Contrary effects of phytoplankton *Chlorella vulgaris* and its exudates on mercury methylation by iron- and sulfate-reducing bacteria

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ABSTRACT

Mercury (Hg) is a pervasive environmental pollutant and poses serious health concerns as inorganic Hg(II) can be converted to the neurotoxin methylmercury (MeHg), which bioaccumulates and biomagnifies in food webs. Phytoplankton, representing the base of aquatic food webs, can take up Hg(II) and influence MeHg production, but currently little is known about how and to what extent phytoplankton may impact Hg(II) methylation by itself or by the methylating bacteria it harbors. This study investigated whether some species of phytoplankton could produce MeHg and how the live or dead phytoplankton cells and excreted algal organic matter (AOM) impact Hg(II) methylation by several known methylators, including the iron-reducing bacteria (FeRB), *Geobacter anodireducens* SD-1 and *Geobacter sulfurreducens* PCA, and the sulfate-reducing bacterium (SRB) *Desulfovibrio desulfuricans* ND132 (or *Pseudodesulfovibrio mercurii*). Our results indicate that, among the 4 phytoplankton species studied, none were capable of methylating Hg(II). However, the presence of phytoplankton cells (either live or dead) from *Chlorella vulgaris* (CV) generally inhibited Hg(II) methylation by FeRB but substantially enhanced methylation by SRB *D. desulfuricans* ND132. Enhanced methylation was attributed in part to CV-excreted AOM, which increased Hg(II) complexation and methylation by ND132 cells. In contrast, inhibition of methylation by FeRB was attributed to the inability of these bacteria to compete with phytoplankton for Hg(II) binding and uptake. These observations suggest that phytoplankton could play different roles in affecting Hg(II) methylation by the two groups of anaerobic bacteria, FeRB and SRB, and may thus offer new insight into how phytoplankton blooms may modulate MeHg production and bioaccumulation in the aquatic environment.

Key words: Methylmercury; *Geobacter sulfurreducens* PCA; *Desulfovibrio desulfuricans* ND132; Algae *Chlorella vulgaris*

1. INTRODUCTION

Mercury (Hg) is a global environmental pollutant. In particular, the bioaccumulation and biomagnification of the neurotoxin methylmercury (MeHg) in food webs are serious health concerns due to human consumption of contaminated seafoods and rice (Mason et al., 1995; Barkay and Wagner-Dobler, 2005; Driscoll et al., 2013; Rothenberg et al., 2014). MeHg is typically produced in anoxic environments through conversion of inorganic Hg by anaerobic microorganisms possessing the two key genes, *hgcA* and *hgcB* (Parks et al., 2013; Hu et al., 2013b; Podar et al., 2015; Liu et al., 2014; Liu et al., 2018). While the genetic basis of microbial Hg methylation is now well known, many environmental factors, such as the presence or absence of various organic and inorganic materials, could significantly influence the rate and extent of Hg methylation by these microbes (Benoit et al., 2003; Schaefer et al., 2011; Lin et al., 2014; Bravo et al., 2017; Zhao et al., 2017; Schwartz et al., 2019; Yin et al., 2020). Specifically, mercuric Hg(II) complexation with naturally dissolved organic matter (DOM) and thiol compounds has been shown to either increase or decrease Hg(II) methylation, depending on the chemical structure and concentration of the organic ligand, as well as the bacterial strain itself (Schaefer et al., 2011; Lin et al., 2014; Zhao et al., 2017; Liu et al., 2016). For example, many thiol compounds (e.g., cysteine, glutathione, and penicillamine) and DOM have been shown to increase Hg(II) methylation by sulfate-reducing bacteria (SRB) (Schaefer et al., 2011; Lin et al., 2014; Zhao et al., 2017; Liu et al., 2016), such as *Desulfovibrio desulfuricans* ND132 (now reclassified as *Pseudodesulfovibrio mercurii*) (Gilmour et al., 2021). On the other hand, only certain thiol compounds (e.g., cysteine) can increase Hg(II) methylation by iron-reducing bacteria (FeRB), such as *Geobacter sulfurreducens* PCA, whereas others (e.g., glutathione,

penicillamine, and DOM) inhibit this activity (Lin et al., 2014; Zhao et al., 2017; Schaefer et al., 2011). Similarly, Hg(II) uptake by microbes could be affected by the presence or absence of different organic ligands or DOM from different sources (Lin et al., 2014; Zhao et al., 2017; Mangal et al., 2019), further highlighting the importance of these different organic ligands in MeHg production in the environment.

Phytoplankton, consisting of mainly phototrophic prokaryotic and eukaryotic algae and cyanobacteria, contributes to about 50% of global primary productivity and represents the base of aquatic food webs (Field et al., 1998; Wang et al., 2020b). Due to its ability to adapt rapidly to environmental changes and robust growth rates, phytoplankton is often considered as the key player in toxic algal bloom and Hg geochemical cycling in aquatic environments (Bianchi et al., 2000; Dokulil and Teubner, 2000). Massive growth of phytoplankton can influence the transfer, transformation, and bioavailability of Hg(II) due to its interactions and strong binding affinities with algal-derived organic matter (AOM) and/or algal cells themselves. At the base of food webs, phytoplankton is also known to adsorb and take up Hg(II) and MeHg (Mason et al., 1995; Mason et al., 1996). While microbial methylation of Hg(II) is now recognized as the main source of MeHg in the aquatic environment, several studies have implicated possible methylation of Hg(II) by phytoplankton and some freshwater cyanobacteria (Deng et al., 2013; Lazaro et al., 2013; Franco et al., 2018). However, the mechanism of MeHg production was unclear and thought to be associated with periphyton (Lazaro et al., 2013; Franco et al., 2018). Indeed, other studies argued that the conversion of inorganic Hg(II) to MeHg or enhanced Hg(II) methylation occurred in periphyton, as the structured periphyton biofilms can host various Hg(II) methylators (Olsen et al., 2016; Schwartz et al., 2019; Bae et al., 2019). This enhanced Hg(II) methylation is generally

attributed to phytoplankton AOM or DOM, thiol compounds, and nutrients in general, which fuels microbial methylation, such as in lake and boreal pond sediments (Bravo et al., 2017; Lei et al., 2019; Ortega et al., 2018; Bouchet et al., 2018). However, it is also recognized that the periphyton structure is critically important in controlling the development of anoxic zones required for Hg(II) methylation by anaerobic bacteria, such as SRB, FeRB, or methanogens. Disruption of the structured periphyton could greatly diminish or inhibit Hg(II) methylation (Olsen et al., 2016), as the growth of phytoplankton requires an oxic environment and can release an ample amount of oxygen. A recent laboratory culture study with *Chlorella pyrenoidosa* and FeRB *G. sulfurreducens* PCA suggested possible symbiotic algae-bacteria interactions leading to substantially enhanced Hg(II) methylation (Zhao et al., 2021). This result, however, contradicted with others, who found that the presence of either live or dead phytoplankton cells, such as *Skeletonema costatum*, strongly inhibited Hg(II) methylation by *G. sulfurreducens* PCA or *G. metallireducens* GS-15 (Ding et al., 2019a; Ding et al., 2019b). Considering global environmental changes such as the increased frequency, magnitude, and duration of phytoplankton blooms (Gregoire and Poulain, 2014; Gregoire and Poulain, 2018), it is therefore important that we reevaluate whether or not phytoplankton can methylate Hg(II); if not, how the presence of phytoplankton cells and AOM may influence MeHg production by the two major groups of anaerobic methylating bacteria, FeRB and SRB, in the environment.

The present study was carried out to determine Hg(II) methylation potential by four selected phytoplankton species, including *Chlorella vulgaris* (CV), *Nostoc* sp. PCC7120, *Microcystis* sp. PCC7806, and *Synechocystis* sp. PCC6803. These phytoplankton species represent those frequently observed in natural aquatic environments (Guiry, 2012; Malviya et

al., 2016) and are commonly used in biogeochemical transformation studies of Hg(II) (Le Faucheur et al., 2014; Gregoire and Poulain, 2014). Subsequently, the effects of the presence of phytoplankton cells and AOM (from CV) on Hg(II) methylation were examined using several known strains of microbial methylators, including FeRB *G. anodireducens* SD-1 and *G. sulfurreducens* PCA, and a SRB strain, *D. desulfuricans* ND132 (Parks et al., 2013; Hu et al., 2013b; Gilmour et al., 2013; Liu et al., 2016). These methylation assays were performed in both phosphate buffered saline (PBS) and the simulated freshwater (SW) to provide insight into the role of phytoplankton in Hg(II) biogeochemical transformations in aquatic ecosystems.

2. MATERIALS AND METHODS

2.1. Phytoplankton and bacteria cultures and preparations

Chlorella vulgaris (CV) UTEX 395, a freshwater green alga, was obtained from the Culture Collection of Algae, University of Texas at Austin, United States, whereas *Nostoc* sp. PCC7120, *Microcystis* sp. PCC7806, and *Synechocystis* sp. PCC6803 (also known as cyanobacteria or blue-green algae) were obtained from the Freshwater Algae Culture Collection (FACC) at the Institute of Hydrobiology, China. All these algae and cyanobacteria cultures were grown at 23 °C in BG-11 medium (obtained from the Culture Collection at University of Texas, Austin), whose chemical composition is available at https://utex.org/products/bg-11-medium. The incubation was conducted with a daily cycle of 8 h dark and 16 h light under cool white fluorescent light at the light intensity of ~ 50 µmol photons m^{-2} s^{-1}. Cells were harvested at the mid-exponential phase (after ~ 20 days) and washed and resuspended in BG-11 solution before Hg(II) methylation assays. A portion of the washed cells (50 mL) was heated to 80 °C for 60 min to inactivate the algal cells

(heat-killed cells) (Le Faucheur et al., 2014; Gregoire and Poulain, 2014), as well as microbes they may harbor (An et al., 2019; Schaefer and Morel, 2009; Schaefer et al., 2011). Sterilization at higher temperatures and pressures was not used in our experiments to avoid potential cell lysis. These heat-killed cells were subsequently used in the control experiments.

Cultures of *Geobacter anodireducens* SD-1, *Geobacter sulfurreducens* PCA, and *Desulfovibrio desulfuricans* ND132 (or *Pseudodesulfovibrio mercurii*) were grown anaerobically at 30 °C, as previously described (Hu et al., 2013b; Liu et al., 2018; An et al., 2019; Schaefer et al., 2011; Gilmour et al., 2021). These bacterial strains were selected, as they are commonly used and studied for Hg(II) methylation assays (Hu et al., 2013b; Liu et al., 2018; An et al., 2019; Schaefer et al., 2011; Gilmour et al., 2011). While *D. desulfuricans* ND132 was isolated from estuarine environments, it was used as a model SRB due to its exceptionally high rates of Hg-methylation (Gilmour et al., 2011; Hu et al., 2013b; An et al., 2019). Experimentally, *G. anodireducens* SD-1 was grown in an acetate-ferric citrate medium, containing 20 mM ferric citrate and 10 mM sodium acetate as the respective electron acceptor and donor (Liu et al., 2018). *G. sulfurreducens* PCA was cultured in nutrient broth basal salts containing 40 mM fumarate and 20 mM acetate, whereas *Desulfovibrio desulfuricans* ND132 was grown in a modified MOY medium containing 40 mM fumarate and 40 mM pyruvate as the respective electron acceptor and donor (Hu et al., 2013b; Smith et al., 2015; An et al., 2019). No sulfate or thiol compounds were added to minimize potential production of sulfide or HgS in the system. All cells were harvested at the mid-exponential growth phase by centrifugation (1500× *g* for 10 min at room temperature), and washed three times by repeated centrifugation and resuspension in either a deoxygenated phosphate buffer saline (PBS) or simulated freshwater (SW) (Dong et

al., 2010; Luo et al., 2017). The PBS consisted of 0.14 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4 at pH 7.4, as commonly used as a non-growth medium in microbial methylation assays (Hu et al., 2013b; Liu et al., 2018; An et al., 2019; Yin et al., 2020). The SW consisted of 1 mM $NaHCO_3$, 0.5 mM NaCl, and 0.3 mM Na_2SO_4 at pH 8.0, and was used to mimic Hg(II) methylation in natural freshwater from the contaminated East Fork Poplar Creek (EFPC) water in Oak Ridge, Tennessee, as previously described (Dong et al., 2010; Luo et al., 2017).

2.2. Hg(II) methylation assays

Hg(II) methylation by four phytoplankton species (either live or heat-killed) was examined first directly in BG-11 culture medium under oxic conditions at the cell concentration of 1×10^6 cells/mL. Hg(II) was added from a working solution (freshly prepared daily from a Hg(II) stock solution of 50 μM as $HgCl_2$), and the final Hg(II) concentration was 25 nM, or otherwise specified. A relatively high Hg(II) concentration was used to represent that observed at contamination sites (Brooks and Southworth, 2011; Miller et al., 2013) and to facilitate laboratory incubation and analysis, as commonly used in Hg biogeochemical transformation studies (Hu et al., 2013b; Liu et al., 2016; Thomas et al., 2020; Zhao et al., 2021). All vials were immediately sealed with PTFE/silicone screw caps and kept in the dark on an orbital shaker. At selected time intervals (4, 24, or 72 h), a set of sample vials (duplicate or triplicate) was taken and immediately analyzed for purgeable elemental Hg(0) due to potential reduction of Hg(II) by phytoplankton. After purging, a sub-sample aliquot was analyzed for total MeHg concentrations using previously established procedures (Liu et al., 2018; An et al., 2019; Yin et al., 2020). The remaining sample in the vial was oxidized in BrCl (5%, v/v) at 4 °C overnight or longer and used for total Hg analysis (described below).

The initial methylation assays showed negligible amounts of MeHg production by any of the phytoplankton species. We therefore subsequently selected CV, one of the most prevalent green algae in freshwater (Safi et al., 2014), as a representative phytoplankton species to examine how the presence of phytoplankton cells might affect MeHg production by several well-studied Hg(II)-methylating bacteria (*G. anodireducens* SD-1, *G. sulfurreducens* PCA, and *D. desulfuricans* ND132) in PBS or SW. Initial assays were performed with *G. anodireducens* SD-1 and *D. desulfuricans* ND132 in PBS in 4 mL amber glass vials (National Scientific). CV cells were also washed with PBS prior to being mixed with washed cells of either *G. anodireducens* SD-1 or *D. desulfuricans* ND132. Hg(II) was added to a final concentration of 25 nM, or otherwise specified. Relatively high Hg(II) concentrations were used here to represent those typically found at contamination sites (e.g., (Gu et al., 2014; Miller et al., 2009) and to facilitate laboratory analysis of Hg, as previously described (An et al., 2019; Hu et al., 2013b; Liu et al., 2016). The final added CV cells and methylating bacterial concentrations were 1×10^6 cells/mL and 1×10^8 cells/mL, respectively. To confirm the effects of phytoplankton cells and AOM on Hg(II) methylation, separate experiments were performed independently (by the third author) in SW to simulate natural freshwater conditions so that we can compare and assess the roles of phytoplankton on MeHg production by different methylating bacteria in the environment. In these experiments, CV cells or cell filtrates were preequilibrated with Hg(II) (25 nM) for 6 h in the dark, following which the washed cells of *G. sulfurreducens* PCA and *D. desulfuricans* ND132 were added to initiate the methylation under anaerobic conditions. Note that cell filtrates were obtained after incubating CV cells for 24 h in SW and then filtering through a 0.2-µm PTFE syringe filter to remove CV cells. The final dissolved organic carbon (DOC) concentrations in the live and dead CV

cell filtrate experiments were 0.22±0.05 and 0.73±0.07 mg/L, respectively. At the predetermined time intervals, samples were taken and analyzed for purgeable Hg(0), MeHg and total Hg concentrations, as described in Section 2.3. Selected samples were also analyzed for Hg(II) and MeHg species distributions (i.e., adsorption, uptake, or soluble) during methylation assays using previously established methods (Parks et al., 2013; Hu et al., 2013b; Liu et al., 2018; An et al., 2019; Yin et al., 2020) (see details in Supplementary Materials).

2.3. Hg(0), MeHg, and total Hg analyses

Hg(0) was determined by directly purging and analyzing the purgeable Hg(0) in vials using a cold vapor atomic absorption spectrometer (CVAAS) (RA-915+, Ohio Lumex Co., Cleveland, OH), as previously described (Liu et al., 2018; An et al., 2019; Yin et al., 2020). Similarly, total Hg (THg) preserved in BrCl (5%, v/v) at 4 °C was analyzed via $SnCl_2$ reduction and detection by CVAAS. The detection limit was about 10 pg Hg (Lu et al., 2019). MeHg concentrations in the assay media were analyzed by taking a sample aliquot (50–100 µL, depending on MeHg concentrations) to a distillation container prefilled with 40 mL Milli-Q water and then placed on a Tekran (Model 2750) distillation apparatus (EPA Method 1630) (Parks et al., 2013; Hu et al., 2013b; Liu et al., 2018; An et al., 2019; Yin et al., 2020). For every sample analysis, a known amount of ^{200}Hg-isotope enriched $Me^{200}Hg$ was also added as an internal standard for correcting potential matrix interferences during distillation and analysis, as previously described (Parks et al., 2013; Zhang et al., 2021). After distillation, the amount of MeHg was determined by ethylation, purging and trapping onto a Tenax trap, followed by thermal desorption and separation on an automated MERX-M system (Brooks Rand), and finally quantified by an inductively coupled plasma mass spectrometer (ICP-MS) (Elan DRC-e, PerkinElmer) (An et al., 2019; Yin et al., 2020; Zhang et al.,

2021). The detection limit was about 1.5 pg Hg (Wang et al., 2020a; Zhang et al., 2021). Additionally, reference standards of Hg(II) and MeHg (from Brooks Rand) were used for calibrations, and randomly selected samples were also spiked with a known amount of Hg(II) or MeHg standards and run with every batch of samples for quality assurance and quality controls (QA/QC). The recovery of spiked Hg or MeHg standards was usually within 100±10%. Control experiments with heat-killed phytoplankton cells, cell filtrates (AOM), PBS and SW (without added bacterial cells) were also performed, and samples analyzed in the same manner. Differences in MeHg production between the untreated and phytoplankton- or AOM-treated samples were tested by the two-sample t-test, as previously described (Zhang et al., 2022).

3. RESULTS AND DISCUSSION

3.1. Hg(II) methylation by phytoplankton

The role of phytoplankton in Hg(II) methylation was evaluated first in the presence of both live and dead cells of *Chlorella vulgaris* (CV), *Nostoc* sp. PCC7120, *Microcystis* sp. PCC7806 and *Synechocystis* sp. PCC6803 in BG-11 medium in the dark at room temperature (~23 °C). Results (Fig. 1a) indicate that none of these phytoplankton cultures (dead or live) were capable of methylating Hg(II) in 72 h, and the measured MeHg concentrations were generally below the method detection limit in all samples. More detailed studies were subsequently performed with CV cells as the representative phytoplankton as commonly used in eco-toxicity studies (Deng et al., 2009; Safi et al., 2014). We also found negligible production of MeHg at varying Hg(II) concentrations (up to 100 nM) and at different time points (up to 72 h) (Fig. 1b). No significant differences were observed in experiments with either live or dead CV cells, again indicating that

the phytoplankton could not produce MeHg. However, both live and dead CV cells could reduce small amounts of Hg(II) to purgeable elemental Hg(0) (2–10 %), particularly within the first 24 h (Fig. S1). Hg(0) production then decreased after 72 h with the live CV cells (Fig. S1), which could be explained by potential reoxidation of Hg(0), as previously observed with bacterial cells and DOM (Gu et al., 2011; Hu et al., 2013b; Lin et al., 2014).

The observation of no MeHg production by phytoplankton cannot be attributed to the toxic effect of Hg(II), as evidenced by no significance differences observed in the growth of CV cells treated with 25, 50, or 100 nM Hg(II) up to 96 h (Fig. S2). Cell numbers (OD_{680}) decreased slightly after 96 h due to depletion of nutrients over time, which is consistent with typical growth curves of green algae (Deng et al., 2009; Peng et al., 2017). Our results are consistent with several recent studies, which have shown negligible Hg(II) methylation by phytoplankton (Bravo et al., 2014; Ding et al., 2019a; Ding et al., 2019b; Cossart et al., 2021). However, a few studies reported possible methylation of Hg(II) by phytoplankton, such as the diatom *Phaeodatylum tricornutum* and freshwater cyanobacteria (Deng et al., 2013; Lazaro et al., 2013; Franco et al., 2018), but the mechanism was unclear (Deng et al., 2013). The study was unable to pinpoint whether MeHg was truly produced by phytoplankton or due to non-axenic experimental conditions with potential bacteria contamination, which may have contributed to Hg(II) methylation. This is mainly because Hg(II) methylation is carried out by certain anaerobic bacteria, such as SRB, FeRB, and methanogens, which require an anoxic environment for growth and methylation to occur (Parks et al., 2013; Hu et al., 2013b; Gilmour et al., 2013). Contrarily, the growth of phytoplankton generally requires an oxic environment, which would inhibit microbial methylation.

3.2. Effects of phytoplankton cells on Hg(II) methylation by FeRB and SRB

While phytoplankton itself cannot methylate Hg(II) (Fig. 1), phytoplankton cells (either live or dead) and their exudates could provide a good environment and growth conditions for methylating bacteria, such as those found in periphyton biofilms (Correia et al., 2012; Leclerc et al., 2015; Olsen et al., 2016; Xiang et al., 2021). As such, the influence of phytoplankton on MeHg production could be especially important during algal bloom when massive amounts of phytoplankton cells are produced. We therefore evaluated how the presence of CV cells and their exudates AOM could modulate MeHg production by several known methylating bacteria, including FeRB *G. anodireducens* SD-1 and *G. sulfurreducens* PCA, and SRB *D. desulfuricans* ND132 (Parks et al., 2013; Hu et al., 2013b; Gilmour et al., 2013; Liu et al., 2016). We determined MeHg production first by reacting Hg(II) (25 nM) with a mixed suspension of CV (10^6 cells mL^{-1}) and SD-1 or ND132 cells (10^8 cells mL^{-1}) in PBS in the dark. We found that the presence of CV cells strongly inhibited Hg(II) methylation by *G. anodireducens* SD-1 but slightly enhanced Hg(II) methylation by *D. desulfuricans* ND132 (Fig. 2). Consistently lower amounts of MeHg (~30–40%) were produced by *G. anodireducens* SD-1 in the presence of CV cells than those without addition of CV cells (Figure 2a). Conversely, MeHg production increased slightly (5–20%) when CV cells were added to the *D. desulfuricans* ND132 culture, compared to those without addition of CV cells (Fig. 2b). Interestingly, we also found that the added inorganic Hg(II) was mostly taken up or adsorbed by the bacteria and/or CV cells in 24 h (Fig. S3). *D. desulfuricans* ND132 cells were particularly effective in taking up Hg(II) rapidly, with >80% of Hg(II) internalized within 4 h (Fig. S3c) as compared to <10% taken up by either *G. anodireducens* SD-1 or CV cells (Figs. S3a,b). Large percentages of Hg(II) were either left in solution or reduced to elemental Hg(0) by *G.*

anodireducens SD-1 (Fig. S3a), as previously observed (Liu et al., 2018). The presence of CV cells decreased Hg(II) reduction by *G. anodireducens* SD-1, but most Hg(II) still remained in solution in the first 4 h (Fig. S3b). This rapid and efficient uptake of Hg(II) by *D. desulfuricans* ND132 may partially explain why more MeHg was produced by ND132 than SD-1 cells either in the presence or absence of CV cells. Only after 24 h, most Hg(II) was taken up by either *G. anodireducens* SD-1 or CV cells, although the DMPS washing technique could not discern who took up more Hg(II) than the other. Different from inorganic Hg(II) sorption and uptake, however, the produced MeHg was mostly adsorbed or remained on *G. anodireducens* SD-1 cells (Fig. S4a) but was mostly exported to the solution phase by *D. desulfuricans* ND132 (Fig. S4c), consistent with previous observations (Lin et al., 2015; Schaefer et al., 2011). These results therefore demonstrate distinctly different effects of CV cells on Hg(II) uptake and methylation by FeRB (*G. anodireducens* SD-1) and SRB (*D. desulfuricans* ND132).

To further illustrate the effects of CV cells on Hg(II) methylation by FeRB and SRB, we compared MeHg production by another FeRB strain, *G. sulfurreducens* PCA, and *D. desulfuricans* ND132 in simulated freshwater (SW), rather than PBS, to mimic the natural aquatic environment where MeHg is produced. In these experiments, the washed CV cells (either live or dead) were preequilibrated with Hg(II) for 6 h to simulate the environment where reactions between Hg(II) and phytoplankton cells occur before methylation. Additionally, the reduced Hg(0) (~ 8 %) by CV cells was purged out prior to the addition of FeRB or SRB bacteria for methylation. Our results again indicate that the presence of either live or dead CV cells strongly inhibited Hg(II) methylation by FeRB *G. sulfurreducens* PCA (Fig. 3a). Compared to samples without addition of CV cells (either dead or live), MeHg production in the presence of CV cells decreased 40–60% by

314 *G. sulfurreducens* PCA. Similar effects were observed with dead CV cells on Hg(II) methylation by
315 *G. sulfurreducens* PCA.

316 Interestingly, however, the presence of CV cells (either live or dead) substantially
317 increased MeHg production by SRB *D. desulfuricans* ND132 (Fig. 3b), much higher than those
318 observed in the absence of CV cells (Figure 2b). Without the addition of CV cells, MeHg
319 production was only about 0.4 nM MeHg produced in 72 h in SW, as compared to about 5.9 and
320 7.7 nM MeHg produced in the presence of the dead and live CV cells, respectively (Fig. 3b). The
321 amount of MeHg produced in SW (without CV cells, Fig. 3b) was also lower than that observed in
322 PBS (Fig. 2b). The result suggests that MeHg production by *D. desulfuricans* ND132 in SW alone
323 was inhibited due to an unfavorable SW water chemistry or an unfavorable methylation
324 environment by *D. desulfuricans* ND132, as previously observed (Gilmour et al., 2011; Luo et al.,
325 2017). The observation may be explained by the fact that *D. desulfuricans* ND132 is a salt-tolerant
326 mesophile, and the optimal NaCl concentration for growth is about 2% (wt/vol). A lower osmotic
327 pressure and a higher pH likely suppressed its activity in taking up and methylating Hg(II) in SW
328 than in PBS. Similarly, lower amounts of MeHg production were observed during growth in
329 sulfidogenic than nonsulfidogenic conditions (Gilmour et al., 2011). The presence of relatively
330 high concentrations of sulfate and sulfide was also found to inhibit MeHg production in
331 contaminated sediments (Hammerschmidt et al., 2008). Therefore, the presence of relatively low
332 chloride but high concentrations of sulfate in SW likely caused inhibition of Hg(II) methylation by
333 *D. desulfuricans* ND132 in SW, compared to PBS. On the other hand, the addition of CV cells could
334 have provided microenvironments, where the presence of abundant nutrients and low-
335 molecular-weight thiol compounds favored microbial activity and methylation (Leclerc et al.,

2015; Bravo et al., 2017). As noted earlier, these thiol compounds can form strong complexes with Hg(II) and enhance Hg(II) methylation by *D. desulfuricans* ND132, but some may inhibit Hg(II) methylation by *G. sulfurreducens* PCA (Schaefer et al., 2011; Lin et al., 2014; Zhao et al., 2017; Liu et al., 2016). Therefore, the growth and activity of *D. desulfuricans* ND132 in the presence of CV cells could be stimulated, leading to increased MeHg production (Fig. 3b). This finding agrees well with a previous report, in which phytoplankton-derived organic compounds were found to greatly enhance Hg(II) methylation rates in boreal lake sediments through an overall increased bacterial activity (Bravo et al., 2017).

3.3. Algal exudate (AOM) and Hg(II) methylation by FeRB and SRB

Algal bloom not only produces massive amounts of phytoplankton cells (dead or live) but also excretes substantial amounts of exudates or soluble AOM as cells become lysed (Leclerc et al., 2015; Bravo et al., 2017; Mangal et al., 2019). It is unclear, however, whether Hg(II) methylation by *G. sulfurreducens* PCA and *D. desulfuricans* ND132 could be enhanced or inhibited in the presence of AOM in water. Our results (Fig. 4a) indicate that AOM isolated from either live or dead CV cells had limited effects on Hg(II) methylation by FeRB *G. sulfurreducens* PCA; the amounts of MeHg produced in the presence of AOM were similar or only slightly higher than that in the control (without AOM). Conversely, MeHg production by *D. desulfuricans* ND132 was markedly increased in the presence of AOM (Fig. 4b), albeit lower than that observed in the presence of live or dead CV cells (Fig. 3b). These results illustrate that the effect of phytoplankton AOM on Hg(II) methylation by FeRB *G. sulfurreducens* PCA also differed from that of SRB *D. desulfuricans* ND132. Compared to that in the presence of phytoplankton cells (Fig. 3b), AOM was clearly responsible in part for the observed enhancement effect of CV cells on Hg(II)

methylation by *D. desulfuricans* ND132. The result again supports the notion that CV cells and AOM provided microenvironments and a wealth of nutrients and thiols for Hg(II) methylation by SRB *D. desulfuricans* ND132.

Collectively this research indicates that all phytoplankton used in this study (i.e., CV, *Microcystis* sp. PCC7806, *Nostoc* sp. PCC7120, and *Synechocystis* sp. PCC6803) were incapable of producing MeHg by themselves, consistent with previous studies (Chen et al., 2014; Leclerc et al., 2015; Bravo et al., 2014; Ding et al., 2019b). However, phytoplankton cells and excreted AOM could play different roles in affecting Hg(II) methylation by the two groups of anaerobic bacteria, FeRB and SRB. The presence of either live or dead cells of phytoplankton inhibits Hg(II) methylation by FeRB, *G. anodireducens* SD-1 and *G. sulfurreducens* PCA, but enhances Hg(II) methylation by SRB *D. desulfuricans* ND132. These contrasting effects of phytoplankton on Hg(II) methylation by FeRB and SRB may be partially explained by the ability of these methylating bacteria to compete with phytoplankton cells or AOM for Hg(II) binding and uptake. Hg(II) was taken up more rapidly and efficiently by SRB *D. desulfuricans* ND132 than FeRB either in the presence or absence of phytoplankton cells. As such, the number of thiol functional groups (or the binding sites) and their structural arrangements on bacterial cell surfaces may be critical in determining cell Hg(II) uptake, transport, and ultimately MeHg production (Zhao et al., 2017; Yin et al., 2020). *D. desulfuricans* ND132 cells contain a much higher thiol content (~ 1.9×10^7 thiols/cell) than that of *G. sulfurreducens* PCA cells (~ 2.2×10^4 thiols/cell) (Zhao et al., 2017; Wang et al., 2016). As a result, *D. desulfuricans* ND132 cells are expected to be more competitive than *G. sulfurreducens* PCA cells in taking up Hg(II). This conclusion is supported by the observation that nearly 94% of Hg(II) was rapidly taken up or adsorbed by *D. desulfuricans* ND132 cells in 4 h,

as compared to only about 16% of Hg(II) adsorbed or taken up by *G. anodireducens* SD-1 cells within the same time period (Fig. S3). Clearly, stronger sorption and uptake of Hg(II) by *D. desulfuricans* ND132 cells could result in more MeHg production than that of *G. anodireducens* SD-1 or *G. sulfurreducens* PCA. Other studies also reported that the structural arrangements of thiols on bacterial cell envelopes may differ significantly (Yu and Fein, 2017; Thomas et al., 2020) and could thus affect Hg(II) uptake and methylation, depending on bacterial species, nutrient availability and concentrations. Therefore, differences in cellular Hg(II) binding and coordination environments may be ultimately responsible for SRB *D. desulfuricans* ND132 cells being more effective than FeRB *G. sulfurreducens* PCA in Hg(II) uptake and methylation.

The effects of AOM on Hg(II) methylation were also bacteria-strain dependent (Fig. 4), consistent with previously observed effects of a terrestrial DOM on Hg(II) methylation by *G. sulfurreducens* PCA and *D. desulfuricans* ND132 (Zhao et al., 2017). DOM was reported to inhibit Hg(II) methylation by *G. sulfurreducens* PCA but enhance methylation by *D. desulfuricans* ND132 with increasing DOM concentrations. DOM or thiol functional groups on DOM are known to bind Hg(II) strongly and form exceptionally strong complexes with Hg(II) (Gu et al., 2011; Haitzer et al., 2002; Liang et al., 2019; Miller et al., 2009), and could thus compete with microbial cells for Hg(II) uptake and methylation (Zhao et al., 2017; Yin et al., 2020). AOM may differ slightly from terrestrial DOM with respect to molecular compositions and chemical structures, as AOM contains more freshly excreted N and C substrates, low-molecular-weight organic acids and thiols (Chen et al., 2014; Leclerc et al., 2015; Bravo et al., 2017; Mangal et al., 2019; Bouchet et al., 2018). These thiol compounds may include, but are not limited to, cysteine, thioglycolic acid, L-cysteine-L-glycine, glutathione, penicillamine, and N-acetylcysteine, most of which have been

shown to enhance Hg(II) methylation by *D. desulfuricans* ND132 (Lin et al., 2015; Liu et al., 2016; Schaefer et al., 2011). However, only certain thiols, such as cysteine and thioglycolic acid, could enhance Hg(II) methylation whereas others inhibit it by FeRB. These results suggest that the thiol-enhanced Hg(II) methylation could not be attributed simply to thiol metabolisms, as several studies reported that HgS clusters and nanoparticles from thiol metabolisms could be utilized for Hg(II) methylation (Zhang et al., 2012; Thomas et al., 2019; Thomas et al., 2020). Overall, these previous studies support the observation why AOM showed little effects on Hg(II) methylation by *G. sulfurreducens* PCA (Fig. 4a), but the presence of phytoplankton cells (either live or dead) always inhibited Hg(II) methylation by FeRB (both *G. anodireducens* SD-1 and *G. sulfurreducens* PCA) (Figs. 2a and 3a). In the latter, Hg(II) could form complexes not only with AOM but also phytoplankton cells, which result in less bioavailable Hg(II) for methylation. Conversely, most thiols and DOM have been shown to greatly enhance MeHg production by *D. desulfuricans* ND132 cells (Schaefer et al., 2011; Graham et al., 2012; Liu et al., 2016; Yin et al., 2020; Zhao et al., 2017). DOM was also shown to enhance Hg(II) methylation by SRB *Desulfobulbus propionicus* 1pr3 (Moreau et al., 2015). These contrary effects of thiols and DOM on microbial methylation also explain why *D. desulfuricans* ND132 is more effective than FeRB *G. sulfurreducens* PCA and *G. anodireducens* SD-1 in methylating Hg(II) in the presence of either live or dead phytoplankton cells or AOM.

We note, however, that our observed inhibitory effects of phytoplankton on Hg(II) methylation by FeRB *G. anodireducens* SD-1 and *G. sulfurreducens* PCA differed from that by Zhao et al. (2021), who reported substantially increased Hg(II) methylation by the same FeRB *G. sulfurreducens* PCA in an alga-bacteria symbiotic culture (i.e., during the growth cycle of both

alga *Chlorella pyrenoidosa* and *G. sulfurreducens* PCA in laboratory). These results and conclusions were debatable, however, since *G. sulfurreducens* PCA is known to be an anaerobe and very sensitive to oxygen (Hu et al., 2013a; Schaefer and Morel, 2009; Schaefer et al., 2011). The growth of *Chlorella pyrenoidosa* would produce an ample amount of oxygen in a planktonic alga-bacteria symbiotic system (Zhao et al., 2021) and would thus inhibit Hg(II) methylation by *G. sulfurreducens* PCA. While it is difficult to pinpoint exact causes of these different observations due to varying experimental and analytical conditions, we note that Hg(II) methylation by Zhao et al. (2021) was performed directly in the algal culture medium at higher algal and Hg(II) concentrations than those used in our study. We used CV cells which were washed and suspended either in the anoxic PBS buffer or simulated freshwater so that the activity and oxygen production by phytoplankton would be minimized, as described in the method section. Our results with two FeRB strains (*G. anodireducens* SD-1 and *G. sulfurreducens* PCA) consistently showed that Hg(II) methylation was inhibited in the presence of either live or dead CV cells (Figs. 2 and 3). Similarly, other studies reported that the presence of either live or dead phytoplankton cells, such as *Skeletonema costatum*, strongly inhibited Hg(II) methylation either by *G. sulfurreducens* PCA or by *G. metallireducens* GS-15 (Ding et al., 2019a; Ding et al., 2019b). One possible explanation of these observed differences is due to the use of the algal culture medium during methylation and potential formation of biofilms and microenvironments in the planktonic cultures used by Zhao et al. (2021). However, disruption of the structured biofilm or periphyton could greatly diminish or inhibit Hg(II) methylation, as previously described (Olsen et al., 2016). Nonetheless, these contrary observations underscore the importance to further validate and

improve our understanding how phytoplankton may influence MeHg production in the environment.

4. CONCLUSIONS

The present study offers additional insight into how and to what extent phytoplankton may impact Hg(II) methylation by itself or by the methylating bacteria it harbors. Although all phytoplankton studied could not methylate Hg(II), both phytoplankton cells and AOM greatly affected microbial methylation which was bacteria-strain specific. For the first time, we report the contrary effects of phytoplankton on Hg(II) methylation by FeRB and SRB (Figs. 2 and 3). The presence of either dead or live CV phytoplankton cells decreased Hg(II) methylation by FeRB *G. anodireducens* SD-1 and *G. sulfurreducens* PCA but enhanced methylation by SRB *D. desulfuricans* ND132. Similarly, AOM showed little effects on Hg(II) methylation by FeRB but substantially increased MeHg production by *D. desulfuricans* ND132, suggesting that phytoplankton-excreted AOM played a key role in modulating microbial methylation. Differences in relative abundances of thiol binding sites on bacterial cell surfaces and cellular Hg(II)-coordination environments between SRB and FeRB were hypothesized to be responsible for them to compete with phytoplankton cells and AOM for Hg(II) binding, uptake, and ultimately for Hg(II) methylation. Overall, these results demonstrate that phytoplankton may play different roles in affecting Hg(II) methylation by the two groups of anaerobic bacteria, FeRB and SRB, thereby controlling MeHg production and bioaccumulation during algal blooms in the aquatic environment.

CRediT authorship contribution statement

Xixiang Yin and Lihong Wang: Investigation, Data curation and analysis, manuscript writing. Xunjun Liang, Jiating Zhao, and Lijie Zhang: Data curation, analysis, manuscript review and editing. Baohua Gu: Supervision and manuscript writing.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi: xxxx.

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Figure Legends

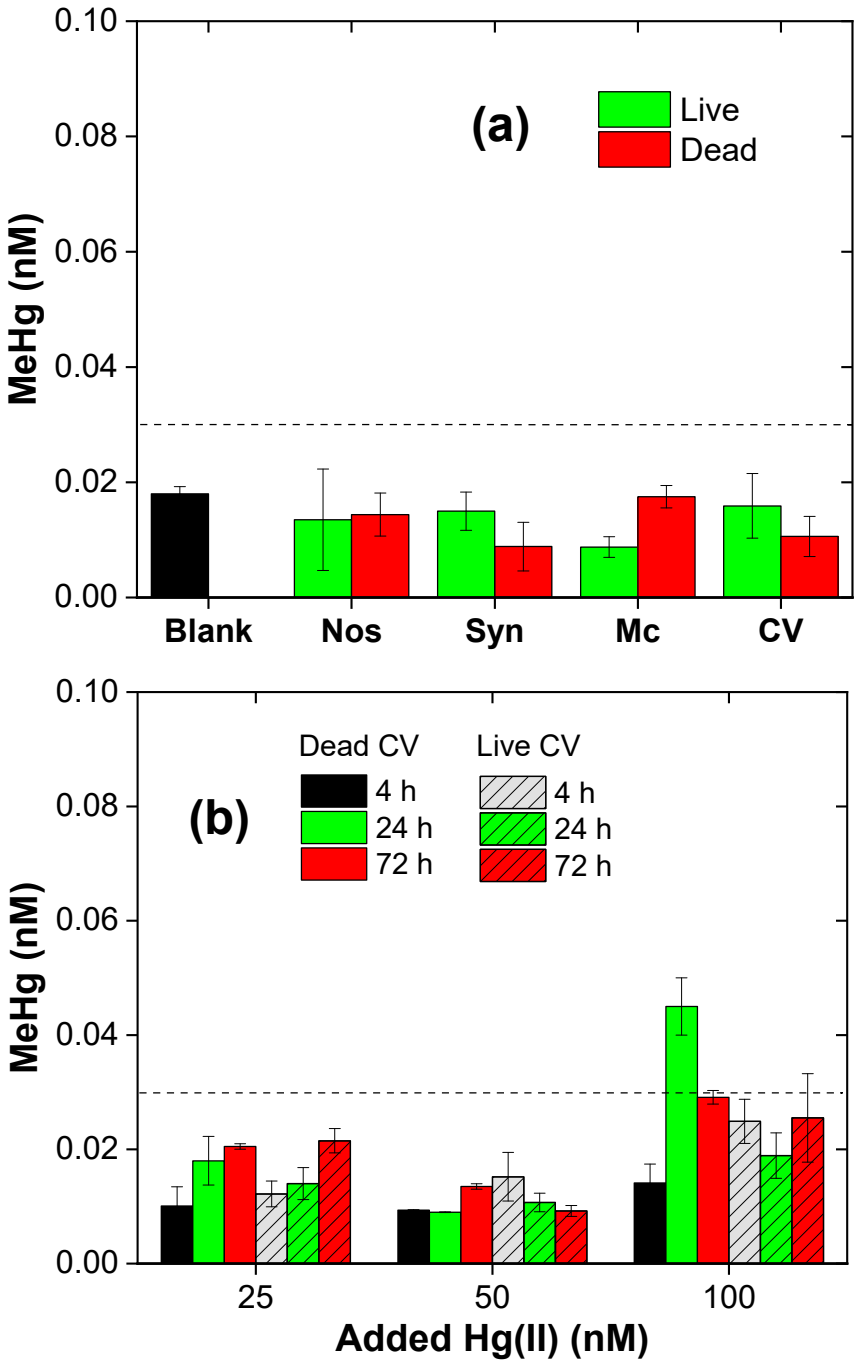
Fig. 1. **(a)** Methylmercury (MeHg) production by both dead and live cells of phytoplankton: *Nostoc* sp. PCC7120 (Nos), *Synechocystis* sp. PCC6803 (Syn), *Microcystis* sp. PCC7806 (Mc), and *Chlorella vulgaris* (CV) in BG11 medium after 72 h. Methylation assays were performed in the dark, and the added Hg(II) concentration was 25 nM. **(b)** MeHg production by dead or live CV cells at varying Hg(II) concentrations (25, 50, and 100 nM). The final OD_{680} of phytoplankton was 0.1 (equivalent to ~1×10^6 cells mL^{-1}). Error bars represent one standard deviation of all replicate samples (*n* =4-6), and dashed lines indicate the method detection limit.

Fig. 2. Methylmercury (MeHg) production by washed cells of **(a)** *G. anodireducens* SD-1 and **(b)** *D. desulfuricans* ND132 in the presence or absence of *Chlorella vulgaris* (CV) cells in PBS. CV (10^6 cells mL^{-1}) and SD-1 or ND132 cells (10^8 cells mL^{-1}) were mixed first, immediately followed by the addition of Hg(II) (25 nM, as $HgCl_2$) for methylation in the dark. Error bars represent one standard deviation of all replicate samples (*n* =2-4). Significant changes following CV treatment are marked as (*) at $P<0.05$ at each timepoint.

Fig. 3. Methylmercury (MeHg) production by washed cells of **(a)** *G. sulfurreducens* PCA and **(b)** *D. desulfuricans* ND132 in the presence or absence of either live (LCV) or dead (DCV) cells of *Chlorella vulgaris* (CV) in simulated freshwater (SW). The added Hg(II) concentration was 25 nM (as $HgCl_2$), which was pre-equilibrated with CV cells (10^6 cells mL^{-1}) for 6 h before the addition of either *G. sulfurreducens* PCA or *D. desulfuricans* ND132 cells (10^8 cells mL^{-1}) for methylation in the dark. Error bars represent one standard deviation from triplicate samples. Significant changes following CV treatment are marked as (*) at $P<0.05$ or (**) at $P<0.01$.

Fig. 4. Methylmercury (MeHg) production by washed cells of **(a)** *G. sulfurreducens* PCA and **(b)** *D. desulfuricans* ND132 in the presence or absence of either the live (LCVF) or dead (DCVF) cell filtrates (or AOM) from *Chlorella vulgaris* (CV) in simulated freshwater. The added Hg(II) concentration was 25 nM (as $HgCl_2$), which was pre-equilibrated with AOM for 6 h before the addition of either *G. sulfurreducens* PCA or *D. desulfuricans* ND132 cells (at 10^8 cells mL^{-1}) for methylation in the dark. Error bars represent one standard deviation from triplicate samples. Significant changes following the treatment are marked as (*) at $P<0.05$ or (**) at $P<0.01$.

Figure 1.


(a)
Live
Dead
MeHg (nM)
0.10
0.08
0.06
0.04
0.02
0.00
Blank
Nos
Syn
Mc
CV
(b)
Dead CV
4 h
24 h
72 h
Live CV
4 h
24 h
72 h
MeHg (nM)
0.10
0.08
0.06
0.04
0.02
0.00
25
50
100
Added Hg(II) (nM)

707 **Figure 2.**

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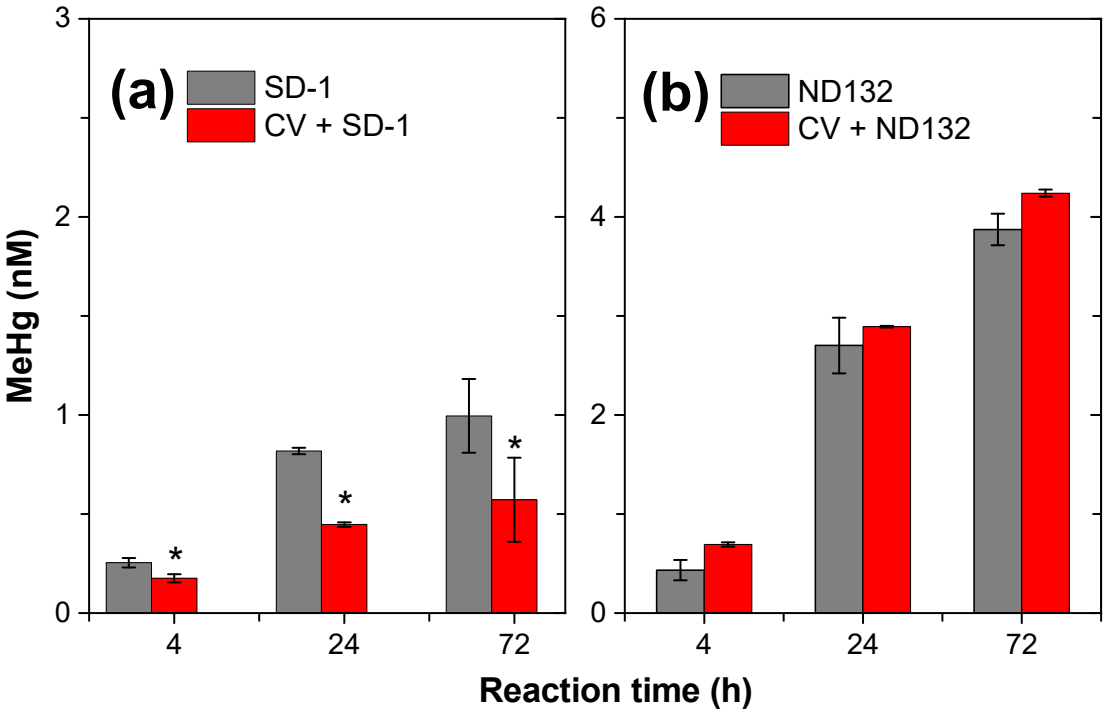

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712 **Figure 3.**

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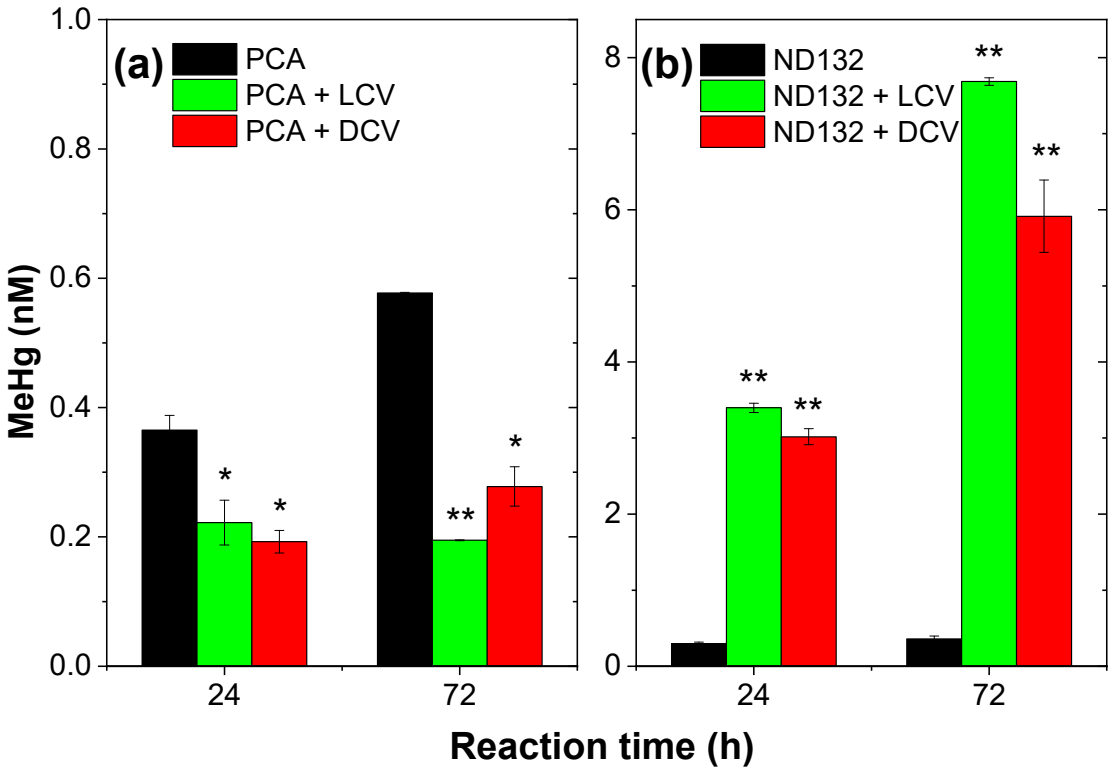

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719 **Figure 4.**

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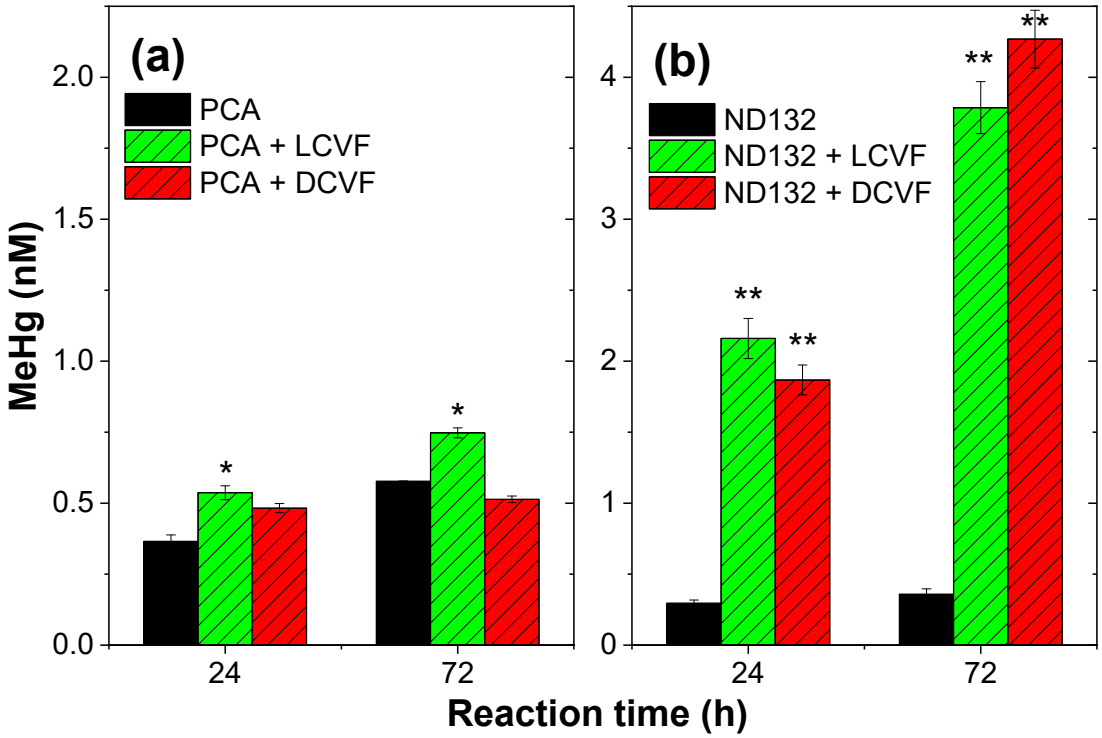

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