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Effects of sulfate reducing bacteria and sulfate concentrations on mercury methylation in freshwater sediments

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ABSTRACT

Methylmercury (MeHg) is the most poisonous form of mercury (Hg) and it enters the human body primarily through consumption of Hg contaminated fish. Sulfate reducing bacteria (SRB) are major producers of MeHg in anoxic sediments. The *dsr*AB gene was isolated from freshwater fish pond sediments. Sequence analyses showed that the SRB in sediments was mainly composed of *Desulfobulbus propionicus* and *Desulfovibrio vulgaris*. The two species of SRB were cultured from freshwater sediments. The addition of inorganic Hg to these freshwater sediments caused an increase in MeHg concentrations at 30 days incubation. MeHg levels were sensitive to sulfate concentrations; a medium sulfate level (0.11 mg/g) produced higher levels than treatments lacking sulfate addition or when amended with 0.55 mg/g. Assessment of bacterial levels by PCR measurements of microbial DNA indicated that the MeHg levels were correlated with cell growth.

1. Introduction

Mercury (Hg) is one of the most common toxic heavy metals, with methylmercury (MeHg) the most poisonous form. MeHg is transferred through food intake and biomagnified in aquatic food webs, which results in high concentrations in fish and other top predators (Orihel et al., 2007). It has been reported that typically 90 to 99% of total mercury (THg) in the environment is associated with sediment, and <1% of the THg accumulates in biota. In contrast 90 to 99% of MeHg accumulates in the biota with 1 to 10% being found in sediment (King et al., 2000). Thus, it is obvious that MeHg is the major Hg species that causes the most concern regarding human exposure.

A number of studies have shown that sulfate reducing bacteria (SRB) are the key mercury-methylating organisms in nature (Achá et al., 2011; Devereux et al., 1996; King et al., 2001). MeHg has been thought to be produced predominantly by SRB in anoxic sediments, although the biochemical pathways that result in Hg methylation remain somewhat unclear. The SRB typically inhabits the anoxic zones of sediment where sulfate is abundant, such as subsurface zones of lakes and anoxic sediments (Gilmour and Henry, 1991). SRB are obligated anaerobes that obtain energy for growth by oxidation of organic substrates. They use sulfate as the terminal electron acceptor and consequently convert sulfate to sulfide (Harmon et al., 2007):

$$SO_4^{2-} + \text{organic matter} \xrightarrow{SRB} HS^{2-} + H_2O + HCO_3^-.$$

Sulfate concentrations of estuarine sediments were found to correlate inversely to Hg methylation activity (Gilmour et al., 1992). The rate of Hg^{2+} methylation in high-sulfate estuarine sediments is far lower than the rate of Hg^{2+} methylation in low-sulfate freshwater sediments. It is mainly caused by precipitation of cinnabar, HgS. The reaction of sulfide with Hg^{2+} to produce insoluble cinnabar decreased the availability of Hg for methylation (Gilmour et al., 1998). In addition, it has been shown that in pure cultures of SRB grown in the absence of sulfate, no MeHg was generated from available inorganic Hg (Pak and Bartha, 1998b). Based on the results of these studies, it can be assumed that Hg methylation is coupled to sulfate reduction catalyzed by SRB.

Previous studies utilized pure cultures of several genera of SRB (such as *Desulfovibrio desulfuricans, Desulfobulbus propionicus, Desulfococcus multivorans, Desulfobacter* sp. strain BG-8, and *Desulfobacterium* sp. strain BG-33, etc.) to determine the Hg methylation potential of the entire SRB population (Gilmour and Henry, 1991; Pak and Bartha, 1998b; King et al., 2000). The community compositions of SRB in marine and freshwater sediments have been investigated using molecular probes that target the 16S rRNA gene or *dsr*AB (dissimilatory sulfite reductase) genes as molecular markers (King et al., 2001; Castro et al., 2002). The freshwater SRB include *Desulfobulbus propionicus* (*D. propionicus*), which is an efficient Hg methylator under sulfate-reducing conditions (King et al., 2000; Stubner, 2004) and *Desulfovibrio vulgaris* (*D. vulgaris*) (Chio et al., 1994; Ekstrom et al., 2003; Zhao et al., 2008). However, the culture of single genus of SRB grown in sediments under different sulfate concentrations has not been reported.

Considering the significant human health risk of oral intake of MeHg via fish consumption, the present study has hence focused on Hg methylation in freshwater fish ponds. Our previous study

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investigated the THg and MeHg concentrations in freshwater fish and associated sediments collected from eighteen freshwater fish ponds around the Pearl River Delta, China. Results indicated that THg concentrations in fish were significantly correlated ($r^2 = 0.60$, $p < 0.05$) with THg levels in sediments. In this paper we report on the identification of the dominant *Desulfobulbus* species in sediments of the fresh water fish ponds. Our studies were extended to determine how authentic cultures of two dominant SRB species affected Hg methylation under different sulfate concentrations in fresh water and to demonstrate the effects of added inorganic Hg on MeHg formation. These studies involved the use of *D. propionicus* and *D vulgaris*.

2. Materials and methods

2.1. Sediment samples

Sediment samples were collected at a depth of 5–20 cm from the freshwater fish pond in Mai Po, Hong Kong. Approximately 60 kg (wet weight) of sediment (moisture content ~30%) was mixed with tap water to obtain a final moisture content of 50%. All sediment samples and tap water were disinfected using an autoclave (TOMY, SS-325i, USA) under high pressure steam at 121 °C for 30 min before using.

2.2. dsrAB gene sequencing

Total DNA was extracted from 0.5 g sediment samples (wet weight) with a FastDNA Spin kit (MP Biomedicals, Illkirch, France). Full length of *dsr*AB genes of SRB were PCR-amplified with the forward primer (5′-(AG)(CG)(GC)CA(CT)TGGAA(AG)CACGG(C/T)GG-3′) and reverse primer (5′- GTGTA(GA)CAGTT(AT)CC(AG)CA-3′) (Michael, 2004; Leloup et al., 2009). *Dsr*B genes of total SRB were PCR-amplified with the forward primer (5′-CAA CAT CGT YCA YAC CCA GGG-3′) and reverse primer (5′-GTG TAG CAG TTA CCG CA-3′) (Dar et al., 2007). Amplification using Phusion High-Fidelity DNA Polymerase (NEB, UK) followed a three-step PCR with 30 s denaturation at 98 °C, 30 s annealing at 50 °C, and 1.5 min elongation at 72 °C. The amplification reactions were performed with a Mastercycler gradient (Eppendorf, Germany). PCR fragments were resolved by electrophoresis in a 1% (w/v) agarose gel to confirm the expected size of the product. The product was sent to Shenzhen Huada Genetics Company (Shenzhen, China) to be sequenced.

2.3. SRB cultures

The SRB cultures used in this study included *Desulfovibrio vulgaris* (ATCC 7757) and *Desulfobulbus propionicus* (ATCC 33891) (ATCC: American Type Culture Collection). They were grown in ATCC medium 1249 using 10 ml tubes with gas-tight caps under anoxic conditions. The pure cultures were used as standards for quantification. SRB cells were spiked into sediment and incubated in the dark at 30 °C.

6 kg of sterile sediments were used as control, and the rest was split into three equal aliquots part, with the first part added with high sulfate concentrations (0.55 mg/g), the second part was added with low sulfate concentrations (0.11 mg/g), and the last part without any addition of sulfate. Mercury ($HgCl_2$) was spiked into the sediments after sulfate (K_2SO_4) was added except the control. The spiked sediment was thoroughly mixed by stirring and then placed into ten glass aquariums ($12 \times 12 \times 24$ cm), each weighing 2 kg. Cells of the authentic SRB strains (10^7/kg) were added into the sediments after 24 h and the level of the water in the aquaria was maintained with tap water to ensure sediment anerobicity.

2.4. Experimental setup

The experimental system was designed to simulate anaerobic freshwater fish pond sediment conditions. Ten experimental conditions were performed in triplicate, with one treatment as the control, the other nine were added with Hg^{2+} ($HgCl_2$). The nine treatments were then divided into three groups including high sulfate (0.55 mg/g), low sulfate (0.11 mg/g) and no sulfate. Each sediment was left without bacterial amendments or was inoculated with either *D. vulgaris* or *D. propionicus*. Sulfate was amended in the form of K_2SO_4 to achieve final concentrations of 0.11 and 0.55 mg/g SO_4^{2-} in sediment. Mercuric chloride was added at Hg^{2+} concentration of 4 mg/kg (dry weight) 24 h after sulfate was added. Due to the possible occurrence of small amounts of sulfate and Hg in the natural sediment, the actual concentrations were determined after each sampling.

2.5. Sample collection

After stabilization period of 24 h, sediment samples were collected daily from day 1 to 8, then on days 10, 12, 14, 17, 21, 25 and 30. All sediment samples were freeze-dried, ground into fine powder and passed through a 0.154 μm sieve.

2.6. Analyses of methylmercury and sulfate

All glassware was soaked in 50% (v/v) HNO_3 for 24 h and rinsed with deionized water before use. The analytical protocol for MeHg was based on the method of Liang et al. (2004). MeHg was extracted from 1 g sediment by 1 ml 25% HNO_3, 1 ml 1 M $CuSO_4$ and 8 ml CH_2Cl_2 in a 50 ml centrifuge tube. The tube was shaken for 30 min, centrifuged at 3000 rpm for 20 min, and then filtered by a phase separation paper, for removing water phase. The organic phase was made up to 40 ml by adding milli Q water to a 50 ml centrifugal tube. The tubes were placed in a water bath at 50 °C, until no visible solvent was left. The remaining liquid was then purged with N_2 to remove excess DCM (60 °C, 10 min). The solution (30 μl) was added to 40 ml vials with Teflon lined septa caps. Samples were buffered (300 μl) to pH 4.9, ethylated with the addition of $NaBEt_4$ (40 μl), topped off with milli Q water, capped, shaken and loaded into the auto sampler. Measurements of MeHg were made using the automated modular mercury system from Brooks Rand (MERX, Brooks Rand Labs, USA). The sulfate concentration of the extract was measured by a barium sulfate turbidimetric technique (APHA, 1975), using an UV-visible spectrophotometer (UV-1601, Shimazu).

2.7. DNA extraction and quantification

Total DNA was extracted from 0.5 g sediment samples (wet weight) with a FastDNA Spin kit (MP Biomedicals, Illkirch, France). DNA concentrations were determined by the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher, USA) at 260 nm. Real-time PCR assays were used to measure the quantity of dissimilatory sulfite reductase gene (*dsr*AB) of SRB in the sediment extracts. The specific primers for *dsr*AB of the *D. vulgaris*, *D. propionicus* and total SRB (Leloup et al., 2009) are listed in Table 1. Amplification followed a three-step PCR with 30 s denaturation at 94 °C, 30 s annealing (temperatures are listed in Table 1), and 30–45 s elongation at 72 °C. Real-time PCR was performed on iCycler iQ Real Time PCR detection system (Bio-Rad, USA) with the iQ SYBR Green Supermix (Bio-Rad, USA). The DNAs extracted from pure culture of *D. vulgaris* (ATCC 7757) and *D. propionicus* (ATCC 33891) were used as standards for the quantification of *D. vulgaris* and *D. propionicus* in the sediments, respectively. Since there was no standard for quantification of the total SRB, CT value was only compared among samples.

Table 1
Primer sequences to identify and quantify bacteria in sediment.

Primer	Sequence	Annealing temperature	Target
1F	5′- TCG TGG CTG TAC CAT GAG AT-3′	60 °C	*Desulfovibrio vulgaris*
1R	5′- CAG TAC TTG TCG GCG ATG TC-3′		
2F	5′- GAT CTG ACC CAC GAT CTG AA -3′	59 °C	*Desulfobulbus propionicus*
2R	5′- GTC AGG CTG TTG CAG ACT TC -3′		
3F	5′-ACS CAC TGG AAG CAC GGC GG-3′	55 °C	Total SRB
3R	5′-GTG GMR CCG TGC AKR TTG G-3′		

2.8. Statistical analyses

All data were initially evaluated using a Kolmogorov–Smirnov test for normality and Levene's Test for homogeneity of variance. Statistical differences in MeHg concentrations in sediments were identified using a one-way analysis of variance (ANOVA). Statistical analyses were performed with SPSS Based 16.0 statistical software.

3. Results and discussion

3.1. SRB identification in sediments

The sequences corresponding to near the full-length of the *dsr*AB gene (~1900 bp) and partial *dsr*B gene (~370 bp) were generated from the sediment DNA isolated from the fresh water pond sediment. The sequencing information of PCR product indicated that the SRB contained in the sediments were mainly composed of the two species: *Desulfobulbus propionicus* and *Desulfovibrio vulgaris*. Based on this identification, authentic cultures of these two species were used in all further studies. These cultures had been previously characterized for their ability to generate MeHg (King et al., 2000; Benoit et al., 2001a,b).

3.2. Effects of amended sediments

During the 30-day incubation period, MeHg concentrations in control were kept at low levels, ranging from 0.16 ± 0.05 to 2.64 ± 0.45 ng/g (d.w.), with the highest value (2.64 ± 0.45 ng/g) observed on day 17. In contrast, MeHg concentrations in Hg^{2+} amended treatment (no sulfate and SRB added) increased approximately 7-fold from days 4 to 25, with the highest value of 38.97 ± 1.15 ng/g recorded on day 25 (Fig. 1). This indicated that more inorganic Hg would be available for methylation even when sulfate and bacteria were not amended. The results implied that adequate inorganic Hg is the prerequisite of MeHg production, and the absence of SRB could not prevent Hg methylation.

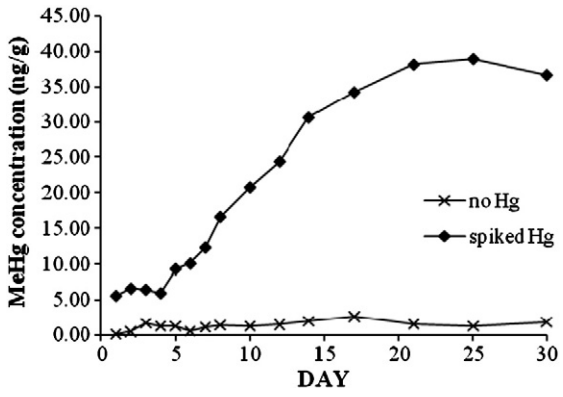

Fig. 1. MeHg concentrations in control and Hg^{2+} amended culture.

The effect of inorganic Hg was well recognized. Isotope experiments proved that MeHg is produced from inorganic Hg (Hg^{2+}), and its loading rate is significantly correlated with the concentration of MeHg in sediments (Orihel et al., 2006). In the present study, MeHg concentration in Hg^{2+} amended treatments was 5-fold higher than that in control on the first day, which indicated Hg methylation commenced from the initial phase. Using pure cultures of SRB, a previous study also indicated that SRB cells were present before methylation occurred and that methylation rate was the highest when inorganic Hg was spiked into actively growing cultures (Benoit et al., 2001b).

3.3. Mercury methylation in SRB cultures

Methylmercury concentrations were determined over time for each sediment inocula. Sediments when augmented with authentic cultures of SRB generated higher final MeHg ($p<0.05$) than the non-inoculated controls (Fig. 2). The final concentrations of MeHg ranged

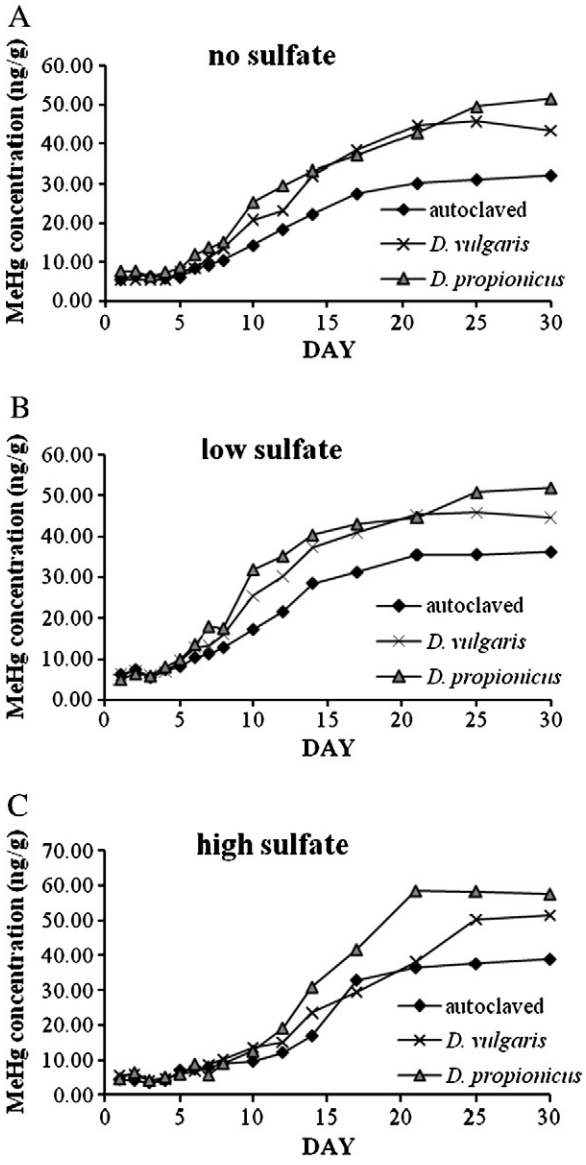

Fig. 2. MeHg concentrations in sediments (A) control (no sulfate), (B) low sulfate (0.11 mg/g) treatments, and (C) high sulfate (0.55 mg/g) treatments. Inorganic Hg was added at the onset of all experiments.

from 39 to 57 ng/g, 36 to 52 ng/g, 32 to 52 ng/g in treatments which contained high, low and no sulfate, respectively. Based on the final MeHg levels, the following trend was observed: sediment inocula (*D. propionicus*) > sediment inocula (*D. vulgaris*) > autoclaved sediment. The results of one-way ANOVA showed that the final MeHg concentration in autoclaved sediment was significantly lower ($p<0.05$) than that in sediment inocula (*D. propionicus*) and sediment inocula (*D. vulgaris*) under each sulfate concentration (Fig. 2A, B, C). However, there was no significant difference ($p>0.05$) obtained between the two SRB sediment inocula, under no sulfate and low sulfate conditions (Fig. 2A, B). Fig. 2A shows that Hg was methylated in the absence of sulfate. The reason is due to the fact that although sulfate was not added, there was enough sulfate already present in the natural sediment to support sulfate reducers. The results demonstrated the ability of the two SRB to methylate Hg under sulfate reducing conditions.

In contrast, the MeHg concentrations in the sediment inocula (*D. propionicus*) were significantly higher ($p<0.05$) than those in the sediment inocula (*D. vulgaris*) from day 12 to 22 in high sulfate amended treatments (Fig. 2C). The Hg methylation capability of *D. propionicus* was significantly higher ($p<0.05$) than that of *D. vulgaris* under high sulfate condition. *D. propionicus* was proved to be an efficient Hg methylator under sulfate-reducing conditions (Benoit et al., 2001b). Earlier reports also suggested it was capable of high rates of Hg methylation (Chio et al., 1994; Pak and Bartha, 1998a). The differential Hg methylation which observed in the present study may be explained by the distinct morphologies and biochemical pathways between the two SRB cultures (Bridou). In addition, it may relate to species specific enzymes such as acetyl-coenzyme A (CoA) and components of organic substrates such as citric acid in sediments. Different SRB groups methylated Hg through diverse metabolic pathways, both SRB species belong to the "incomplete oxidizer" strains (Ekstrom et al., 2003). However, *D. vulgaris* can utilize CoA to methylate Hg better than *D. propionicus* (Ekstrom et al., 2003). In the presence of sulfate, *D. vulgaris* oxidizes lactate to pyruvate, and yields CO_2, acetate, and reducing equivalents for sulfate reduction, but it is unable to use acetate (Pak and Bartha, 1998b).

3.4. Mercury methylation in sulfate amendment

Low sulfate concentrations were common characteristics of freshwater sediments (approximately 5 to 40 mg/L in pore water) (Feng and Hsieh, 1998). In the present study, sulfate amendments (0.11 and 0.55 mg/g) are higher than the background sulfate level (probably 0.06 mg/g) of freshwater sediments seemed to affect Hg methylation under different treatments (Fig. 3). The MeHg concentrations in sediments amended with different sulfate concentrations are shown in Fig. 3. It was noted that Hg was methylated in autoclaved control (Fig. 3A), which may be caused by abiotic methylation process. Mercury methylation can be formed in the aquatic environment by abiotic factors under suitable conditions (pH, temperature and the presence of complexing agents, such as chloride) (Celo et al., 2006). The final concentrations of MeHg in autoclaved control ranged from 32 to 38 ng/g which were lower (not significant, $p>0.05$) than those in sediment inocula (*D. vulgaris*) (43 to 52 ng/g) and sediment inocula (*D. propionicus*) (52 to 57 ng/g) (Fig. 3).

After a stabilization period of 4 to 7 days, MeHg concentrations increased sharply in all treatments (except control). In sediment inocula (*D. vulgaris*) (Fig. 3B), MeHg concentrations in high sulfate treatments was significantly lower ($p<0.05$) than that in low and no sulfate treatments during day 10 to 21. After day 23, MeHg levels in high sulfate treatments were higher (not significant, $p>0.05$) than those in low and no sulfate treatments, and approached equilibrium towards the end of the experiment. For the sediment inocula (*D. propionicus*) and autoclaved sediment (Fig. 3C and A), MeHg concentrations in high sulfate treatments were significantly lower ($p<0.05$) than that in other two treatments during day 8 to 14 and 10

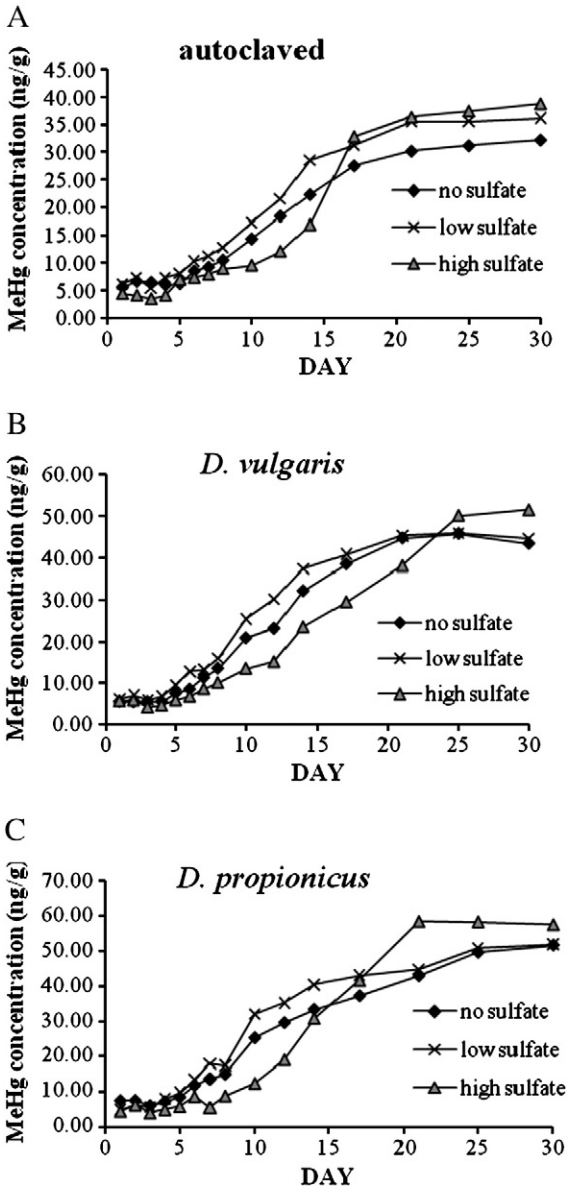

Fig. 3. MeHg concentrations in sediments (A) control (autoclaved), (B) with *D. vulgaris* cells, and (C) with *D. propionicus* cells. Inorganic Hg was added at the onset of all experiments.

to 14, respectively. It indicated that low sulfate concentrations can stimulate Hg methylation but high levels inhibit MeHg production. The results are in line with previous studies which noted much lower sulfate concentrations (60–80 μM) in freshwater lakes and the addition of sulfate to 200 μM (0.19 mg/g SO_4^{2-} sediment) level was able to stimulate Hg^{2+} methylation. However, at levels above 200 μM, the product of H_2S inhibits Hg methylation (Gilmour et al., 1992; Pak and Bartha, 1998b).

Though Hg methylation was inhibited by high sulfate amendment, MeHg concentrations in high sulfate were still higher ($p>0.05$) than those in low and no sulfate treatments towards the end of experiments. This was caused by sulfate depletion (Fig. 4), due to the increase of MeHg (Harmon et al., 2007). The sulfate reducing rates (SRR) in the two SRB cultures were quantified in pure cultures by King et al. (2000), and the calculated SRB of *D. vulgaris* and *D. propionicus* were 15.23 and 16.98 nmol/ml/h, respectively. When sulfate concentrations were lower than 200 μM, Hg methylation was enhanced. In addition, studies of SRB pure culture have shown that *D. propionicus* will methylate HgS^0 under propionate fermentative conditions (Benoit et al., 2001a,b).

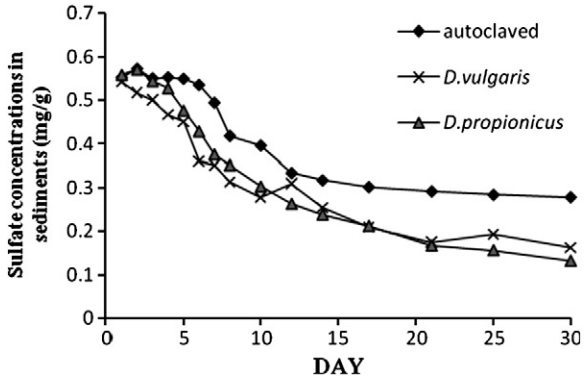

Fig. 4. Sulfate concentrations of SRB cultures in high sulfate treatments with time.

3.5. DNA concentration in sulfate amendment

DNA concentration was quantified by PCR amplification, the growth curves for the two SRB in different sulfate treatments are shown in Fig. 5 (A and B). The growth rates of the two SRB cultures were delayed by approximately 6 days in treatments with and without sulfate. Such delay was also observed by Harmon et al. (2007). This delay probably accounted for the initial Hg methylation in corresponding treatments (Fig. 3B and C). It has been proved that MeHg production is related to SRB cell density (Benoit et al., 2001b). Following the same trend of MeHg levels, DNA concentrations in treatments without sulfate were the lowest. On the contrary, DNA concentrations in high sulfate treatments were higher (not significant, $p>0.05$) than those in low sulfate treatments. It indicated that addition of sulfate amended enhanced SRB growth under anaerobic conditions. The redox between sulfate and organic substrates provided energy for SRB growth (Benoit et al., 2001b). However, there was no obvious increase of total SRB growth in autoclaved control (Fig. 5C). As there was no standard for total SRB quantification, CT value was used instead of the concentrations of total SRB shown in the figure.

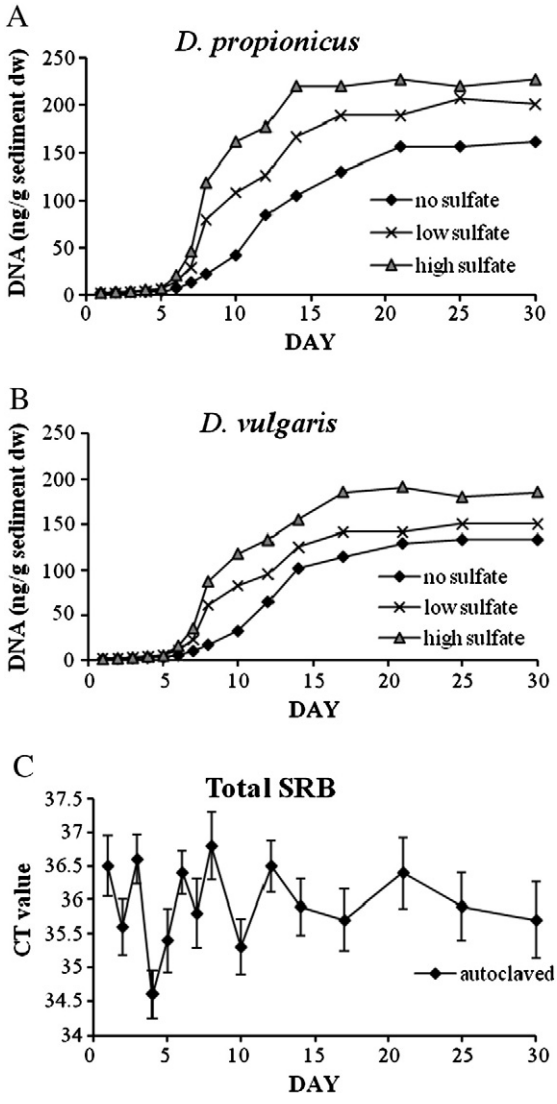

Fig. 5. (A) DNA concentrations of *D. vulgaris* in sediments. (B) DNA concentrations of *D. propionicus* in sediments. (C) CT value of total SRB DNA in autoclaved treatments.

4. Conclusions

In this study, two SRB species: *Desulfobulbus propionicus* and *Desulfovibrio vulgaris* in sediment of freshwater fish ponds were identified. The two SRBs purchased from ATCC were then cultured in freshwater sediments under various sulfate levels and inorganic Hg amendments. After a 30-day incubation period, MeHg concentrations increased in all treatments except the control. The results demonstrated that inorganic Hg is the prerequisite of MeHg production. Both of the two SRB species can facilitate Hg methylation, with *D. propionicus* possessing higher capability than *D. vulgaris* under high sulfate condition (0.55 mg/g). However, it was noted that Hg methylation was inhibited under the highest sulfate treatments (0.11 mg/g). DNA quantification indicated that Hg methylation increased with DNA concentrations, and the addition of sulfate stimulated the SRB growth in sediments.

Acknowledgments

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