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ORIGINAL PAPER

Effect of nitrate injection on the bacterial community in a water-oil tank system analyzed by PCR-DGGE

Diogo Jurelevicius · Irene von der Weid · Elisa Korenblum · Erika Valoni · Mônica Penna · Lucy Seldin

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Abstract Sulfide production by sulfate-reducing bacteria (SRB) is a major concern for the petroleum industry since it is toxic and corrosive, and causes plugging due to the formation of insoluble iron sulfides (reservoir souring). In this study, PCR followed by denaturing gradient gel electrophoresis (PCR-DGGE) using two sets of primers based on the 16S rRNA gene and on the *aps* gene (adenosine-5-phosphosulfate reductase) was used to track changes in the total bacterial and SRB communities, respectively, present in the water-oil tank system on an offshore platform in Brazil in which nitrate treatment was applied for 2 months (15 nitrate injections). PCR-DGGE analysis of the total bacterial community showed the existence of a dominant population in the water-oil tank, and that the appearance and/or the increase of intensity of some bands in the gels were not permanently affected by the introduction of nitrate. On the other hand, the SRB community was stimulated following nitrate treatment. Moreover, sulfide production did not exceed the permissible exposure limit in the water-oil separation tank studied treated with nitrate. Therefore, controlling sulfide production by treating the produced water tank with nitrate could reduce the quantity of chemical biocides required to control microbial activities.

Keywords Nitrate treatment · PCR-DGGE · Sulfate-reducing bacteria · Sulfide production · Water-oil tank

D. Jurelevicius · I. von der Weid · E. Korenblum · L. Seldin (✉)
Laboratório de Genética Microbiana, Departamento de Microbiologia Geral, Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Bloco I, Ilha do Fundão, CEP 21941.590 Rio de Janeiro, RJ, Brazil
e-mail: lseldin@micro.ufrj.br; lucy@seldin.com.br

E. Valoni · M. Penna
CENPES, PETROBRAS, Rio de Janeiro, RJ, Brazil

Introduction

Souring is one of the main problems in petroleum industry due to the toxicity and corrosiveness of the sulfide (H_2S) produced by sulfate-reducing bacteria (SRB). In addition, it lowers the economic value of the produced oil and imposes safety hazards. Insoluble iron sulfides may also cause plugging of the oil reservoir [3, 8, 16]. A number of methods for controlling sulfide production in different oil production facilities have been proposed in order to reduce microbial activity, including the use of biocides such as glutaraldehyde, antraquinone, and tetrakishydroxymethylphosphonium sulfate (THPS) [2, 6, 12]. However, the efficacy of these biocides is usually questionable as cases of microbial resistance have already been reported [6], and also they may be a risk to human health and to the environment.

An alternative approach for the control of SRB-sulfide production in water-oil systems is the use of repeated injection of nitrate [4, 11, 20]. The effect of nitrate may cause competition between SRB and heterotrophic nitrate-reducing bacteria (NRB) for common electron donors [5], the presence of chemolithotrophic NRB which not only remove sulfide but also suppress sulfide formation by the SRB [10], and the direct inhibition of SRB when nitrite is accumulated during nitrate reduction by NRB. In addition, some SRB may switch their energy metabolism to reduce nitrate instead of sulfate [8].

Previous studies based on cultivation of bacteria and also on molecular techniques have been used to demonstrate that nitrate introduction to oil fields waters increase the number of NRB and controls sulfide production [5, 11, 19]. Nucleic acid-based analyses of bacterial communities have been used to overcome biases of cultivation-dependent methods and to provide data concerning diversity and metabolic activity from bacterial communities present in inhospitable environments. Therefore, the aim of this study was

to assess for the first time the impact of the introduction of nitrate on the complex microbial community, including the SRB, present in the water-oil separation tank on a Floating, Storage and Offloading (FSO) unit of an offshore platform in Brazil. The water-oil tank microbial community was analyzed during 2 months (15 nitrate injections) and the impact of the treatments was evaluated by molecular methods based on DNA extraction from produced water samples, PCR and DGGE.

Materials and methods

Water-oil separation tank

The separation tank used in this study is located at larboard on the FSO unit of an offshore platform, 180 km east of Rio de Janeiro, Brazil. This FSO receives 8,000 m^3 of oil and 715 m^3 of produced water per day. Whenever this produced water presented more than 1% of oil, it was treated in the separation tank and then transferred to a clean tank. The separation tank includes at least one inlet for introducing water-oil emulsion, and a separate discharge port for conducting hydrocarbon-free aqueous component from the tank. The volume capacity of the separation tank is 2,700 m^3. The input flow rate of produced water into this tank is low, resulting in a high residence time of 10-30 days. This stagnation of the produced water may result in a perfect condition for bacterial development. Before the beginning of this study, THPS (70 ppm) was usually added to the system to control the SRB activity. This condition was considered as the negative control of the experiment (without nitrate). The characteristics of the separation tank studied here were: temperature−34°C; pH−6.5; potential redox– −265 mV; sulfate–1,411 mg l^{-1}; sulfide–1.6 mg l^{-1}. The operators at FSO turned off the biocide feed 2 days prior to sampling. The nitrate treatment (5 mM $NaNO_3$) was repeated 15 times throughout a 2-month period (usually one application/4 days).

Produced water samples

Water samples were collected in sterile glass bottles, which were filled completely to prevent contact with air, and sealed with rubber stoppers. They were transported on ice to the laboratory and stored at −20°C. Samples were collected before and after each nitrate injection.

Chemical analysis

The analytical method used in this study was based on standard methods described elsewhere [1]. Samples were taken by collecting the needed aliquot in a beaker just before performing the analysis. Sulfide analysis was carried out by iodometric titration. This procedure is described in detail in Method 4500-S^{-2} F.

DNA extraction

For DNA extraction, 30 ml of each sample were centrifuged for 20 min at 12,800*g*, the pellets were suspended in 500 µl of TE 1X [18] and then the extraction was performed as described by Pitcher et al. [17]. Agarose gel electrophoresis of the total DNA was performed in 0.8% agarose gels in a Tris-Borate-EDTA (TBE) buffer [18] at 70 V for 4 h at room temperature.

Amplification by PCR

The 16S rRNA gene sequences were amplified from the DNA extracted from the water samples by PCR using the universal primers (U968f + GC clamp and L1401r) and the PCR conditions described by Heuer and Smalla [7]. The 50 µl PCR reaction mix contained 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 20 nmol dNTPs, 0.02 µmol of each primer, 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI, USA), 5 µg formamide, 5 µg BSA and 2 µl of the DNA sample. The amplification conditions applied were as follows: denaturing step of 94°C for 3 min, followed by 35 cycles of 1 min at 94°C, annealing for 1 min at 55°C and extension for 1 min at 72°C, followed by a final extension at 72°C for 10 min. In addition, specific primers for SRB based on *aps* gene (adenosine-5-phosphosulfate reductase) were used as described by Zinkevich and Beech [21]. A GC clamp was attached to the reverse primer. The 50 µl PCR reaction mix contained 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 3.25 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of each primer, 2.5 U of Taq DNA polymerase (Promega) and 2 µl of the DNA sample. The amplification conditions applied were as follows: denaturing step of 95°C for 2 min, followed by 35 cycles of 1 min at 95°C, annealing for 1 min at 62°C and extension for 1 min at 72°C, followed by a final extension at 72°C for 10 min. Negative controls (without DNA) were run in all amplifications and the presence of PCR products was checked by 1.4% agarose gel electrophoresis followed by staining with ethidium bromide.

DGGE

DGGE was performed using a Dcode DGGE system (Bio-Rad Laboratories, Richmond, VA, USA). Polyacrylamide gels in 1X TAE buffer [18] containing a linear denaturing gradient were loaded with the PCR products (15–20 µl) mixed with 2× loading dye. The gradients were formed with 6% (w/v) acrylamide stock solutions [14] that contained no denaturant and 100% denaturant (the 80% denaturant

solution contained 7 M urea and 40% [v/v] formamide deionized with AG501-X8 mixed-bed [Bio-Rad]). The concentration of the denaturant ranged from 35 to 65 and 35 to 70% when 16S rDNA and *aps* PCR products were used, respectively. The gels were electrophoresed for 16 h at 60°C and 65 V (16S rRNA PCR products) and for 5 h at 60°C and 200 V (*aps* PCR products). Gels were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA) for 40 min prior to imaging, using the IMAGO system (B&L System).

Statistical analysis

The sulfide analyses were performed in triplicate. Basic statistics were calculated and results were expressed as mean values and the standard deviation given as required.

Results and discussion

The effect of nitrate injection on the microbial community has already been evaluated in oil fields, oily wastes and oil industry production waters [4, 11, 13, 16, 19], but has never been studied in a water-oil separation tank in a FSO unit of an offshore platform in Brazil. Different responses to this treatment have been achieved, from the lack of enhancement of the bacterial community members including the SRB [11, 19], to the increase in the number of the heterotrophic nitrate reducing population [5] and the collapse of SRB population [15]. The present work showed the influence of the nitrate injection in the complex bacterial community present in a water-oil tank using molecular methods. Results from PCR-DGGE analysis of total bacterial community based on 16S rDNA (Fig. 1a, b) showed the existence of a dominant bacterial community present in the water-oil tank. The different DGGE profiles were stable from the beginning of the experiment to the end of 2 months (Fig. 1a, b), with minor shifts in the diversity of the dominant bacterial population. The changes in the different DGGE patterns were basically in the increase of intensity and/or the appearance of some bands observed only at specific moments (Fig. 1a, b). However, these alterations were not permanent throughout the 2-month period of nitrate treatment. These minor changes in the DGGE patterns could be explained by the constant supply of produced water inside the separation tank. Therefore, these results suggest that the treatments did not significantly affect the dominant bacterial populations, but it is still possible that the interval of 4 days between each nitrate treatment was too short to cause any observable changes in populations. Moreover, the stability of the DGGE patterns observed here also confirms that the different samplings are representative of the tank studied. The same results were observed by

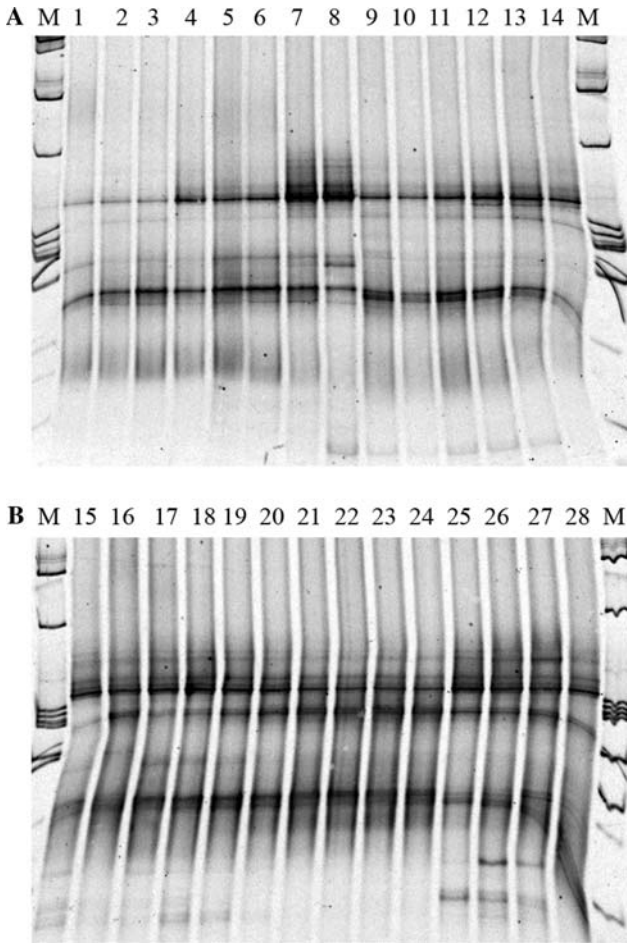

Fig. 1 DGGE patterns obtained with PCR-DGGE based on 16S rRNA gene in response to the different nitrate treatments. **1**—produced water samples treated with biocide (THPS); **2**–48 h after the interruption of THPS introduction; **3–28** h after the first to the 15th nitrate injection (dates of treatment are shown in Fig. 3): **a 3**–4 h, 1°; **4**–24 h, 1°; **5**–72 h, 1°; **6**–4 h, 2°; **7**–24 h, 2°; **8**–72 h, 2°; **9**–72 h, 8°; **10**–4 h, 9°; **11**–24 h, 9°; **12**–48 h, 9°; **13**–86 h, 9°; **14**–6 h, 10°; **b 15**–82 h, 13°; **16**–4 h, 14°; **17**–24 h, 14°; **18**–48 h, 14°; **19**–74 h, 14°; **20**–96 h, 14°; **21**–168 h, 14°; **22**–186 h, 14°; **23**–4 h, 15°; **24**–28 h, 15°; **25**–51 h, 15°; **26**–75 h, 15°; **27**–99 h, 15°; **28**–165 h, 15°. (M) 1 kb ladder (Promega)

Kjellerup et al. [11] when they monitored the microbial souring in produced water biofilm systems.

On the other hand, results from PCR-DGGE analysis of SRB community based on *aps* gene (Fig. 2b) showed an increase in SRB community in response to the injection of nitrate over the 2 months of experiment. The comparison between the DGGE profiles obtained when the DNAs extracted from the different water samples were amplified by PCR using both primers (based on 16S rRNA and *aps* genes) can also be observed in Fig. 2a and b, respectively. It becomes clear that this increase in the SRB community abundance was only observed when a specific primer (*aps*) was used. Therefore, the increase of the SRB community was not high enough to allow its detection in the 16S

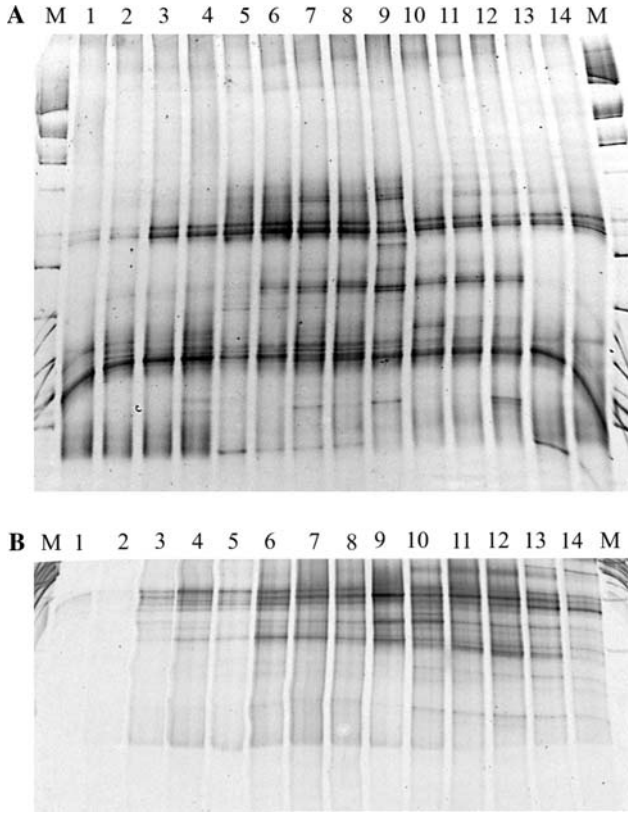

Fig. 2 Comparison between DGGE profiles obtained with **a** PCR-DGGE based on 16S rRNA gene and b PCR-DGGE based on *aps* gene in response to the different nitrate treatments. **1**—produced water samples treated with biocide (THPS); **2**–48 h after the interruption of THPS introduction; **3–14** h after the first to the seventh nitrate injection (dates of treatment are shown in Fig. 3): **3**–24 h, 1°; **4**–4 h, 2°; **5**–72 h, 2°; **6**–4 h, 3°; **7**–71 h, 3°; **8**–4 h, 4°; **9**–24 h, 4°; **10**–4 h, 5°; **11**–93 h, 5°; **12**–5 h, 6°; **13**–48 h, 6°; **14**–6 h, 7°. (M) 1 kb ladder (Promega)

rRNA-based DGGE, indicating that this community has not achieved a condition of predominance within the total bacterial population after the introduction of nitrate.

The amendment of 5 mM nitrate in the separation tank in intervals of about 4 days was sufficient to control sulfide production, maintaining the sulfide concentration under the permissible exposure limit (P.E.L) (10 ppm). However, complete cessation of sulfide production has never been observed. The nitrate concentration used here is similar to that reported for other laboratory investigations in which the sulfide production was controlled [4]. Figure 3 shows that the level of sulfide varied along the 2 months but it was kept below 10 ppm, as previously observed when THPS biocide was used. The only increase of the sulfide amount was detected at the end of the experiment, when a 10-day-period was observed between the two last nitrate injections. This indicates that no long-term effects should be expected for this treatment, and continuous nitrate addition may be required to control sulfide production. In contrast, other studies have shown total inhibition of SRB activity in oil-field produced water [5], presumably as a result of increased NRB activity. As an increase in SRB population was observed here but not the sulfide production, this may be attributed to a switch of the energy metabolism of the SRB population to reduce nitrate instead of sulfate. Moreover, the maintenance of the sulfide level could be explained by the presence of chemolithotrophic NRB which may oxidize sulfide, as suggested before [10]. Although SRB are often considered to be strict anaerobes, several recent studies have shown significant SRB activity in the presence of nitrate and under microaerophilic conditions [9].

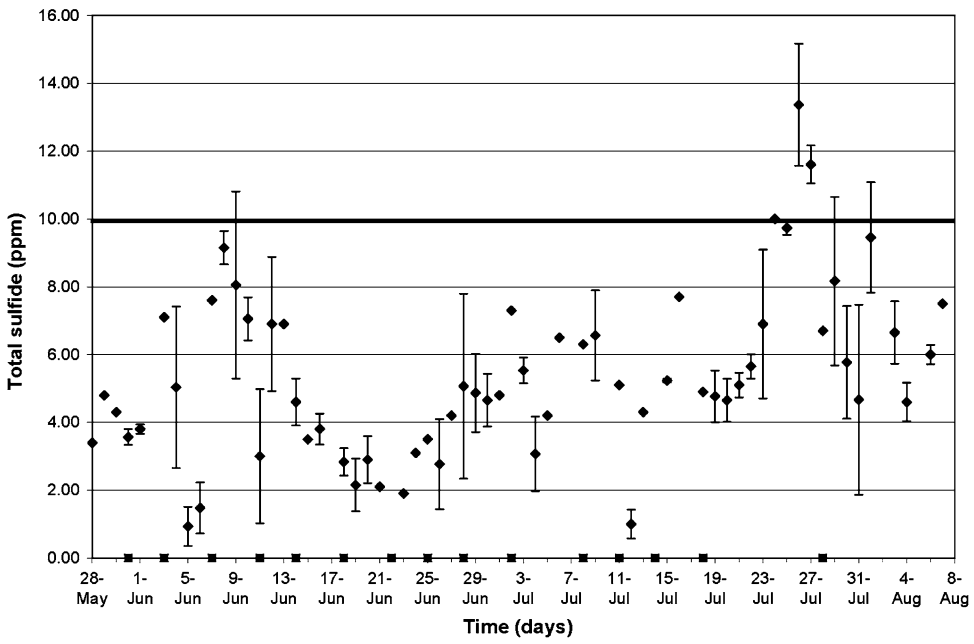

Fig. 3 Concentration of sulfide in the production water along the 2 months when the water-oil separation tank was treated with nitrate. *Filled square* 5 mM nitrate treatment in the water-oil tank (nitrate injection time points are highlighted at *x*-axis); *filled diamond* Sulfide average concentration over time. Each data point is an average of three sulfide concentration values, and *error bars* indicate the standard error

In conclusion, hydrogen sulfide production was lowered in the water-oil separation tank studied treated with nitrate, but no long lasting sulfide inhibition should be expected. PCR-DGGE analysis of total bacterial community based on 16S rRNA gene showed that the appearance and/or the increase of intensity of some bands in the gels were not permanently affected by the introduction of nitrate treatment along the 2-month period. On the other hand, the SRB community was considerably stimulated following nitrate treatment, as the intensity of bands in the DGGE profiles increased when the *aps* primers were used. Therefore, controlling sulfide production by treating the produced water tank with nitrate, besides being less expensive and an environment friendly treatment, would greatly reduce the quantity of chemical biocides required to control microbial activities. However, in the absence of nitrate (or other controlling agent) the SRB community still present in the water-oil separation tank in the FSO unit may rapidly reduce sulfate and consequently produce sulfide.

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