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Effects of organoclays on soil eubacterial community assessed by molecular approaches

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ABSTRACT

The aim of this study was to evaluate the effects of the commercial organoclays, CLOISITE 30B, NANOFIL 804 and DELLITE 26C on soil eubacterial community. An enrichment test was carried out on Nutrient Broth containing the organoclay and the microorganisms previously isolated from soil. Four transfers were made, each after 7 days incubation. The molecular analyses on the eubacterial community were performed before treatment and 7 days after each transfer. DNA was extracted, amplified with eubacterial primers, finally analysed by amplified ribosomal DNA restriction analysis (ARDRA) and denaturing gradient gel electrophoresis (DGGE). The profiles of the samples treated with each organoclay showed the absence, the appearance and an increase in the intensity of some bands. These bands were excised from the gels, and the related microorganisms were identified by DNA sequencing, as *Pseudomonas putida*, *Alcaligenes xylosoxidans*, *Pseudomonas monteilii* and *Pseudomonas aeruginosa*. NAN804 treatment did not have any influence on soil eubacterial community, CLO30B had a slight toxic effect only on *P. putida*, instead the DEL26C treatment had a stronger toxic effect on *P. putida* and a slight toxic effect on *P. monteilii*. Finally, all the tested organoclays stimulated the growth of both *A. xylosoxidans* and *P. aeruginosa*.

1. Introduction

In recent years, organoclays have attracted great interest due to their academic and industrial importance [1,2]. Organoclays are an important type of modified clay material, which are synthesized by introducing cationic surfactants such as quaternary ammonium compounds (QACs) into the interlayer space through ion exchange [3,4]. As a result of the hydration of inorganic cations on exchange sites, a clay mineral surface is hydrophilic, thus making natural clays ineffective sorbents for adsorbing organic compounds [5,6]. The treatment of clays with cationic surfactants makes them hydrophobic, increasing their adsorption capacity for organic pollutants characterised by low water solubility or polarity [7–9].

There are currently many applications for organoclays, which can be used as precursors to nanocomposite formation or as sorbents in pollution prevention and environmental remediation, for example, in the treatment of spills, waste water and hazardous waste landfills.

McBride et al. [10] were the first authors to report the ability of organoclays to sorb organics in water. They found the sorption of 2,4-dichlorophenoxyacetic acid (2,4-D) from aqueous solution on Bentone 24, which is a montmorillonite ion exchanged with a dimethyl benzyl octadecyl ammonium ion. Many other authors [11–15] have since confirmed that organoclays are good sorbents in removing pesticides from water. Sanchez-Camazano and Sanchez-Martin [12] reported a study of the adsorption of azinphosmethyl and dichlorvos (organophosphorus pesticides) by montmorillonites at different degrees of saturation with the cations hexadecyltrimethylammonium, dodecyltrimethylammonium and tetramethylammonium in aqueous media.

As regards soil, organoclays have been proposed for decontamination purposes [16–18] and also as carriers for the controlled release of pesticides to reduce the leaching process in soil and minimize the risk of ground water contamination [19–22]. The mobility of pesticides in soil is reduced by hydrophobic and polar interactions between the agrochemical and the alkyl chains of the organoclay. Undabeytia et al. [19] developed controlled release formulations of the herbicide norflurazon by using organoclays. They modified the surface of the clay mineral montmorillonite from hydrophilic to hydrophobic by preadsorbing it with organic cations. They found that formulations based on organoclays showed slow release, reducing leaching and good herbicidal activity at the optimal depth for weed control.

While the use of different types of organoclay as adsorbents for organic pollutants is well known [23–25], few studies have been performed regarding the influence of organoclays on the growth and activity of microorganisms in soil [26]. Several authors [26,27] have reported that cationic surfactants are toxic for many bacteria, in particular, Nye et al. [26] described the inhibitory effect

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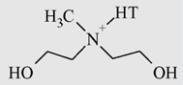
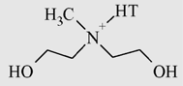
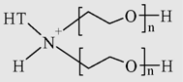
Table 1
Characteristics of montmorillonites used.

Type of clay	Commercial name	Interlayer cations	Modifier structure	Producer	Code
Montmorillonite	CLOISITE 30B	$(CH_3)N^+(HT)$ $(CH_2CH_2OH)_2$		Southern Clay Products (USA)	CLO30B
Montmorillonite	DELLITE 26C	$(CH_3)N^+(HT)$ $(CH_2CH_2OH)_2$		Laviosa Chimica Mineraria S.p.A. (Italy)	DEL26C
Montmorillonite	NANOFIL 804	$HN^+(HT)$ $(CH_2CH_2OH)_2$		Süd-Chemie (Germany)	NAN804

HT, hydrogenated linear alkyl chains, R, C_{8-18}.

of cationic surfactants on bacteria occurring in soils treated with QACs.

Since the applicability of organoclays as adsorbents can be restricted as a result of their being harmful to the bacteria responsible for biodegradation, in this paper, we investigated the influence of three different commercial organoclays (CLOISITE 30B, NANOFIL 804 and DELLITE 26C) on soil eubacterial community by amplified ribosomal DNA restriction analysis (ARDRA) and denaturing gradient gel electrophoresis (DGGE) approaches.

2. Materials and methods

2.1. Materials

The clays (CLOISITE 30B, NANOFIL 804 and DELLITE 26C) used were commercial organo-modified montmorillonites and were supplied by Southern Clay Products (USA), Süd-Chemie (Germany) and Laviosa Chimica Mineraria S.p.A. (Italy), respectively. The characteristics of the modified montmorillonites are listed in Table 1. Sodium cloisite, a natural montmorillonite, was used as control.

2.2. Enrichment culture

In order to isolate soil microorganisms, 10 g of soil were dissolved in 90 ml of sterile physiological solution (0.9% NaCl) and stirred for 1 h. After centrifugation, the supernatant was used as an inoculum in Nutrient Broth (Oxoid, Milan, Italy) and placed in a thermostat at 30 °C to let it grow.

Straight after, in order to verify which microorganisms were able to grow in presence of organoclays, an enrichment test was carried out as follows. The samples (50 ml of Nutrient Broth, 1 ml of fresh microbial culture, previously isolated from soil and 3 g of organoclay) were incubated aerobically at 30 °C in the dark on a rotary oscillator. After 7 days incubation, an aliquot of the samples was transferred to fresh Nutrient Broth containing the organoclay. Four transfers were made, each after 7 days incubation.

The molecular analyses on the eubacterial community were performed before treatment and 7 days after each transfer. Analyses were carried out twice for each treatment.

The above procedure was also carried out without clays and with sodium cloisite to perform two different control experiments.

2.3. DNA extraction

DNA was extracted directly from 250 μl of the samples [28]. Samples were homogenised in 1 ml of extraction buffer [100 mM Tris, pH 8; 100 mM EDTA; 100 mM NaC1; 1% (w/v) polyvinylpyrrolidone; 2% (w/v) sodium dodecyl sulphate] for 30 s at 1600 rpm in a mini-bead cell disrupter. Cell debris was removed by centrifugation (5 min at 14,000 × *g*). Proteins were eliminated after sodium acetate precipitation. Nucleic acids were precipitated with cold isopropanol, then washed with 70% ethanol. DNA extracts were purified with a polyvinylpyrrolidone spin column. The quality and the integrity of the DNA was checked by electrophoresis on 1% agarose gel.

2.4. Amplification of eubacterial 16S rDNA

The 16S ribosomal DNA (rDNA) fragments were obtained by amplifying the DNA using the universal eubacterial primers p0f (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and p6r (5′-CTA CGG CTA CCT TGT TAC GA-3′) as reported by Lane [29] in a PCR Px2 thermocycler (Hybaid) to obtain a product of about 1.5 kb fragments. The PCR conditions were: a hot start of 90 s at 95 °C; four cycles consisting of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 4 min; subsequently, the annealing temperature was lowered by 2 °C until it reached 55 °C for a further four cycles, and the final 14 cycles consisted of 92 °C for 30 s, 50 °C for 30 s and 72 °C for 4 min, followed by a final 10 min elongation at 72 °C and 10 min at 60 °C. The reaction mixture contained 40 ng of DNA, 400 μM of each primer, 400 μM of dNTP, 1.5 mM $MgCl_2$ and 2.6 Units of Taq DNA polymerase in a buffered final volume of 50 μl (Invitrogen). The PCR products, 5 μl subsamples, were examined by electrophoresis on 1× TAE agarose gel (0.8%, w/v) stained with ethidium bromide (0.5 μg ml^{-1}), with appropriate DNA size standards (Mass Ruler™, DNA Ladder Mix, Fermentas) to evaluate the size and approximate quantity of the generated amplicons. The length of the expected amplified fragment was about 1.5 kb.

2.5. Amplification of eubacterial 16S rDNA fragments for DGGE analysis

Eubacterial 16S rDNA was amplified using the primer sets GC-968f (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TA-3′) and 1401r (5′-GCG TGT GTA CAA GAC CC-3′), as described by Felske et al. [30] to obtain products of about 450 bp. The DNA template (80 ng) was amplified with 5 Units μl^{-1} Taq DNA polymerase, 10 μM of each primer, 10 mM of each dNTP, 10 mM of $MgCl_2$, 500 μg ml^{-1} of BSA and reaction buffer 1× (Invitrogen) in a final reaction volume of 50 μl. The PCR conditions were: 94 °C for 90 s, followed by 33 cycles at 95 °C for 20 s, 56 °C for 30 s, 72 °C for 45 s and a final extension step at 72 °C for 7 min. Amplicons were analysed as described above.

2.6. Amplified ribosomal DNA restriction analysis

The amplified 16S DNA products (400 ng) were digested for 3 h at 37 °C with 10 U of HaeIII restriction endonuclease (New England BioLabs). Restricted DNA was analysed by electrophoresis on 3.5% agarose gel at 30 V overnight and stained with ethidium bromide. Bands were detected from digital images (Polaroid Gel Cam, Elect; Polaroid Type 667 Film ISO 3000) by UV light gel transillumination (λ 312 nm).

2.7. Denaturing gradient gel electrophoresis

16S rDNA-DGGE was performed using the DCode System (Universal Mutation Detection System, BIO-RAD). An amount of 300 ng of amplicons was loaded in duplicate (top filling method) on 6% polyacrylamide gel (acrylamide/bisacrylamide, 40%, 37.5:1; BIO-RAD) containing a denaturant gradient of 46–56% parallel to the electrophoresis direction made of urea and formamide (100% denaturant contains 7 M urea and 40% formamide). Gels were electrophoresed at a constant temperature (60 °C) and voltage (75 V) for 16 h, followed by 2 h coloration using SYBR Green I nucleic acid gel stain 1:1000 diluted in the running buffer (FMC Bio Products, Rockland, ME, USA). Bands were detected from digital images (Polaroid Gel Cam, Elect; Polaroid Type 667 Film ISO 3000) by UV light gel transillumination (λ 312 nm).

Bands to be sequenced were excised from the DGGE gels, placed in 50 μl sterile H_2O and stored at −80 °C. Before PCR amplification, the samples were thawed for 1 h at room temperature, frozen again at −80 °C for 1 h and finally thawed at 4 °C overnight to elute the DNA fragments. The eluted DNA (2 μl) was used as a template in PCR amplification with the primer set 968f–1401r (without the GC clamp).

3. Results and discussion

Total DNA was directly extracted from the samples, 16S rDNA fragments were amplified by PCR with eubacterial primers and analysed by ARDRA.

The restriction patterns of amplified eubacterial 16S rDNA digested with HaeIII are shown in Figs. 1–3. In ARDRA gels, a total number of 10–12 bands were detected. Patterns proved to be very similar in terms of number and intensity of bands. However, the profiles of the samples after the second, third and fourth treatment with each organoclay showed the appearance of some bands (Figs. 1–3, white box) and an intensification of others (Figs. 1–3, black box). At the same time, some faint bands present in the samples before treatments disappeared after each organoclay treatment (Figs. 1–3, striped arrows).

Furthermore, molecular analysis of the eubacterial community was performed by PCR amplification of 16S rDNA genes from the total DNA of each sample and separation on parallel denaturing gradient gel by electrophoresis (Figs. 4–6). Both ARDRA and DGGE results showed a similar trend in the three organoclays tested.

Some common bands were detected in DGGE gels, for example, band 1 was present before and after the first, second and third CLO30B treatment, but it disappeared after the fourth (Fig. 4). The same band became very faint after the first DEL26C treatment and disappeared soon after (Fig. 5) while it was not influenced by the NAN804 treatment (Fig. 6). The last DEL26C treatment caused the disappearance of band 3 (Fig. 5), which, however, was not influenced by the CLO30B (Fig. 4) and NAN804 treatments (Fig. 6).

In all DGGE patterns, band 2 appeared after the second treatment with each organoclay and remained stable until the end of the test. Finally, band 4, observed in all DGGE gels, appeared after the first treatment and became more pronounced at the end of the test.

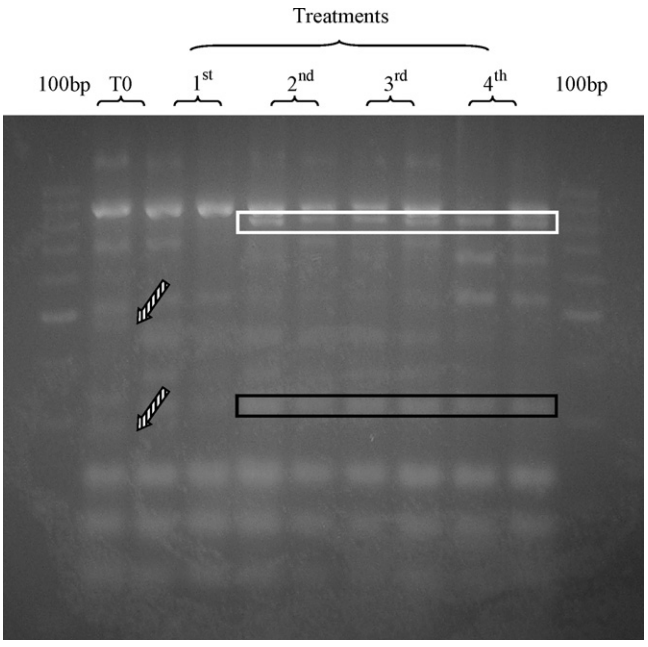


Fig. 1. ARDRA gel of eubacterial community treated with CLOISITE 30B.

Bands 1, 2, 3 and 4 were identified by DNA sequencing (Biodiversity s.r.l., Brescia, Italy) using NCBI library as *Pseudomonas putida* (96%), *Alcaligenes xylosoxidans* (98%), *Pseudomonas monteilii* (100%) and *Pseudomonas aeruginosa* (88%), respectively.

The effects of each organoclay on the identified microorganisms are summarized in Table 2.

Two control experiments without clays and with sodium cloisite were performed to verify if the results, mentioned above, were due to the presence of organoclays. In Figs. 7 and 8, respectively, DGGE gel of eubacterial community not treated with clays and ARDRA gel of eubacterial community treated with sodium cloisite are reported. Both analysis haven't showed any difference in the microbial consortium after the enrichments. Thus, we can suppose

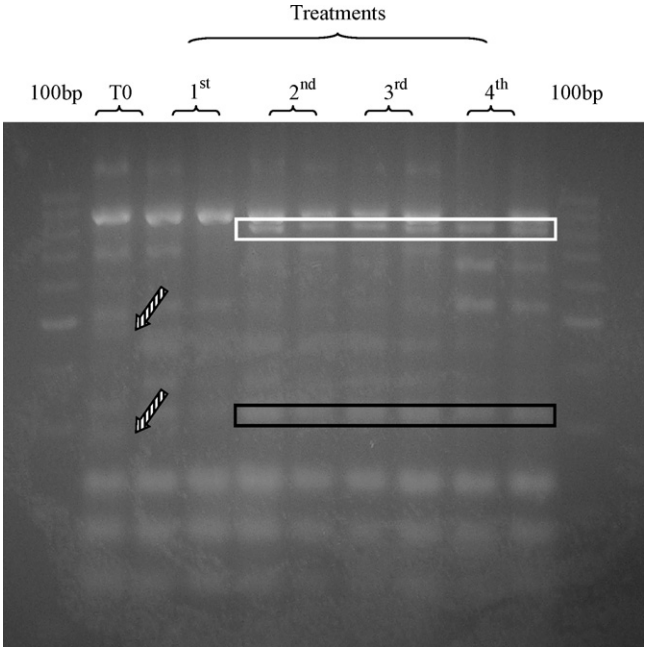


Fig. 2. ARDRA gel of eubacterial community treated with DELLITE 26C.

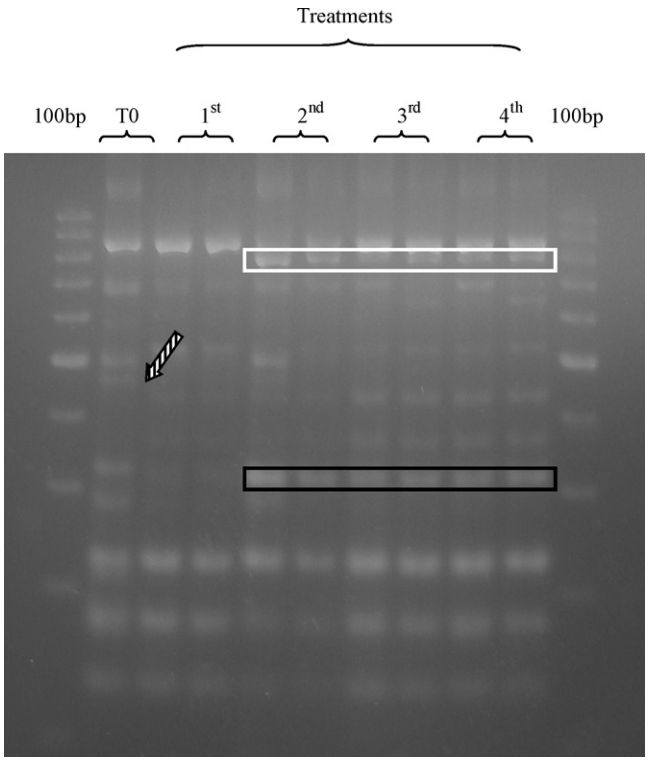


Fig. 3. ARDRA gel of eubacterial community treated with NANOFIL 804.

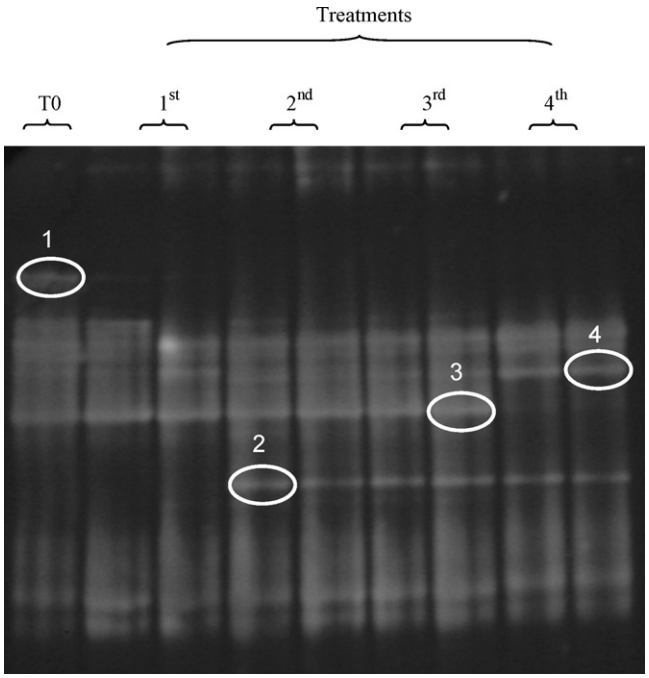


Fig. 5. DGGE gel of eubacterial community treated with DELLITE 26C.

that the microorganisms identified above are influenced only by organoclay effects.

P. putida and *P. aeruginosa* are two of the most important fluorescent species [31]. *P. aeruginosa* is the type species of the genus *Pseudomonas* and is a typical opportunistic pathogen [32]. *P. putida* is particularly interesting because recently, on the basis of a great deal of research, workers have described its role in aromatic hydrocarbon degradation [33–35].

Elomari et al. [36] proposed the name *P. monteilii* for a new species of aerobic, Gram-negative, rod-shaped, nonspore-forming, motile bacteria. The strains of *P. monteilii* produce fluorescent pigments, catalase and cytochrome oxidase, and possess the arginine dihydrolase system. They are capable of respiratory but not fermentative metabolism.

Finally, *A. xylosoxidans*, formerly known as *Achromobacter xylosoxidans*, is a nonfermenting Gram-negative bacillus found in soil and water. Strains of *A. xylosoxidans* produce flat, spreading and rough colonies and have peritrichous flagellae. They are oxidase-positive, catalase-positive, oxidize glucose to produce acid and (as the species name indicates) oxidize xylose readily.

The tested organoclays showed both toxic and stimulatory effects toward some microorganisms.

In fact, after the first DEL26C treatment, *P. putida* became too faint to be detected in the gel. In CLO30B treatment, the same bac-

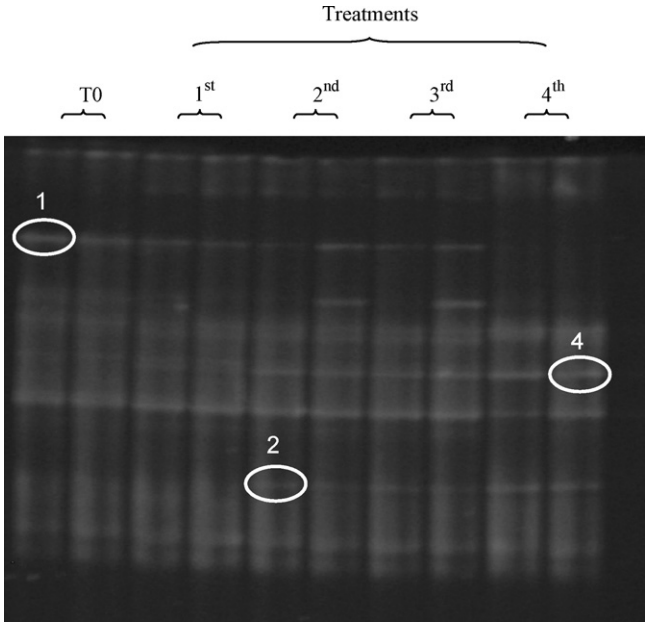


Fig. 4. DGGE gel of eubacterial community treated with CLOISITE 30B.

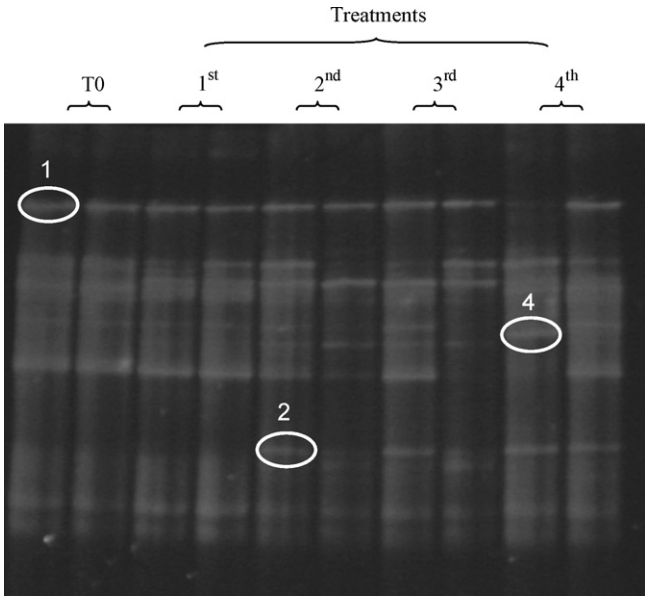


Fig. 6. DGGE gel of eubacterial community treated with NANOFIL 804.

Table 2
Effects of organoclays on microorganisms identified in this study.

	CLOISITE 30B treatments				DELLITE 26C treatments				NANOFIL 804 treatments			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th	1st	2nd	3rd	4th
Pseudomonas putida	√	√	√	×	√	×	×	×	√	√	√	√
Alcaligenes xylosoxidans	×	√	√	√	×	√	√	√	×	√	√	√
Pseudomonas monteilii	√	√	√	√	√	√	√	×	√	√	√	√
Pseudomonas aeruginosa	√	√	√	√	√	√	√	√	√	√	√	√

√, band detected in the gel; ×, band not detected in the gel.

terium hasn't been detected after the last application. *P. monteilii* was only slightly influenced by the presence of DEL26C. In DGGE gel, the corresponding band disappeared only after the last application.

Our results are in agreement with those of Masuda et al. [37], who reported the ability of *P. monteilii* to persist even in unfavourable growth conditions. They isolated a strain of *P. monteilii* from field, sewage and pond water samples, which was tolerant to and degraded bisphenol A (BPA), a highly biotoxic compound, which kills many microorganisms at a low concentration (1000 ppm). Masuda et al. [37] demonstrated that this strain was able to grow in a minimum medium containing BPA as the sole carbon source.

However, there are several studies, which, in accordance with our results, also support the toxicity of organoclays. He et al. [38] and Yang et al. [39] reported the synthesis of a series of new organoclays with antibacterial activity using Ca-montmorillonite and the cationic surfactant chlorhexidine acetate by ion exchange. Chlorhexidine acetate is a bisbiguanide antiseptic and disinfectant, which is bactericidal or bacteriostatic against a wide range of Gram-positive and Gram-negative bacteria [40]. Herrera et al. [41] have revealed that several montmorillonite clays, exchanged with the cationic surfactant cetylpyridinium (CP), are able to adsorb and inactivate bacteria in aqueous solution. The antimicrobial activity of CP is due to its ability to alter the permeability of cellular membranes allowing intracellular ions and low-molecular-weight metabolites to diffuse out [42].

The antibacterial properties of QACs have been known for a long time and are closely linked with the chemical properties of cationic surfactants. They are positively charged and are attracted to negatively charged substances such as bacterial proteins essential for the structure and enzymatic activities of the cell. QACs denature these proteins exerting their antibacterial activity.

On the other hand, organoclay treatments showed a selective pressure toward other microorganisms. All DGGE gels highlighted that the growth of *A. xylosoxidans* was stimulated after the second treatment, suggesting that this microorganism uses the cationic surfactants as carbon and energy sources. Our results agree with those reported by other authors regarding the ability of this bacterium to use different substrates as its carbon and energy sources. In fact, Erdlenbruch et al. [43] found that this bacterium was the first organism able to use ethanesulfonate as its sole source of carbon and energy. It also grew with unsubstituted C3–C5 alkanesulfonates and isethionate. In addition, Jenčová et al. [44] reported that *A. xylosoxidans*, isolated from soil contaminated with polychlorinated biphenyls (PCBs), was able to use 2-clorobenzoate (2-CB) and 2,5-dichlorobenzoate (2,5-DCB) as its sole sources of carbon and energy. Shin et al. [45] isolated this bacterium from agricultural soil, and found it was able to utilize and grow on pentachloronitrobenzene (PCNB) (100 ppm) as its sole carbon source. Reinecke et al. [46] found that *A. xylosoxidans*, isolated from sewage sludge, used the synthetic chelating agent iminodisuccinate (IDS) as its sole carbon source for growth and was able to degrade it. The mechanism by

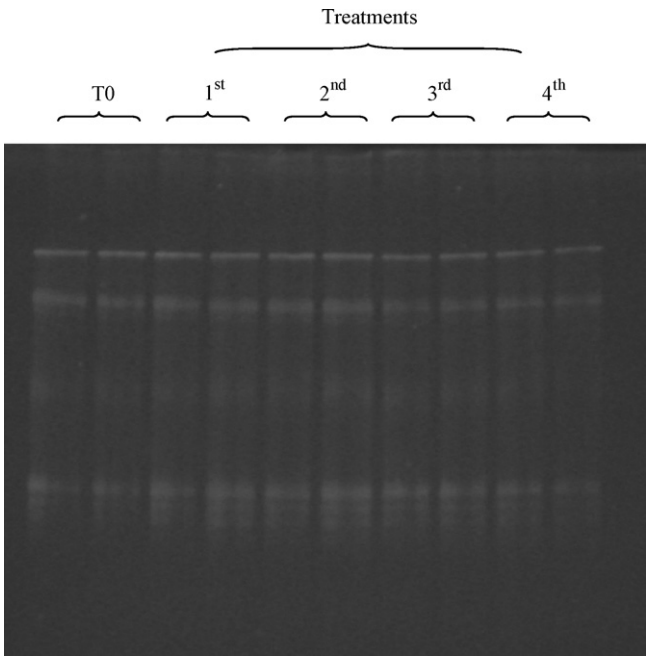


Fig. 7. DGGE gel of eubacterial community not treated with clays.

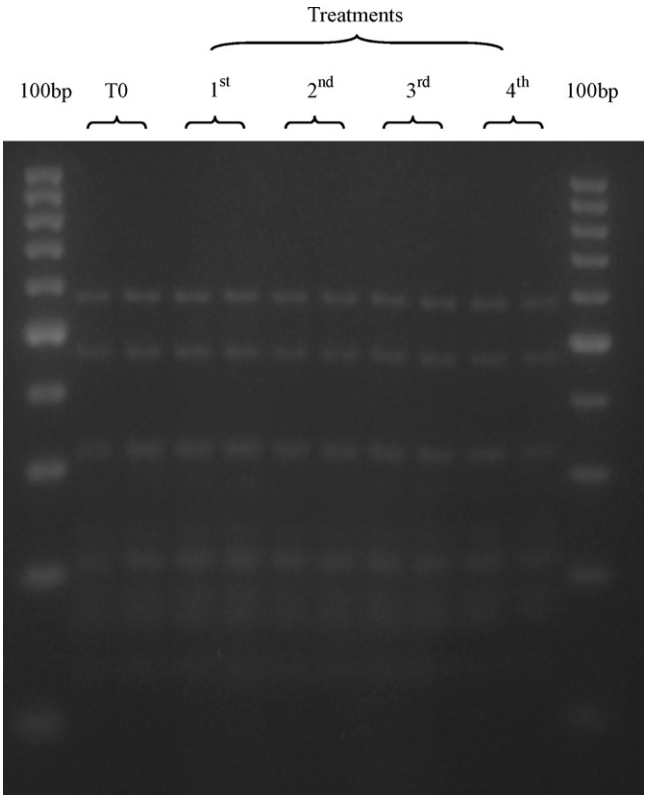


Fig. 8. ARDRA gel of eubacterial community treated with sodium cloisite.

which IDS is cleaved has not yet been identified but the authors supposed a cleavage mechanism catalyzed by monooxygenases or lyases.

Finally, *A. xylosoxidans* is an ubiquitous bacterium present in soil and water, able to degrade xenobiotics and potentially other persistent chemicals in the environment. For this reason, it could be useful in bioremediation processes.

Each organoclay used in this study caused a selective pressure toward the microorganism *P. aeruginosa*. The corresponding band in DGGE gels appeared more evident after the last organoclay application. Texier et al. [47,48] showed that it may be possible to use cells of *P. aeruginosa* to remove and separate lanthanide ions from aqueous effluents. These bacterial cells enabled rapid removal of lanthanide ions and showed high affinities at low metal ion concentrations.

Finally, since CLO30B and DEL26C are modified by the same cationic surfactant, we gather that the slight difference in their behavior toward soil microorganisms is attributable to the different techniques used in the preparation of organoclays.

Instead, the presence of H in place of CH_3 in the organic cation of NAN804 seems to be important in avoiding the toxic effect of this organoclay on soil microorganisms.

4. Conclusions

Three commercial organoclays were chosen for this study and their effect on the eubacterial community was investigated. Based on the results of the present study, the following conclusions can be drawn.

In terms of toxicity, the NAN804 treatment did not have any influence on soil eubacterial community, CLO30B had a slight toxic effect only on *P. putida*, instead the DEL26C treatment had a stronger toxic effect on *P. putida* and a slight toxic effect on *P. monteilii*. It is possible to hypothesize that the presence of cationic surfactants, used to modify the montmorillonites, produced this toxic effect.

On the other hand, all the tested organoclays stimulated the growth of both *A. xylosoxidans* and *P. aeruginosa*. We can suppose that the small amount of cationic surfactants (5 wt%) was not toxic in this case but that these two microorganisms probably used such cationic surfactants as a carbon source to support their growth.

The microorganisms, identified in this study, are common in soil or water playing an important role in decomposition, biodegradation and the carbon and nitrogen cycles. They are able to utilize a wide range of carbon sources, including molecules, such as xenobiotics, which few other organisms can break down. Consequently, they are important organisms in bioremediation.

Therefore, we conclude that, as the organoclays tested can both inhibit and stimulate the various microorganisms involved in bioremediation, it is important to perform further specific studies in order to identify which microorganisms are responsible for the degradation of each particular xenobiotic.

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