

Video Article

Development of Sulfidogenic Sludge from Marine Sediments and Trichloroethylene Reduction in an Upflow Anaerobic Sludge Blanket Reactor

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Abstract

The importance of microbial sulfate reduction relies on the various applications that it offers in environmental biotechnology. Engineered sulfate reduction is used in industrial wastewater treatment to remove large concentrations of sulfate along with the chemical oxygen demand (COD) and heavy metals. The most common approach to the process is with anaerobic bioreactors in which sulfidogenic sludge is obtained through adaptation of predominantly methanogenic granular sludge to sulfidogenesis. This process may take a long time and does not always eliminate the competition for substrate due to the presence of methanogens in the sludge. In this work, we propose a novel approach to obtain sulfidogenic sludge in which hydrothermal vents sediments are the original source of microorganisms. The microbial community developed in the presence of sulfate and volatile fatty acids is wide enough to sustain sulfate reduction over a long period of time without exhibiting inhibition due to sulfide.

This protocol describes the procedure to generate the sludge from the sediments in an upflow anaerobic sludge blanket (UASB) type of reactor. Furthermore, the protocol presents the procedure to demonstrate the capability of the sludge to remove by reductive dechlorination a model of a highly toxic organic pollutant such as trichloroethylene (TCE). The protocol is divided in three stages: (1) the formation of the sludge and the determination of its sulfate reducing activity in the UASB, (2) the experiment to remove the TCE by the sludge, and (3) the identification of microorganisms in the sludge after the TCE reduction. Although in this case the sediments were taken from a site located in Mexico, the generation of a sulfidogenic sludge by using this procedure may work if a different source of sediments is taken since marine sediments are a natural pool of microorganisms that may be enriched in sulfate reducing bacteria.

Video Link

The video component of this article can be found at <https://www.jove.com/video/52956/>

Introduction

One of the most important contributions to environmental biotechnology was the design of bioreactors in which the sludge used (inoculum) was able to perform under sulfate reducing conditions. Sulfate reduction (SR) allows the treatment of wastewater streams that contain high concentrations of sulfate in addition to the simultaneous removal of COD, heavy metals and organic pollutants, a fact that makes SR a desirable characteristic of the sludge¹. Some examples of effluents contaminated with sulfate come from tannery, paper, pharmaceutical and chemical manufacturing industries¹. However, most of the literature refers to sulfidogenic sludge when methanogenic granular sludge has been adapted to sulfidogenesis². This adaptation is commonly attained by manipulating the COD/SO₄²⁻ ratio in the bioreactor and adding chemicals to inhibit methanogens in the sludge^{2,3}. In addition to the long time that may require the formation of the sulfidogenic granules, the competition between methanogens and sulfate reducers and the tolerance of the sludge to high concentrations of sulfide are some of the main problems that may arise if the sulfidogenic sludge used in the bioreactor is obtained from the adaptation of predominantly methanogenic sludge to sulfate reducing conditions. In this work, we describe the procedure to obtain a predominantly sulfidogenic sludge from hydrothermal vents sediments (Punta Mita, Nayarit, Mexico) in an upflow anaerobic sludge blanket reactor (UASB), then we evaluate its sulfate reducing activity over time and conduct an experiment to evaluate its application on reductive dechlorination. The location of the sediments was chosen because it has been reported that in that site there is formation of sulfides due to the sulfate reducing activity exhibited by the microbial community inhabiting that particular place⁴.

There are several advantages in obtaining this sulfidogenic sludge from sediments over adapting methanogenic granular sludge to sulfidogenesis. Some of these advantages are: (1) it is not necessary to form granules for the bioreactor to operate, (2) the sludge tolerates relatively high concentrations of sulfide compared to others UASB that operate with adapted methanogenic sludge, and (3) there is no

competition for substrate with methanogens even if acetate is used in the mixture of volatile fatty acids that is included in the culture medium to promote the formation of the sludge.

This procedure was followed to promote sulfidogenesis because marine sediments are a natural pool of a wide variety of microorganisms such as sulfate reducing bacteria, fermenting bacteria and dehalogenating bacteria just to mention a few^{5,6}. The type of consortium developed from marine sediments by using this protocol may exhibit efficiency in sulfate reduction and therefore, high sulfate reducing activity over time and higher tolerance to sulfide at concentrations higher than the reported as toxic to methanogens and sulfate reducing bacteria. On the other hand, it is likely that the dehalogenating capability is also shown in the sediments by following the protocol proposed here but it may depend on the original microbial community. This assumption is done based on the fact that reductive dechlorination can occur either by respiration or cometabolism, both conditions that may be promoted in the marine microbial community⁷. The cultivation of the sediments to obtain the sludge was conducted by using a mixture of acetate, propionate and butyrate as substrate because these volatile fatty acids are used by several strains of sulfate reducing bacteria. These acids are also the type of carbon compounds frequently found in marine sediments, according to several reports in literature on carbonaceous material in sea sediments^{5,6}.

Finally, some of the most toxic compounds that are found in groundwater and other water bodies around the world are the chlorinated solvents such as trichloroethylene (TCE) or perchloroethylene (PCE). These compounds are toxic not only to the human being but also to microorganisms, particularly TCE, which is still considered a priority pollutant by the Environmental Protection Agency in the US⁸. In this work we proposed an experiment in which the sulfidogenic sludge is tested on its capability of reducing TCE at concentrations that are in the range reported for chlorinated compounds biodegradation under methanogenic conditions^{9,10}. It is worth mentioning that most of the research on biodegradation of chlorinated compounds has been conducted under methanogenic conditions^{9,10}. We consider that the experiment with TCE proposed in this protocol is a good example of the potential applications of the sludge. The objective of this experiment was to evaluate the tolerance of the sludge to the TCE and the TCE effect on the sulfate reducing activity. Taking into account that most of the research on biodegradation of chlorinated compounds is carried out under methanogenic conditions, this protocol suggests the formation of a sludge may be used to simultaneously: (1) remove sulfate, (2) remove COD and (3) remove chlorinated compounds. A further step could be to evaluate the sludge on the simultaneous removal of TCE and heavy metals (in addition to sulfate and COD), two conditions that cannot be evaluated under methanogenic conditions.

Protocol

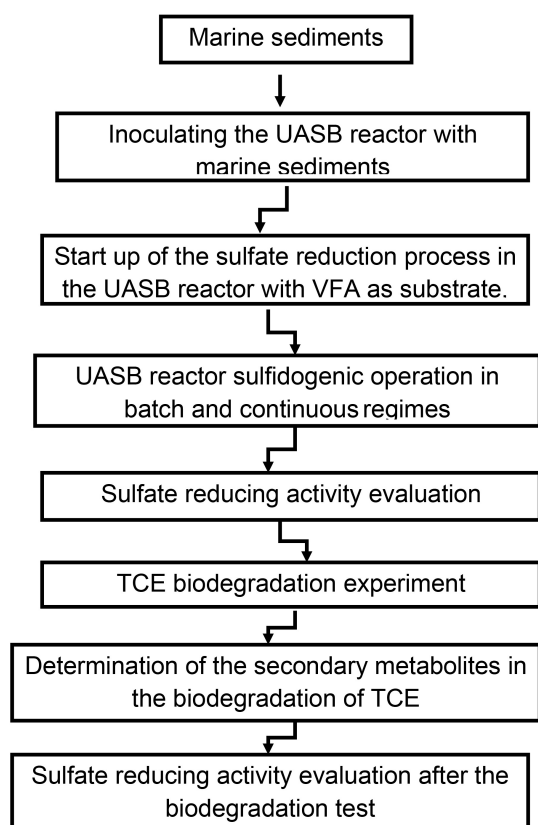


Figure 1. Scheme for the steps of the protocol. [Please click here to view a larger version of this figure.](#)

1. Collect Marine Sediments for the Formation of the Sludge

1. Identify an approachable subsea area either close to hydrothermal vents (due to the presence of sulfides, which may indicate a higher sulfate reducing activity) or to an area where debris of organic matter are detectable.

2. For the purpose of this work, take approximately 3 or 4 kg of sediment and drain the water off the samples. Place the samples in dark plastic bags. No refrigeration is needed.
3. Once in the lab, keep the bags with the samples in the refrigerator if they are not going to be used immediately. For the purpose of this work, samples can be in the refrigerator for weeks or months before using them.
4. Take a large portion of the sediment sample (*i.e.*, 1 or 2 kg) and use an appropriate mesh (0.2 cm) to eliminate from the sediments the large debris of carbonaceous material that may be found or some rocks that may be present.
 Note: In this case a mesh of 0.20 cm diameter (0.0767 in) was used but it may be of a different size according to the size of the particles in the sample.
 1. After passing the sediment through the mesh, mix the portion selected to promote that the portion is homogeneous.
 2. Take separated smaller samples (*i.e.*, 2 to 3 g) to determine the volatile suspended solids (VSS) content by following the standard methods¹¹.
 Note: See **Figure 2** for steps 1.2 to 1.4.

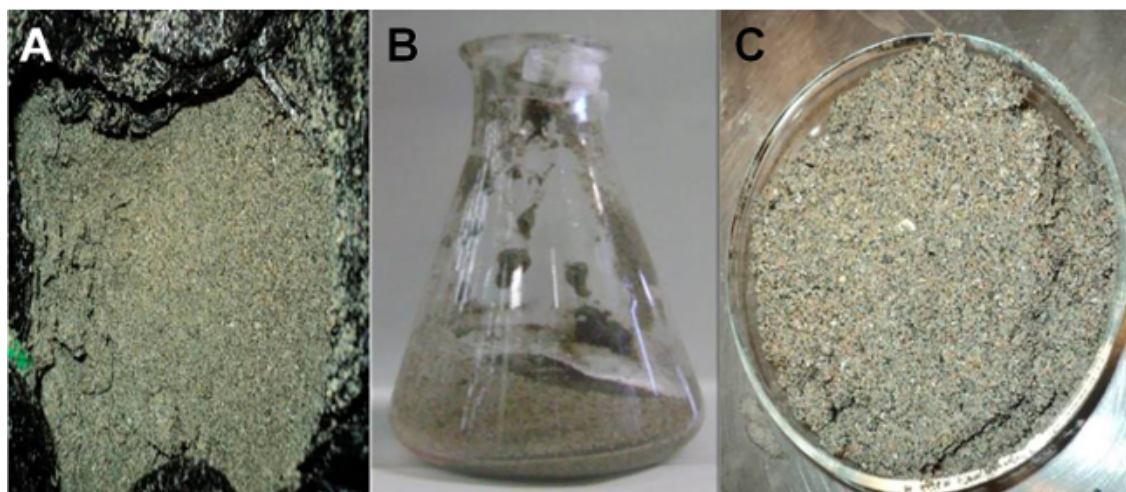


Figure 2. Photographs of the sediment samples. (A) Sediment samples just after being taken. (B) Sediment sample after passing through the mesh. (C) Sample taken for weighing prior to volatile suspended solids (VSS) determination. The Petri dish does not need to be sterilized. [Please click here to view a larger version of this figure.](#)

2. Bioreactor Set Up

1. For the purpose of this work, use a UASB glass reactor with a total working volume of 3 L. Alternatively, use a 1 or 2 L volume glass reactor.
2. Based on the VSS content of the sediments calculate the amount of sediment to be used as inoculum to obtain 5 g of VSS in 1 L.
3. Take into account that if the amount of sediment after calculation is too large, then approximately 25% to 30% volume of the bioreactor should be occupied by the sediments instead.
 1. Record the VSS content since it will change when the microbial community is enriched in the bioreactor. The VSS content is needed for the calculations of sulfate reducing activity in the bioreactor.
4. Ensure that the final concentration of the basal medium and buffer solution in the bioreactor is similar to the reported by Guerrero-Barajas *et al.* (2014)¹².
 1. Ensure that the final volumes of the sediments, basal medium, buffer solution and volatile fatty acids are equal to the final working volume of the reactor. The basal medium recipe¹² contains the appropriate concentrations for the trace metals and vitamins solution.
 2. Prepare a stock solution of basal medium and buffer solution in an appropriate concentration for the working volume of the reactor used (*i.e.*, 2, 3 or 4 fold more concentrated than the reported in step 2.4) to ensure that when it is diluted, it is at the concentration reported by Guerrero-Barajas *et al.* (2014)¹².
 Note: The stock solution for the basal medium is always necessary, however, the buffer solution is only needed at the start up. It is not necessary to add buffer solution after this time.
 3. Prepare a stock solution of volatile fatty acids: acetate, propionate and butyrate in a 2.5:1:1 COD proportion. Take into account for the calculations the sodium acetate included in the basal medium. The final COD concentration in the reactor must be 2.7 g/L.
 Caution: Prepare this solution in a fume hood. Wear nitrile gloves and goggles for the preparation of this solution. Take into account the stoichiometry of the reactions of sulfate with the volatile fatty acids that is shown in **Figure 3**.
 4. Prepare a stock solution of sodium sulfate (Na_2SO_4) in an appropriate concentration to deliver to the reactor a final concentration of 4,000 mg/L of the sulfate ion (SO_4^{2-}). Alternatively, include the amount of sulfate required in the basal medium instead of adding it from a stock solution as long as the final sulfate (SO_4^{2-}) concentration is right.
5. Place the sediments in the reactor mixed with a portion of the basal medium to make sure they reach the bottom of the reactor.
 1. Add the rest of the basal medium and buffer solution mixed with the volatile fatty acids solution and the sulfate solution. Make sure that the solution of volatile fatty acids is poured into the liquid. Note: Conduct this step in a fume hood.
 2. Set the connections and pipelines of the reactor to the recycling pump. Set the recycling flow rate at 60 ml/min. Set the bioreactor in the temperature chamber at 34 °C. Regularly check that the temperature variations are small (*i.e.*, 34 ± 1.7 °C)
 3. Set the connections to the gas displacement column.

Note: See **Figure 4** for steps 2.1 to 2.5.

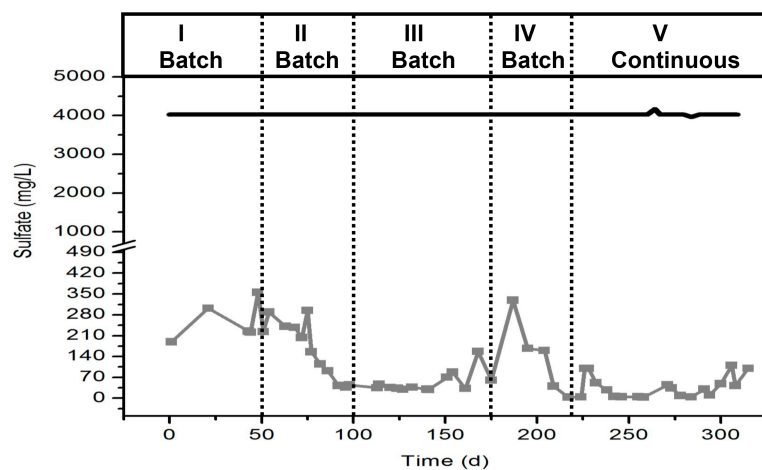


Figure 3. Stoichiometry of sulfate reduction with VFA (acetate, propionate and butyrate). [Please click here to view a larger version of this figure.](#)



Figure 4. UASB reactor. (A) Initial time. **(B)** Continuous regime after 300 days of operation. [Please click here to view a larger version of this figure.](#)

3. Operation of the Reactor to Promote Sulfidogenesis and Growth of the Microorganisms

Note: Allow for the inoculum to consume the volatile fatty acids and sulfate. For this purpose, wait for one week to carry out the first analysis for sulfate, sulfide and COD consumption.

1. After one week of incubation take a sample of 5 to 7 ml of the liquid to conduct analysis for COD, sulfate and sulfide content and pH following standard methods ^{11, 13}.
 1. Analyze sulfide in the liquid spectrophotometrically (at a wavelength (λ) of 670 nm) by following the methylene blue method ¹³.
 1. Place 5 ml of a zinc acetate solution (2% w/w) in a 25 ml volumetric flask, add quickly 200 μ l of the sample to the zinc acetate solution.
 2. Add 2.5 ml of an *N,N*-dimethyl-*p*-phenylenediamine oxalate (DMP) solution (0.2% w/w in 20% H_2SO_4) and 125 μ l of the iron (III) ammonium sulfate solution (10% w/w in 2% H_2SO_4) and complete with distilled water the 25 ml in the volumetric flask. Wait 30 min for the reaction to occur, time at which the blue color is stabilized. ¹³.
Note: Wait at least 15 min, but not more than 60 min to test the samples in the spectrophotometer. Conduct the reading of the blue final solution in the spectrophotometer.
 2. Analyze sulfate according to standard methods ¹¹. Here, quantify sulfate as barium sulfate by using a turbidimetric method.
 1. Place 5 ml of a conditioning solution (hydrochloric acid HCl 1:1) in a volumetric flask of 25 ml, add 1 ml of the previously centrifuged sample (at 11,320 $\times$ g), complete the 25 ml of the volumetric flask with distilled water and add 1 g of barium chloride.
 2. Mix the solution for 1 min in a vortex. Wait for 4 min for the barium sulfate to form and read the sample in the spectrophotometer at a wavelength (λ) of 420 nm ¹¹.
 3. Analyze COD according to standard methods ¹¹. Alternatively, use a COD determination kit.
 1. Prior to the COD determination, centrifuge the sample thoroughly (at 11,320 $\times$ g) to remove the remaining sulfide that may interfere in the determination. If necessary, centrifuge twice: the first time immediately after taking the sample and the second time wait 6 or 8 hr and then conduct the COD analysis.
 2. Add 2 ml of sample to a reaction vial of the COD determination kit, seal the vial and homogenize the mixture by gentle agitation. Prepare a blank by adding 2 ml of distilled water to another reaction vial and homogenize the mixture.
 3. Place the vials in the digestion reactor at 150 $^{\circ}C$ for 2 hr. Remove the vials and let them cool down in the dark. Take the readings of the vials in the spectrophotometer at a wavelength of 620 nm.
4. Obtain the gas volume from the gas displacement column.
2. Wait for up to another 5 to 7 days until the sulfate is consumed. Sulfate and COD must be consumed in approximately 85% to 90% before a new fed batch is started.
3. Once sulfate (and COD) are consumed, completely repeat step 2.4. Supply fresh medium and new nutrients for each batch.
4. Repeat steps 3.1 and 3.2. At this point each batch should last between 7 and 10 days.
5. When 3 to 4 batches have been completed, repeat step 2.4 but increase the COD concentration to 4 g/L.
6. Repeat step 3.1 and step 3.2.
 1. Repeat step 3.3 but increase the COD concentration to 6 g/L.
 2. Repeat 3.6 and 3.6.1 gradually increasing COD concentration until it is 10 g/L.
Note: Make the graph that presents the sulfate concentration (mg/L) versus time (d).
7. When sulfate consumption is over 80% in less than 24 hr and this occurs for more than one week, switch the operation of the reactor to continuous mode. For the continuous mode set the hydraulic retention time (HRT) at 24 hr and maintain the sulfate concentration at 4 g/L and the COD at 10 g/L.
Note: Over time the sulfate consumption **should be** faster.

4. Sulfate Reducing Activity Test

1. Prior to this test make sure that the bioreactor under continuous regime presents less than 10% variation in the sulfate concentration remaining.
2. At any given day, stop the reactor after one HRT cycle and conduct step 2.4. For step 2.4.3 use a COD concentration of 10 g/L.
3. Once the bioreactor is fed, take 5 to 7 ml samples of the liquid and perform analysis for COD, sulfate, sulfide (step 3.1) and pH every hour. Record the gas volume produced.
4. Calculate the sulfate reducing activity according to the literature ¹⁴.

$$SRA = \frac{mH_2S}{VSS * t}$$

SRA = sulfate reducing activity (mg COD- H_2S)/gVSS*d

mH_2S = sulfide concentration expressed as mg COD- H_2S

VSS = volatile suspended solids concentration

t = time (d or hr)

5. Make the corresponding graphs that show the percentage of sulfate consumption versus sulfide concentration over time in mg/L. Make the graphs that show percentage of COD consumption over time. Make the graphs that show pH variation over time.

5. Trichloroethylene (TCE) Reduction Test

1. Prior to this test make sure that the bioreactor is working under continuous regime and presents less than 10% variation in the sulfate concentration remaining. Do not start this test if sulfate reduction in the bioreactor is less than 90%.
2. Prepare a stock solution of trichloroethylene (TCE) taking into account that the final concentration of this compound in the liquid phase of the bioreactor must be 300 μM . Consider the partitioning of the compound to the headspace by using the Henry's Law dimensionless constant (H') for TCE at 34 $^{\circ}\text{C}$. H' at 34 $^{\circ}\text{C}$ for TCE is 0.4722.

$$H' = \frac{\text{gas phase concentration}}{\text{liquid phase concentration}}$$

Caution: Prepare this solution in a fumehood and wear gloves and goggles.

1. For example, for a 5,000 μM stock solution, calculate as followed:

$$H = \frac{\text{gas phase concentration}}{5000 \mu\text{M}}$$

TCE gas phase concentration = $(0.4722) \times (5,000) = 2,139 \mu\text{M}$. Include this concentration in the preparation of the stock solution since this amount of TCE will be in the headspace.

Then in the liquid (water) of the stock solution, the actual TCE concentration will be: $5,000 + 2,139 = 7,139 \mu\text{M}$. TCE density = 1.43 g/ml. Convert the 7139 μM to mg and then by using the density of TCE calculate the volume of TCE for the stock solution.

Note: The concentration of the TCE stock solution may be lower than 5,000 μM , i.e., 3,000 or 1,000 μM , this depends on how much volume of this solution may be delivered to the bioreactor according to its liquid phase volume.

3. Prepare standard curves in the gas chromatograph for TCE, *cis*-1,2-dichloroethylene, *trans*-1,2-dichloroethylene, vinyl chloride and ethene. Prepare the *cis*-1,2-dichloroethylene and *trans*-1,2-dichloroethylene standard curves from a stock solution of these compounds by following the same procedure described in 5.2 for the TCE stock solution. Prepare the standard curves for vinyl chloride and ethene by diluting the concentration of each gas from the standards (gas cylinders).
 1. Prepare the standard curves of these compounds in a range of 20 to 300 μM . Use the method reported by Guerrero-Barajas *et al.* (2011)¹⁵ for the analysis of these compounds in the gas chromatograph.

Caution: Prepare these standard solutions in a fumehood and wear gloves and goggles.
4. At any given day, stop the reactor after one HRT cycle and conduct step 2.4. For step 2.4.3 use a COD concentration of 10 g/L.
5. Once the bioreactor is fed, add the TCE directly to the liquid in the bioreactor from the stock solution prepared in 5.2, the final TCE concentration in the liquid phase of the bioreactor must be 300 μM . Set the HRT to 12 hr.
 1. At the end of one HRT cycle take samples of the liquid (500 to 1,000 μL) and conduct analysis for COD, sulfate and sulfide (steps 3.1.1, 3.1.2 and 3.1.3). Take samples of the headspace (100 to 250 μL) and conduct analysis for TCE, *cis*-1,2-dichloroethylene, *trans*-1,2-dichloroethylene, vinyl chloride and ethene in the gas chromatograph.
6. Repeat step 2.4. For step 2.4.3 use a COD concentration of 10 g/L.
7. Do not repeat any TCE reduction test until the bioreactor presents over 90% sulfate reduction and less of 10% variation in both, sulfate reduction and sulfate remaining in the bioreactor.
8. Repeat 5.4, 5.5 and 5.6 two or three times more.
9. Take sediment samples (0.5 g) to conduct identification of the microorganisms just after a TCE reduction test has finished. Do this after 2 or 3 TCE reduction tests.

6. Sulfate Reducing Activity Test after TCE Reduction Experiment

1. Repeat step 4 completely.

7. Identification of the Microorganisms

1. Take samples of sludge of approximately 0.5 g each and conduct RNA total extraction according to standard method¹².
2. Amplify the 16S rRNA gene with reverse transcription and conduct the polymerase chain reaction (RT-PCR) amplification one step¹².
3. Design the primers to amplify or use as an initial approach the ones suggested in literature¹¹. Follow the amplification procedure suggested in literature¹².
4. Construct the 16S rRNA libraries. PCR amplicons can be cloned by using a cloning-kit¹¹. Typically, 10 colonies from each plate (each colony representing one PCR product) can be cloned. Prepare the plasmid DNA for sequencing according to the procedure suggested in literature¹².
5. Conduct the sequencing of fragments. Re-amplify approximately 1,400 bp of the PCR external products with the protocol for PCR amplification previously described (step 7.4) and clone according to the procedure suggested in literature¹². Isolate the recombinant plasmid from *E. coli* colonies as suggested in literature¹². Do conduct the partial procedure for sequencing with M13 universal primers¹².

- Conduct the sequences analysis. Align the nucleotide sequences by using the Clustal X and manually adjust in the text editor. Perform BLAST searches of the NCBI database. (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>)¹².
- Obtain the nucleotide sequence accession numbers. Deposit the nucleotide sequences of the clones identified in the EMBL nucleotide sequence database (Gen-Bank/EMBL/DDBJ) under the corresponding accession numbers (*i.e.*, JQ713915eJQ713925 for sequences from amplicons)¹².

Representative Results

A typical behavior of the sulfate reduction in the bioreactor is shown in **Figure 5**. It is important to notice that during the first weeks of operation sulfate reduction will be slow. However slow, the consumption of over 90% of sulfate over time indicates that the inoculum is developing a microbial community capable of reducing sulfate and therefore, enriched in sulfate reducing bacteria. The different periods in the figure indicate that sulfate reduction was increasing its rate over time. At the beginning, the batch took 20 days for sulfate to be reduced (Period I), then its reduction rate was increasing, and it took approximately 10 days to reduce the fed sulfate (Period II). Period III presented less variation in sulfate reduction and this was achieved in an average of 10 days as it is seen in **Figure 5**. After this period, the reduction of sulfate took an average of 4 days (Period IV) and in period V the 4,000 mg/L of sulfate were consumed in less than 24 hr. The performance in the bioreactor was set under continuous mode after 200 days at HRT of 24 hr.

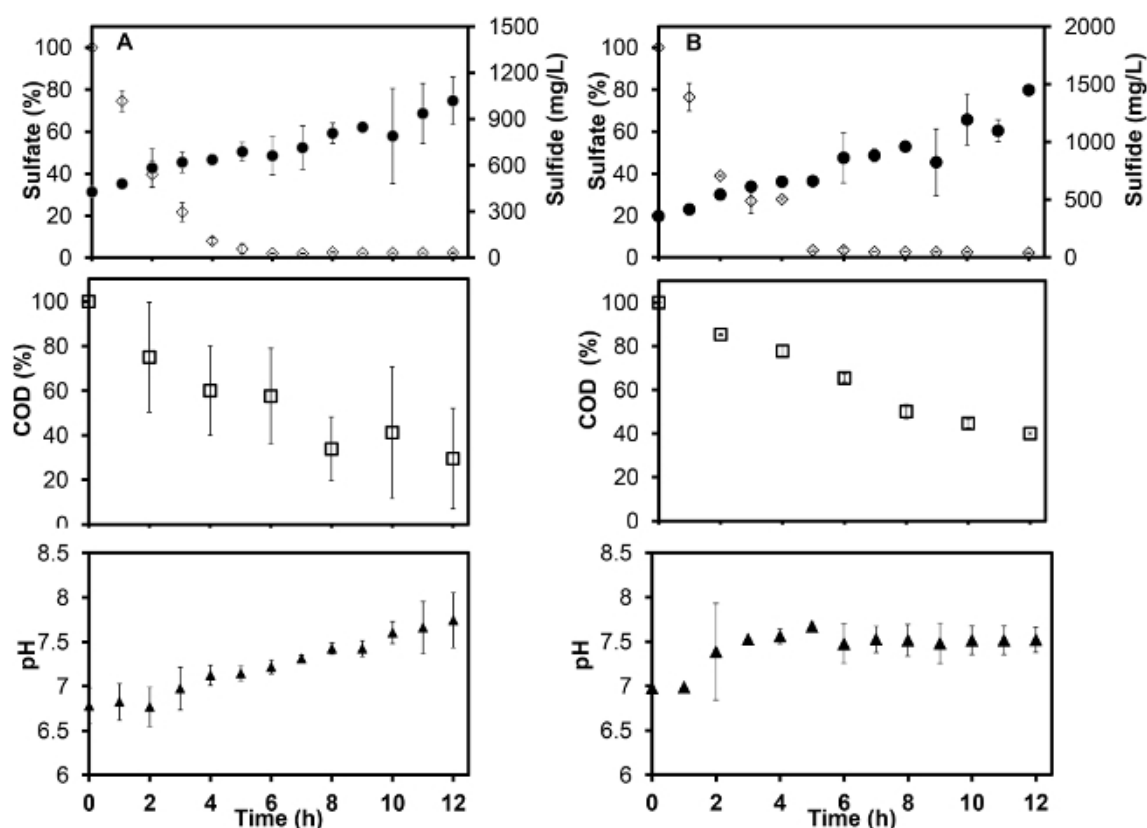


Figure 5. Sulfate (SO_4^{2-}) concentration over time during the sulfidogenic sludge formation in the bioreactor. The continuous line refers to influent sulfate concentration. Squares refer to sulfate concentration in the effluent. This figure has been taken from Guerrero-Barajas *et al.* (2014)¹² with the corresponding copyright permission. [Please click here to view a larger version of this figure.](#)

Representative good results on sulfate reduction, sulfide concentration, COD consumption and pH variations over time are shown in **Figure 6A**. These results were obtained in experiments conducted once the bioreactor was under continuous regime almost for a year. At this point the microbial community was developed in the bioreactor and a black sludge was formed as it can be seen in **Figure 4B**. In **Figure 6A** it can be seen that sulfate was reduced in 4 hr and sulfide reached a maximum concentration of $1,200 \pm 30$ mg/L and 188 ± 50 mg COD- $\text{H}_2\text{S/g VSS}^*\text{d}$ was the sulfate reducing activity. It is worth to mention that the concentration of sulfide obtained in the bioreactor is considered high and toxic to microorganisms, and in this case the bioreactor did not cease the sulfate reducing activity.

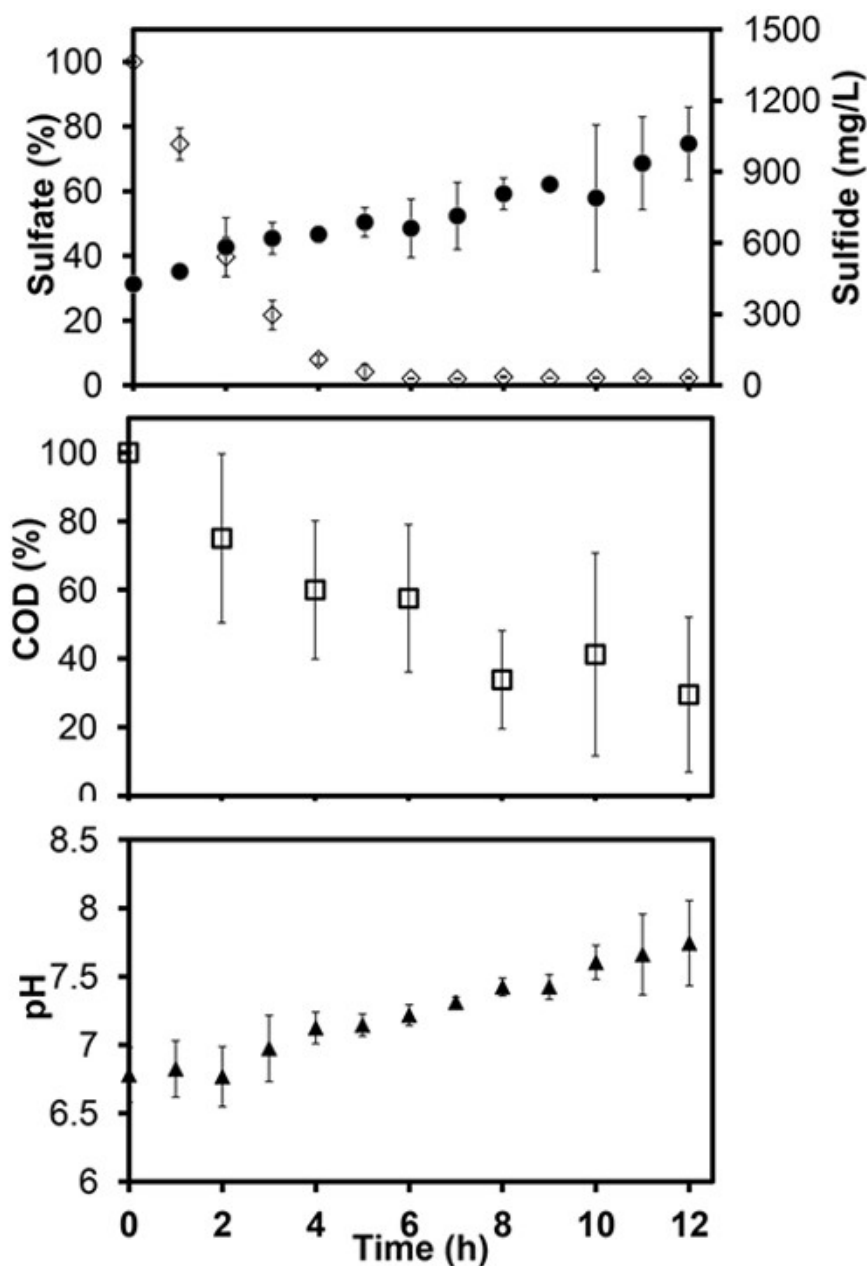


Figure 6. Performance of the bioreactor before (A) and after (B) the TCE addition. Sulfate (SO_4^{2-}) (#), Sulfide (H_2S) (•). Data presented are the average and standard deviation of $n=3$. This figure has been taken from Guerrero-Barajas *et al.* (2014)¹² with the corresponding copyright permission. [Please click here to view a larger version of this figure.](#)

For the experiment in which the sludge was tested on the capability to reduce TCE, the results obtained are shown in **Table 1**. The sulfate reducing activity obtained was slightly lower than the obtained before the TCE addition and approximately 80% of the TCE was reduced to ethene (see **Table 1**). These results should be the expected according to the time in which the reactor had been operating under sulfate reducing conditions. It is unlikely that TCE reduction occurs if the sludge is taken when the reactor is operating in Period I or II (see **Figure 5**) because the sulfate reduction is not steady yet.

Parameter	Value during the TCE reduction test
Percentage of SO_4^{2-} removal (%)	98 (± 0.06)
Sulfide (H_2S) concentration (mg/L)	971 (± 72)
Percentage of conversion of SO_4^{2-} to H_2S (%)	68 (± 2)
Percentage of COD removal (%)	93 (± 0.1)
pH range	7.1 - 7.7

Gas production (ml/d)	200 (± 55)
Sulfate reducing activity (mg COD-H ₂ S/ g VSS*d)	161 (± 7)
*TCE final concentration (μM)	77 (± 8)
Vinyl Chloride concentration (μM)	16 (± 0.3)
Ethene concentration (μM)	202 (± 81)
Percentage of TCE removal (%)	74.3 (±14)

Table 1. Results on the performance of the sulfidogenic sludge during the TCE biodegradation experiment. This table has been modified from Guerrero-Barajas *et al.* (2014). *TCE initial concentration: 300 μM. Data presented are the mean and standard deviation of n=3.

After the TCE reduction test, the results for sulfate reduction, sulfide concentration, COD consumption and pH variations over time are shown in **Figure 6B**. These results show that sulfate was reduced in 5 hr and sulfide concentration reached 1,400 ± 35 mg/L, along with a sulfate reducing activity of 248 ± 22 mg COD-H₂S/g VSS*d. The slightly higher sulfate reducing activity — compared with 188 ± 50 mg COD-H₂S/g VSS*d — indicates that the microbial community was not inhibited by the TCE.

Results on the microorganisms identified in the sludge used in this protocol are presented in **Table 2**. Sulfate reducing bacteria, fermenting bacteria and dehalogenating bacteria were identified in the sludge developed by using this protocol. The results are not surprising because the bioreactor was operating during one year under sulfate reducing conditions and several days with TCE. Genera of bacteria such as *Desulfovibrio*, *Desulfomicrobium*, *Desulfotobacterium*, *Clostridium*, *Dehalobacter* and *Sulfurospirillum* have been related to sulfate reduction and biodegradation of chlorinated compounds. Furthermore, the identification of these microorganisms in the sludge confirms that the protocol was successful in developing a sulfidogenic sludge that may be used for simultaneous removal of sulfate and trichloroethylene, which is one of the most toxic chlorinated compounds.

Bacteria reported	High similarity	max ident
gi 386685641 Uncultured <i>Desulfovibrio</i> sp.	<i>Desulfovibrio desulfuricans</i>	96%
gi 386685640 Uncultured <i>Desulfomicrobium</i> sp.	<i>Desulfomicrobium norvegicum</i>	99%
gi 386685639 Uncultured <i>Desulfomicrobium</i> sp.	<i>Desulfomicrobium baculatum</i>	99%
gi 386685638 Uncultured <i>Desulfomicrobium</i> sp.	<i>Desulfomicrobium hypogaeum</i>	99%
gi 386685637 Uncultured <i>Desulfotomaculum</i> sp.	<i>Desulfotomaculum acetoxidans</i>	99%
gi 386685636 Uncultured <i>Clostridium</i> sp.	<i>Clostridium celerecrescens</i>	99%
gi 386685635 Uncultured <i>Desulfovibrio</i> sp.	<i>Desulfovibrio halophilus</i>	98%
gi 386685634 Uncultured <i>Dehalobacter</i> sp.	<i>Dehalobacter restrictus</i>	99%
gi 386685633 Uncultured <i>Desulfotobacterium</i> sp.	<i>Desulfotobacterium hafniense</i>	99%
gi 386685632 Uncultured <i>Sulfurospirillum</i> sp.	<i>Sulfurospirillum multivorans</i>	97%
gi 386685631 Uncultured <i>Sulfurospirillum</i> sp.	<i>Sulfurospirillum halorespirans</i>	97%

Table 2. Consortium identified in the sludge of the bioreactor and its similarity to other bacteria. Max ident: maximum identity.

Discussion

There are several applications of sulfidogenesis in environmental biotechnology, one of the most used applications of the metabolism of sulfate reducing bacteria in consortia with fermenting bacteria is in wastewater treatment. UASB reactors are among the main engineered approaches to industrial wastewater treatment with high sulfate concentrations. In this work, we present a protocol to obtain sulfidogenic sludge from marine sediments in a UASB reactor. The critical steps within the protocol to obtain a sulfidogenic sludge from marine sediments are: (1) promoting the homogeneity of the sediment sample to be placed in the reactor, (2) feeding the right concentration of sulfate and volatile fatty acids (COD/SO₄²⁻) ratio, (3) analyzing the sulfate, sulfide, COD and pH periodically, (4) refreshing the basal medium, which contains trace metals and vitamins every time a batch is started. It is important to consider that the development of the sludge depends greatly on the microbial community of the original sample and on the right mass balances (sulfate and COD). Although it takes time for the sulfate reducing activity to develop, the bioreactor requires continuous monitoring to avoid leaks, accidents, or temporal lack of energy that may stop the recirculation pump or shut down the temperature control. Special attention must be paid in the performance of the analysis of sulfide, sulfate and COD.

Marine sediments in general are a natural source of a wide variety of microorganisms; it is likely that sludge can be formed with marine sediments taken from different depths. In this protocol it was chosen to use hydrothermal marine sediments for two main reasons: (1) these shallow vents are located close to the coast and (2) these type of sites are more abundant in sulfides, which is an indication of sulfate reduction and therefore, of the presence of sulfate reducing bacteria. If anything, taking the sediments from any other place in the subsea floor will only require more time for the sludge to develop, although we think this is very unlikely.

Also, it is necessary to conduct several TCE reduction tests to promote the presence of dehalogenating microorganisms in the sludge to be further identified by following the procedure suggested in this protocol or any other molecular biology technique. It is important to consider that

the development of dehalogenating microorganisms also depends on the source of the sludge, although literature refers to the presence of this type of microorganisms in several sea sediments around the world⁷. A sulfate reducing activity assay is recommended after the exposure of the sludge to the toxic compound in order to evaluate the viability of the sulfidogenic sludge after a stressful condition, e.g., the presence of TCE. If the sulfate reducing activity of the sludge decreases it will be necessary to maintain it several weeks in a batch mode fed with sulfate and COD to promote again the increase of sulfate reducing activity. This can be done by following the steps suggested in the protocol to promote sulfidogenesis and growth of the microorganisms. It is important to mention that it was attempted to obtain a sulfidogenic-dehalogenating sludge by adding TCE and sulfate since the beginning of the culturing of the sediments, but no activity (either sulfate reducing or dehalogenating) was ever observed. It was assumed that the low content of microorganisms present in the early stages of the culture did not tolerate the TCE (even at 20 μM concentration), however, the addition of TCE since the beginning of the enrichment can always be attempted with other sediments.

A good result in this case was the reduction of TCE to ethene, however, depending on the microorganisms that could have developed in the sludge the reduction of TCE may yield mainly dichloroethenes and vinyl chloride (VC). In this case the data obtained for the TCE reduction were recorded after each HRT cycle in the bioreactor instead of sampling for TCE and its intermediate products constantly during the test. It was done in that way in order to avoid evaporation of the chlorinated compounds due to frequent sampling. Therefore, there are no graphs of TCE concentration over time to show but the concentrations at the end of each experiment. It is important to avoid evaporation of the volatile chlorinated compounds to report accurate concentrations of them and to distinguish if the changes can be attributed to biological reduction instead of losses. The presence of ethene and the absence of common intermediates of TCE such as *cis*-1,2-dichloroethylene in the final products after the TCE reduction is particularly interesting. It has always been reported that the complete TCE reduction to ethene can be carried out only by *Dehalococcoides* sp. and in this case these microorganisms were not detected among the genera identified in the sludge. We attribute the TCE reduction to the cometabolism that may have arisen in the sludge among the dehalorespiring, the fermenting and the sulfate reducing bacteria. A shortcoming in this consortium is that it promotes the transient formation of VC, a compound more toxic than TCE, however, it is present at relatively low concentrations. It may be possible to say at this point that a very unusual cometabolic activity was developed by the consortium and that a new TCE reduction pathway under sulfate reducing conditions can be further investigated in the presence of the halo-respiring microorganisms identified in this work. On the other hand, it has been previously reported that dehalogenating genes are frequently detected in subsurface marine sediments but there are no reports on TCE biodegradation with subsea sediments combined with sulfate reducing conditions, this is, using sulfate as an alternate electron acceptor.

The protocol can be modified in the substrates used for sulfate reduction. For example, in this work we used a mixture of acetate, propionate and butyrate because they are used by many sulfate reducers and in the sea are easily found short chain fatty acids that serve as electron donors for sulfate reduction and also they are used by fermenting bacteria^{5,6}. We would recommend combinations such as: acetate-butyrate, propionate-butyrate or only butyrate. However, we do not recommend the utilization of lactate because when it was used, we experienced the development of sulfate reducing bacteria, but the accumulation of acetate in the bioreactor and the aim of this method is to obtain a sludge that can use acetate. Acetate accumulation is a shortcoming in many of the sulfidogenic reactors reported in literature. Also, we have not attempted the usage of alcohols. On the other hand, we do recommend — if desired — starting with lower concentrations of sulfate (i.e., 1 g/L) and gradually increasing the concentration while maintaining the appropriate mass balance, i.e., the correct concentration of COD in the bioreactor (COD/SO₄²⁻ ratio). It is not suitable, however, to start with higher concentrations of sulfate (higher than 4 g/L), although it may be increased over time along with the corresponding COD. The COD/SO₄²⁻ ratio can be in a range between 0.67 and 2.5 to start, and it can be modified once the sludge is developed.

One of the limitations of this method is that the structure of the sludge depends on the physical characteristics of the sediment used and although in this method the sludge formed allowed a good fluidization of the reactor and formation of biofilm on the glass, we cannot predict how it would actually work using sediments of different locations. It may be possible to use smaller reactors to conduct preliminary tests and observe if it is feasible. If the intention is to operate with the sludge in a bioreactor, we do not recommend starting with microcosms. The suggestion is to set the reactor from the beginning, though with a smaller volume for the first attempt.

The method presented here is significant in that the sludge formed tolerates higher sulfide concentrations than other sulfidogenic sludges that are obtained through adaptation of methanogenic granular sludge to sulfidogenesis. The method promotes the formation of a sludge with a consortium that includes sulfate reducing bacteria and fermenting bacteria and is easily adapted to biodegrade toxic compounds. In this protocol we used TCE as an example of a toxic compound which has only been biodegraded under methanogenic conditions in UASB reactors. For example, if the purpose of the reactor is to biotransform chlorinated solvents it could be useful to gradually increase the solvent concentration, i.e., higher TCE concentration in the tests, while decreasing the sulfate concentration to stimulate dehalogenation over sulfate reduction. Although, according to the results obtained in this work, we still think that sulfate cannot be completely removed from the culture if sulfate reducing conditions are desirable. Moreover, sulfate reducing conditions should be maintained since some dehalorespirers are also sulfate reducers. The sludge generated under the conditions described in this protocol could be eventually used to remove simultaneously COD, sulfate, chlorinated toxic compounds and heavy metals. The heavy metals can precipitate with the sulfide produced by the sludge.

Finally, the sulfidogenic sludge formed can be tested for: (1) the tolerance of higher sulfide concentrations (e.g., to precipitate heavy metals), (2) the biodegradation of other organic pollutants, or (3) the consumption of hydrocarbons as electron donors instead of volatile fatty acids to further explore its applications in environmental biotechnology. It is important to consider that once the sludge is formed it can be used as seed sludge to inoculate other reactors. In which case, the sulfate reduction process will occur immediately and thus, it will not be necessary to wait a year to fully operate the bioreactor.

Disclosures

The authors Selene Montserrat García-Solares, Claudio Garibay-Orijel and Claudia Guerrero Barajas submitted (as inventors) in 2012 the application to obtain a patent entitled: "Process for the anaerobic treatment of industrial wastewater with high content of sulfate and chlorinated compounds". This submission has been approved to the stage of the formatting. The submission has Instituto Politécnico Nacional as the rights owner.

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