Bioremediation of acid mine drainage coupled with domestic wastewater treatment

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ABSTRACT

Acid mine drainage (AMD) – characterized by high acidity and elevated sulfate and metal concentrations – represents a big environmental concern. Biological sulfate reduction has become an alternative to the classical physicochemical methods. In this study, domestic wastewater (DW) was tested as a cost-effective carbon-source for the remediation of AMD. Sediments from Tinto River, an extreme acidic environment with an elevated concentration of metals, were used as inoculum. Three anaerobic bioreactors with different microbial supports were fed with a 1:10 (v:v) mixture of synthetic AMD:DW. Around 50% of the organic matter present in the DW co-precipitated with the metals from the AMD previous to feeding the reactor. Therefore, the reactors had to be supplemented with an extra carbon-source (acetate) to achieve higher S elimination. Elevated removal efficiencies of chemical oxygen demand (COD) (>88%), sulfate (>75%), Fe (>85%) and other dissolved metals (>99% except for Mn) were achieved. Bacterial communities were examined through denaturing gradient gel electrophoresis and scanning electron microscopy. Higher biodiversity was found in the bioreactors compared with that of the inoculum. Dominant species belong to two metabolic groups: fermentative (Clostridium spp., Delftia spp., Paludibacter spp. and Pelotomaculum spp.) and sulfate-reducing bacteria (Desulfomonile spp., Desulfovibrio spp., Desulfosporosinus spp. and Desulfotomaculum spp.).

Key words | acid mine drainage, bioremediation, sulfate-reducing bacteria, Tinto River

INTRODUCTION

Acid mine drainage (AMD) is characterized by a low pH and high concentrations of sulfate and heavy metals. When a sulfide-bearing material is exposed to oxygen and water, iron- and sulfur-oxidizing chemolithoautotrophs, such as Acidithiobacillus ferrooxidans, can accelerate AMD production by assisting in the breakdown of sulfide minerals. Although it occurs naturally, it is mainly associated with mining activities. AMD reaches waterways and groundwater, producing long-term harm to water quality and biodiversity. Globally, AMD has become a major environmental concern because of its toxicity, extent and worldwide distribution (Neculita et al. 2007).

Biological treatment of AMD with sulfate-reducing bacteria (SRB) has been under research as a promising alternative to chemical methods. SRB are able to degrade a large amount of different substrates to reduce sulfate to hydrogen sulfide (Odom et al. 1993), preferring simple organic compounds or hydrogen as electron donors. The hydrogen sulfide produced reacts with dissolved metal ions forming low solubility metal sulfide precipitates. The SRB concurrently consume hydrogen ions and produce carbon dioxide which generates alkalinity, thereby increasing pH levels (Kaksonen et al. 2003a).

Application of this biological feature to AMD remediation is usually carried out in bioreactors because important variables such as pH, hydraulic retention time (HRT), sludge retention time (SRT) and organic loading, can be better controlled and optimized for long term performance. In active sulfate-reduction bioreactors, the main variable when evaluating the cost-effectiveness of this method is the electron donor used. Several simple compounds (e.g. lactate, ethanol) (Kolmert & Johnson 2002; Kaksonen et al. 2003a) and complex substrates (e.g. molasses, manure) (Choudhary & Sheoran 2011) have been used. In this study domestic wastewater (DW) is used as a cost-effective source of organic matter and macronutrients (nitrogen and phosphorous) for.
SRB. In prior studies (Johnson & Younger 2006; Strosnider & Nairn 2010), DW has been used for the remediation of AMD, although little attention was paid to the role of sulfate reduction. Microbial communities are responsible for this process, so a goods election of the inoculum is crucial. Previous studies in the extreme acidic environment of Tinto River (Huelva, Spain) have suggested the presence and activity of a sulfate-reducing microbial community in its anaerobic zones (Sánchez-Andrea et al. 2011), therefore Tinto River sediments were used as inocula.

The aim of this study was first, to assess the feasibility of coupling AMD bioremediation with the treatment of DW through a sulfate reduction process in anaerobic bio-reactors. Next, to evaluate three different inocula made with different support material by following the efficiency of the process (sulfate reduction, metal precipitation and pH attenuation). Then, to investigate the influence of hydraulic resident time (HRT), influent pH and extra addition of electron donors. And finally, to examine bacterial diversity and its shifts at different times through denaturing gradient gel electrophoresis (DGGE), and through scanning electron microscopy (SEM).

MATERIALS AND METHODS

Site sampling and inocula preparation

Sediments used as inoculum were collected from the J/L dam (37.691207N, 6.560587W) at Tinto River basin (Huelva, southwestern Spain) in November 2010 as described previously (Sánchez-Andrea et al. 2011).

An enrichment culture was grown in a 1 l anaerobic bottle containing 100 g of sediment and 500 ml of the following sterilized medium (in g/l): 3.5 sodium lactate, 2 yeast extract, 2 MgSO₄·7H₂O, 1.5 Na₂SO₄, 0.5 K₂HPO₄·3H₂O, 0.05 l-Cysteine, 0.4 Fe(NH₄)₂(SO₄)₂·6H₂O, 0.1 CaCl₂; pH was adjusted to 5.5. The culture was incubated statically at 30°C. After one month of incubation, an anaerobic bottle of 1 l containing 500 ml of medium and 150 g of granular activated carbon (Chemviron F-400, Aguas de Levante, Spain) was inoculated with 10% inoculum from the aforementioned enrichment.

SRB inocula for the three reactors were prepared as follows. For reactor 1 (R1) granular activated carbon (GAC) from the second enriched culture described above was placed in the reactor. For reactor 2 (R2), sediment from the first enriched culture described was mixed in a 1:1 proportion with melted agar (1% at 41°C). For reactor 3 (R3), 500 ml of the supernatant from the second enrichment culture was concentrated to 100 ml through centrifugation, and mixed with 100 ml of melted agar (2% and 41°C). Both gels obtained were cut with a sieve of 2 mm diameter. The three different inocula (made by cells adhered to GAC and cells immobilized in agar) were incubated in batch mode for 3 weeks in the AMD:DW medium, which was replaced weekly (Figure 1).

Experimental design

Synthetic AMD was made with a composition close to that observed in Tonto River (Gonzalez-Toril et al. 2003) (in mg/l): 11449 FeSO₄·7H₂O or 8522 Fe₂(SO₄)₃·H₂O, 660 ZnSO₄·7H₂O, 196 Mn(NO₃)₂·4H₂O, 196 CuSO₄·5H₂O, 40 CoCl₂·6H₂O, 9 NiSO₄·6H₂O; pH of the synthetic AMD was 2.5. DW was collected from the campus wastewater treatment plant of the Autonomous University of Madrid and it was settled prior to being used. DW had a mean dissolved chemical oxygen demand (COD) of 400 mg-COD/l. The pH of the DW ranged from 7.4 to 8.9.

Three laboratory-scale upflow anaerobic sludge bed (UASB) reactors (0.5 l) were fed with the previous inocula prepared and operated for 118 days at 30 ± 1°C (Figure 1). The reactors were fed with a 1:10 ratio (v:v) of AMD:DW, experimentally implemented, with the addition of acetate (150 mg/l) from day 71 on. The AMD:DW mix was prepared twice a week (stored at 4°C) and precipitates were removed from the feed tank once a week. The experiment was divided into five phases (Figure 1). Phase I corresponded to the adaptation of the inocula and stabilization of the system. In phase II the HRT was fixed at 48 h. During phases I and II, the pH of the influent varied from 4.8 to 6.4. From phase III onwards, the influent pH was adjusted to 5 with the addition of HCl or NaOH when necessary. In phase IV the addition of acetate began and finally in phase V the HRT was reduced to 24 h. COD, sulfate, total Fe and pH were measured twice a week during phases I to IV and three times during phase V. Metals were measured twice for every operational phase. Rates of sulfate reduction, dissolved COD removal and total Fe precipitation were determined as the difference between feed and effluent concentrations. Samples of the biomass were taken on days 0, 67, and 118 for DGGE analysis, and on days 0 and 118 for SEM.

Reactor tracking

For chemical analyses, effluent samples were centrifuged at 13,000g for 10 min and the supernatants taken for analysis.
of COD, sulfate and dissolved total Fe (Fe_{tot}). The COD was determined according to method 5220D, *Standard Methods* (APHA 1995) using a COD Reactor (Hach, USA) and a spectrophotometer (Pharma Biotech Novaspec II, Sweden). Sulfate concentration was determined by ion chromatography with a suppressed conductivity detector (790 Personal IC with an A Supp 5 250/4.0 column Omega Metrohom, Switzerland). Measurements for pH were obtained with an Orion 2-Star portable pH meter (USA). Iron species (ferric, ferrous and total) were determined by reflectometry (Iron test method 1.16983.0001, Reflectoquant, Merck, Germany). For the determination of dissolved metals, samples were stabilized with nitric acid and were determined by inductively coupled plasma (ICP-MS Elan 6000 Perkin Elmer Sciei, USA).

For biological tracking, microbial samples for 16S rRNA analyses were taken at different times throughout the experiment: at the starting point (day 0) from the sediment and the enrichment culture before support preparation (AC), at day 67 (before acetate supplementation) and day 118 (end of the experiment) from the three reactors (R1, R2 and R3). DNA extraction and DGGE was carried out for bacterial V3 to V5 variable regions of the 16S rRNA gene as described previously (Sánchez-Andrea et al. 2011). A total of 57 bands were successfully sequenced using a Big-Dye sequencing kit (Applied Biosystems) following the manufacturer's instructions. A comparative analysis of all the sequences was done using the BLAST routine from NCBI employing the GenBank database (http://www.ncbi.nlm.nih.gov/Blast). Sequences were deposited in GenBank under accession numbers: JQ517512-JQ517538. Samples for SEM were treated as previously described (Sánchez et al. 2008).

**RESULTS AND DISCUSSION**

**Water quality**

The mixture of DW and AMD, *per se*, started the remediation process chemically. Several minutes after mixing them, flocks appeared due to the coagulant properties of metal cations, which cause the co-precipitation of suspended and colloidal organic matter and metals; 51% of Fe, 31% of Mn, 59% of Co, 49% of Ni, 68% of Cu and 34% of Zn were removed by this mechanism.
Throughout the experiment, R1 and R2 showed similar evolution patterns for COD, sulfate and Fe\textsubscript{tot} removal (Figure 2). However, the efficiencies were slightly higher for R1 than R2 during phase I to III (COD, sulfate and Fe removal: 62%, 39%, 51% for R1 and 46%, 25% and 41% for R2). Significant fluctuations in the removal efficiencies were observed during these phases, possibly due to the variations of the DW influent. As observed, the mean COD removal efficiencies were 62% and 46% for R1 and R2, even with electron acceptor excess, which might reflect

![Figure 2](https://iwaponline.com/wst/article-pdf/66/11/2425/441082/2425.pdf)

**Figure 2** Removal efficiencies (%) and loading rates (mg/d) of COD, sulfate and Fe\textsubscript{tot} for R1-AC (a), R2-sediment-agar (b) and R3-liquid culture-agar (c).
the non-degradable nature of some compounds of the domestic water. Supporting this is the fact that after acetate supplement, removal efficiencies increased notably for the three parameters (88, 75 and 85%), and became more stable. According with these results, a fraction of the DW organic matter (24 ± 3%) seems to be scarcely biodegradable, limiting sulfate reduction. For this reason, sulfate reduction efficiency seems to be coupled to the COD:SO$_4^{2-}$ ratio (1:1) more than the theoretical ratio established as 0.67 mg-O$_2$:1 mg-SO$_4^{2-}$.

The removal efficiencies for R1 and R2 were similar during phases IV and V, thereafter, the highest removal rates were achieved at phase V when HRT was decreased from 48 to 24 h. Removal rates were very close for the two reactors with values as high as 350 mg-COD/l d, 340 mg SO$_4^{2-}$/l d and 150 mg total Fe/l d. The reduction of HRT did not affect the COD and Fe removal efficiencies, only sulfate removal efficiency decreased from 82 to 68%. The sulfate removal rates were observed similar to those reported by Greben (Greben & Maree 2005) although they were lower than those seen in other studies of AMD bioremediation at low pH (Kolmert & Johnson 2001; Kaksonen et al. 2003b; Greben & Maree 2005). As these studies used simple organic substrates, lower rates can be explained by the use of a complex source of organic matter, which is not readily available for the SRB. Moreover, R1 and R2 were performing well, thus, HRT could have been lowered.

As the performance of R3 during phase I was very low, the reactor was re-inoculated with fresh inoculum (days 11 to 32). The highest removal rates were achieved during phase IV, with removal efficiencies that were slightly lower than the ones for R1 and R2. However, at phase V, R3 efficiencies dropped drastically. The overloading of the reactor, confirmed by a decrease in the pH, or the diffusion limitation of the nutrients inside the cell, suggested by SEM observations, could be the reasons.

Throughout the experiment, an increase of the pH effluent was observed in the three reactors. This increase is caused by the inorganic carbon formed during anaerobic respiration (Kaksonen et al. 2003b). Effluent pH stayed at approximately 6.0 for the three reactors through phases I to III. After the addition of acetate, pH values increased up to 6.3 and were more stable, except for R3 at phase V when pH dropped to 5.7.

**Metal removal**

Metal removal efficiencies were elevated (above 99%) for Fe, Co, Ni, Cu and Zn for R1 and R2, releasing an effluent with low metal concentrations. The performance of R3 was also very satisfactory for metal removal (higher than 98%) with the exception of Fe (Table 1). Mn removal was low for the three reactors, even reaching negative values, which can be explained by an accumulation in the reactor during previous phases. Poor performance of Mn removal is explained by its chemistry: Mn has to be oxidized from Mn$^{2+}$ to Mn$^{4+}$ before being removed as MnO$_2$ (Hallberg & Johnson 2005).

**DGGE**

The microbial community and its variation throughout the experiment were analyzed by DGGE.

Methanogenic populations were not investigated due to the low activity that they might present at the conditions tested in this experiment. Sulfate-reducers dominate when competing with each other over limited resources and moreover at low pH and high sulfate and metal content. Comparing the band pattern from time 0 with the end of phases III and IV (days 67 and 118), an increase in the microbial diversity in the samples from the reactor is observed (Figure 3) possibly due to a diversification of the community to adapt to a complex source of nutrients, like DW, and the colonization of carriers by bacterial species present in the DW.

The sequences obtained (Table S1 in the Supplementary Material) fell into two main metabolic bacterial groups: (i) fermentative bacteria, which would metabolize complex substrates: Clostridium spp. (bands C1, C2, C3 and C4), Delftia spp. (band H5), Paludibacter spp. (bands E2, E3, F1, H1, H2 and H3), and Pelotomaculum spp. (syntrophic bacterium, band D6) and (ii) SRB, which would use the intermediate molecules produced by fermenters or the added acetate: Desulfoxonile spp. (bands D4, D5, E7 and E8), Desulfovibrio spp. (bands F4, H6, H7, H8, and H9), Desulfitomaculum spp. (band G7) and Desulfospiribacillus spp. (Clostridia, bands E4, E5, E6 and G2).

Several bacterial sequences identified in this study have a high similarity to those identified in a recent study of Tinto River sediments (Sánchez-Andrea et al. 2011). For instance, bands E2, E3, F1, H1, H2, H3 share a high similarity with HQ730741 (uncultured Paludibacter sp. detected by DGGE) and band A5 shares a high similarity with HQ730647 (uncultured Peptostreptococcaceae identified by cloning). These organisms were found in those sediment strata with reducing redox potential and less acidic pH (5.3–5.4). Other bands (E4, E5, E6, E7) are related with HM745405 (Desulfospiribacillus spp.), a sequence retrieved...
from a close area of the Iberian Pyritic Belt (Gonzalez-Toril et al. 2014). Bands F4 and H8, identified in R2 and R3 at day 118, share a high similarity with FJ873799 (Desulfovibrio desulfuricans strain BSR-22). This organism has been reported as the predominant SRB species in a sulfate reducing bioreactor working at pH 4–4.5 (Bijmans et al. 2013).

Furthermore, other identified bacteria, for instance bands D4 and D5, seem to be related with AY167450 (uncultured Desulfomonile) found in an acidic environment.

**SEM**

Samples from the three reactors were examined through SEM at the start and end of the experiment (Figure S1 in the Supplementary Material, available online at http://www.iwaponline.com/wst/066/477.pdf). In R1, initial colonization with cocci and coccobacilli as predominant organisms, was observed over the surface of the activated carbon used as a support. At the end of the experiment, surface images showed the formation of a dense biofilm. In R2, Tinto River sediments used as inoculum showed, from beginning to end, little colonization and non-biofilm formation. An electron back-scattered filter of the inner part of the bacterial support in R3 was used to differentiate between organic and metallic structures showing that cells were coated with metallic compounds. Metal sulfides could have precipitated onto bacterial cell walls, isolating them from the surrounding environment and preventing the access of reactants to the cell (Utgikar et al. 2005). This may have been the reason for the poor performance of R3 throughout the experiment and the cause for the collapse in phase V. This phenomenon, called encapsulation (Remoundaki et al. 2008), may have
also occurred in R1 and R2, however either bacterial growth could have overcome the rate of cell encapsulation or a better inner structure may have allowed internal bacterial development protected from the outer metal precipitation.

CONCLUSIONS

Results show that it is possible to bio-remediate AMD by its co-treatment with DW in anaerobic bioreactors. Around half of the dissolved metal ions were chemically removed along with organic matter just by mixing AMD and DW. Good heavy metal removal efficiencies were achieved during the operation. Additional substrate supply made elevated pollutant removal efficiencies possible (COD > 88%; sulfate > 75%; Fe\text{tot} > 85%; and dissolved metals >99% except Mn), leading to a good effluent quality with neutral pH. No important differences were found between R1 and R2, but the agar matrix used for R3 was the least efficient, collapsing in the last phase of the experiment. Two groups of bacterial metabolism were found by DGGE: fermenters and SRB.

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Irene Sánchez-Andrea and David Triana contributed equally to this work.

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