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Metabolic and molecular methods to evaluate the organoclay effects on a bacterial community

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ABSTRACT

The aim of this work was to evaluate the influence exerted by two different commercial organoclays (DELLITE 43B and DELLITE 67G) on a model microbial consortium using microbial metabolic characterization with BIOLOG system and denaturing gradient gel electrophoresis (DGGE) molecular approach. The information obtained from the molecular analyses, in their complex, account for the differences in species composition induced on the reference consortium by the contact with the organoclays under study. DELLITE 43B resulted to produce a marked selective effect, stimulating the quantitative increase especially of *Pseudomonas pseudoalcaligenes*. A weaker effect was found for DELLITE 67G. On the other hand, Biolog analyses indicated a depressing action exerted by DELLITE 43B on the metabolic activity of the model microbial consortium as a whole. The presence of *P. pseudoalcaligenes* and *B. borstelensis* in the bacterial community after the treatments confirmed that a positive change in the microbial structure consortium occurred.

1. Introduction

Organoclays are clay minerals whose surface is made hydrophobic after the replacement of the inorganic exchangeable cation by an organic one, such as organic quaternary ammonium. Thanks to this substitution, organoclays have shown to be excellent sorbents for many types of pesticides, being proposed for decontamination purposes. In addition, organoclays can be used as potential sorbent additives in remediation of soils and groundwaters because of their geotechnical compatibility (Witthuhn et al., 2005). They can be also used as carriers for controlled release of pesticides. Carrizosa et al. (2000) assessed the sorption capacity of different organoclays for bentazone, demonstrating their efficiency to immobilize the herbicide in a contaminated soil and protect soil and water by using them as pesticide carriers in slow release formulations.

Andrades et al. (2004) studied the potential use of clay minerals modified with the organic cation hexadecylpyridinium (HDPY) for immobilising pesticides, and as barriers aimed at protection of soils and waters against pollution by hydrophobic pesticides.

Celis et al. (2005) confirmed the usefulness of hexadecyltrimethylammonium-exchanged Arizona montmorillonite (HDTMA-SA) formulations of hexazinone to reduce herbicide leaching while maintaining weed-control efficacy.

While the use of different types of organoclay as adsorbents for organic pollutants is well known, the interactions of these materials with microbial growth and activity in soil is poorly documented. A description of structure, composition, density and diversity of soil microbial communities is important to better understand the soil functioning (Borzì et al., 2007).

The presence of organic quaternary ammonium in the clay interlayer could limit the application of organoclays for decontamination because of the toxic effect on the biodegrading microflora. In fact, quaternary ammonium compounds (QACs) are amphoteric surfactants that are widely used for the control of bacterial growth in clinical and industrial environments showing a broad-spectrum antimicrobial activity (Brannon, 1997; Shimizu et al., 2002).

Quaternary ammonium compounds such as aqueous hexadecyltrimethylammonium (HDTMA) bromide added to soils caused increased lag periods and decreased rates and extents of mineralization of test compounds as a result of selective toxicity towards Gram-negative soil microorganisms. Toxic effects were more pronounced at higher HDTMA treatment levels and with more complex test substrates (Nye et al., 1994).

Abbate et al. (2009) showed that organoclays can either stimulate and inhibit different types of microorganisms. They demonstrated that Cloisite 30B had a slight toxic effect on *Pseudomonas*

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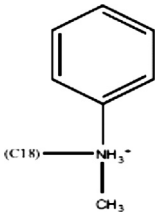
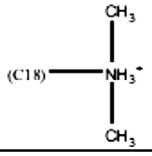
Table 1
Characteristics of organoclays under study.

Type of clay	Commercial name	Organic modifier	Modifier structure	Producer	Code
Montmorillonite	DELLITE 43B	Dimethyl benzylhydrogenated tallow ammonium		Laviosa Chimica Mineraria S.p.A. (Italy)	DEL43B
Montmorillonite	DELLITE 67G	Dimethyl dihydrogenate tallow ammonium		Laviosa Chimica Mineraria S.p.A. (Italy)	DEL67G

putida, instead Dellite 26C treatment had a stronger toxic effect on *P. putida* and a slight toxic effect on *P. monteilii*. Cloisite 30B, Dellite 26C and Nanofil 804 stimulated the growth of both *Alcaligenes xylosoxidans* and *P. aeruginosa*.

The aim of this work was to evaluate the influence exerted by two different commercial organo-modified montmorillonites (DELLITE 43B and DELLITE 67G) on a model microbial consortium, using microbial metabolic characterization with Biolog system and molecular approach with Denaturing Gradient Gel Electrophoresis (DGGE) and real time PCR. Biolog system is a well-known methodology for the metabolic fingerprinting of microorganisms, based on the evaluation of their capacity to attack a standard array of substrates (Garland, 1997). It has been originally used for the identification of pure bacterial cultures and subsequently for the characterization of mixed populations. Recently, Biolog system proved to be useful for the evaluation of substrate composition and/or environmental factors' influence on microbial populations (De Nittis et al., 2010; Bertolone et al., 2011). In our paper, it was used to assess the organoclays' effect on the general metabolic activity of a model microbial consortium, as an integration of the information about its species modifications provided by the molecular approach.

2. Materials and methods

2.1. Organoclays

The organoclays used were DELLITE 43B and DELLITE 67G, commercial organo-modified montmorillonites, supplied by Laviosa Chimica Mineraria S.p.A. (Italy). Their characteristics are listed in Table 1. DELLITE HPS, a High Purified Sodic montmorillonite, was used as control.

2.2. Microbial culture

The model microbial culture on which to test the organoclays' effect was provided by extracting the bacterial population from a compost obtained from an urban composting plant, chosen as a typical source of a rich and varied mixed microbial consortium. Ten grams of such compost were suspended in 90 ml of sterile physiological solution (0.9% NaCl) and stirred for 1 h. After centrifugation, the supernatant was inoculated in Nutrient Broth (Oxoid, Milan, Italy) and incubated at 30 °C in the dark for 36 h to allow good microbial multiplication.

The effect of organoclays' contact on the original compost population was carried out on Nutrient Broth. One ml of the microbial culture at the end of the incubation period was inoculated in 50 ml of Nutrient Broth with 3 g of one of the two organoclays under study and aerobically incubated at 30 °C in the dark on a rotary shaker. After 7 days incubation an aliquot of the resulting culture was transferred to fresh substrate containing the organoclays. Four subsequent transfers have been made.

Molecular analyses and Biolog analyses of metabolic characterization were performed on the original compost population and on the resulting one 7 days after each transfer, namely after 7, 14, 21, 28 days of contact with the organoclays. Contact with DELLITE HPS, a High Purified Sodic montmorillonite, was used as control.

Each analysis was carried out in duplicate for each treatment.

2.3. Molecular analyses

2.3.1. DNA extraction

DNA was extracted directly from 250 μl of the samples (Martin-Laurent et al., 2001). Samples were homogenised in 1 mL of extraction buffer [100 mM Tris, pH 8; 100 mM EDTA; 100 mM NaCl; 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.

2.3.2. 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. (1997) 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.

2.3.3. Denaturing gradient gel electrophoresis (DGGE)

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).

2.3.4. Design of Pseudomonas pseudoalcaligenes-specific amplicon

An alignment of *P. pseudoalcaligenes* 16S rRNA gene was analysed to identify conserved regions suitable for developing a real-time PCR assay for detection of gene sequences specific to this bacterium.

Specific forward Ppf (20-mer [5′-GTAATGGTGGGCACTCTAAG-3′]) and reverse Ppr (20-mer [5′-GAATACGATCGGTTTTATGG-3′]) primers were designed to amplify a 169-bp amplicon from *P. pseudoalcaligenes* 16S rRNA gene to allow its quantification.