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ORIGINAL PAPER

# Isolation and characterization of an aerobic bacterial consortium able to degrade roxarsone

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**Abstract** Roxarsone is an organoarsenical compound used as food additive in the poultry industry. Roxarsone has the potential risk to contaminate the environment, mainly by the use of poultry industry manure as fertilizer, releasing inorganic arsenic to the soil and water. The aim of this work was to isolate and characterize a bacterial consortium capable to degrade roxarsone under aerobic conditions. A bacterial consortium was cultured from a soil sample obtained from a field fertilized with poultry litter containing roxarsone. The consortium was cultured in the presence or absence of roxarsone. Roxarsone degradation and growth kinetics were determined by incubation of the bacterial consortium in the presence of roxarsone at room temperature and under aerobiosis. Both consortiums were characterized molecularly by denaturing gradient gel electrophoresis analysis and metabolically using Biolog Ecoplates. Inorganic arsenic was assessed by precipitation with silver nitrate. The consortium was also analyzed by scanning electron microscopy. The results showed that growth rate of the bacterial consortium was 1.4-fold higher in presence of roxarsone and 81.04 % of the roxarsone was transformed after 7 days of incubation. Molecular characterization revealed the presence of different bacterial groups, being alphaproteobacteria and firmicutes the groups that showed the highest count in both consortiums. The metabolic profile of the consortium did not change in the presence of roxarsone, but it showed a greater ability to oxidize amines, suggesting production of functional amines to decrease the stability of the aromatic ring resonance energy, the principal problem associated with aromatic compounds degradation.

**Keywords** Roxarsone · Arsenic · Biotransformation · Soil · Bacterial consortium

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## Introduction

Roxarsone (3-nitro-4-hydroxyphenylarsonic acid) is an organoarsenical compound used, for decades, after its approval by the US Food and Drug Administration (FDA) in 1944, in the broiler poultry industry as a feed additive to promote growth and to control coccidial intestinal parasites. Approximately 70 % of broiler chickens produced in the USA are fed roxarsone (Chapman and Johnson 2002), which is excreted largely unaltered into the manure. Very little information is available on the molecular mechanisms of action of roxarsone to improve chicken growth. Only recently, Li et al. (2011) reported that its effect could be due mainly to modification of gene expressions.

According to Garbarino et al. (2003), nearly 900 metric tons of roxarsone are released annually into the environment in the USA, whose arsenic content ranges from 14 to 48 mg $kg^{-1}$. Concordantly, studies carried out by Wershaw et al. (1999) concluded that approximately 1,000 metric tons/year of roxarsone and its degradation products are added to the environment, as part of fowl manure used

as fertilizer. Furthermore, studies performed by Hancock et al. (2001), in fresh fowl manure, detected total As concentrations of 27 mg $kg^{-1}$, most of it being organic arsenic, but in soil samples of agricultural fields, where chicken manure was used as fertilizer, mainly inorganic arsenic was detected.

Roxarsone can be rapidly degraded during compost production from manure (Garbarino et al. 2003), while being stored or when applied as fertilizer (Christen 2001; Jackson et al. 2003), and its toxic degradation products include inorganic arsenic, such arsenate (As V) and arsenite (As III), as well as a variety of organic arsenical compounds. When reaching the soil, roxarsone is rapidly transformed into arsenate by soil microorganisms. Depending on the moisture level of the soil, when As (V) is in an oxygen poor environment, it can be easily transformed, by microorganisms, to As (III) or dimethylarsenate, which can be easily mobilized and rapidly absorbed by most soils (Nachman et al. 2005).

It has been reported that the risk of human contamination as a consequence of consuming animals fed with this arsenical compound is minimal because its accumulation in the animal is slow and it is mainly excreted unchanged in feces and urine. In fact, according to U S Environmental Protection Agency (EPA 1988), human problems caused by arsenic can be associated with water and fish consumption but rarely to domestic animals or meat. Nevertheless, a recent study by Nachman et al. (2013), in samples from chicken breasts for human consumption in the USA, showed that animals with detectable roxarsone (presumably representing treated chicken) had higher inorganic Arsenic concentrations than chicken without detectable roxarsone.

Besides arsenical compounds, there is a wide variety of substances, either naturally present or man-made, that pollute the environment. Among them, we can include heavy metals, dyes, pesticides, rubber chemicals and diverse industrial wastes and different approaches have been devised in order to reduce them. These strategies include, for example, adsorption to remove heavy metals or dyes (Jain et al. 2003; Gupta et al. 2006, 2007a, 2009, 2010, 2011; Mittal et al. 2008), photochemical degradation (Gupta et al. 2007b), biosorption (Mittal et al. 2010) and advanced oxidation process (AOP) (Karthikeyan et al. 2012).

Microbial transformation is one of the natural processes helping to remove chemical compounds from the environment, being one of the most cost-effective methods among remedial approaches. In the case of waters, Gupta et al. (2013) classify biological routes (mediated mainly by microorganisms) as secondary water treatment technologies. Nevertheless, there are few reports about the isolation and identification of microorganisms involved in the transformation of the arsenical compound roxarsone. Studies by Stolz et al. (2007) demonstrated that *Clostridium* species present in fowl manure rapidly transform roxarsone into inorganic arsenate under anaerobic conditions, pointing out that it can be easily lixiviated and added to aquifers and springs providing water to the population. On the other hand, Garbarino et al. (2003) demonstrated the biotransformation of roxarsone to inorganic arsenic and other metabolites, but they did not isolated nor identified the microorganisms involved.

The main object of this work was to search for and characterize a consortium capable to degrade roxarsone in order to promote its possible use as a biological treatment of manure before using it as fertilizer, thus avoiding arsenic contamination of soils, superficial and underground waters. For this purpose, we studied the microbial transformation of roxarsone by a bacterial consortium isolated from soil previously treated with poultry litter (sampled at an agricultural field in the Bio Bio region Chile, during autumn 2011) characterizing it and evaluating its growth kinetics and metabolic activity.

## Materials and methods

### Sampling

A soil sample was collected from soil fertilized for years with poultry litter from a poultry industry using roxarsone. Sampling took place in the vicinity of Florida, Bio Bio Region, Chile (36°47′31.02″ South and 72°44′13.57″ West) in May 2011. Samples from superficial soil (5 cm) were obtained and stored at 4 °C until further analysis. All further analyses were done at the Laboratory of Environmental Microbiology, Department of Microbiology, Faculty of Biological Sciences, University of Concepcion, Concepcion, Chile.

### Enrichment of bacterial consortium

Five grams of soil was inoculated in 100 mL of basal medium (Stolz et al. 2007). The sample was incubated at room temperature (nearly 25 °C) with agitation (100 rpm) and under aerobic condition, during 7 days, in the dark. This process was repeated three times, every 7 days. Then, an inoculum of the bacterial consortium was transferred into basal medium as above plus 0.5 mM roxarsone for its stabilization, while another was cultured in the absence of roxarsone (control).

### Scanning electronic microscopy

The bacterial consortium plus 0.5 mM roxarsone and without roxarsone (negative control) were incubated for

168 h, to be studied, after harvesting, under scanning electron microscopy (SEM). Treatment of the samples involved washing, fixing and drying of cells. Harvested cells were washed thrice with phosphate-buffered saline (PBS, pH 7.4) and layered onto polylysine-coated cover slips. Fixation was done using modified Karnovsky's fixative (2 % paraformaldehyde and 3 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4). Cells were again washed with PBS and distilled water. Fixed cells were dehydrated through a series of ethyl alcohol (30, 50, 70, 90 and 100 %) and finally layered with *t*-butyl alcohol for freeze-drying and sputter coating. Samples were visualized under a JEOL JSM 6380LV SEM.

### DNA extraction, 16 s rDNA amplification and denaturing gradient gel electrophoresis (DGGE) analyses

Total DNA of the bacterial consortium grown in basal medium, in the presence or absence of roxarsone was extracted using the Ultra Clean soil DNA extraction kit (MO BIO Laboratories, Inc.) following the protocol provided by the manufacturer. Both total DNAs were amplified with ARNr 16 s universal primers EUB 9-27 and EUB 1542 (Brosius et al. 1978). Nested PCR was performed using the primer pair 341f and 534r-GC-clamp (Muyzer et al. 1993) attached to the forward primer. Hot-start PCR was carried out in a 50 µL reaction mixture containing 5 µL of 10× (Sigma) with 15 mmol of $MgCl_2$ $L^{-1}$, 1 µmol of each primer $L^{-1}$, 200 µmol of deoxynucleoside triphosphates $L^{-1}$, 1 U of Taq DNA polymerase (Sigma) and 0.2–1.0 µL of DNA extract. The touchdown temperature program consisted of 6 min at 94 °C; 30 cycles of 15 s at 94 °C 30 s at the annealing temperature and 2 min and 30 s at 72 °C; and a final extension at 72 °C for 3 min. During the first 20 cycles, the annealing temperature was decreased by 0.5 °C in each cycle from 50 to 40 °C. For nested PCR with the primer pair 341f and 534r, the temperature program consisted of 2 min at 94 °C and 30 cycles of 15 s at 94 °C 1 min at the annealing temperature and 1 min 30 s at 72 °C. The annealing temperature was decreased during the first 20 cycles by 0.5 °C in each cycle from 65 to 55 °C, and a final extension of 3 min at 72 °C was added. PCR products were checked for concentration, purity and appropriate size by agarose gel electrophoresis and Gel Red nucleic acid staining (Biotium) (Campos et al. 2011).

Denaturing gradient gel electrophoresis (DGGE) was performed with a DGGE 1001 system (C.B.S. Scientific Company Inc.). Fifteen microliters of PCR products V3 region was applied directly onto 6 % (wt/vol) polyacrylamide gels in 13 TAE (40 mM Tris 20 mM acetate 1 mM EDTA) with denaturant gradient from 20 to 60 % (where 100 % denaturant contains 7 M urea and 40 % formamide). Electrophoresis was performed at a constant voltage of 200 V at 60 °C for 6 h. After electrophoresis, gels were stained for 20 min with SYBR Gold nucleic acid gel stain (Molecular Probes), as specified by the manufacturer, and visualized on a transiluminator (UVP Inc) (Campos et al. 2011).

### Analysis of DGGE profiles

Magnified sections of DGGE gels were photographed with a ChemImager 4000 imaging system (Alpha Innotech). Bands of OTUs (operational taxonomic units), defined as those having an intensity of at least 5 % of the most intense band in the sample, were scored as present or absent at each position in the gel using the Gel-Pro Analyzer 4.0 software package (Applied Maths). For comparison of banding profiles, a binary matrix was constructed based on the presence (1) or absence (0) of individual bands in each lane. The binary data representing the banding patterns were used to generate a pairwise Dice distance matrix (León et al. 2012). The distance matrix was used for constructing a multidimensional scaling diagram (MDS), a two dimensional map with artificial *x*- and *y*-axis, where each DGGE fingerprint is placed as one point in a way that similar samples are plotted together. Clustering analysis and MDS were performed using the PRIMER V.6 software package (Clarke and Gorley 2001).

### Fluorescence in situ hybridisation and DAPI staining

For the analysis by FISH (Amann et al. 1995) were used probes specific EUB338 ALF968 BET42a and GAM42a for Bacteria the alpha beta and gamma subclasses Proteobacteria, respectively (Manz et al. 1992) and LGC354 (A, B and C) for Firmicutes (Meier et al. 1999). All samples used for these probes were fixed using the protocol for Gram-negative bacteria with paraformaldehyde, which renders most Gram-positive bacteria unlabeled as previously described (Manz et al. 1992). DAPI staining (46-diamino-2-phenylindoldihydrochloride-dilactate) was applied after fixation of the sludge with paraformaldehyde by adding DAPI to a final concentration of 1 mg $mL^{-1}$ for 30 min in the last washing step.

### Bacterial degradation of roxarsone

The bacterial consortium was growth in basal medium with roxarsone (0.5 mM) during 168 h. Basal medium with 0.5 mM roxarsone was used as negative control. Aliquots (1 mL) were obtained each 12 h and filtered (0.22 µm Millipore). Then, 200 µL of each aliquot were transferred, in triplicate, to 96-wells plates. Roxarsone degradation was

quantified, using a microplate spectrophotometer (Epoch, BIOTEK), by means of spectrograms integration (310–500 nm) by the trapezoid method, using the Gen5 software (BIOTEK).

### Growth kinetics of bacterial consortium

Cell growth was determined, in each of the triplicates, at intervals, during the 168 h incubation, by optical density measurements (at 600 nm) of the 96-well plates using a microplate spectrophotometer (Epoch, BIOTEK). The curves obtained were analyzed through mathematical modeling approaches: Gompertz, Logistic, exponential Malthusian, exponential plateau and Weibull (Zwietering et al. 1990). The best model was selected by the Fisher test and comparing the coefficients of determination ($R^2$). Graphs and models were made using GraphPad Prism version 5.0 (GraphPad software, USA).

### Analytical methods

Inorganic arsenic detection was carried out through a modification of the silver nitrate ($AgNO_3$) technique described by Simeonova et al. (2004). Experiments were carried out using basal medium supplemented with arsenate (0.1 mM) or arsenite (0.1 mM) as positive controls and basal medium with roxarsone (0.5 mM) and without arsenical inorganic compounds was used as negative control. For bacterial consortium analyses, the samples were filtered (0.22 µm Millipore), titrated with 0.1 M $AgNO_3$ and incubated at room temperature for 3 h. The production of a yellow precipitate indicates the presence of arsenite, and the production of a brown precipitate indicates the presence of arsenate.

### Community-level physiological profiles (CLPP)

The metabolic characterization was carried out using Biolog Ecoplates (Biolog) (Garland and Mills 1991). The 96-well Biolog Ecoplate contained three replicate wells of 31 carbon substrates and, for each replicate, a control well without a carbon substrate was included. Aliquots of each consortium capable to degrade roxarsone were taken, diluted and adjusted to $1 \times 10^6$ cell $mL^{-1}$ (counted by means of a Neubauer chamber). Then, the 96-well Biolog Ecoplates were inoculated with 150 µL from each aliquot and incubated at 25 °C. The optical density ($\lambda = 590$ nm) of each well was determined at time 0 and every 24 h thereafter up to 120 h using a microplate reader (Epoch, BIOTEK).

The CLPP was determined by calculating average well color development (AWCD), Richness (S), Evanness Index (E) and Shannon–Weaver Diversity Index (H') (Harch et al. 1997; Garland 1997; Gomez et al. 2006).

The carbon sources were divided by category such as: carbohydrates ($N = 10$), carboxylic acid ($N = 9$), amino acid ($N = 6$), polymers ($N = 4$) and amines ($N = 2$) (Garland and Mills 1991). Then, AWCD was calculated for each category during the time (120 h), and a principal component analysis (PCA) was performed using the version 15 MINITAB software (USA).

Finally, the carbon sources were divided by category such as: aromatic ($N = 4$) and no aromatic ($N = 27$). Then, AWCD results were analyzed using the GraphPad Prism 5 software.

### Statistical analysis

Bacterial roxarsone degradation, growth rates (K) of the models that best conformed to the kinetics of bacterial consortiums growth and results of CLPP were analyzed through Student's $t$ tests using the MINITAB version 15 (USA) software. $P$ values $< 0.05$ were considered as statistically significant.

## Results and discussion

### Morphological and molecular characterization of the bacterial consortium

A microbial consortium was isolated after 1 month of selective enrichment by repeated subcultures. Once obtained, the consortium was divided into two subcultures, one cultured in absence of roxarsone (control) and the other in presence of roxarsone. SEM analyses showed that both bacterial consortiums, either with or without roxarsone, present heterogeneous communities with predominance of bacillary species after 168 h of incubation (Fig. 1).

In order to assess the composition of the bacterial community of the consortiums, DGGE were performed for cultures with and without roxarsone. DGGE bands profile of samples cultured in the presence of roxarsone showed the presence of 11 OTUs (Fig. 2). The closest GenBank matches for 16S rDNA sequences revealed the presence of 4 bacterial groups, namely Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Firmicutes. Identities were confirmed with 98–100 % similarity (Table 1).

OTUs profile demonstrated that most sequences detected (7 out of 11) belong to Alphaproteobacteria, a group described as able to degrade aromatic compounds (Buchan et al. 2000; Shaw and Burns 2004; Fuchs et al. 2011). It is worthwhile mentioning that OTUs 3, 6, 8 and 10 were present only when roxarsone was present, suggesting that roxarsone stimulated the growth of certain microorganisms.

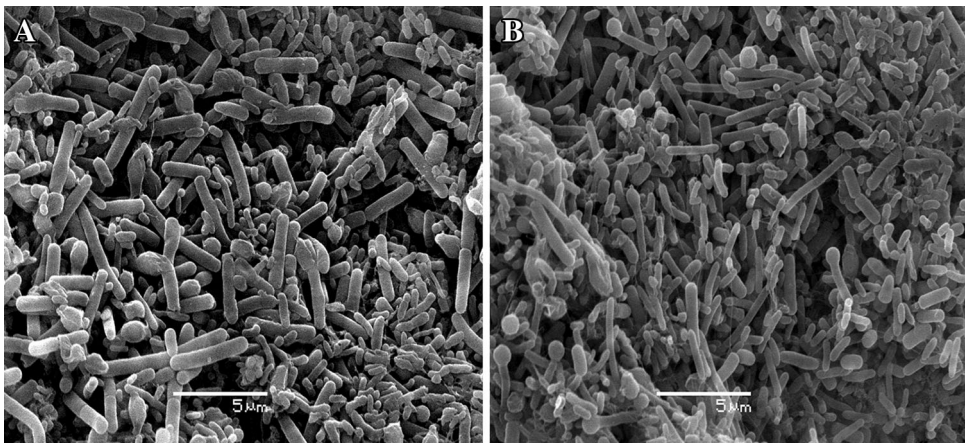


**Fig. 1** Scanning electron microscope (SEM) of bacterial consortium after 48 h of incubation. **a** Bacterial consortium without roxarsone. **b** Bacterial consortium with roxarsone

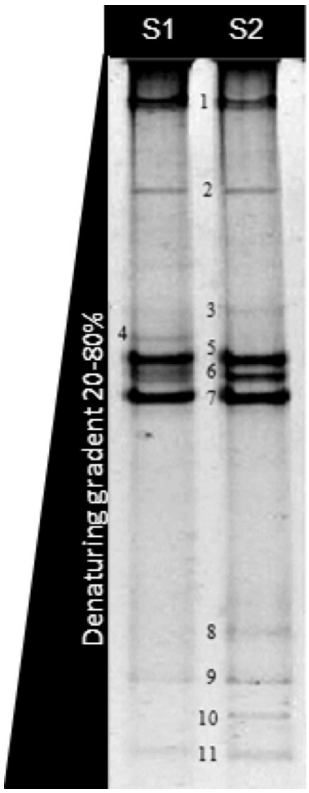


**Fig. 2** DGGE of 16S rDNA products amplified with the primers P2 and P3 (GC-clamp) with a denaturing gradient (20–80 %). *S1* Bacterial consortium without roxarsone. *S2* Bacterial consortium with roxarsone

**Table 1** Analysis of 16S rDNA sequences obtained by DGGE

| Band | Closest sequence relative | GenBank access | Bacterial group |
|---|---|---|---|
| 1 | Uncultured Rhizobiales bacterium | JQ254341 | Alphaproteobacteria |
| 2 | *Bacillus* sp. | DQ268774 | Firmicutes |
| 3 | *Lysobacter* sp. | JX097006 | Gammaproteobacteria |
| 4 | Uncultured Burkholderiales bacterium | HM486305 | Betaproteobacteria |
| 5 | *Rhizobium* sp. | JN089717 | Alphaproteobacteria |
| 6 | *Agrobacterium* sp. | JF262579 | Alphaproteobacteria |
| 7 | *Rhizobium* sp. | JN089717 | Alphaproteobacteria |
| 8 | *Sphingomonas* sp. | AY806752 | Alphaproteobacteria |
| 9 | *Rhizobium* sp. | GU477469 | Alphaproteobacteria |
| 10 | *Aurantimonas* sp. | JQ346806 | Alphaproteobacteria |
| 11 | *Bacillus* sp. | JX485844 | Firmicutes |

In addition, DGGE profile OTUs were analyzed using the Bray–Curtis correlation. A distance matrix was calculated ,and a cluster analysis was performed which resulted in a multidimensional scaling (MDS). The similarity percentage (Fig. 3), calculated from the banding patterns, indicated that roxarsone affected the structure of the consortiums, showing a decrease in the percentage of similarity from 70 to 50 % in the presence of roxarsone. Concordantly, Jiang et al. (2013), studying soil bacterial communities, showed significant effects in their diversity and metabolism in the presence of roxarsone.

The microbial abundance and major phylogenetic groups were examined through DAPI staining and hybridization with specific oligonucleotide probes (EUB 338). The microbial populations and communities were analyzed in both bacterial consortiums. Total cell numbers were determined by DAPI staining, and domain bacteria numbers were counted by hybridization with EUB 338 probe. The results showed that the total cell counts of the bacterial consortium in the presence or absence of roxarsone were $8.2 \times 10^7$ and $7.2 \times 10^7$ cells $mL^{-1}$, respectively. Counts for members of the Bacteria domain only, were $6.1 \times 10^7$ and $5.3 \times 10^7$ cells $mL^{-1}$ in the presence or absence of roxarsone, respectively. Due to the fact that 75 % of the DAPI counts were hybridized to the EUB 338 bacterial probe, we assume that bacteria were the dominant group in the both consortiums.

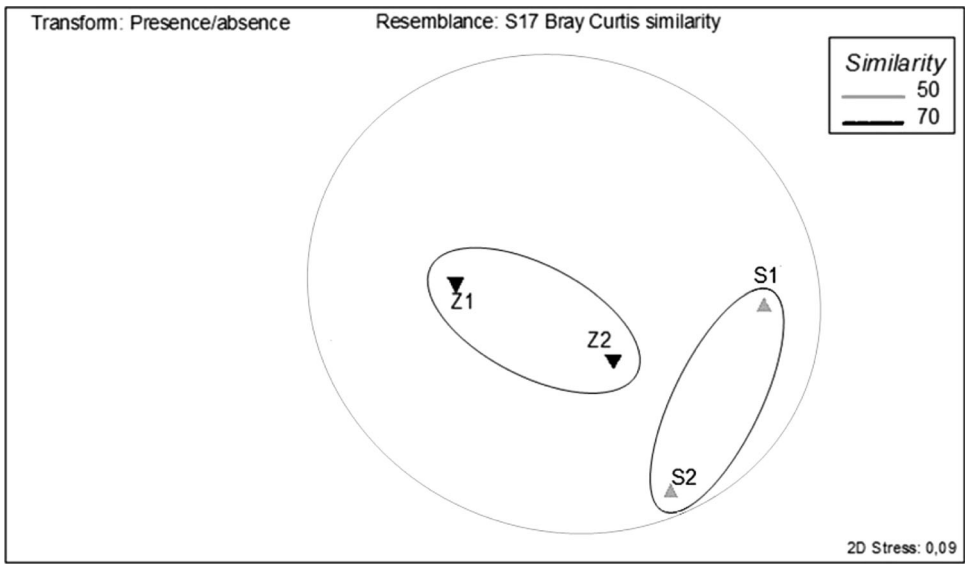


**Fig. 3** Multidimensional scaling (MDS) of the DGGE data matrix of Eubacteria rDNA fragments from bacterial consortium without ROX (*Z1* and *Z2*) and bacterial consortium with ROX (*S1* and *S2*). Similarly, index was evaluated for percentage

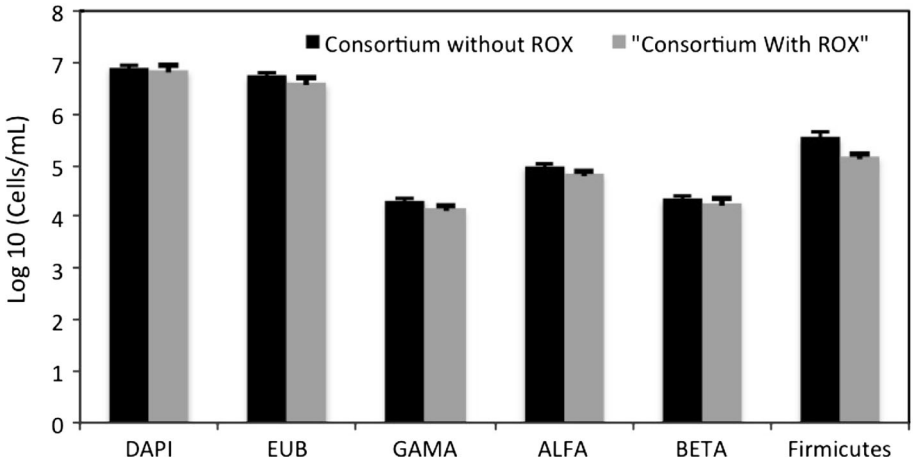


**Fig. 4** Bacterial community composition in sediments samples from consortium without ROX and consortium with ROX determined by FISH. Probes specific: EUB338 for Bacteria and ALF968 BET42a and GAM42a for alpha beta and gamma subclasses Proteobacteria, respectively. LGC354 (A, B and C) was used for Firmicutes

Major phylogenetic groups present in the bacterial consortiums were investigated (Fig. 4). Alphaproteobacteria and Firmicutes were the groups showing the highest counts in both consortiums. In addition, Firmicutes were the group that showed the highest counts in the consortium both in the presence or absence of roxarsone. Betaproteobacteria and Gammaproteobacteria subgroups showed low counts, possibly due to the low content of detrital and humic substances in the soil as these are regarded as a favorite nutrients for proteobacteria (León et al. 2012).

## Consortium growth and roxarsone degradation

In order to determine the effect of roxarsone on growth of the consortium, its growth kinetics was studied in the presence and absence of roxarsone by means of the Weibull algorithm (Zwietering et al. 1990) (Fig. 5). The model demonstrated that after of 168 h of incubation the bacterial consortium cultured in the presence of roxarsone showed a better growth than the control, with growth rates of 0.01689 and 0.01190 OD $h^{-1}$, respectively. Student's *t* test (with 95 % confidence) demonstrated statistically significant differences between both conditions ($P = 0.039$), which represents a 1.4-fold increase in *k* when roxarsone was present. It is possible that the bacterial consortium was using roxarsone as an additional carbon source.

Spectrophotometrical analyses evaluating the use of roxarsone demonstrated that the bacterial consortium, after 168 h of incubation at 25 °C under aerobic conditions, were able to degrade 81.04 % of this compound present in the culture medium, (data not shown). These results were similar to those by Stolz et al. (2007) for soil samples but under anaerobic conditions, reaching oxidation percentages close to 100 % after 9 days to incubation. Theses authors did not report roxarsone transformation under aerobic conditions. In addition, Cortinas et al. (2006) studied roxarsone degradation, in soil samples, under anaerobic and aerobic conditions, reporting the absence of roxarsone bioconversion under aerobic or

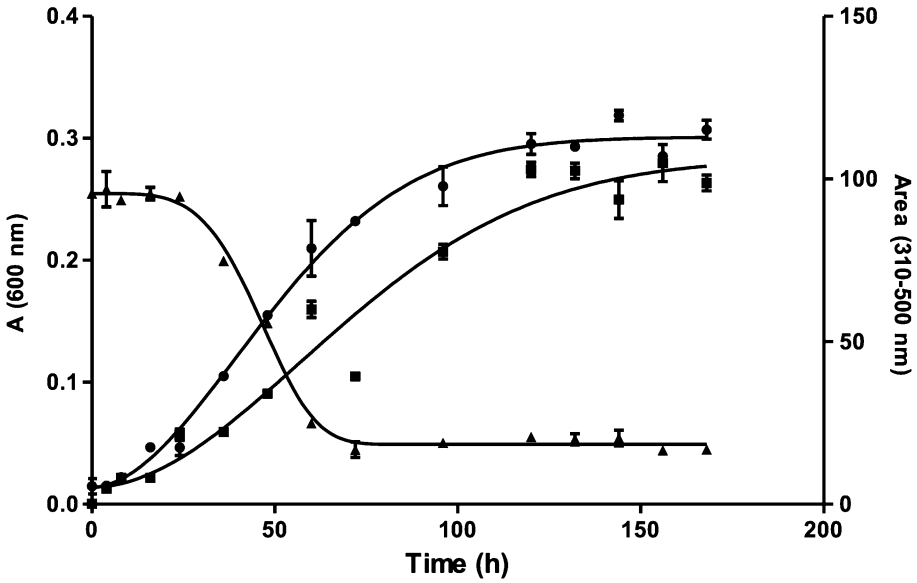


**Fig. 5** Growth kinetics of bacterial consortium in the presence and absence of ROX and ROX degradation. Rate of roxarsone degradation was 0.09582 OD nm $h^{-1}$ ($R^2 = 0.9935$). Growth rate of bacterial consortium was 0.09574 OD $h^{-1}$ ($R^2 = 0.9561$) and bacterial consortium with roxarsone was 0.01689 OD $h^{-1}$ ($R^2 = 0.9829$). Kinetics and degradation were adjusted with Weibull growth. Degradation of roxarsone (*solid line with triangle*), bacterial consortium without roxarsone (*solid line with square*) and bacterial consortium with roxarsone (*solid line with circle*)

denitrifying conditions. However, under anaerobic conditions, they reported 41 and 46 % roxarsone removal with and without sulfate addition, respectively, and an increase of biotransformation to 71 % when lactate was added. Therefore, this work demonstrates that a bacterial consortium can also, effectively, biotransform roxarsone under aerobic conditions, and easier and cost-effective condition, if compared with anaerobic conditions to treat roxarsone-contaminated soils

Roxarsone degradation kinetics by the bacterial consortium was adjusted using the Weibull growth algorithm showing a value of $R^2 = 0.9935$, indicating the fitness of the model. A marked slope of the degradation curve was apparent between 24 and 60 h of incubation, a time span coinciding with a bacterial improved growth in the presence of roxarsone. This supports the possible use of roxarsone as a carbon source by the consortium, it being degraded at a rate of 0.09582 OD nm $h^{-1}$ (Fig. 5).

The presence of inorganic arsenic compounds resulting from roxarsone degradation by the bacterial consortium was searched using the silver nitrate technique, demonstrating the presence of these compounds, specifically arsenate, after 168 h of incubation (data not shown). Since arsenate is the most abundant inorganic species resulting from roxarsone degradation this result was expected. (Cortinas et al. 2006; Stolz et al. 2007; Andra et al. 2010). Also, the production of considerable amounts of arsenate and several other roxarsone transformation intermediates was recently confirmed by D'Angelo et al. (2012), while litter is accumulated in broiler houses.

## Metabolic analysis of the bacterial consortium

The metabolic profile of the bacterial consortium in the presence or absence of roxarsone was investigated by

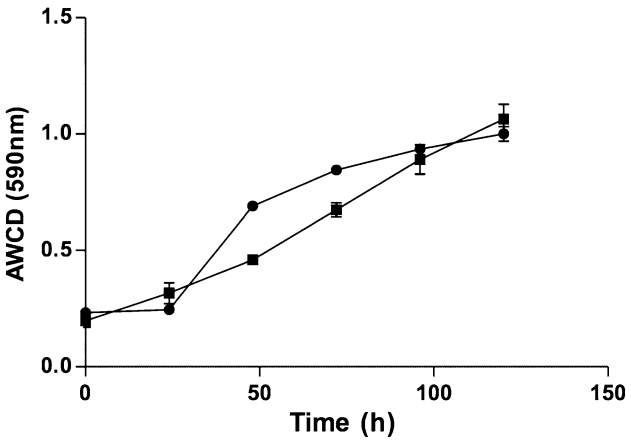


**Fig. 6** The average well color development (AWCD) of all carbon sources as a measure of bacterial functional diversity. Bacterial consortium without roxarsone (*solid line with circle*) and bacterial consortium with roxarsone (*solid line with square*)

calculating the average well color development (AWCD). AWCD did not show a significant difference (Student's $t$ test, $P = 0.458$) at the end of the 120 h incubation. But, during development of the AWCD assay, there was a significant difference at 48 and 72 h of incubation (Student's $t$ test, $P = 0.002$ and $P = 0.019$, respectively) showing that color development in the bacterial consortium without roxarsone, meaning that the oxidative capacity is negatively affected by roxarsone during the first hours of incubation (Fig. 6). Since results from the molecular characterization of the consortium cultured in the presence of roxarsone showed changes in its diversity (see above), if these results are associated with AWCD results, its is possible to propose that the community is adapting to the presence of roxarsone at this time span, including the

disappearance of some bacteria (supported by the decrease in diversity of the consortium) and finally favoring the growth of others.

When analyzing AWCD for groups of substrates, using the Student's *t* test (Fig. 7a, b), the bacterial consortium in the presence or absence of roxarsone were not significantly different for carbohydrates ($P = 0.160$), carboxylic acid ($P = 0.795$) and polymers ($P = 0.478$). Nevertheless, there was a statistically significant difference for amino acids ($P = 0.04$) and amines ($P = 0.002$), being amino acid AWCD greater in the absence roxarsone while amines AWCD was greater in its presence. Additionally, no statistically significant difference was observed when substrates were grouped as aromatic ($P = 0.191$) and non-aromatic ($P = 0.122$).

The CLPP in Ecoplates was determined using various ecological indexes, such as Richness (S), Shannon–Weaver Diversity Index (H') and Evanness Index (E), during the 120 h of incubation (Garland 1997). Regarding the S Index, the bacterial consortium without roxarsone had the capacity to metabolize 29 of the 31 substrates tested. But, in its presence, the metabolized substrates decreased to 24, probably due to the metabolic pressure exerted by roxarsone on some carbon sources; that is to say, that the various species of the consortium able to degrade toxic organic compounds have different metabolic responses in the presence or absence of roxarsone. No statistically significant difference was observed when comparing the H' Index for the bacterial consortium in the presence or absence of roxarsone (Student's *t* test, $P = 0.265$), meaning that the metabolic diversity of the consortium did not change, despite roxarsone presence. The E Index revealed a significant difference, being greater in the consortium with roxarsone (Student's *t* test, $P = 0.01$), meaning that the

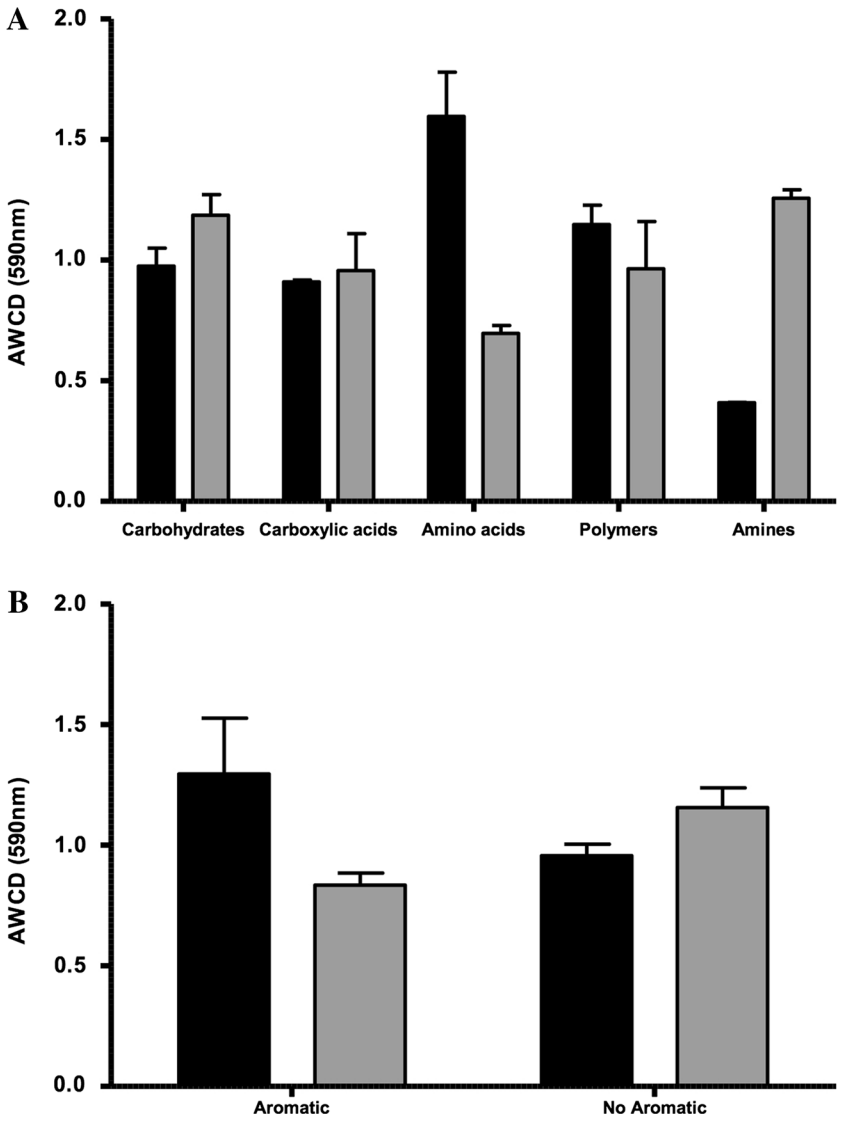


**Fig. 7** Relative average well color development (AWCD) calculated from absorbance values of Biolog EcoPlate for bacterial consortium with and without roxarsone. The absorbance was measured after 120 h incubation. **a** The substrates were divided in five categories: carbohydrates ($N = 10$), amines ($N = 2$), amino acids ($N = 6$), carboxylic acids ($N = 9$) and polymers ($N = 4$). **b** The substrates were divided into two categories: aromatic ($N = 4$) and non-aromatic ($N = 27$). Bacterial consortium without roxarsone (*black rectangle*) and bacterial consortium with roxarsone (*gray rectangle*)

equitability of substrate utilization among all substrates utilized increases in the presence of roxarsone (Fuller et al. 1997).

Principal component analysis (PCA) was performed utilizing the AWCD of substrate groups (carbohydrates, carboxylic acids, amino acids, polymers and amines) for the bacterial consortium in the presence or absence of roxarsone (data not shown). The PCA of the bacterial consortium lacking roxarsone (explaining 91.6 % of the variance) presented a first component (PC1) with a significant positive correlation with carbohydrates, amino acids, polymers and amines, while the second component (PC2) had a significant positive correlation with carboxylic acids and amines and negative correlation with carbohydrates and polymers. On the other hand, the PCA of the bacterial consortium in the presence of roxarsone (explaining 95.8 % of the variance) changed its correlation in the different components, showing that while PC1 had a positive correlation with carbohydrates, carboxylic acids, amino acids and polymers; PC2 had a positive correlation with amines and negative correlation with carbohydrates, carboxylic acids, amino acids and polymers. In brief, these results demonstrate that the presence of roxarsone favors the oxidation of amines rather than the metabolization of other carbon sources.

These results also demonstrated that in the absence roxarsone, the oxidation of amines requires other carbon sources while in the presence of roxarsone, the bacterial consortium changed the oxidation patterns and the capacity to oxidize amines becomes independent of the oxidation of other carbon sources. Even more, amines oxidation when roxarsone was present showed a negative correlation with the other carbon sources. Probably, the independence of amines oxidation and increasing capacity to metabolize this carbon source is related to roxarsone degradation, occurring firstly by the reduction in the nitro group, then by oxidative fission of the aromatic ring (most important stage) and finally by the breaking of the C–As (Wershaw et al. 1999).

Additionally, the oxidation of aromatic and non-aromatic compounds did not change despite roxarsone presence, suggesting that the reduction in the nitro group, producing a functional amine, should occur first, followed by the decrease in the stability of the aromatic ring resonance energy, the principal problem associated with aromatic compounds degradation (Carmona et al. 2009; Fuchs et al. 2011). Even more, by means of a prediction model software available online (http://www.chemicalize.org) described by Miller and Savchik (1979), it was possible to evidence a decrease in roxarsone polarizability (18.73) to 4-hydroxy-3-aminophenylarsonic acid, the functional roxarsone amine (17.75), confirming the need for the reduction in the nitro group.

## Conclusion

The results demonstrate the ability of a bacterial consortium, isolated from an agricultural soil, to degrade roxarsone under aerobic conditions with the subsequent release of inorganic arsenic, mainly arsenate. Despite roxarsone-induced changes in the diversity of the consortium, the general metabolic profile of the consortium did not change in the presence of this organoarsenical compound, except for a greater ability to oxidize amines. The oxidation of the aromatic ring is a fundamental reaction for roxarsone degradation, but it will not occur unless there is a reduction in the nitro group of this compound.

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