = 5$). Tukey test showed significant difference for the two results ($p < 0.05$). The exposure group 'Microalgae 10' had a similar average tAs concentration to 'Microalgae 5' at 6.6 ± 0.4 mg/kg ($n = 5$).

The BCF for 'Microalgae Control' and 'Microalgae 5' were comparable at 688 and 678 L/kg, respectively, whereas 'Microalgae 10' had a BCF of 514 L/kg.

3.2. As species in microalgae

Five different As species were detected in *D. lutheri* (Fig. 3). Among the extracted As species in 'Microalgae Control', iAs was predominant at 0.26 ± 0.07 mg/kg ($n = 5$), accounting for 22% of tAs (Fig. 4). Other major species were DMA and AsSug SO_4, with average concentrations of 0.20 ± 0.02 mg/kg and 0.12 ± 0.01 mg/kg ($n = 5$), respectively. The profile seemed to change when *D. lutheri* was exposed to 5 μg/L As(V). The main As species found in 'Microalgae 5' was AsSug SO_4 at 2.2 ± 0.1 mg/kg ($n = 5$), comprising 33% of tAs (Fig. 3 and 4). DMA was the second most abundant at 1.38 ± 0.07 mg/kg ($n = 5$). When *D. lutheri* was exposed to 10 μg/L As(V), iAs was the most prevalent species at 2.0 ± 0.3 mg/kg ($n = 5$), though not markedly higher than AsSug SO_4, which had an average concentration of 1.7 ± 0.1 mg/kg ($n = 5$) (Fig. 3). There were two AsSug detected in *D. lutheri*, namely AsSug SO_4 and AsSug OH. In all exposure groups, AsSug SO_4 was more abundant, approximately two magnitudes higher in concentration than AsSug OH (Fig. 3). In this study, AB was notably not detected in *D. lutheri*. Also, a relatively high fraction of tAs was unextracted (20–49%).

The accumulation of iAs and MA seemed to increase as the exposure concentration increased, resulting in significantly different concentrations in the exposure groups (Tukey test; $p < 0.05$) (Fig. 3). For DMA, the concentration found in 'Microalgae 5' was significantly higher (seven times) than in 'Microalgae Control' (Tukey; $p < 0.05$). DMA in 'Microalgae 10', even though having a slightly higher average concentration, was not significantly different from 'Microalgae 5' (Fig. 3). Levels of AsSugs were significantly different between exposure groups (Tukey; $p < 0.05$). Comparing 'Microalgae Control' and 'Microalgae 5', a 19-fold difference was noted for AsSug SO_4, while a 12-fold difference was observed for AsSug OH. Both AsSugs were significantly lower in 'Microalgae 10' than in 'Microalgae 5' (Fig. 3).

3.3. Total As concentrations in blue mussels

The total As concentrations ranged from 9.5 to 12.2 mg/kg dw for Group A mussels ($\bar{x} = 10.9 \pm 0.8$; $n = 15$), 9.3–13.0 mg/kg dw for Group B mussels ($\bar{x} = 11.0 \pm 1.3$; $n = 15$), 9.5–14.0 mg/kg dw for Group C mussels ($\bar{x} = 11.0 \pm 1.1$; $n = 15$), and 8.2–12.7 mg/kg dw for Group D mussels ($\bar{x} = 10.6 \pm 1.2$; $n = 15$) (Fig. 5, Table S1). The number of samples pertain to pooled samples composed of nine mussels. Verification of PAMAS data showed that the mussels were feeding actively on the microalgae at a rate of approximately 6×10^5 cells per h per mussel

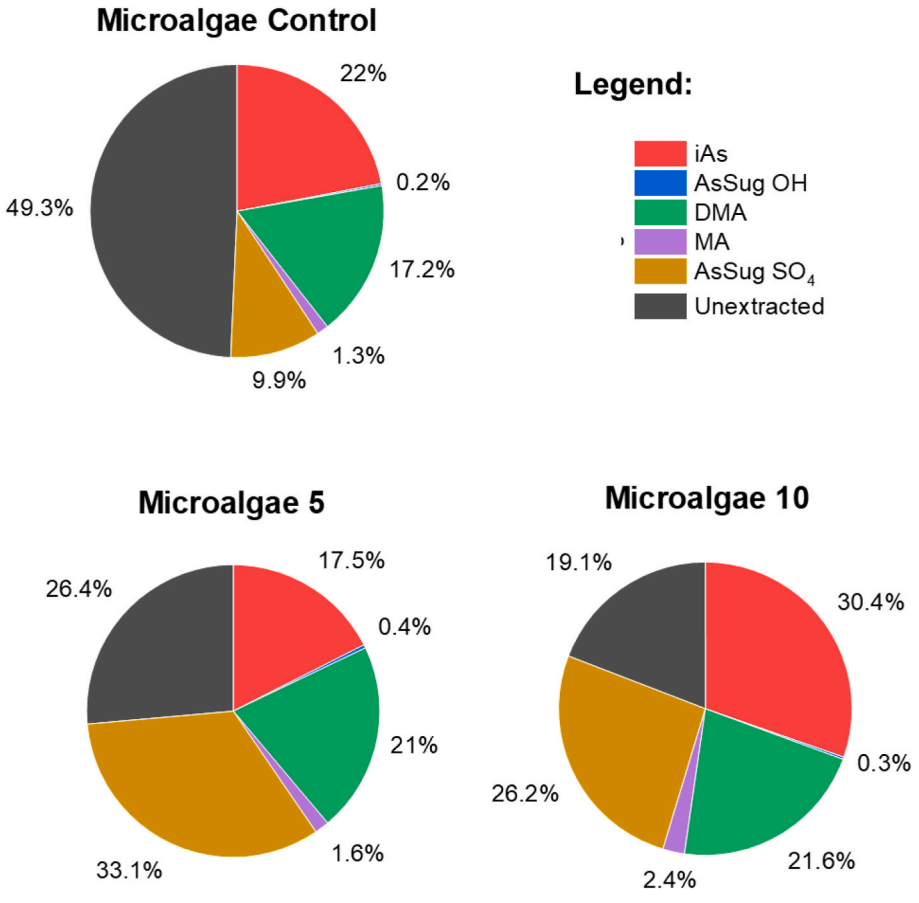

Fig. 4. Arsenic species profile in *Diacronema lutheri* grown under different treatments. Arsenic species fraction (% = concentration of As species/total As x 100). Treatments: 'Microalgae Control' – *D. lutheri* cultured without added As(V), 'Microalgae 5' – *D. lutheri* exposed to 5 μg/L As(V), 'Microalgae 10' – *D. lutheri* exposed to 10 μg/L As(V). Arsenic species: iAs – inorganic arsenic, MA – methylarsonate, DMA – dimethylarsinate, AsSug SO_4 – sulfate-arsenosugar, AsSug OH – glycerol-arsenosugar.

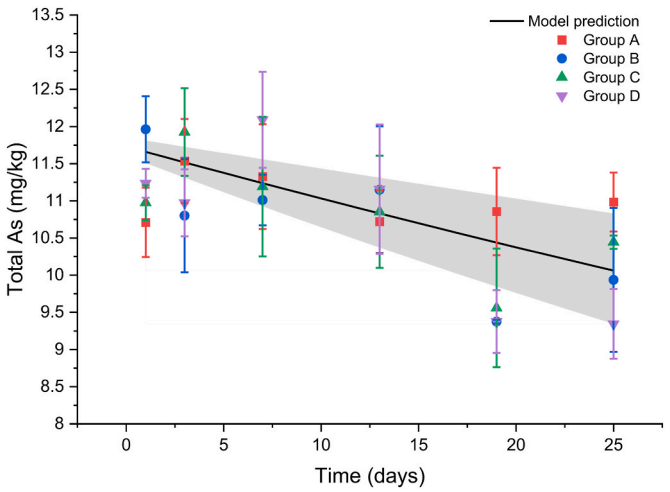

Fig. 5. Model prediction (linear model, gamma distribution) based on total arsenic concentrations over time for the different treatment groups.

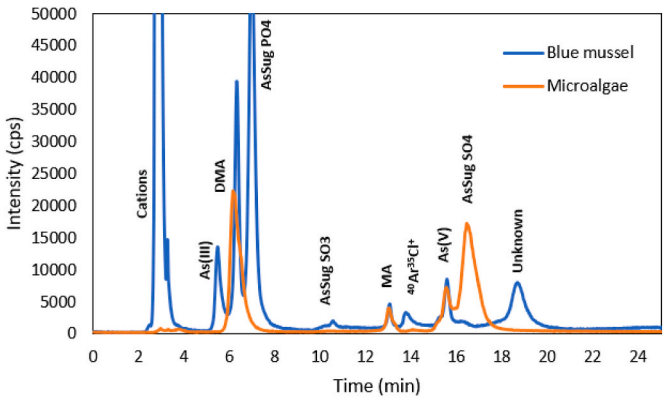

Fig. 7. Overlapping anion-exchange chromatograms of blue mussel and microalgae (*Diacronema lutheri*) extracts using HPLC-ICP-MS. Samples depicted here were taken from the Control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(data not shown).

The best general linear model to describe the change of tAs over time assumed a Gamma distribution of the data and used a negative inverse link function. This model found a significant non-linear decrease over time (Fig. 5). There was no clear effect of either CI or treatment on the decrease rate (Supplementary information in Table S2 and Fig. S1).

3.4. As species in blue mussels

A total of 11 As species were detected in the blue mussels (Figs. 6 and 7), and the respective As concentrations for the different time points are given in Tables S3–S13. AB was the predominant species in all exposure groups, ranging from 1.4 to 2.4 mg/kg dw ($\bar{x} = 1.9 \pm 0.2$; $n = 60$), accounting for approximately 18% of tAs (Fig. 6). The second most abundant species, AsSug PO_4, ranged from 0.6 to 1.9 mg/kg dw ($\bar{x} = 1.1 \pm 0.3$; $n = 60$), comprising approximately 13% of tAs. The other forms of AsSug found were AsSug OH and AsSug SO_3, with average concentrations of 0.5 ± 0.1 mg/kg dw ($n = 60$) and 0.012 ± 0.004 mg/kg dw ($n = 60$), representing 4% and 0.1% of tAs, respectively. Low levels of other methylated As species, including DMA and MA, were found, with a combined contribution of 7% of tAs (Fig. 6). The concentrations of iAs ranged from 0.06 to 2.2 mg/kg dw ($\bar{x} = 0.4 \pm 0.4$; $n = 60$), which generally accounted for less than 8% of tAs. Unknown peaks were also detected in chromatograms (Fig. 7), representing around 2% of tAs (Fig. 6). The fraction of unextracted As comprised almost half of the tAs in blue mussel samples (Fig. 6).

3.5. Seawater

Total As concentrations in seawater samples are given in Table S14. The inlet seawater had an average tAs concentration of 2.5 ± 0.2 µg/L ($n = 6$) (salinity: 35.4 ± 0.1 psµ; temperature: 12.5 ± 0.4 °C; pH: 8.03 ± 0.05), which is in the range of tAs concentrations reported for marine waters (0.5–3 µg/L) (Neff, 2002; Missimer et al., 2018). Seawater samples collected from Groups A, B, and C had tAs concentrations ranging from 1.0 to 4.0 µg/L ($\bar{x} = 2.2 \pm 0.7$; $n = 24$) (salinity: 35.4 ± 0.3 psµ; temperature: 11.7 ± 0.5 °C; pH: 8.05 ± 0.05). The tAs concentrations of the inlet seawater and those from the three exposure groups were not significantly different (Tukey test; $p < 0.05$). Seawater samples from Group D were significantly different from the rest, with tAs concentrations ranging from 4.0 to 7.5 µg/L ($\bar{x} = 6.1 \pm 0.8$; $n = 38$). Verification of seawater As(V) concentrations after water change (to check if target exposure concentration was achieved) and before water change (to check if there were changes in concentration since the water was replaced) showed no significant difference throughout the exposure period.

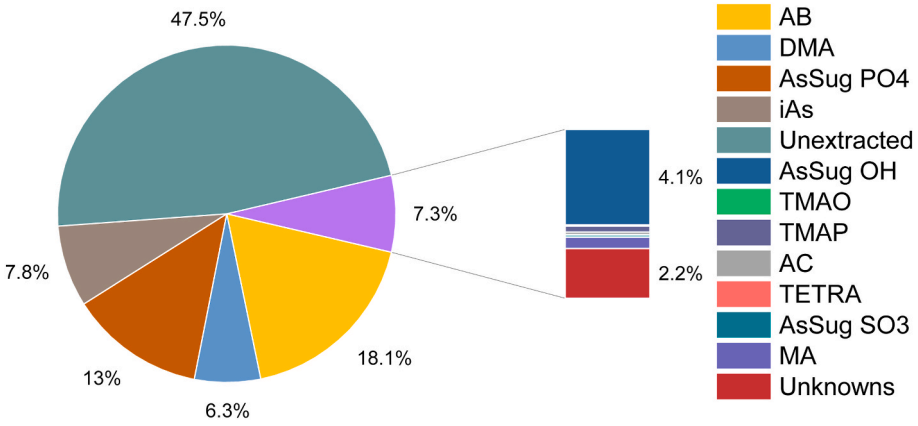

Fig. 6. An example of arsenic species profile in blue mussel from Group A (control). Arsenic species fraction, % = (concentration of As species/total As) x 100. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

4.1. Exposure of D. lutheri to inorganic As

4.1.1. Arsenic uptake

The current work utilized exposure concentrations which are representative of natural As concentrations found in seawater, i.e. 0.5–3 μg/L (Neff, 2002; Missimer et al., 2018). While extremely high levels of As can exist especially in contaminated sites (Whaley-Martin et al., 2012), the use of lower concentrations gives a better insight into As biotransformation occurring naturally in unpolluted sites. In the present study, the concentrations used were notably lower than previous reports, where levels ranged from 100 μg/L to as high as 10 mg/L (Cullen et al., 1994; Miyashita et al., 2011; Wang et al., 2013; Huang et al., 2021).

For the microalgae, the tAs concentration in the control group was different from the exposed groups, whereas the tAs concentrations in both exposed groups were similar. The bioaccumulation potential of a compound or metal in aquatic environments can be assessed by BCF (DeForest et al., 2007). For aquatic organisms, BCF has an inverse relationship with aqueous exposure concentrations (McGeer et al., 2003; DeForest et al., 2007), implying that BCF decreases as exposure concentration increases. The results from the present work may suggest that As accumulation of *D. lutheri* may not be as efficient at high exposure concentrations since BCF was lowest in the highest exposure group (Microalgae 10). Earlier studies hypothesized that after surpassing a certain tolerance limit, the organisms' ability to bioaccumulate metals may become restricted (Debelius et al., 2009; Huang et al., 2021; Das et al., 2022), which could explain the results found in the current work.

4.1.2. Arsenic speciation

Among the As species detected in *D. lutheri*, iAs was present in notable concentrations. The prevalence of iAs has been reported in several microalgal species, and its accumulation in exposure studies has usually been related to unrealistically high exposure concentrations (Duncan et al., 2015). AsSug were also one of the most prevalent As species in the microalgae exposure groups. Microalgae are known to contain AsSug, and the occurrence varies e.g. depending on the type of microalgae (Duncan et al., 2015). The microalgal synthesis of AsSug can be influenced by factors such as nutrient medium, exposure duration, and As concentration (Duncan et al., 2013; Papry et al., 2022). In a study involving *Dunaliella tertiolecta* and *Thalassiosira pseudonana*, the use of f/2 medium favored the production of AsSug PO_4 (Duncan et al., 2013). In contrast, AsSug PO_4 was not detected in the current work even when f/2 medium was used, suggesting that different species of microalgae have inherent differences in biotransfomation abilities.

The unextracted fraction of tAs was significant in this study (Fig. 4). As the analytical methods employed were mainly targeted to extract water-soluble As species, the unextracted fraction could be AsLipids. Previous studies have shown that some microalgal species contained AsLipids comprising at least 50% of tAs (Duncan et al., 2015). Although AsLipids were not determined in the current work, it is likely that they were present since *D. lutheri* was reported to have 29% lipid content (Mayer et al., 2022), and that lipid content has been usually correlated with AsLipid levels (Sele et al., 2012; Al Amin et al., 2020). Microalgae collected from the North Atlantic Ocean also contained AsLipids (Glabonjat et al., 2021). The absence of AB in *D. lutheri* corroborates observations in *D. tertiolecta* (Duncan et al., 2010) and *T. pseudonana* (Duncan et al., 2013). AB has not been found in microalgae to date. In a review which tried to elucidate AB transformation at the base of the marine food chain, traces of AB were first seen in herbivorous zooplankton which fed on microalgae (Caumette et al., 2012). It has been suggested that AsSug from microalgae is metabolized to AB in zooplankton, indicating that microalgae only contain precursors for AB formation in higher trophic aquatic organisms.

4.1.3. Arsenic biotransformation

Microalgae tend to take up As(V) (AsO_4^{3-}) from seawater due to its chemical and structural resemblance to phosphate (PO_4^{3-}) (Edmonds and Francesconi, 2003; Glabonjat et al., 2018). The ions exhibit a competitive behavior in terms of uptake via phosphate transporter systems, and an inverse relationship has been observed in some studies (Wang et al., 2014; Duncan et al., 2015; Wang et al., 2015). Phosphate-deficient conditions reportedly favored more efficient uptake of iAs in *Microcystis aeruginosa* (Wang et al., 2014). Contradicting results were seen in other species of microalgae, such as *Thalassiosira* sp. and *Chaetoceros* sp., where varying the P/As ratio did not produce significantly different results in terms of As uptake (Wang et al., 2015). Phosphate concentration was also observed to influence the formation of other As species, such as AsLipids (Glabonjat et al., 2021), DMA Duncan et al. (2015), and AsSug (Duncan et al., 2015). The use of f/2 medium in this work yielded an average phosphate concentration of 26.0 ± 0.3 μM ($n = 3$) in the exposure groups, which is significantly higher than phosphate concentrations reported for the world's oceans (<0.1–3.2 μM) (Glabonjat et al., 2021). Despite this, AsSug PO_4 was not detected in *D. lutheri*. Clearly, the effect of phosphate concentrations on As uptake and speciation is far more complex and involves interactions among several variables, as also seen by others (Papry et al., 2022).

Previous studies have shown that when microalgae are exposed to elevated As levels, detoxification mechanisms may become overburdened, causing a reduced degree of methylation (Cullen et al., 1994; Duncan et al., 2015; Huang et al., 2021). In the present study, exposure to 10 μg/L As(V) yielded higher concentrations of MA in 'Microalgae 10' compared to 'Microalgae 5' (Fig. 3). Formation of MA is one of the first steps in the biomethylation process of As, as shown by the Challenger pathway (Fig. 1). However, comparing DMA, similar concentrations were seen for both microalgae groups exposed to iAs, which could suggest that biomethylation was inhibited. This was more evident for the AsSug, with lower levels found in 'Microalgae 10' than in 'Microalgae 5'. Since the formation of AsSug is one of the latter steps in the Challenger pathway, the results could imply that biomethylation has become less efficient for the microalgae exposed to 10 μg/L. It has been shown that when detoxification mechanisms are overwhelmed, the microalgae will accumulate iAs instead (Duncan et al., 2015), which can explain the results in the current work. Higher proportion of iAs was noted in the 10 μg/L exposure group, comprising 30% of tAs (Fig. 4). It is uncertain, however, whether a 10-μg/L As exposure concentration is high enough to burden the detoxification mechanism of *D. lutheri*. This may be verified by investigating photosynthetic biomarkers such as chlorophyll (Chl *a*) content (Cabrita et al., 2018; Das et al., 2022), or oxidative stress biomarkers (Koechler et al., 2016; Pikula et al., 2019; Tripathi and Poluri, 2021). These biomarkers were not assessed in the current study.

4.2. Exposure to As of blue mussels

4.2.1. Arsenic uptake through the dissolved phase

The tAs concentrations in blue mussels exposed to 5 μg/L As(V) in seawater (Group D) suggest no or very limited As accumulation via the dissolved phase. The results are in accordance with an earlier study involving blue mussels collected off the coast of Western Australia (Gailer et al., 1995), where a 10-day exposure to 100 μg/L As(III) and As (V) in seawater did not produce significantly different tAs concentrations. This concentration is substantially higher (20 times) than the exposure level in the current study. Similarly, Hunter et al. (1998) did not observe a distinct change in As concentrations after exposing the common shrimp *Crangon crangon* to 100 μg/L As(V) in seawater for 24 days. In contrast, the water-borne exposure of the Bombay oyster *Saccostrea cucullata* to 1–20 mg/L As(III) and As(V) resulted in increased tAs levels proportional to the exposure concentrations (Zhang et al., 2015). Also, significant differences in tAs concentrations were noted when the clam *Asaphis violascens* was exposed to 1–20 mg/L As(III) and As(V) in

seawater (Zhang et al., 2019). All studies which reported increased tAs levels employed exposure concentrations which were 200–4000 times higher than the current study, sometimes resulting to mortality rates of 20%–100% depending on the exposure concentration and length of exposure (Zhang et al., 2015). It was further observed that organic As species were prevalent at low exposure concentrations while iAs prevailed at extremely high levels of exposure (Zhang et al., 2015; Zhang et al., 2019).

4.2.2. Arsenic uptake through the diet

The use of linear mixed models showed that 'time' seemed to be the only factor which was affecting the tAs levels in blue mussels. The tAs concentration appeared to decrease throughout the exposure period (Fig. 5). It appears that the blue mussels were losing As instead of gaining, resembling a depuration process. This can suggest that the acclimation period was not long enough or that the feeding rate was not sufficient for the mussels. A depuration process can occur when mussels are relocated from a site with a higher degree of contamination to a location which is relatively less polluted (Hervé-Fernández et al., 2010). In the present study, for the initial two-week acclimation where the mussels were kept close to a pier at 3 m depth, the mussels might have been exposed to higher natural levels of As concentrations than when kept in tanks filled with seawater pumped from a depth of 160 m. It is possible that there are differences in As concentrations in the water column (Cuong et al., 2008; Yuan et al., 2021), or other sources of As such as phytoplankton and detritus available to the blue mussels during the initial acclimation. It could also be the case that the microalgae were exposed to too low As concentrations to produce a detectable response in the blue mussels, hence, calling for further fine-tuning of the experimental design. In hindsight, statistical power could have been improved by pooling less samples, thus having more replicates to be analyzed.

A study on the flux of As through the Mediterranean mussel (*Mytilus galloprovincialis*) reported that As can also be released through the byssus (Ünlü and Fowler, 1979). Radiotracer techniques revealed that mussels were still excreting byssal threads which contained low but significant amounts of ^{74}As even after they have been removed from the radioactive seawater. The lack of appropriate radioisotopes and safety concerns on radioactive wastes limit the application of radiotracer techniques. For other elements, the use of a double stable isotope method has been proposed, which combines the benefits of radiotracer techniques and stable isotopes (Lin et al., 2021). However, this cannot be applied for studying As since it only has one stable isotope (^{75}As).

4.2.3. Arsenic species profile

The As species profile of blue mussels from different treatment groups did not vary significantly. The predominant species was AB (Fig. 6), but the proportion was relatively low (18%) compared to previous studies where AB comprised 30%–40% of tAs in blue mussels (Molin et al., 2012; Tibon et al., 2021). Nonetheless, lower percentages were also reported, e.g. in mussels from a contaminated harbor in Canada, where AB accounted for 4%–21% of tAs, and iAs comprised up to 36% of tAs (Whaley-Martin et al., 2012). Similarly, earlier studies found proportions of iAs ranging from 40% to 50% of tAs in blue mussels collected from Norwegian fjords (Sloth and Julshamn, 2008; Gomez--Delgado et al., 2023). In the present study, iAs generally comprised less than 5% of tAs.

The presence of AsSug in blue mussels have been reported in literature and their occurrence varies (Dahl et al., 2010; Whaley-Martin et al., 2012; Tibon et al., 2021). The relative abundance of AsSug PO_4 in this study is in accordance with previous observations for blue mussels from Norway (Tibon et al., 2021). In the present study, the blue mussels fed on *D. lutheri*, which contained AsSug SO_4 as one of its major species while AsSug PO_4 was not detected. The absence of AsSug SO_4 in blue mussels may suggest that it was metabolized and may have been converted to other forms. The limited occurrence of other methylated species corroborates earlier studies in blue mussels (Molin et al., 2012; Tibon et al., 2021). Unknown As species (chromatographic peaks) were detected in blue mussels, as also observed in previous studies (Dahl et al., 2010; Whaley-Martin et al., 2012). Earlier reports suggest that this maybe a thio-analogue of an AsSug (Schmeisser et al., 2004; Soeroes et al., 2005), but this was not confirmed in the present study. Furthermore, around 50% of tAs in blue mussels was unextracted (Fig. 6), which is comparable to extraction efficiencies reported in previous studies (Dahl et al., 2010; Whaley-Martin et al., 2012). The unextracted As may include AsLipids, since AsLipids have been reported in other species of mussels (*Mytilus galloprovincialis*) (Freitas et al., 2020).

5. Conclusion

Low-trophic marine organisms such as microalgae and blue mussels play a substantial role in As cycling in the environment. In this study, it was demonstrated that the marine microalgae *D. lutheri* can take up As from seawater and transform it to methylated As species and AsSug. Exposure of *D. lutheri* to 10 μg/L As(V) showed an increase in proportion of iAs and lower proportions of organic As species, indicating that the methylation process was affected and further suggesting that detoxification mechanisms were overwhelmed. This suggests that the uptake and transformation of As in microalgae is influenced by As levels in the medium/environment, but other factors, e.g. nutrient levels, may also play a role. Exposure of blue mussels to As(V) via seawater showed no accumulation of As. Thus, the dominant pathway for As accumulation under natural/low-As conditions is likely via ingestion of food. However, there was also no accumulation of As in blue mussels fed with *D. lutheri* exposed to As(V). Linear mixed models showed that the blue mussels were slowly losing As, resembling a depuration process, which may be attributed to differences in As concentrations in the mussels' natural environment and the exposure regime. Both *D. lutheri* and blue mussels contained notable proportions of simple methylated species (i.e. MA and DMA) and AsSug. The findings suggest that low-trophic marine organisms consist mostly of simple methylated As species and AsSug, which are the precursors for AB formation in higher trophic aquatic animals. The use of low-trophic marine organisms as feed ingredients can have potential implications with regards to feed and food safety since AsSug, together with AsLipids, are regarded as potentially toxic.

Author contributions statement

Jojo Tibon: Conceptualization, Data curation, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Ana I. Gomez-Delgado**: Conceptualization, Data curation, Methodology, Formal analysis, Writing – review and editing. **Antonio Agüera**: Conceptualization, Data curation, Formal analysis, Writing – review and editing. **Tore Strohmeier**: Conceptualization, Writing – review and editing. **Marta S. Silva**: Conceptualization, Methodology, Writing – review and editing. **Anne-Katrine Lundebye**: Conceptualization, Writing – review and editing. **Martin M. Larsen**: Data curation, Methodology, Writing – review and editing. **Jens J. Sloth**: Writing – review and editing, Supervision. **Heidi Amlund**: Writing – review & editing, Supervision. **Veronika Sele**: Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Pertinent data are available both in the manuscript and supplementary file. Additional data can be made available on request.

Acknowledgements

The authors would like to thank Annhild Engevik, Cathinka Krogness, Øivind Strand, Kjetil Stensland, and Sjur Åge Skår for the assistance in the exposure experiments at the IMR Research Station in Austevoll. Furthermore, the authors thank Sabrina June Hvid (AU Ecoscience) for the assistance in the analysis of seawater samples. This work was part of the project "Metals, arsenic and arsenic species in novel marine feed resources" (Project no. 15333), which was funded by the Ministry of Trade, Industry and Fisheries (NFD), Norway.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122176.

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