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Microbial aerobic and anaerobic degradation of acrylamide in sludge and water under environmental conditions—case study in a sand and gravel quarry

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Abstract Polyacrylamides (PAMs) are used in sand and gravel quarries as water purification flocculants for recycling process water in a recycling loop system where the flocculants remove fine particles in the form of sludge. The PAM-based flocculants, however, contain residual amounts of acrylamide (AMD) that did not react during the polymerization process. This acrylamide is released into the environment when the sludge is discharged into a settling basin. Here, we explore the microbial diversity and the potential for AMD biodegradation in water and sludge samples collected in a quarry site submitted to low AMD concentrations. The microbial diversity, analyzed by culture-dependent methods and the denaturing gradient gel electrophoresis approach, reveals the presence of Proteobacteria, Cyanobacteria, and Actinobacteria, among which some species are known to have an AMD biodegradation activity. Results also show that the two main parts of the water recycling loop—the washing process and the settling basin—display significantly different bacterial profiles. The exposure time with residual AMD could, thus, be one of the parameters that lead to a selection of specific bacterial species. AMD degradation experiments with 0.5 g L^{-1} AMD showed a high potential for biodegradation in all parts of the washing process, except the make-up water. The AMD biodegradation potential in samples collected from the washing process and settling basin was also analyzed taking into account on-site conditions: low (12 °C) and high (25 °C) temperatures reflecting the winter and summer seasons, and AMD concentrations of 50 μg L^{-1}. Batch tests showed rapid (as little as 18 h) AMD biodegradation under aerobic and anaerobic conditions at both the winter and summer temperatures, although there was a greater lag time before activity started with the AMD biodegradation at 12 °C. This study, thus, demonstrates that bacteria present in sludge and water samples exert an *in situ* and rapid biodegradation of AMD at low concentration, whatever the season, and in both the aerobic and anaerobic parts of the water recycling system.

Keywords Acrylamide biodegradation · Microbial diversity · Proteobacteria · Actinobacteria · Polyacrylamide · Flocculant

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Introduction

Polyacrylamides (PAM) are linear anionic, cationic, or neutral acrylamide (AMD) polymers (or copolymers) that are widely used as flocculants in the industrial field (wastewater, food industry, chemical industry, etc.) to improve water treatment (Haveroen et al. 2005). This is particularly the case in sand and gravel quarries. Most industrial quarries use water recycling loops with PAM to remove fine particles and clarify the process water since PAM flocculants enhance the settling of solids in the clarifier. Although PAM is considered as nontoxic, industrial preparations may nevertheless contain residual AMD monomers that can negatively impact the environment (Guézennec et al. 2014). AMD is classified as a carcinogenic (level 2), mutagenic (level 2), and reprotoxic (level 3) compound (World Health Organization 1985; Molak 1991). It is listed in the Registration, Evaluation,

Authorisation and Restriction of Chemicals regulation as a carcinogenic category 1B and mutagenic category 1B substance (European Parliament 2006b). Any AMD released into the environment may have toxic effects on living organisms as it can interact with sulfhydryl proteins and thus disturb biological functions (Cavins and Friedman 1968). The amount of residual AMD can vary substantially depending on what measures were taken during the manufacturing process to maximize the extent of polymerization. Within the EU, all PAMs are required to contain less than 0.1 % (*w*/*w*) of residual AMD (European Parliament 1999) unless they are classified and labeled as a category 2 carcinogen (European Parliament 2006a). Knowledge about the fate of PAM and associated residual AMD in hydrosystems (transfer, reactivity, etc.) is limited or nonexistent where the use of PAM as a flocculating agent for the treatment of waters is concerned. However, AMD release into the environment has been highlighted by the legal authorities of several European countries (France, Spain, Norway, and Germany, among others) as a major environmental issue in the vicinity of quarries (UNPG 2013). Consequently, knowledge of the fate and dissemination of AMD in the environment and the remediation of AMD-contaminated sites are crucial for quarry operators. It is essential that AMD is effectively removed from the environment in order to avoid contamination of water.

The main AMD degradation pathway is associated with microbial degradation. Soil microorganisms, mainly bacteria, have been shown to use AMD as a source of nitrogen and/or carbon so leading to its degradation (Kay-Shoemake et al. 1998a, b). Well represented among the several bacteria that have been isolated from contaminated sites, mainly in soil, sediment, wastewater, and aquatic environments, is species within the genera *Bacillus* (Shukor et al. 2009) and *Pseudomonas* (Shanker et al. 1990; Nawaz et al. 1993; Sathesh Prabu and Thatheyus 2007). More recent studies have demonstrated that PAM and AMD degradation activities are also supported by many other bacterial species (see Guézennec et al. 2014, for a review); for example, microbial amidases catalyze the degradation of AMD to acrylic acid and ammonia (Sharma et al. 2009).

Laboratory-scale studies on the biodegradation of AMD have concentrated mainly on isolating and characterizing bacteria able to grow and degrade AMD in the contaminated environment. The experiments were thus performed with high AMD concentrations, usually >1,000 mg L^{-1}, under optimal (especially temperature) conditions for bacterial growth so as to better characterize the isolated bacteria and their potential for AMD degradation. For example, Brown et al. (1980) worked at 20 °C, Shukor et al. (2009) at 25–30 °C, and Buranasilp and Charoenpanich (2011) at 25 °C. However, *in situ* growth conditions are usually quite different from the optimal growth conditions in laboratory media or buffers; AMD concentrations can be significantly lower, and on-site temperatures will vary according to the season. It is, therefore, important to study the ability of microorganisms to degrade AMD *in situ* at low concentrations and low temperatures, without any molecule amendment such as nutrients. Labahn et al. (2010), using both high (up to 100 mg L^{-1}) and low (0.05 mg L^{-1}) concentrations of AMD, were one of the first to have carried out on-site experiments at different temperatures (25 °C and 15 °C). They also noted an anaerobic biodegradation of AMD.

The present study was carried out in a French sand and gravel quarry site, previously worked on by Touzé et al. (2014), where small amounts of AMD are introduced into the process slurry through the use of PAM-based flocculants containing residual acrylamide monomers. Touzé et al. (2014) had already confirmed the presence of a low concentration of AMD (0.41 to 5.66 μg L^{-1}) in the process water and sludge, as well as a variation in temperature according to the season. This study was thus aimed at characterizing the AMD biodegradation activity in the water and sludge samples collected on site through combining a microbial characterization of the samples (using culture-dependent and molecular [denaturing gradient gel electrophoresis] approaches) with assays of AMD biodegradation under both artificial (i.e., optimal) and natural conditions, the latter with a low AMD concentration and two process temperatures.

Material and methods

Site description

Our study was carried out on samples of water and sludge from a French sand and gravel quarry site that uses PAM-based flocculants in its recycling loop of the process water. The site, which has been fully described by Touzé et al. (2014), excavates unconsolidated sand, gravel (90 % quartz and 10 % feldspar), and pebbles (75 % quartz and 20 % other siliceous rocks). Treatment of the aggregate follows the normal processes of grinding, crushing, and screening, followed by a washing step. The process water leaving the washing unit is loaded with fines (<100 μm) that must be removed before the water is re-used in the aggregates washing step. The water circuit of the quarry is briefly summarized in Fig. 1, which also shows the sampling points of the present study. At the outlet of the washing unit (point P3), the water undergoes a flocculation step (injection of a concentrated PAM-based flocculant solution) followed by a settling step. The clarified water from the settling tank overflow (point P4) is then pumped to a buffer tank (clean-water tank) in which it is mixed with make-up water (point P1), the combined water being used to wash the aggregate (point P2). The fines are removed by underflow from the settling tank (point P5) and pumped to the settling basin (point P7) through a pipeline (2 km). The quarry

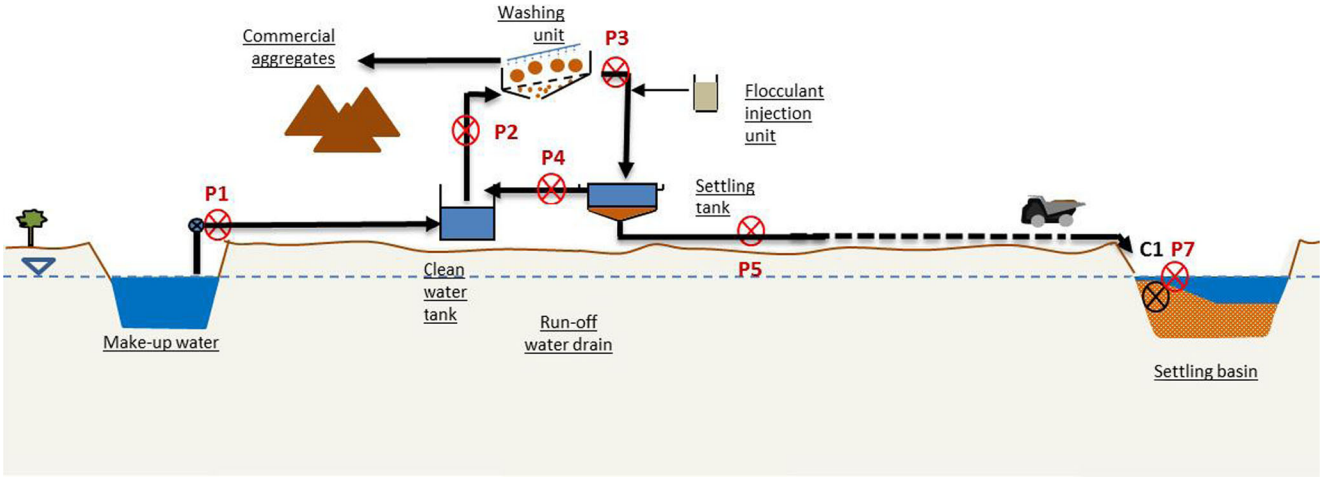

Fig. 1 Diagram of the quarry field site showing the process steps and sampling locations (Touzé et al. 2014)

recycles 80 % of its process water. The make-up water needed for the process is drawn from a pond next to the installation. The flocculant is an anionic polyacrylamide powder with a low charge density and high molecular weight (between 10 and 12×10^6 Da), which is prepared (i.e., dissolved) on site (4 g/L stock solution). The PAM feed is regulated automatically at an injection rate ranging from 350 to 700 g PAM/t fine particles. Touzé et al. (2014) demonstrate that the residual AMD present in the flocculants used by the quarry spreads out into the quarry's water circuit.

Sample collection

The water and sludge samples were collected at several points in the washing circuit (Fig. 1). Make-up water was sampled from the pond located near the installation (P1) and process water was collected both from the overflow canal of the settling tank (P4) and from the clean water tank (P2) by grabbing. Process sludge was collected both before the PAM injection point (P3) and after the PAM injection in the underflow of the settling tank (P5).

A boat was used in order to collect sludge samples from the settling basin. Unconsolidated sludge and water near the entrance of the basin (P7) were collected according to ISO standard 5667-4 (ISO 1987), and cores of consolidated sludge (C1) were collected at the same location according to ISO standard 5667-12 (ISO 1995). The coring was carried out to a depth of 1.5 m using an Eijekampf manual core sampler with three successive 50-cm stainless-steel tubes enabling the collection of undisturbed material.

All the tools used to collect each sample type were sterilized by autoclaving prior to the sampling campaign. The water and unconsolidated sludge samples were collected in July 2012, preserved in sterile amber glass bottles and stored at 4 °C. The consolidated sludge cores were sampled in November 2012, maintained in the sterile stainless-steel tubes, and also stored at 4 °C. The experiments were performed as soon as possible after sampling.

Microbial characterization using a culture-dependent method

Aliquots of the water samples (1 mL), sludge samples, and core samples (1 g) were mixed with 9 mL of saline solution (0.9 % *w*/*v* NaCl) and homogenized, following which serial tenfold dilutions were performed in saline solution. Counts of viable bacterial cells were carried out by plating 0.1 mL of each dilution on agar plates.

The following selective media were used:

- Plate count agar (30 °C for 72 h) for total microorganisms
- Malt extract agar (30 °C for 72 h) for yeasts
- Actinomycete isolation agar (Becton, Dickinson and Company, Sparks, Maryland) supplemented with 5 g L^{-1} glycerol (30 °C for 3–7 days) for actinomycetes
- Potato dextrose agar supplemented with 0.2 g L^{-1} chloramphenicol (30 °C for 3–5 days) for fungi
- HiCrome™ Bacillus Agar (HiMedia Laboratories, Mumbai, India) supplemented with Polymyxin B (30 °C for 48 h) for *Bacillus* spp.
- Columbia agar supplemented with 200 μg mL^{-1} streptomycin (30 °C for 7–14 days) for *Sphingomonas* spp. (Vanbroekhoven et al. 2004)
- Ashdown's selective agar supplemented with 4 mg L^{-1} gentamycin (35 °C for 48 h) and *Burkholderia cepacia* selective agar (30 °C for 4 days) for *Burkholderia* spp. (Miller et al. 2002; Howard and Inglis 2003; Glass et al. 2009)
- *Pseudomonas* CFC agar supplemented with 10 mg/L cetrimide, 10 mg L^{-1} fucidin and 50 mg L^{-1} cephalosporin (30 °C for 48–72 h) for *Pseudomonas* spp.

– Xan-D medium (30 °C for 3–5 days) for *Xanthomonas* spp. (Lee et al. 2009)

Except for not ready-to-use media, all media were purchased from Biokar Diagnostics (Beauvais, France).

Microbial characterization using denaturing gradient gel electrophoresis

The total genomic deoxyribonucleic acid (DNA) of the water and sludge samples was extracted by the FAST DNA Spin Kit for Soil (MP Biomedicals, Illkirch, France). For the water samples, DNA extraction was performed on concentrated microbial biomass collected after filtration of approximately 500 mL of water using a 0.2 μm-pore size membrane. For the unconsolidated sludge and the consolidated sludge core (C1) samples, DNA extraction was performed on approximately 1 g of sample.

The V1–V3 regions of the bacterial 16S rRNA genes were amplified using the primers used by Nexidia Company BACTF1 (5′-GTA TTA CCG CGG CTG CT-3′) and BACTR1 (5′-AGT TTG ATC ATG GCT CAG-3′) with a GC clamp attached to the reverse primer. PCR amplifications were performed in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, USA) using the HotStarTaq Plus DNA Polymerase (QIAGEN, Courtaboeuf, France). Thermocycling conditions were (a) an initial denaturation at 95 °C for 10 min, followed by (b) 35 cycles of 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min, and then (c) a final elongation at 72 °C for 10 min. Five microliters of the product was analyzed by standard agarose gel electrophoresis before denaturing gradient gel electrophoresis (DGGE) analysis to confirm product size and the negative control.

The DCode System for DGGE (BioRad Laboratories, Hercules, USA) was used for biodiversity analysis. PCR samples (20 μL of PCR products) were loaded onto 10 % polyacrylamide (37.5:1, acrylamide to bisacrylamide ratio) denaturing gels with a gradient from 40 to 55 % in 1× TAE. One hundred percent of denaturant corresponded to 7 M urea and 40 % (*v*/*v*) formamide. Electrophoresis was performed at a constant voltage of 100 V at 60 °C overnight. After electrophoresis, the gels were stained with an aqueous solution of GelRed™ Nucleic Acid Gel Stain (Interchim, Montluçon, France) and analyzed with a Gel Doc™ XR+system System (BioRad Laboratories, Hercules, USA).

Bands in the DGGE gels were excised under UV illumination and the DNA was extracted using a GenElute™ Gel Extraction kit (Sigma-Aldrich, Saint-Quentin-Fallavier, France). The extracted DNA was re-amplified with the specific primers described above for sequencing. The band sequences were then compared against the GenBank nucleotide data library using the Blast software at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/).

Determination of AMD biodegradation potential

The water samples (10 mL) and sludge samples (10 g), collected from P1, P2, P3, P4, P5, and P7, were inoculated in 90 mL of the mineral salt medium (MSM) described by Rousseaux et al. (2001), with some modifications, for a make-up (per liter of distilled water) of:

> 1.6 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g $MgSO_4$ $7H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2$, 10 mL of a sodium citrate stock solution (concentration 100 g L^{-1}), 1 mL of a salt stock solution*, 1 mL of a vitamin stock solution**, 1 mL of a $FeSO_4$ $6H_2O$ stock solution***, and 0.02 g L^{-1} of phenol red (used as pH indicator)****
> *The mineral salt stock solution contained 2 g L^{-1} boric acid, 1.8 g L^{-1} $MnSO_4$ H_2O, 0.2 g L^{-1} $ZnSO_4$, 0.1 g L^{-1} $CuSO_4$, and 0.25 g L^{-1} Na_2MoO_4.
> **The vitamin stock solution contained 100 mg L^{-1} of thiamine hydrochloride and 40 mg L^{-1} of biotin.
> ***The $FeSO_4$ $6H_2O$ stock solution contained 5 g L^{-1} $FeSO_4$ $6H_2O$.
> ****The pH of the medium was adjusted to 7.0±0.2.

Acrylamide (Merck, Hohenbrunn, Germany) was added separately to the growth flask at a final concentration of 0.5 g L^{-1}. The cultures were incubated aerobically in the dark at 30 °C for 72 h on an orbital shaker at 140 rpm.

The deamination of AMD leads to ammonia release, thus, enabling the amidase activity to be determined by quantifying the concentration of released ammonia (Kay-Shoemake et al. 1998a, b). This release of ammonia contributes to an increase of the growth medium pH, resulting in a color change of the pH indicator (phenol red) from red to pink. The increase in pH was monitored automatically with a multichannel pH meter (Consort D130). The bacterial growth was monitored by measurement of the absorbance at 600 nm.

AMD biodegradation in conditions representative of in situ conditions

Batch experiments for aerobic and anaerobic degradation of acrylamide were conducted in the laboratory using samples collected from the quarry site. The tests were performed in 500-mL glass flasks spiked with 50 μg L^{-1} AMD and incubated in the dark (to avoid AMD photodegradation) at both 12 °C and 25 °C with gentle shaking. The two temperatures correspond to the average winter and summer temperatures in the quarry's water circuit.

For the quarry water and sludge samples, 400 mL of sample was spiked with AMD to test the biodegradation.

For the cores of consolidated sludge, 10 g of sample was diluted in 400 mL of sterile saline solution (0.85 % *w*/*v* NaCl) before AMD amendment. The aerobic experiments were done with samples from P4 and P7, while the anaerobic experiments were done with unconsolidated sludge samples from P7 and consolidated sludge from the core samples (C1) of the settling basin. For the anaerobic experiments, the flasks were submitted to vacuum, N_2 flushing, and Na_2S amendment before AMD amendment.

AMD degradation activity was measured by regularly sampling 10 mL of the solution in the flasks. These samples were filtered with 0.2 μm pore-size membranes (PVDF filters) and analyzed using the method developed by Togola et al. (2014). The analyses, with a quantification limit of 20 ng L^{-1}, were performed immediately after sampling in order to avoid AMD degradation. All the degradation tests were performed in duplicate, and a control experiment was done by measuring AMD degradation in a sterile NaCl (0.85 %) solution.

Results

Microbial diversity of the sample collection

Enumeration of the mesophilic aerobic flora was performed for the water and sludge samples collected from the water recycling system (Table 1). Although the make-up water (P1) showed the lowest microbial concentration (3.36 log CFU mL^{-1}), the concentrations in the other water and sludge samples of the washing process were fairly similar with the total count values at 30 °C being greater than 5.0 log CFU mL^{-1}. These results clearly indicate that the soil and gravel introduced microorganisms into the washing process. However, it is important to highlight that the microbial concentration in the unconsolidated sludge from the settling basin (P7) is less than that in the sludge from the washing process. This can be explained by a decantation of microorganisms during storage in the settling basin.

The microbial diversity of the quarry site samples was determined by a culture-dependent method. The main populations of bacterial genera involved in the local biodegradation of environmental pollutants, such as aromatic compounds (Haritash and Kaushik 2009; Seo et al. 2009; Perez-Pantoja et al. 2012), acrylamides, and polyacrylamides (Guézennec et al. 2014), were estimated from all the collected samples and are given in Fig. 2. No yeast and mold, *Xanthomonas* spp., *Sphingomonas* spp., or *Burkholderia* spp., were detected in the samples. The bacterial composition of the make-up water from the pond located near the installation (P1), with *Pseudomonas* spp. and Actinomycetes representing approximately 57 and 20 %, respectively, of the total microbial flora, differs from the compositions determined for the other samples from the washing process, where the bacterial compositions tend to be similar with a concentration of *Pseudomonas* spp. at around 30 % and the majority of microorganisms not belonging to the target bacterial species. The proportion of *Pseudomonas* spp. in the cored sludge sample collected in the settling basin is lower than the proportions assessed in the other samples.

Another widely used tool to study bacterial community structures in complex environments is the DGGE analysis of the 16S rRNA gene. The DGGE approach was used to investigate the changes in bacterial diversity and community dynamics in the water and sludge samples collected from the quarry site. The diversity profiles obtained from the total DNA extracted from the water, sludge, and core samples (Fig. 3) were obtained by DGGE analysis after amplification of the V1–V3 region of the 16S rRNA gene. The reproducibility of these profiles was confirmed by performing three replicate runs, with the profiles proving to be highly reproducible between different gels and PCR runs (data not show).

Figure 3 clearly shows a variability in the bacterial community structures between the make-up water (P1), the water and sludge samples collected from the washing process (P2, P3, P4, and P5), the unconsolidated sludge from settling basin (P7), and the consolidated sludge from the core samples (C1). A remarkable shift is seen between the samples collected from the washing process and the samples collected in the settling

Table 1 Microbial population of water and sludge samples from the quarry site

Sample point	Sample name	Total aerobic microbial population (log CFU mL^{-1} or *CFU g^{-1})
P1	Make-up water	3.36
P2	Process water from the clean water tank	5.17
P4	Process water from the settling tank overflow	5.15
P3	Sludge from the washing unit outlet	5.87
P5	Sludge from the underflow of the settling tank	5.81
P7	Unconsolidated sludge from the settling basin	3.53
C1	Cores of consolidated sludge	5.58*

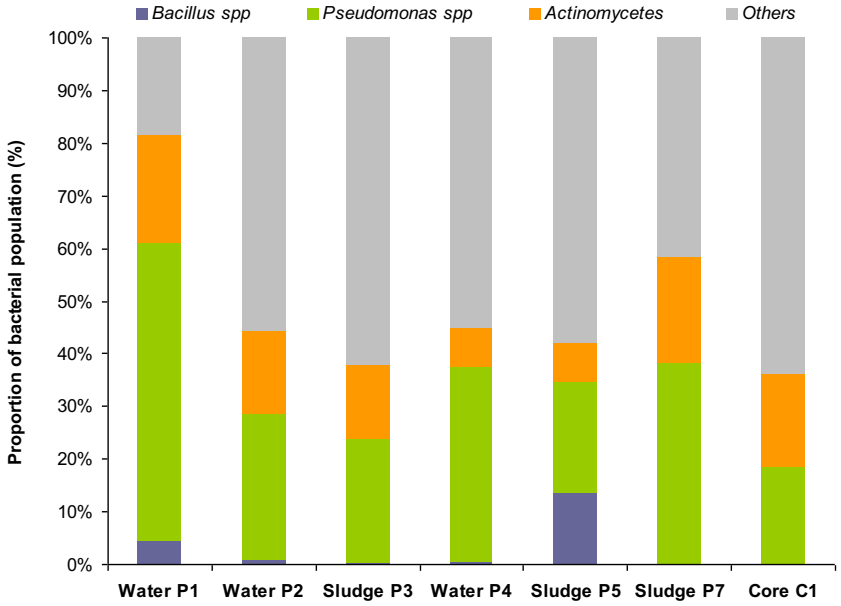

Fig. 2 Proportion of *Bacillus* spp., *Pseudomonas* spp., and *Actinomycetes* in the water samples (P1, P2, and P4), sludge samples (P3, P5, and P7), and core sample (C1) collected from the quarry site

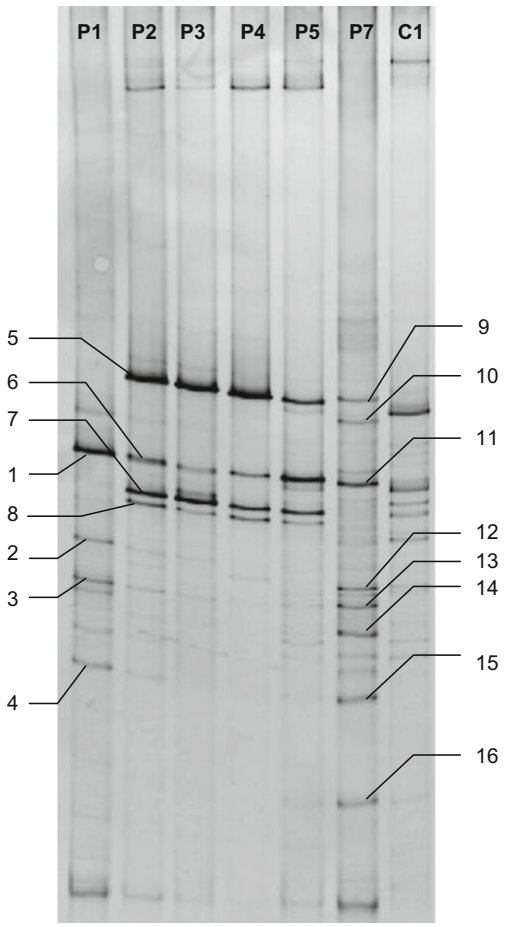

Fig. 3 Representative DGGE profiles of the water (P1, P2, and P4), sludge (P3, P5, and P7), and consolidated sludge (C1) samples after PCR-amplified of V1–V3 domains of the bacterial 16S rRNA gene. Bands that were excised for sequence analysis are labeled with the same number as in Table 2

basin where the banding patterns are completely different from the initial pattern of the make-up water (P1) and the pattern of the samples from the washing process (P2 to P5). It is important to highlight that the make-up water was not exposed to AMD (Touzé et al. 2014). The DGGE profile of the biomass changed after the addition of sand and gravel, and the number of bands increases after the washing unit granulation. The DGGE profile changes again after the sludge is stored in the settling basin, with the positions of the major bands also shifting between the unconsolidated and the consolidated sludge, suggesting a modification of the dominant species. One hypothesis to explain the modification of DGGE profiles in samples collected from the basin is that environmental conditions such as anaerobic conditions or AMD exposure time induce a modification of the bacterial diversity and an enrichment of AMD-degrading bacteria and/or anaerobic bacteria. The number of dominant species in the unconsolidated sludge sample collected from the settling basin (P7) is much higher than those in the samples collected during washing process.

Several bands were excised from the three DGGE gels and re-amplified with the primers BACTF1 and BACTR1 described earlier. The results of the DGGE band sequences are presented in Table 2. Some bands in the different PCR DGGE profiles were not sequenced and there were difficulties in obtaining identification, while in other cases, the retrieved sequences shared the same similarity value with several closely related bacterial species. Most of the sequences, however, are similar to the 16S rRNA gene sequences reported for cultured or uncultured bacteria present in soil or marine sediment samples. All the bacterial species belong to a Proteobacteria, Actinobacteria, or Cyanobacteria class and are strictly or facultatively aerobic. These results indicate the

Table 2 Sequence similarities of excised DGGE bands

Band number	Species	Identity (%)	Phylogenetic group	Respiratory type
1	*Prochlorococcus marinus*/*Cyanobium gracile*	96	Cyanobacteria	Facultative anaerobic
2	*Curvibacter gracilis*	97	β-Proteobacteria	Facultative anaerobic
3	*Arcicella* sp.	99	Sphingobacteria	Aerobic
4	*Ornithinibacter aureus*	94	Actinobacteria	Aerobic
5	*Pedomicrobium* sp.	100	α-Proteobacteria	Aerobic
6	*Candidatus pelagibacter*	97	α-Proteobacteria	Aerobic
7	Uncultured cyanobacterium	97	Cyanobacteria	Facultative anaerobic
8	*Curvibacter delicatus*	97	β-Proteobacteria	Facultative anaerobic
9	IH*	–	–	–
10	Uncultured cyanobacterium	97	Cyanobacteria	Facultative anaerobic
11	*Cyanobium gracile*	97	Cyanobacteria	Facultative anaerobic
12	IH*	–	–	–
13	*Streptomyces cattleya*/*Kineosporia mesophila*	96	Actinobacteria	Facultative anaerobic
14	IH*	–	–	–
15	*Ornithinibacter aureus*	94	Actinobacteria	Aerobic
16	*Phycicola gilvus*	93	Actinobacteria	Aerobic

*IH insufficient sequence homology to enable identification (identified as "Uncultured bacterium")

presence of bacteria that are closely related to known pollutant-degrading genera. They also confirm the presence of Actinomycetes, as was observed with the culture-dependent method.

Characterization of AMD degradation potential

The AMD biodegradation activity of the samples collected from the quarry site was evaluated using an indicator MSM method. The method is similar to that used by Santoshkumar et al. (2010) for screening bacteria capable of degrading aliphatic nitriles, but was not applied to the cores of consolidated sludge (C1) due to the small amount of sample that could be recovered with the coring step.

The degradation of AMD initiates a release and accumulation of ammonia in the culture medium, so contributing to an increase of the mineral medium pH from an initial value of 7.0 to 8.8, and it is through the pH evolution of the MSM inoculated with the water and sludge samples that degradation was determined (Fig. 4). Thus, after 50 h of incubation, AMD degradation was observed in all the samples except for the make-up water collected in the pond near the installation (P1). The contained microorganisms therefore display an AMD biodegradation activity, using the sodium citrate contained in the MSM as a carbon source and the liberated ammonia as a nitrogen source for their growth during incubation.

The water samples and sludge samples exhibited different abilities to metabolize AMD. Here it should be highlighted that the sludge collected from the outlet of the washing unit (P3) and weakly exposed to PAM naturally contained bacteria able to degrade AMD. Moreover, the samples of sludge collected before the addition of PAM (P3) and at the outlet of the settling tank (P5) demonstrated greater deamination activity than the samples of process water collected from the overflow canal of the settling tank (P4) and from the clean water tank (P2) (Fig. 4). For these four samples, which exhibit similar DGGE profiles (Fig. 3), the AMD degradation activity would appear to be strongly related to the initial bacterial concentration of the samples (Table 1). This data also suggest that other bacterial species—potentially present among the cultivated mesophilic aerobic flora—may be involved in the process of AMD degradation. The weaker degradation of AMD observed with the sample of unconsolidated sludge collected near the entrance of the pond (P7) may be explained by the fact that this sample has a lower aerobic bacterial population than other samples collected from the quarry site (Table 1).

Aerobic AMD biodegradation under growth conditions similar to the in situ conditions

The aerobic biodegradation of AMD in samples collected at two different points of the recycling loop system was studied at laboratory scale under conditions close to those found on site in terms of temperature and AMD concentration (Fig. 5). Samples from P4 and P7 were chosen as they come from the two main parts of the system (i.e., the washing process itself [P4] and the settling basin [P7]), and as they are quite different from the point of view of their bacterial diversity (Fig. 3).

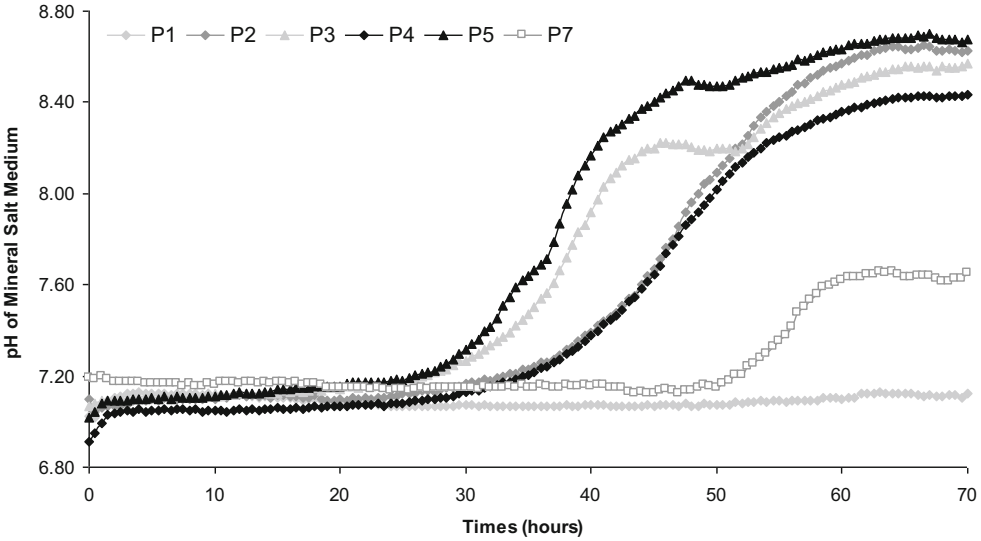

Fig. 4 AMD biodegradation measured in terms of the pH evolution of the mineral salt medium inoculated with the water P1 (*light gray diamond*), P2 (*gray diamond*), and P4 (*black diamond*) and sludge P3 (*light gray triangle*), P5 (*black triangle*), and P7 (*square*) samples collected from the quarry site. Tests were performed with 0.5 g L^{-1} AMD and incubated at 30 °C. The results represent the mean calculated from duplicate

Moreover, the P4 sample comes from a very well aerated part of the process, and the P7 sample is supposed to be in the most aerobic part of the settling basin—P7 can in fact be considered as at the limit between aerobic and anaerobic conditions. The AMD concentration used in these tests was 50 μg L^{-1} in order to be in agreement with that measured on site, and the AMD biodegradation was tested at both 25 °C (summer temperature) and 12 °C (winter temperature).

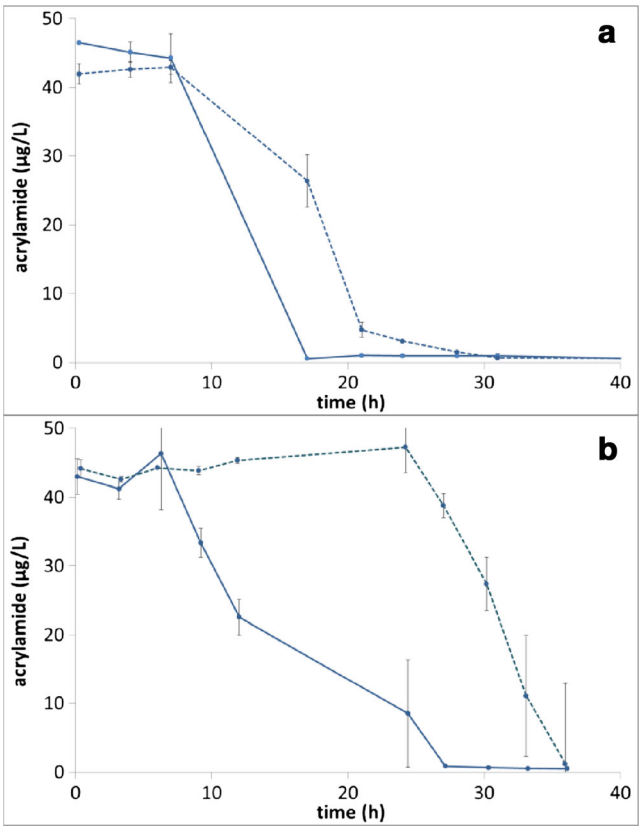

Fig. 5 Aerobic biodegradation of acrylamide in **a** a sample collected from the settling tank (P4) and **b** a sample collected from the settling basin (P7). The batch tests were spiked with 50 μg L^{-1} AMD and incubated at 25 °C (*line*) and 12 °C (*broken line*). The *data points* are an average of replicate samples

These *ex situ* tests showed a similar AMD degradation response (Fig. 5) to that observed on site (Fig. 4), with AMD degradation being observed for both the sample collected from the settling tank (P4; Fig. 5a) and the sample collected from the settling basin (P7; Fig. 5b), and at both temperatures. The activity obtained for P7, however, was weaker than that obtained for P4. The AMD concentration in the P4 sample was below the detection limit after 17 h of incubation at 25 °C, whereas total removal of AMD in the sample collected from the settling basin (P7) was not observed until after 28 h of incubation at 25 °C. This could be explained by the fact that, as described above, the P4 sample comes from a more aerated part of the recycling loop and potentially contained microorganisms more adapted to the aerobic biodegradation of AMB. The incubation temperature also has an important effect on AMD degradation, with the lag time before activity started being between 17 and 24 h for the samples incubated at 12 °C as against 7 h for those incubated at 25 °C.

An abiotic control performed under the same conditions exhibited no AMD degradation (data not shown), which demonstrates that the AMD degradation detected in the biotic tests is a microbial-driven process.

The aerobic biodegradation of AMD was also tested in the presence of carbon sources (acetate and glucose). The tests were done at 25 °C with water collected from the settling tank (P4) after 40 h of incubation. The water amended with glucose and acetate showed no removal of AMD (data not show) suggesting that glucose or acetate are preferentially

metabolized and could act by a repression mechanism on amidase gene expression.

Anaerobic AMD degradation under conditions similar to the in situ conditions

Given the short residence time of the water and sludge samples under aerobic conditions in the washing unit, we also evaluated the ability of samples collected from the settling basin to degrade AMD under anaerobic conditions. After decantation into the settling basin, the particles contained in the sludge settle rapidly at the bottom of the basin to form a layer of consolidated sludge. In an earlier study carried out in the sand and gravel quarry, Touzé et al. (2014) found a significant amount of AMD in core samples from the settling basin. Their results seem to indicate that AMD degrades little, or not at all, once the sludge has settled. As the settling basin is characterized by water stagnancy, and thus total or partial anaerobic conditions, we tested the biodegradation of AMD by incubating unconsolidated sludge (P7) and consolidated sludge (C1) from the basin under anaerobic conditions at 12 °C and 25 °C. In the flasks incubated with consolidated sludge (C1), the AMD concentrations remained constant with no AMD degradation being detected, whereas AMD degradation was observed in the flasks incubated with unconsolidated sludge (P7; Fig. 6). The lag time was, however, greater (20 h at 25 °C and 44 h at 12 °C) than that observed for the aerobic degradation.

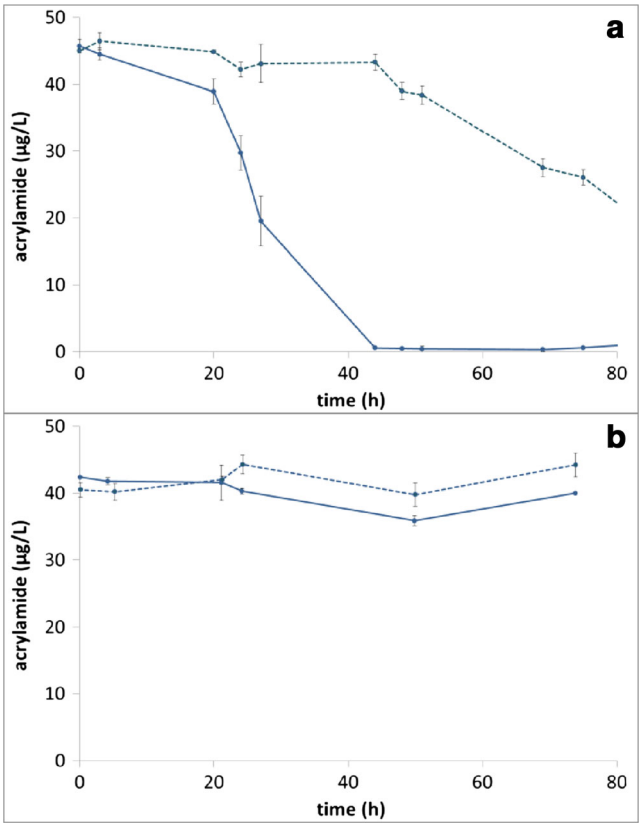

Fig. 6 Anaerobic biodegradation of acrylamide: **a** unconsolidated sludge from the settling basin (P7) and **b** consolidated sludge from the settling basin (C1). The batch tests were spiked with 50 µg L^{-1} AMD and incubated at 25 °C (*line*) and 12 °C (*broken line*). The *data points* are an average of replicate samples

Discussion

The objectives of the study was to map an AMD contaminated quarry site by combining analysis of the microbial diversity and an evaluation of the capability of the bacterial communities present in the quarry's water circuit to degrade AMD. Although, several studies have shown the abilities of soil microorganisms to reduce AMD concentrations (see Guézennec et al. 2014, for a review), the interactions of AMD with soil and aquatic bacterial communities remain relatively little studied (Labahn et al. 2010). Soil microorganisms are considered to be the best indicators of soil pollution in that they are sensitive to low concentrations of pollutants, and modifications of their diversity reflect the toxic effects of contaminants (Schloter et al. 2003). Characterization of the bacterial diversity and evaluation of the AMD biodegradation capacities of the bacteria could provide new insights for improving the management of potential pollution.

In this study, we combined culture-dependent and molecular (DGGE) approaches in order to characterize the bacterial diversity of water and sludge samples collected from the recycling loop system of the quarry site. The culture-dependent and DGGE approaches are complementary methods in that they enable the detection of different bacterial genera. The results seem to indicate that most of the microorganisms in the washing process were introduced from the soil and gravel. Most of the bacterial species found in the samples belong to Proteobacteria, Actinobacteria and Cyanobacteria—of these, Proteobacteria and Actinobacteria are known to degrade natural polymers, pollutants, and polycyclic aromatic hydrocarbons in environmental samples (Fuentes et al. 2014). The bacterial community of the samples shows a remarkable change after the washing process. The evolution of the DGGE profiles indicates that the structure of the bacterial community is dynamic rather than static throughout the sand and gravel washing process, with the remarkable change in the DGGE profiles of samples collected from the settling basin suggesting that the bacterial community was modified by environmental conditions such as anaerobic conditions and AMD exposure time. It has to be noted that the potential impact of PAM on the microbial communities is negligible as (a) no PAM was detected in the water process as PAM rapidly/immediately interacts with soil particles (Touzé et al. 2014) and (b) anionic PAM has been demonstrated to have no significant impact on microbial communities and their

Table 3 Aerobic biodegradation of AMD in various environmental samples

References	AMD concentration	Temperature	Lag time	Time for complete AMD degradation
This study	50 μg L^{-1}	25 °C	7 h	17 h
		12 °C	7–17 h	31–41 h
Labahn et al. (2010)	50 μg L^{-1}	15 °C	No degradation after 72 h	
		25 °C	0–12 h	24 h
Croll et al. (1974)	10 μg L^{-1}	Not indicated	5–220 h	50–300 h
Brown et al. (1980)	0.5–5.0 mg L^{-1}	20 °C	75 h	125 h
Ver Vers (1999)	1.5–2.5 mg L^{-1}	Not indicated	0	From 480 to more than 1,100 h

metabolic potential (Sojka et al. 2006). The incoming rate of the acrylamide monomer is related directly to the feed rate of the polyacrylamide flocculants added to the settling tank. The bacteria were exposed to AMD for only a limited period during the washing process. Mnif et al. (2014) have demonstrated that the sorption of AMD on the sludge used for this study is negligible; thus, being extremely soluble in water and poorly adsorbed onto minerals and organic matter, the acrylamide monomer is highly bioavailable. In the settling basin, the microorganisms are in contact with AMD over a long period and it is possible that this induces a selective pressure on the bacterial communities, leading to a selection of AMD-degrading microorganisms. Another interesting result is that the number of dominant species increases in the settling basin sample (P7). We therefore postulate that, in our case, the degradation of AMD involved different populations. The bacterial composition is again modified in the core of consolidated sludge (C1) collected from the settling basin; in this case, the modification could be due to the establishment of anaerobic and reducing conditions (Bell et al. 1990; Navel et al. 2011).

Our study has demonstrated that microorganisms present in water and sludge samples collected from the water recycling loop of a sand and gravel quarry submitted to PAM, and thus AMD amendment, are capable of removing the AMD without carbon or nitrogen amendments. Although soil, which is contained in sand and gravel, is uncontaminated by AMD, it naturally harbors bacteria that are capable of AMD biodegradation.

Many earlier studies (Shanker et al. 1990; Shukor et al. 2009; Buranasilp and Charoenpanich 2011) focused on the ability and selection of some bacteria to grow in the presence of high concentrations of AMD (more than 1,000 mg L^{-1}). However, in environments or sites contaminated with residual AMD, the concentrations of AMD are much smaller and so we felt it necessary to study the ability of microorganisms to degrade AMD at low concentrations. Our results show that the microbial population at the studied quarry site can degrade AMD at 50 μg L^{-1}. Moreover, the abiotic control demonstrates that the AMD degradation in these samples is mainly a microbially driven process; similar observations were obtained by Labahn et al. (2010) with samples of water and sediment collected from the Rocky Ford Highline Canal. In addition, AMD biodegradation is rapid as complete degradation was obtained after less than 24 h at 25 °C, with only a fairly short time lag before degradation starts, which is faster than the acrylamide biodegradation activities obtained in other studies (Table 3). Another interesting result is that we observed an AMD biodegradation activity at low temperature (12 °C); only a few other studies have shown such an activity at this temperature (e.g., Brown et al. 1980; Table 3). Sharma et al. (2009) demonstrated that the enzyme involved in bacterial AMD degradation is an amidase and that the expression of the gene encoding this enzyme can be constitutive or inducible by AMD. As the activity detected in our study is rapid and starts after only a short lag time, it could be that the samples collected from the quarry site have a significant proportion of microorganisms with a gene encoding potential amidase. The fact that AMD biodegradation activity was detected at 12 °C is a good point as it means that on-site AMD biodegradation can occur regardless of the season. Taking into account that the washing process is based on water recycling, our results could indicate that the microbial part of the site has been selected and enriched in microorganisms capable of degrading AMD at low concentrations whatever the season (and so for a large range of temperatures). This is in agreement with the fact that no potential AMD biodegradation activity was detected with the make-up water from P1 (i.e., the only sample that was never in contact with PAM and AMD).

Although many studies (Table 3) have focused on AMD biodegradation under aerobic conditions, Labahn et al. (2010) are one of the few that have demonstrated the anaerobic biodegradation of AMD. Anaerobic degradation of AMD was also observed in the present study in the sample collected from the settling basin (P7). The degradation kinetics, however, was lower than that observed under aerobic conditions, which strongly suggests that aerobic AMD biodegradation is the main biodegradation pathway. Touzé et al. (2014) demonstrated that AMD concentrations decrease mainly in the washing process of the recycling loop, but that low AMD concentrations are still present in the settling basin. This can be explained by the abundant presence of mainly aerobic bacteria

such as Proteobacteria and Actinobacteria. Moreover, our present results from the same quarry suggest that even if AMD is not totally biodegraded in the washing process, there still is a potential for AMD biodegradation under aerobic and anaerobic conditions in the settling basin. This could greatly limit, or even avoid, further contamination of groundwater. The degradation tests we performed clearly show that the microorganisms in the samples are able to use AMD as a carbon and nitrogen source, which (a) is consistent with the work of other authors (Nawaz et al. 1994, 1998; Shukor et al. 2009) and (b) indicates that these microorganisms do not degrade AMD in the presence of other carbon sources that are easier to use.

Finally, the collected samples containing different groups of AMD-degrading bacteria give an indication of the microbial diversity of this quarry site and of the high potential for AMD degradation. Experiments based on our results are now underway to isolate and characterize the bacterial species mainly involved in AMD biodegradation. If successful the use of these species may allow us to optimize the *in situ* degradation of AMD released in quarry sites.

Conclusion

Our study has been able to map the recycling loop of a sand and gravel quarry in terms of microbiology, biodiversity and bioactivity. The specific biodiversity and high-potential aerobic AMD biodegradation activity of the washing process is quite different from that of the settling basin, characterized by a significantly different biodiversity and a generally anaerobic biodegradation activity. The presence of microorganisms has to be taken into account when explaining the chemistry of the site and the environmental impact of the use of PAM as a flocculant in such an industry. Successful bioremediation depends of the presence of specific microorganisms capable of degrading acrylamide under the *in situ* conditions. The studied quarry site contains microorganisms that are naturally present in sand and gravel and that are able to aerobically and anaerobically degrade AMD, even at low concentration. Isolation of the bacterial strains involved in the biodegradation activities will be the next step in developing a bioremediation process.

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