Chemosphere 349 (2024) 140798

Contents lists available at ScienceDirect

Chemosphere

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

Influence of extracellular polymeric substances on arsenic bioaccumulation and biotransformation in biofilms

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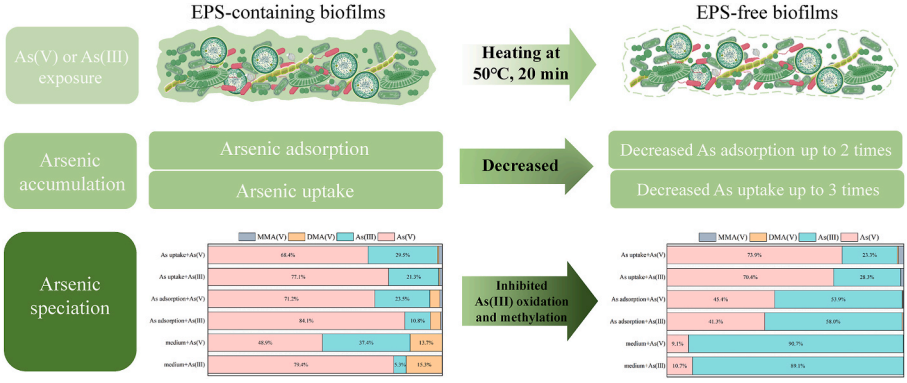
HIGHLIGHTS

- EPS increased As uptake and adsorption by biofilms.
- EPS enhanced As(III) oxidation and methylation in biofilms.
- EPS removal changes the response mode of biofilm to As metabolism.
- The proportion of arsenic speciation in biofilms is similar regardless As(III) or As(V) exposure.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Prof. X. Cao

Keywords:
Biofilms
EPS
As speciation
Biosorption
Methylated

ABSTRACT

It is well recognized that biofilms can biosorb and biotransform heavy metals in aquatic environments. However, the effects of extracellular polymeric substance (EPS) on inorganic arsenic (As) bioaccumulation and biotransformation in biofilms are still unrevealed and need to be investigated. In order to explore the above scientific issues, the As accumulation and speciation in EPS-containing or EPS-free biofilms and growth medium under As(V)/As(III) exposure conditions were measured. After the removal of EPS, the amount of As uptake (As_{up}) and As adsorption (As_{ad}) in biofilms were significantly reduced, no matter whether exposed to As(V) or As(III). FTIR analysis further suggested that the interaction between these functional groups with As was limited after the removal of EPS. In the EPS-containing biofilms, the As_{ad} was mainly As(V) with low toxicity. However, after the removal of EPS, the As_{ad} was mainly As(III) with high fluidity, and no methylated As was found. Moreover, the removal of EPS inhibited As(III) oxidation and methylation by biofilms, resulting in the decrease of As(V) and methylated As in the growth medium. The findings of this study emphasized the essential impact of EPS on the biosorption and biotransformation of As in biofilms. This study provides a unique understanding of the role of biofilms in As biogeochemical cycle, and water quality purification function in water environments.

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https://doi.org/10.1016/j.chemosphere.2023.140798
Received 25 June 2023; Received in revised form 19 November 2023; Accepted 22 November 2023
Available online 28 November 2023

1. Introduction

As a toxic contaminant, arsenic (As) is found worldwide in nature, the continuous exposure to As poses serious public health risks for millions of people worldwide (Chen and Costa, 2021; Duker et al., 2005). Arsenic exists in nature in a variety of valence states (e.g., −3, 0, +3, +5), of which As(III) and As(V) are the most common forms (Smedley and Kinniburgh, 2002). And As(III) is typically considered more poisonous and mobile than As(V) (Nriagu and Pacyna, 1988; Duker et al., 2005).

Natural biofilms are complex biological communities, which are made up of varied organisms like autotrophs (including green algae, diatoms, and cyanobacteria) and heterotrophs (including bacteria, fungus, and protozoa), as well as abiotic components like extracellular polymeric substances (EPS) (Flemming et al., 2016). The ecological health of freshwater ecosystems can be significantly improved and maintained by biofilms (Battin et al., 2016; Wu et al., 2014). Biofilms could accumulate heavy metals (such as copper, zinc, As, and others), which inevitably influence their adsorption, uptake and transformation in the environment (Behra et al., 2002; Guo et al., 2020, 2021; Stolz et al., 2006; Yan et al., 2021).

The effects of biofilms on the As adsorption, uptake or transformation have been investigated in many studies (Barral-Fraga et al., 2020; Carolina et al., 2014). The accumulation of As by biofilms was accomplished through two steps, including rapid extracellular passive adsorption and slow intracellular uptake (Leong and Chang, 2020). As (III) will activate As oxidation mechanism after the entrance into prokaryotes and microalgae, which will serve as the detoxifying function by using arsenite oxidase (Aio/Arx) (Nagvenkar and Ramaiah, 2010; Naveed et al., 2020b; Wang et al., 2015). Enzymes from outside of the cells, such as carbonic anhydrase and extracellular phosphatase, can also oxidize As(III) on the algal surface (Qin et al., 2009; Zhang et al., 2014). Similarly, prokaryotes and microalgae are also able to reduce As(V) to As(III), both intracellularly and extracellularly (Wang et al., 2015; Zhou et al., 2020). The intracellular reduction of As(V) can be achieved by cytoplasmic arsenate reductase enzyme (ArsC), reducing As(V) to As(III) (Wang et al., 2015; Yan et al., 2016). Extracellularly, the secretions of the biofilms (such as quinones, humic substances, riboflavin, and other organic matter) can reduce As(V) to As(III) (Jiang et al., 2009; Zhou et al., 2020). In biofilms and microalgal cells, the detoxification process also involves the production of methyl-As (Barral-Fraga et al., 2018; Yan et al., 2016), as well as more complex organic compounds (e.g. As sugars and As lipids) (Huang, 2014).

EPS is an important component of biofilms, which plays a crucial role in the accumulation and transformation of contaminants (Flemming and Wingender, 2010; Huang et al., 2022). EPS on biofilms provides a coating of porous organic material around the cell surface, containing abundant hydroxyl (-OH), carboxyl (-COOH), carbonyl (C═O), sulfhydryl (-SH), amino (-NH_2) and other functional groups responsible for metal adsorption (Huang et al., 2022; Wang et al., 2015; Yan et al., 2010; Zhu et al., 2018). Furthermore, EPS contains a variety of reducing agents (e.g. proteins and polysaccharides), as well as mediators of electron transfer (e.g. flavin and quinone), which can interact with As (Tourney and Ngwenya, 2014; Zhou et al., 2020). Studies have investigated the adsorption and transformation behavior of heavy metals by microbial cells with or without EPS (Baker et al., 2010; Naveed et al., 2020b; Ueshima et al., 2008; Zhou et al., 2020). The observed effects of EPS on the accumulation of metals, however, are contradictory. In comparison to cells without EPS, *Synechocystis* PCC6803 cells with EPS showed increased As adsorption and decreased As uptake (Naveed et al., 2020b). While the presence of EPS in *Pseudomonas putida* had no discernible influence on Cd adsorption (Ueshima et al., 2008). EPS can also influence the transformation of metals, *Escherichia coli* or *Bacillus subtilis* can promote As(V) reduction after the removal of EPS (Zhou et al., 2020), while *Synechocystis* PCC6803, cells containing EPS significantly increased As(III) oxidation and As(V) reduction (Naveed et al., 2020b).

Bacteria and microalgae with or without EPS play different roles in metal accumulation and transformation. Biofilms are intricate communities made up of bacteria and microalgae, which makes the effects of biofilm-EPS on As accumulation and biotransformation more complicated. However, previous research on As biotransformation by biofilms is insufficient, previous studies on the effects of EPS on As accumulation and transformation were conducted with the single organism, therefore, the effects of biofilm-EPS on As transformation remain unclear. We speculated that the removal of EPS in biofilms can reduce the As accumulation, promote the reduction of As(V) when exposed to As(V), and stimulate As(III) to be oxidized under As(III) exposure. A series of batch experiments were conducted to verify our speculation. The objective of this study is: (1) to investigate As accumulation and speciation change in biofilms and the alteration of As concentration and species in the medium; and (2) to reveal the role of EPS on the bioaccumulation and biotransformation of As in biofilms. The findings of this study will contribute to further insights into As transformation and fate in the water-biofilm system and provide a better understanding of the effects of biofilm-EPS on As biogeochemical cycles in aquatic environments.

2. Materials and methods

2.1. Chemicals and regents

$Na_3AsO_4{\cdot}12H_2O$ (Sinoreagent, China) and $NaAsO_2$ (Innochem, China) were used to prepare inorganic arsenate and arsenite stock solutions, respectively. All studies were conducted using a 750 mg L^{-1} As stock solution. The standard stock solutions of As(III), As(V), MMA, and DMA were purchased from the National Institute of Metrology, China. The spectrally pure potassium bromide (KBr) was purchased from Sigma-Aldrich (USA). Deionized water was used to make all of the solutions (Thermo, USA). All reagents were of analytical grade.

2.2. Cultivation of natural biofilms

Water containing natural microorganisms was collected from the Houxi River in Xiamen, China (24°37′46.22″N, 117°59′50.37″E) in 2022, the physico-chemical properties of river water are shown in Table S2. To cultivate natural biofilms, a glass microcosm aquarium (60 cm × 40 cm × 45 cm) was employed in a laboratory (Hua et al., 2012). On pre-cleaned glass microscope slides (100 mm × 50 mm × 1 mm) mounted on polypropylene racks, biofilms developed under white light with 50 mmol photons m^{-2} s^{-1} intensity, with a 12:12 h day/night cycle at 25 ± 2 °C. Weekly nutrient replenishment was performed by renewing roughly a third of the water in the system with the river water. Biofilms were scraped from glass microscope slides after maturing and suspended in 0.85% NaCl. The electron microscope observed that the biofilm had a dense structure and the EPS held the microorganisms together. And the chlorophyll-a content and dry weight of the biofilms were assessed at 3 d intervals, with no statistically significant differences observed between the last three measurements, the maturity of biofilms was confirmed. The organic matter-containing supernatant and any remaining culture medium were then removed from the suspensions by centrifuging them at 4000 rpm for 10 min. The suspensions were finally diluted to 4 mg mL^{-1} (based on dry weight) for the succeeding experiment.

2.3. Preparation of biofilms without EPS

EPS was removed following the method described by Naveed et al. (2020b) and Zhu et al. (2019). Briefly, the biofilms suspension was resuspended in 0.85% NaCl, heated at 50 °C for 20 min, immediately cooled to room temperature in ice bath, and then centrifuged at 11,000 rpm for 15 min. To obtain EPS, the supernatant was filtered using a 0.22 μm cellulose acetate membrane, and the filtrate was then kept at −20 °C

for subsequent analysis. The precipitation was collected as EPS-free (EPS-F) biofilms for As accumulation and transformation experiments. To ensure that EPS was effectively eliminated, the primary EPS components were examined. The phenol sulfuric acid digestion method, Bradford method, and Qubit kit were used to measure the polysaccharides, proteins, and DNA, respectively. The total yield of extracted EPS was calculated by adding polysaccharides, proteins, and DNA.

2.4. As accumulation and transformation in biofilms with and without EPS

Batch studies were used to investigate the role of EPS in the accumulation and transformation of As in natural biofilms. After reaching maturity, the biofilms were divided into two groups, one group for EPS extraction (EPS-F biofilms) and the other for EPS-containing biofilms (EPS-C biofilms). For each group, the biofilms were exposed to As(V) and As(III) at 340 μg L^{-1} for 60 h in a 500 mL conical flask containing 300 mL of modified BG11 medium (TN = 10 mg L^{-1}, TP = 0.2 mg L^{-1}), respectively. The exposure concentration was set based on the criterion maximum concentrations (CMC) of the U.S. Environmental Protection Agency for acute As exposure to aquatic life in freshwater, which is 340 μg L^{-1} (EPA, 2014). As-free treatment was set as the control group. Abiotic control was used to assess the stability of As(V) and As(III) at the beginning and end of the experiment, in which As was added without biofilms. The initial biomass of biofilms concentration was set as 1.2 mg mL^{-1}.

After inoculation, 20 mL samples were collected at 0 h, 6 h, 12 h, 24 h, 36 h, 48 h, and 60 h, and the samples were then separated into supernatant and biofilm precipitation by centrifugation (4000 rpm, 10 min). Inductively coupled plasma mass spectrometry (ICP-MS, 7700a Agilent) and HPLC–ICP-MS (Agilent LC1100 series and Agilent ICP-MS 7700a; Agilent Technologies) were used to analyze the supernatant for the total amount of As and As speciation. The biofilms precipitation (with and without EPS) was separated into two sections after being washed twice with 10 mL phosphate-buffered saline (PBS, 10 mM, pH = 7.2–7.4). One was freeze-dried to determine the amount of accumulated As in the biofilms, and the other was utilized to determine the total amount and speciation of As uptake (As_{up}) and As adsorption (As_{ad}) in the biofilms. All the samples for As analysis were filtered with a 0.22 μm cellulose acetate membrane and kept at −20°C for no more than 10 d. The detailed extraction methods of accumulated As, As_{ad} and As_{up} of biofilms are shown in 2.5, respectively.

After 60 h As exposure, 20 mL biofilms samples were collected for re-extraction of EPS according to the procedures in 2.3, and the composition and total yield of EPS were measured to confirm the absence of EPS in biofilms during the EPS-removed experiment. 5 mL biofilms samples were also collected and observed using scanning electron microscope (SEM, Hitachi S-4800, Japan) for surface morphological changes of the biofilm-EPS after As exposure for 60 h, this was performed to analyze the resynthesis of EPS in the biofilms. Additionally, the biofilm's EPS properties and surface functional groups were examined using Fourier infrared spectroscopy (FTIR, Nicolet 6700, Thermo, USA) both before and after EPS extraction and As exposure.

2.5. Analysis methods

As accumulation in biofilms. The amount of retained As intracellularly (uptake minus metabolism and excretion) and extracellularly is referred to as the biofilms accumulation (Zhang et al., 2020). The As accumulation in the biofilms was extracted by HNO_3 and $HClO_4$ mixed solution digestion (Naveed et al., 2020b). The procedure was carried out in detail as follows: the freeze-dried biofilms samples were put in a 50 mL digestion tube and incubated overnight with a 3 mL mixture of HNO_3 and $HClO_4$ (2:1, v/v). The digestion solution was concentrated to the size of soybean grains at 180 °C after the biofilms had been digested to transparency at 140°C (about 3 h). After chilling, ultrapure water was used to dilute the digestion solution to 15 mL. Following 0.22 μm cellulose acetate membrane filtration, the As concentration was measured by ICP-MS. As a standard sample, Laver (GBW08521) was digested with biofilms, the measured value (39.4 ± 0.9 μg g^{-1}) was 94.7–98.7% of the authorized value (41 ± 3 μg g^{-1}) and the recovery rate conformed to standard.

Extracellular and intracellular As in biofilms. According to the procedure described by Levy et al. (2005), As_{ad} in the biofilms was extracted with acidic phosphate, and subsequently, As_{up} was extracted by ultrasound according to the procedure of Rubio et al. (2010) and Zhen et al. (2020). To extract As_{ad}, the biofilms were resuspended in 10 mL of 100 mM KH_2PO_4/K_2HPO_4 buffer solution (pH = 5.95). After shaking the suspension for 30 s, standing for 20 min, and centrifuging at 4000 rpm for 10 min, the supernatant obtained was the As_{ad}, and the extraction was repeated once. The two eluents were combined, filtered (0.22 μm cellulose acetate membrane), and then kept at −20°C for As concentration and As speciation measurements within 10 d. The remaining biofilm samples were then washed gently with 10 mL of Milli-Q water, and centrifuged for 10 min at 4000 rpm, then further freeze-drying to extract As_{up} of the biofilms. The freeze-dried biofilms samples were then ground into a powder with the addition of 10 mL PBS, followed by ultrasonic at 4°C for 1 h and then centrifuged at 4000 rpm for 10 min, and extracted twice with 10 mL PBS solution. The As_{up} eluents were treated the same as As_{ad}. Triplicates were set for every experiment.

Biofilms biomass. Chlorophyll-a content typically represents the amount of autotrophs (e.g. microalgae) in the biofilms (Zhao et al., 2022). After 15 min of dark adaptation, phytoplankton pulse-amplitude-modulated fluorometer was used to measure the concentration of chlorophyll-a (PHYTO-PAM, Heinz Walz GmbH, Germany) (Zhang et al., 2023). The total biomass of biofilms was represented as dry weight (DW) (Graba et al., 2013), 10 mL sample of biofilms after it had been centrifuged at 4000 rpm for 10 min, frozen, and dried for DW determination (three decimal places to 0.001).

Observation of the biofilms surface structure. SEM was used to examine the impact of As exposure on the surface of biofilms after the experiment (Michel et al., 2007). 5 mL biofilms were collected and centrifuged at 4000 rpm at 4°C for 5 min, and then washed twice in PBS. 2.5% glutaraldehyde is used to fix these cells overnight at 4°C, after which biofilms were dehydrated in a gradient of ethanol solutions for 10 min at various concentrations of 30%, 50%, 70%, 80%, 90%, and 100% (twice) ethanol. SEM was used to analyze the cells after they had been dried for 24 h in a critical point drier and coated with a thin coating of gold.

Determination of As. ICP-MS was employed to measure the As concentration of the medium and biofilms in helium gas mode. The measured masses were ^{75}As, ^{72}Ge, and ^{103}Rh, with the latter two being the internal standards of signal stability (Wang et al., 2013a). Based on the method of Wang et al. (2013a), HPLC-ICP-MS was used to analyze As speciation of culture medium and biofilms. The PRP-X100 anion exchange column (10 μm; 250 × 4.1 mm; Hamilton, CA, USA) was used to separate the different As species (As(III), As(V), DMA and MMA). The mobile phase consisted of 10 mM $NH_4H_2PO_4$ and 10 mM NH_4NO_3, and the pH value was adjusted to 6.25 with HNO_3 or ammonia. The mobile phase was run at a constant velocity of 1.0 mL min^{-1} at 25 °C.

Functional groups on the surface. The changes of functional groups on the surface of biofilms cells were revealed by FTIR. All the biofilm samples cultured for 60 h were used as experimental subjects. The freeze-dried biofilms (1 mg) were manually homogenized with 99 mg of oven-dried KBr to form particles with a diameter of 16 mm (Abu et al., 1991). The scanning range of FTIR spectroscopy is 500–4000 cm^{-1}. The background spectrum obtained from the pure KBr scan is automatically subtracted from the sample. All samples have three replicates.

2.6. Statistical analysis

Data were processed with EXCEL 2019 and IBM SPSS 26.0 and expressed as the mean ± SD of three replicates, One-way ANOVA was performed to evaluate the difference among treatments. Differences were considered significant when $P < 0.05$. ORIGIN Pro 2023 was used for data fitting and image processing.

3. Results

3.1. Characterization of EPS from biofilms

After extracting EPS using a heating method, the components were detected, and the removal efficiency of EPS was assessed by SEM. The composition of EPS was represented in Table S1, the EPS extracted from initial biofilms was mainly composed of polysaccharides and proteins, which were 10.91 mg g^{-1} and 1.31 mg g^{-1} dw, respectively. Further, SEM examination revealed that the EPS biofilms had a thick layer of EPS coating on their surface (Fig. S1a). In contrast, after EPS extraction, EPS was barely observed on the surface of the biofilms (Fig. S1b), proving that EPS removal was effective.

To further understand EPS regeneration, the biofilm samples were examined by SEM, and the EPS of the biofilms was extracted after being exposed to As(III)/As(V) for 60 h. After 60 h, the composition of EPS in the control group did not vary significantly from the original biofilms ($P > 0.05$), and the EPS content was 4.06 times higher than that of EPS-removed biofilms (Table S1). The SEM image of EPS-removed biofilms was shown in Fig. S1d, which was in an EPS-deficient state. No matter whether exposed to As(III) or As(V) for 60 h, the EPS content of EPS-removed biofilms did not change significantly. While there was no significant change of EPS content after exposure to As(III), the EPS content of intact biofilms significantly decreased following exposure to As(V) ($P < 0.05$) (Table S1), which was also consistent with the results as shown in SEM images (Fig. S1e). The results of SEM and EPS re-extracted showed that EPS-free biofilms were still in an EPS-lacking state after As exposure for 60 h (Fig. S1), indicating an EPS-limited status in the EPS-free biofilms during the entire experiment.

3.2. Biomass of biofilms with and without EPS in the presence of As

The content of chlorophyll-a and DW of biofilms were used to measure the effects of EPS on the biomass of the biofilms after As(III)/As(V) exposure (Fig. 1). The chlorophyll-a and DW in the control group did not significantly vary during the experiment (Fig. S2), showing that the biofilms were in a relatively stable state. After exposure to As(V), the content of chlorophyll-a decreased compared with control, the decrease in EPS-C biofilm was up to 33.8% after 60 h exposure. After the removal of EPS, the decrease of chlorophyll-a was inhibited compared with EPS-C biofilm (30.1%). However, no such inhibition was observed under As (III) exposure after EPS removal, although the content of chlorophyll-a was decreased with exposure time. The DW change trend of biofilms was noticeably different from chlorophyll-a (Fig. 1c and d). The DW of biofilms significantly increased after As(V)/As(III) exposure in the early stage (0–6 h) in both treatments compared to the control group ($p < 0.05$). There was no noticeable variation in DW during the middle of the exposure (12–36 h), while DW started to decline at 36 h.

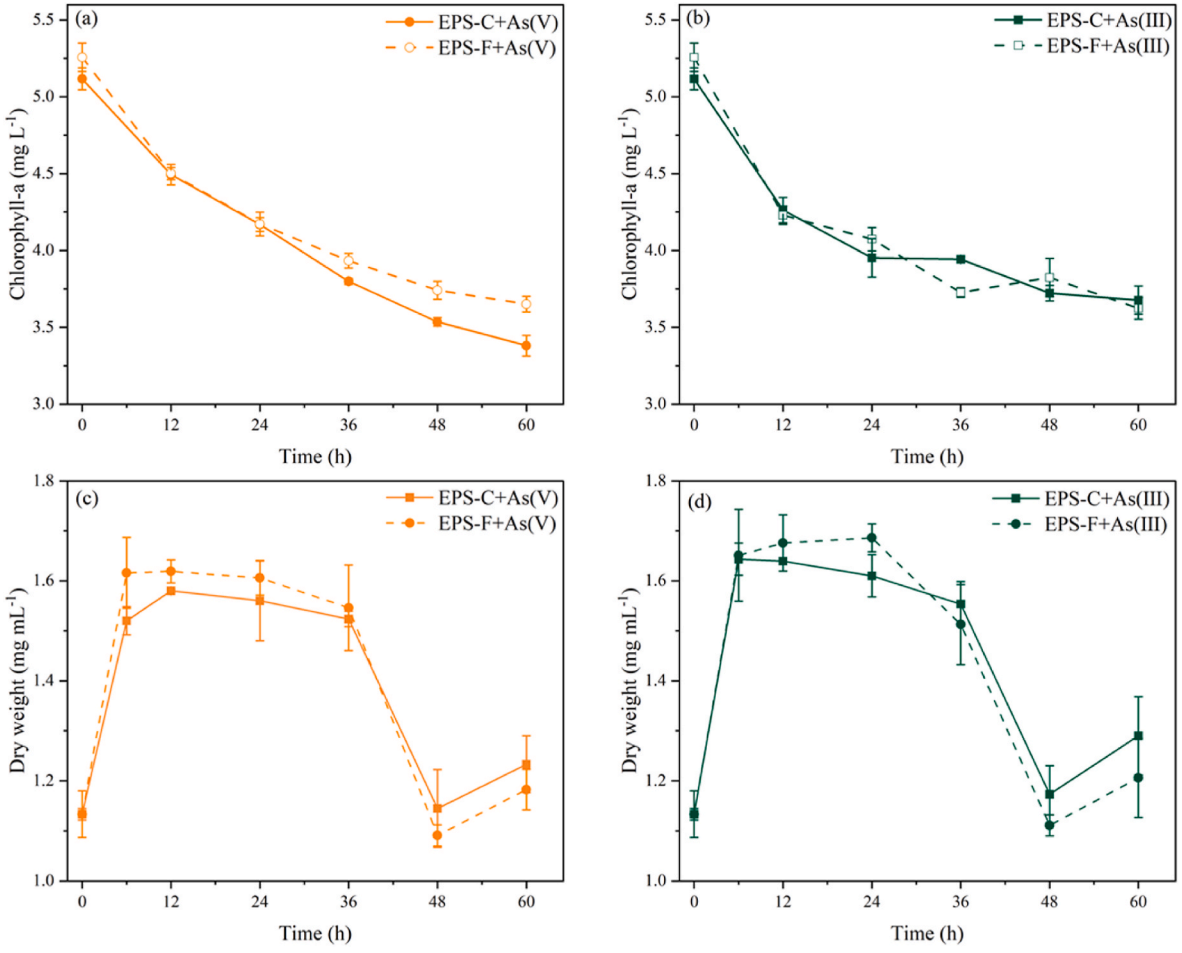

Fig. 1. Chlorophyll-a and dry weight of biofilms. Chlorophyll-a of EPS-C and EPS-F biofilms exposure to As(V) (a) or As(III) (b), dry weight of EPS-C and EPS-F biofilms exposure to As(V) (c) or As(III) (d). Data are expressed as means ± standard errors (n = 3).

3.3. Functional groups of biofilms with and without EPS

FTIR analysis was used to reveal the functional groups and binding sites of the biofilms surface interacting with As. As shown in Fig. 2a, the EPS-C biofilms has a wide absorption peak at 3404.93 cm^{-1}, which is mainly related to –OH or –NH; the absorption peaks at 2923.20 cm^{-1} and 2852.32 cm^{-1} were from $–CH_2$- on the saturated carbon chain; the absorption peaks at 1651.60 cm^{-1} and 1540.93 cm^{-1} were observed from the C═O and $–NH_2$ in the typical protein amide I and II bands, respectively; the absorption peak at 1092.45 cm^{-1} is related to C–O–C in polysaccharide derivatives, the coexistence of the peaks at 1651.60 cm^{-1} and 1560.93 cm^{-1} shows a large number of carboxyl functional groups in biofilm. In summary, the active functional groups on the surface of biofilms are –OH, –NH, $–CH_2$-, $–NH_2$, C═O, C–O–C and –COOH. When the wavelength change ≥4 cm^{-1}, the wavelength is considered shifted. In the control group of EPS-F biofilm, no shift in absorption peak was observed, showing that the functional group did not change after removal of EPS. (Fig. 2a).

Compared with the control (Fig. 2a), the FTIR spectra of EPS-C biofilms treated with As (III) and As(V) showed peaks shifted (Fig. 2bc). After exposure to As(V) or As(III), the absorption peak of –OH or –NH at 3404.93 cm^{-1} shifted to 3396.00 cm^{-1} and 3385.84 cm^{-1}, respectively, while the absorption peaks at 1092.45 cm^{-1} representing C–*O*–C shifted to 1085.01 cm^{-1} and 1085.10 cm^{-1}, respectively. Regardless of As(III) or As(V) exposure, the FTIR spectra showed that the absorption peaks did not shift and the functional groups did not change significantly after the removal of EPS (Fig. 2c).

3.4. Arsenic accumulation in EPS-C and EPS-F biofilms

The amount of As in the control group showed that there was no As contamination in the initial biofilm (6.54 μg g^{-1} dw). The amount of total As accumulation, As_{ad} and As_{up} in the biofilms were significantly reduced after EPS removal under As(V) or As(III) treatment ($P < 0.05$) (Fig. 3). Compared with EPS-C biofilms, the decrease of the total As_{ad}, and As_{up} was up to 2.04 and 3.45 times after EPS removal when exposed to As(V), respectively. When exposed to As(III), the As_{ad}, and As_{up} reduced up to 2.16 and 3.73 folds after EPS removal, respectively. The amount of As_{ad} of EPS-C biofilms after exposure to As(III) was higher than that after exposed to As(V). Moreover, the amount of As_{ad} is higher than As_{up} in all treatments. The concentration of As in the medium was measured to further verify the As amount in biofilms, the consistent results was also observed (Fig. S3).

3.5. As speciation in biofilms with and without EPS

The speciation of As_{ad} and As_{up} in biofilms was determined to investigate As biotransformation in biofilms (Figs. 4 and 5). The speciation of As_{ad} in biofilms was shown in Fig. 4. No matter whether under As(V) or As(III) exposure, four As speciation were detected in As_{ad} on EPS-C biofilms, including As(V), As(III), MMA(V) and DMA(V), while no organic As was detected in As_{ad} on EPS-F biofilms. The proportion of As (III) in the As_{ad} on the EPS-C biofilms decreased with As(V) or As(III) exposure time, while the proportion of As(III) in the As_{ad} increased within 36 h and then decreased after EPS removal. As(V) was the majority speciation of As_{ad} on EPS-C biofilms, regardless of exposed to As (V) or As(III), accounting for 61.1–71.2% and 53.6–84.1% of As_{ad}, respectively. While the speciation of As_{ad} on EPS-F biofilms was predominantly As(III), regardless of exposed to As(V) or As(III) exposure, accounting for 53.9–75.8% and 58.0–79.1%, respectively (Fig. 4). Further, only DMA(V) (up to 1.1 μg g^{-1}) was detected in As_{ad} on EPS-C biofilms, and no organic As was detected in As_{ad} on EPS-F biofilms (Fig. 4).

In all treatments, As_{up} in biofilms consisted of As(V), As(III), MMA (V) and DMA(V). And As(V) was the main speciation of As_{up} in the biofilms, and MMA was the main organic As detected (up to 0.65 μg g-1) (Fig. 5). When exposed to As(V), As(V) accounts for 63.6–75.3% of As_{up} in EPS-C biofilms and 62.4–73.9% in EPS-F biofilms, respectively. When exposed to As(III), As(V) accounts for 64.9–77.1% of As_{up} in EPS-C biofilms and 50.9–75.9% in EPS-F biofilms, respectively (Fig. 5).

3.6. As species in growth medium

As shown in Fig. 6, the species of As in the growth medium were analyzed. In the abiotic group, the proportion of As(V) didn't show change in the growth medium when exposed to As(V), while less than 5% of As(III) was chemically oxidized to As(V) within 12 h under As(III) exposure and only 9.6% at 60 h (Fig. S4). No matter exposed to As(III) or As(V), three As species including As(V), As(III) and DMA(V) were detected in the medium of EPS-C biofilm treatments, while no organic As detected in EPS-F biofilm treatments.

When exposed to As(V), As(V) in the growth medium was rapidly decreased (within 6 h) and fluctuated with the extension of time in EPS-C biofilm treatments, and the DMA content increased with exposure time. After the removal of EPS, As(V) in the growth medium was also rapidly decreased (within 6 h) while As(V) maintained a lower proportion in the EPS-F biofilm treatments (6.5–12.7%) compared with the EPS-C biofilm treatments. Moreover, the As species in the growth medium was mainly As(III) regardless of EPS-C or EPS-F biofilm treatments. When exposed to As(III), the proportion of As(V) and DMA(V) in the medium increased with time in the EPS-C biofilm treatments, and As(III) in the medium was slowly decreased. However, the proportion of As(V) in the growth medium was consistently low (9.3–19.6%) under As(III) exposure after EPS removal.

4. Discussion

4.1. Accumulation of As by biofilms with and without EPS

The total accumulated As in biofilms is accomplished by two actions, including extracellular adsorption and intracellular uptake (Leong and Chang, 2020; Zhang et al., 2020). Compared with EPS-F biofilms, the

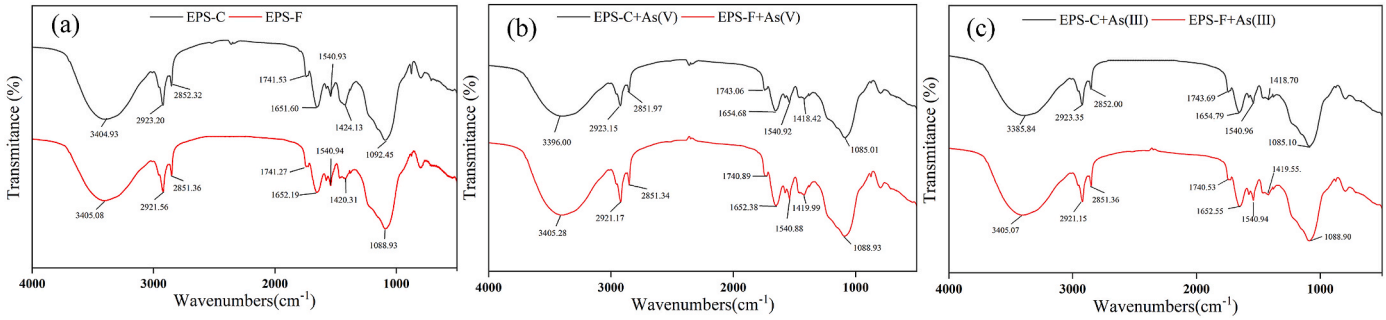

Fig. 2. FTIR spectra of control (EPS-C and EPS-F biofilms) (a). FTIR spectra of EPS-C and EPS-F biofilms treated with As(V) (b) and treated with As(III) (c).

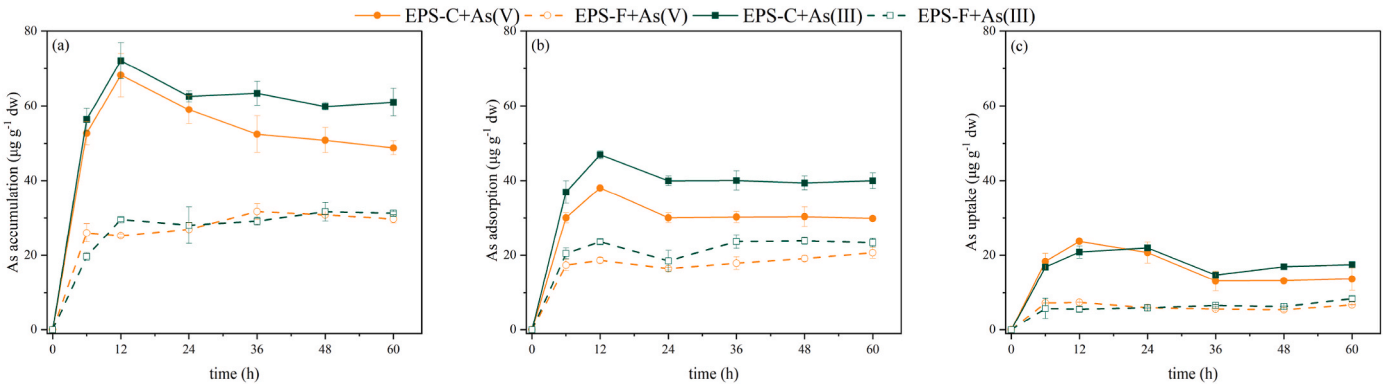

Fig. 3. The amount of As accumulation (a), As adsorption (As_{ad}) (b) and As uptake (As_{up}) (c) in ESP-C and EPS-F biofilms after exposure to As(V) and As(III) for 60 h. Data are expressed as means ± standard errors (n = 3).

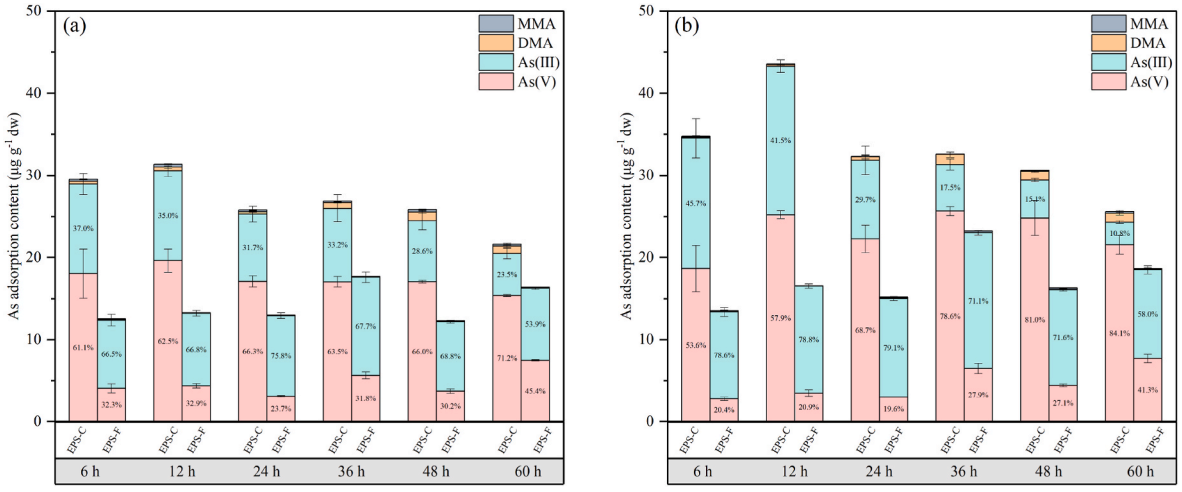

Fig. 4. The content of different As speciation adsorbed on EPS-C or EPS-F biofilms treated with As(V) (a) and As(III) (b). Data are expressed as means ± standard errors (n = 3).

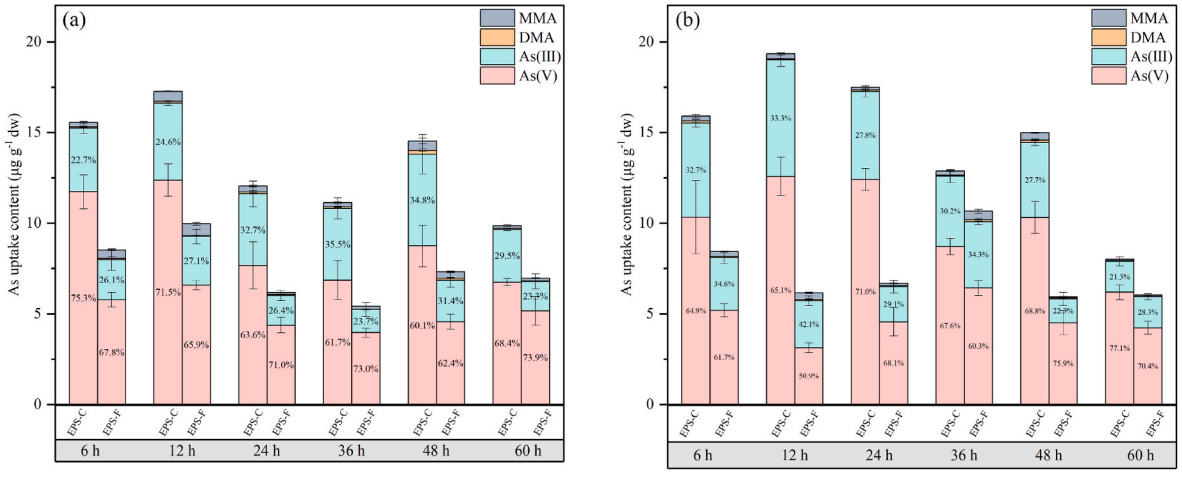

Fig. 5. The content of different As speciation uptake in EPS-C and EPS-F biofilms treated with As(V) (a) or As(III) (b). Data are expressed as means ± standard errors (n = 3).

amount of As_{ad} and As_{up} in EPS-C biofilms increased significantly, no matter whether exposed to As(III) or As(V) (Fig. 3), which was supported by previous studies that the EPS of biofilms play an important role in the accumulation of As (Sheng et al., 2010; Flemming and Wingender, 2010). EPS provides plenty of binding sites (Naveed et al., 2020b; Zhang et al., 2020), which explains the significant reduce of As_{ad} in the EPS removed biofilms (Fig. 3b).

However, conflicting results were reported in previous studies on microalgae, which showed that the As exposure induced an increase of the amount of As_{up} after EPS removal (Naveed et al., 2020b; Zhang et al.,

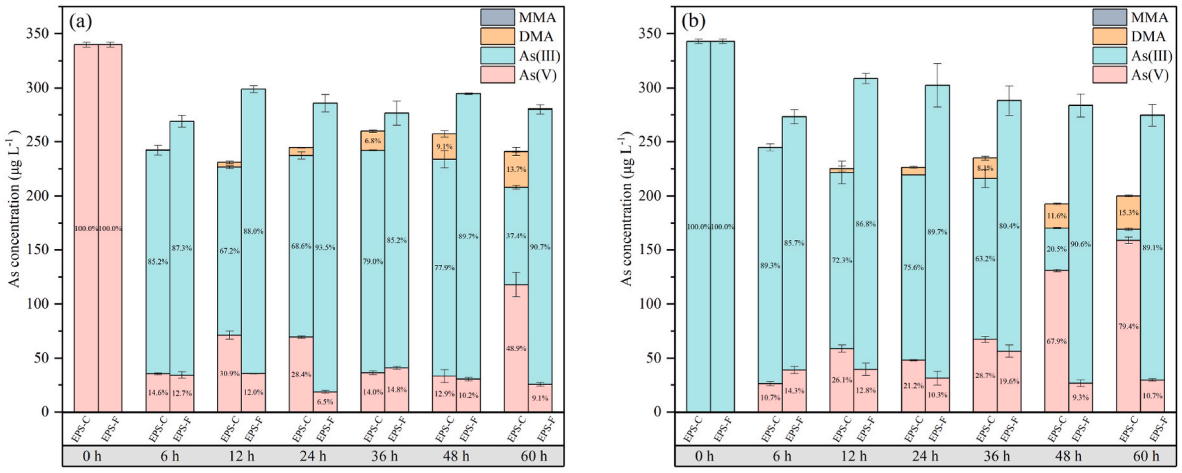

Fig. 6. As species in growth medium with EPS-C or EPS-F biofilms treated with As(V) (a) or As(III) (b). Data are expressed as means ± standard errors (n = 3).

2020). The decrease of As_{up} in biofilms after removing EPS in this study may be mainly due to the better adaptive capacity of the algal-bacterial complex as biofilm, which can quickly convert As in the biofilm into As (III) and excrete it after losing the barrier of EPS. This point is further confirmed by the higher proportion of As(III) both in the culture medium and the As_{ad} in the biofilm. The amount of As_{ad} in EPS-C biofilms was higher under As(III) exposure than As(V) exposure, which suggests differences in adsorption capacity under different As exposure (Ferreira et al., 2018). However, the difference induced by varied As species exposure was eliminated after EPS removal. Although the As_{up} decreased after EPS removal, the response pattern between As(III) and As(V) was indiscriminate, which was inconsistent with previous studies conducted in microalgae (Naveed et al., 2020b; Zhang et al., 2020), indicating the uptake pathway might be different in biofilms compared with microalgae.

The important role of biofilms on the As accumulation is further supported by FTIR spectroscopy. The intact biofilms surface is primarily made up of functional groups found in protein and polysaccharide molecules (Fig. 2a), including –OH, –NH, $–CH_2$, $–NH_2$, C═O, C–O, and C–O–C (Leceta et al., 2013; Naveed et al., 2020a, 2020b; Zivanovic et al., 2007). The shift of absorption peak under As(III) or As(V) exposure indicates that the functional groups interact with As are –OH, –NH and -C-O-C in EPS-C biofilms, which is consistent with other research (Zhou et al., 2020; Zhu et al., 2018; Zhang et al., 2020; Wu et al., 2023). Since the main components of the cell wall of algae in the biofilm are polysaccharides and proteins, the functional groups remained the same as those on the intact biofilm after EPS removal. ,The absorption peaks of functional groups in EPS-F biofilms did not shift under As exposure, indicating that the interaction between these functional groups with As was limited after the removal of EPS.

4.2. Arsenic speciation changes in biofilms

In all treatment groups, the four As species (As(V), As(III), MMA(V) and DMA(V)) constitute of the As_{up} in the biofilms (Fig. 4), indicating that there are multiple As metabolism pathways (e.g. reduction, oxidation, and methylation) in the biofilms. Under As(V) exposure, As(V) was reduced to As(III) as Lopez et al. (2018) had reported, while As(III) oxidation was observed when exposed to As(III). Similar to previously observed in *Chlorella* sp., *Microcystis aeruginosa*, and *Chlorella pyrenoidosa* (Levy et al., 2005; Wang et al., 2013a; Zhang et al., 2020), As_{up} of biofilms was also further methylated into less toxic MMA(V) in this study (Fig. 4). When exposed to As(V) or As(III), though different biotransformation pathways may be involved in biofilms, As_{up} is dominated by As(V) with less toxicity in EPS-C and EPS-F biofilms, which was same as *Dunaliella salina* (Wang et al., 2013b), *Synechocystis* sp. PCC6803 (Yin et al., 2011; Naveed et al., 2020b).

Although no significant change of the As_{up} species between EPS-C and EPS-F biofilms was observed, the speciation change of As_{ad} further manifested the effects of EPS on the As biotransformation in biofilms. As(V) was the dominant species of As_{ad} in EPS-C biofilms after exposure to either As(V) or As(III), with DMA(V) increasing over time (Fig. 5), which is consistent with other studies that the As on the biofilms surface was mainly As(V) (Guo et al., 2020; Lopez et al., 2018; Yang et al., 2011). However, when EPS was removed, As(III) was the dominant species of As_{ad} and without organic As formation, no matter exposed to As(V) or As(III). As_{ad} speciation in EPS-C biofilms is mainly As(V) with low toxicity and exists methylation, while As_{ad} in EPS-F biofilms is primarily As(III) with high mobility, and no organic As was detected. These suggested that biofilms may adopt different protective strategies after the removal of EPS under As exposure. The EPS-C biofilms have a great capability for adsorption, and As would stay for an extended period, so biofilms tends to convert As into As(V) and DMA, which is lesser toxic. In contrast, EPS-F biofilms tend to convert As into As(III) with higher fluidity, then As is excreted from the biofilm in time.

4.3. Arsenic species change in growth medium

Arsenic in the growth medium could be accumulated by biofilms, and then via intracellular and extracellular pathways to be oxidized, reduced, or methylated. After biotransformation, As was partially retained in biofilms, and most of it was released into the medium (Fig. 6).

When exposed to As(V), as reported by Kulp et al. (2004) and Wang et al. (2013a), the As(V) in the medium can be rapidly reduced to As(III) in biofilms, and then efflux to the growth medium, regardless of with or without EPS (Fig. 6). The reduced As(III) can also be methylated to DMA (V) in EPS-C biofilms, and then release to the growth medium. However, in EPS-F biofilms, As(V) in the medium was maintained at a lower level and no methylated As was produced during the experiment, indicating that the presence of EPS promoted oxidation and methylation of As(III) in biofilms, which was confirmed by the results of Naveed et al. (2020a). Similarly, after exposure to As(III), As(III) was oxidized to As(V) gradually or methylated to DMA in EPS-C biofilms, and then efflux to the medium; when EPS was removed, As(V) in the medium remained at a lower level without As methylation during the whole experiment, suggesting that As(III) oxidation and methylation in biofilms are inhibited after EPS removal.

5. Conclusion

This is the first systematic investigation of the effects of EPS on the

iAs bioaccumulation and biotransformation in biofilms. This study demonstrated that the adsorption and uptake of As in biofilms can be remarkably reduced by the removal of EPS. Either As(V) or As(III) exposure, As(V) was the dominant adsorbed As speciation in the EPS-Containing biofilms, and the DMA increased with time. However, As (III) dominates the adsorbed As after the removal of EPS, and no DMA was found. Moreover, the different dynamics of As species in the growth medium between EPS-containing and EPS-free biofilms suggested that the removal of EPS inhibited the oxidation and methylation of As(III) in biofilms. With the widespread distribution of biofilms in nature, this study improves our understanding of the biogeochemistry of As and our exploration of biofilm-based bioreactor techniques for As removal. However, As biotransformation in the biofilm could be affected by many influencing factors, future studies should conducted under different environmental conditions, and more specific mechanisms of As biotransformation in biofilms are needed to further explore the regulation effects of EPS.

CRediT authorship contribution statement

Donghua Qiu: Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Ziyue Yu:** Investigation, Methodology, Writing - review & editing. **Xin Zhang:** Methodology, Writing - review & editing. **Ce Wen:** Investigation, Writing - review & editing. **Changzhou Yan:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

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

The authors do not have permission to share data.

Acknowledgement

This project was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (No. XDA23030203).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.140798.

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