World J Microbiol Biotechnol (2009) 25:553–560
DOI 10.1007/s11274-008-9923-z

ORIGINAL PAPER

Degradation of 2,4,6-tribromophenol and 2,4,6-trichlorophenol by aerobic heterotrophic bacteria present in psychrophilic lakes

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Received: 25 June 2008 / Accepted: 17 November 2008 / Published online: 30 November 2008

Abstract Halogenated compounds have been incorporated into the environment, principally through industrial activities. Nonetheless, microorganisms able to degrade halophenols have been isolated from neither industrial nor urban environments. In this work, the ability of bacterial communities from oligotrophic psychrophilic lakes to degrade 2,4,6-tribromophenol and 2,4,6-trichlorophenol, and the presence of the genes *tcp*A and *tcp*C described for 2,4,6-trichlorophenol degradation were investigated. After 10 days at 4°C, the microcosms showed the ability to degrade both halophenols. Nonetheless, bacterial strains isolated from the microcosms did not degrade any of the halophenols, suggesting that the degradation was done by a bacterial consortium. Genes *tcp*A and *tcp*C were not detected. Results demonstrated that the bacterial communities present in oligotrophic psycrophilic lakes have the ability to degrade halophenolic compounds at 4°C and the enzymes involved in their degradation could be codified in genes different to those described for bacteria isolated from environments contaminated by industrial activities.

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Keywords Bromophenol · Biodegradation · Bacterial consortium · Psychrophilic

Introduction

Halophenolic compounds are part of a group of pollutants that are incorporated into the environment, largely due to industrial activities. 2,4,6-trichlorophenol (246TCP) is widely used as a wood preservative, herbicide, insecticide, and fungicide (Aranda et al. 2003; Xun and Webster 2004; Sánchez et al. 2004), whereas 2,4,6-tribromophenol (246TBP) is used in the wood industry to control fungi (Gutiérrez et al. 2002) and as a flame retardant (Hassenklöver et al. 2006). Due to their constant use, these compounds are common pollutants of soils and freshwater. Fulthorpe and Schofield (1999) reported that microorganisms recently developed the ability to degrade halogenated aromatic compounds as a response to their use as pesticides, even as sole carbon and energy source (Kharoune et al. 2002; Sánchez et al. 2004; Yamada et al. 2008). Nevertheless, several studies have shown that bacteria isolated from environments not exposed to this type of pollutant are able to degrade organochlorinates (Kamagata et al. 1997; Sánchez et al. 2004). Furthermore, in nature, several organisms have been described as producers of chlorinated and brominated organic compounds (Gribble 1992, 1998; Whitfield et al. 1999; Ahn et al. 2003; Vetter and Janussen 2005). Thus, bacterial communities associated with these organisms might be exposed to the presence of halophenolic compounds of biological origin.

In the aerobic degradation of chlorophenol compounds, the first steps are carried out by mono- and dioxygenase enzymes. *tcp*A and *tcp*C genes have been described for the degradation of 246TCP by *Cupriavidus necator* JMP134.

These genes codify the enzymes 2,4,6-trichlorophenol monooxygenase (TCP-MO) and 6-chlorohydroxyquinol-1,2-dioxygenase (HQDO), respectively. The enzyme TCP-MO is involved in the dehalogenation process of 246TCP, whereas the enzyme HQDO participates in cleavaging of the aromatic ring (Louie et al. 2002; Matus et al. 2003; Sánchez and González 2007). These enzymes were described for the 246TCP degradative pathway. However, Aranda et al. (2003) suggested that the enzymes involved in the degradation of halophenolic compounds do not discriminate between chlorinated or brominated substrates.

The degradation properties of most of the bacteria that have shown the ability to degrade halophenols have been characterized at temperatures between 20 and 30°C (Fulthorpe et al. 1996; Kamagata et al. 1997; Kharoune et al. 2002; Aranda et al. 2003; Godoy et al. 2003; Matus et al. 2003; Sánchez et al. 2004). However, little information is available regarding the activity of the aerobic psychrophilic bacteria participating in the metabolism of halophenols such as 246TBP.

The aim of this work was to study the ability to degrade 246TBP by aerobic heterotrophic bacterial communities from psychrophilic and oligotrophic lakes, (Soto 2002; Woelfl et al. 2003) of the Chilean Patagonia; and also to investigate if this ability is due to the presence of the *tcp*A and *tcp*C genes described for 246TCP degradation.

Materials and methods

Study sites and sampling

Samples (1l) were taken from four lakes (Alto Reino, Las Dos Torres, Venus, Orilla Camino) in the Chilean Patagonia (44–46° S, 72–73° W). The samples were stored at 4°C until being processed in the laboratory. The presence of organohalogenated compounds in the water samples was determined by gas chromatography with an electron capture detector (GC-ECD) and gas-mass chromatography (GC-MS). For this, 10-ml samples were extracted with n-hexane (three times) and concentrated to 500 µl. The chromatographic analysis (GC-ECD) was carried out with a Shimadzu 9A with a Shimadzu C-R7A Chromatopac integrator. An HP-5 capillary column (30 m × 0.53 mm) (J&W Scientific) was used. Carrier N_2 (30 psi), split less 1:100, injection temperature 275°C, oven temperature 210°C. The GC-MS was carried out in an Agilent 5890 Series II gas chromatograph with a Hewlett Packard 5,972 mass detector equipped with a capillary column (HP-5MS; 30 m × 0.25 mm) (J&W Scientific). Lindane, DDT, myrex, aldrin, dieldrin and heptachlorine, 2,4,6-trichlorophenol and 2,4,6-tribromophenol were used as standards for GC-MS.

Bacterial counts

The total bacterial community in the water samples was determined by fixing 50 ml of water with 3% formalin. Fixed samples were stained with acridine orange (125 µg/ml) and the bacterial counts were done by triplicate using epifluorescent microscopy, (Hobbie et al. 1977). The viable bacterial counts were done by inoculating, by triplicate, 20 µl aliquots of serial dilutions on the surface of petri dishes with R2A agar (HeMedia), and incubated at 4 or 30°C for 48–120 h (Herbert 1990).

Halophenol degradation test in water microcosms

The ability of the bacterial populations present in the water samples to degrade 246TBP and 246TCP was studied by preparing microcosms in 250 ml Erlenmeyer flasks. For this purpose, to 75 ml of the water sample, 25 ml of threefold concentrated mineral saline medium (MSM) was added (Aranda et al. 2003). In addition, each Erlenmeyer flask was added with 20 µg/ml of 246TCP or 246TBP. To determine if the degradation of halophenols is carried out by biological process, a negative control with autoclaved water sample was performed as described above. The Erlenmeyer flasks were then incubated at 4 or 20°C and stirred constantly (100 rpm). Every 48 h, 1 ml aliquots were taken for UV spectroscopy to analyze the aromatic ring cleavage (Aranda et al. 2003) and viable bacterial counts (Herbert 1990). After the complete degradation of halophenols, the microcosms were again added with 246TBP or 246TCP (20 µg/ml) and incubated at 4°C, and bacterial counts and degradative kinetics were evaluated as described above. Also, 1 ml of the samples was stored to confirm the complete degradation of halophenols. The presence of halogenated compounds was evaluated through high resolution liquid chromatography (HPLC-DAD) (Agilent Series 1100, California, USA) and a diode arrangement detector using a Lichospher 100 RP-18.5 µm column (250 × 4 mm) (Merck). About 20 µl of the samples obtained at the beginning and end of the experiment were injected. The chromatographic conditions were mobile phase acetonytrile 50% (vol/vol) in 30 mM ammonium acetate, flow 1 ml/min, column temperature 38°C.

Metabolic profile study of the bacterial microcosms

The diversity of carbon sources that can be used by the bacterial communities present microcosms were determined by using the Biolog Ecoplate™ system (Biolog Inc., CA, USA). Once the complete degradation of halophenols was detected, each well of the microplate was inoculated with 100 µl of 246TBP of degrading microcosm, and incubated at 4°C for 10 days. Richness was considered as

the number of oxidized carbon substrates and the microbial activity, expressed as average well-color development were determined as described by Gomez et al. (2006).

Bacterial isolation and degradation study

Aliquots (100 µl) were taken from the microcosms in which a decrease in the concentration of 246TBP or 246TCP was detected. These samples were spread onto R2A agar, and incubated for 72 h at 4 or 30°C. Later, all the different macroscopic colonies were selected and characterized according to their staining affinity as Gram positive or Gram negative.

Since to only a Gram negative nor fermentative bacteria was isolated, the biochemical properties of the isolated strains were investigated using the codified system API 20 NE (Bio Merieux). Then, the ability of isolated bacterial strains to degrade 246TBP or 246TCP was evaluated. For this, the isolated strains were cultured in R2A broth for 48 h and washed 3 times with MSM. The bacterial strains were inoculated at a cellular density of 1×10^7 CFU/ml in Erlenmeyer flasks with 100 ml MSM as sole carbon source or supplemented with glucose (0.3 mM) (Aranda et al. 2003), and 20 µg/ml of 246TBP or 246TCP and incubated at 4 and 30°C. Viable bacterial counts were done daily for five days and the degradation of the halogenated compounds was evaluated through UV spectroscopy.

The same strains inoculated as pure strains were also inoculated as a mixture of all of them in 100 ml of MSM in a 250 ml Erlenmeyer flask containing 20 µg/ml of 246TBP or 246TCP, at a density of 1×10^7 CFU/ml of each one of them, in order to evaluate their degrading ability as sole carbon source of the mixture.

DNA extraction

The DNA from the bacteria in the microcosms was purified using the Power Soil DNA Isolation Kit (Mo Bio Laboratories). DNA was extracted from *Cupriavidus necator* JMP134 and from *Cupriavidus* sp. PZK strain (Matus et al. 2003) as a positive control for the amplification of *tcp*A and *tcp*C genes using the Ultra Clean Microbial DNA Isolation Kit (Mo Bio Laboratories). The amount of DNA was determined spectrophotometrically by using a 260/280 ratio.

Amplification of *tcp*A and *tcp*C genes

The DNA obtained from the water samples and the isolated strains were checked for the presence of the genes described for the degradation of 246TCP. This was done by using the degenerated primers (TftDF1/TftDR1 and HQF/HQR) described by Louie et al. (2002) and the specific primers (MON-F/MON-R and HQ-F/HQ-R) reported by Godoy et al. (2003) and Matus et al. (2003) for *tcp*A and *tcp*C genes, respectively. The amplification conditions for the primers MON-F/MON-R and HQ-F/HQ-R and TftDF1/TftDR1 and HQF/HQR were the same as those described by Godoy et al. (2003) and Matus et al. (2003) and by Louie et al. (2002), respectively.

Results and discussion

Physico-chemical characterization of the study areas

The lakes of the study area are glacial in origin, deep, and oligotrophic (Woelfl et al. 2003), with highly transparent waters and low levels of suspended solids and dissolved organic carbon (Soto 2002).

The results obtained from the GC-ECD and GC-MS analyses of the water samples suggest the presence of two unidentified halogenated phenolic compounds whose retention times were 10.4 and 24.7 min with 247 and 303 units of mass, respectively (data not showed). No industrial activity exists in the area surrounding the water bodies in which these halogenated compounds were found. Hence, their presence could be related to biological activity since some organisms are known to produce a chemical defense system when faced with competitors (Gribble 1992; Bantleon et al. 1994; Peters et al. 2003). Moreover, the combustion of vegetable biomass during forest fires could form a variety of halogenated organic compounds including phenolics (Gribble 1998). However, anthropogenic contamination could not be ignored, since some persistant organic pollutans have the ability to be transported from distant areas (Castro-Jiménez et al. 2008).

Viable and total bacterial counts

The total bacterial count by eplifluorescence microscopy was 1×10^5 cells/ml, whereas the viable bacteria did not exceeded 1×10^3 CFU/ml (Table 1). According to these results, the proportion of bacteria that can be recovered for cultivation was less than 1%. Amann et al. (1995) reported that, in aquatic environments, that it is possible to cultivate only a small proportion (no more than 1%) of the population of microorganisms since most of the bacteria are found in an inactive or dormant metabolic state.

Halophenol degradation in the water samples

Under both temperature conditions, there was an adaptation period during which no degradation of the assayed halogenated compounds was detected (Fig. 1). Sánchez et al. (2004) reported that bacterial communities in soils not

Table 1 Total and viable bacterial counts of water from patagonic lakes

	Lake			
	Alto Reino	Las Dos Torres	Venus	Orilla Camino
Culturable bacterial count[a]	2×10^2 (2×10^1)[c]	1.2×10^2 (1.4×10^1)	1.6×10^2 (3.3×10^1)	2.5×10^2 (2.8×10^1)
Total bacterial count[b]	4.6×10^5 (1.9×10^4)	4.2×10^5 (3.1×10^4)	3.7×10^6 (4.3×10^5)	4.5×10^5 (4.7×10^4)
Culturable bacteria (%)	0.1	0.1	0.1	0.1

[a] Counts expressed in CFU/ml; [b] Counts expressed in cells/ml, [c] Values in parenthesis correspond to standard deviations

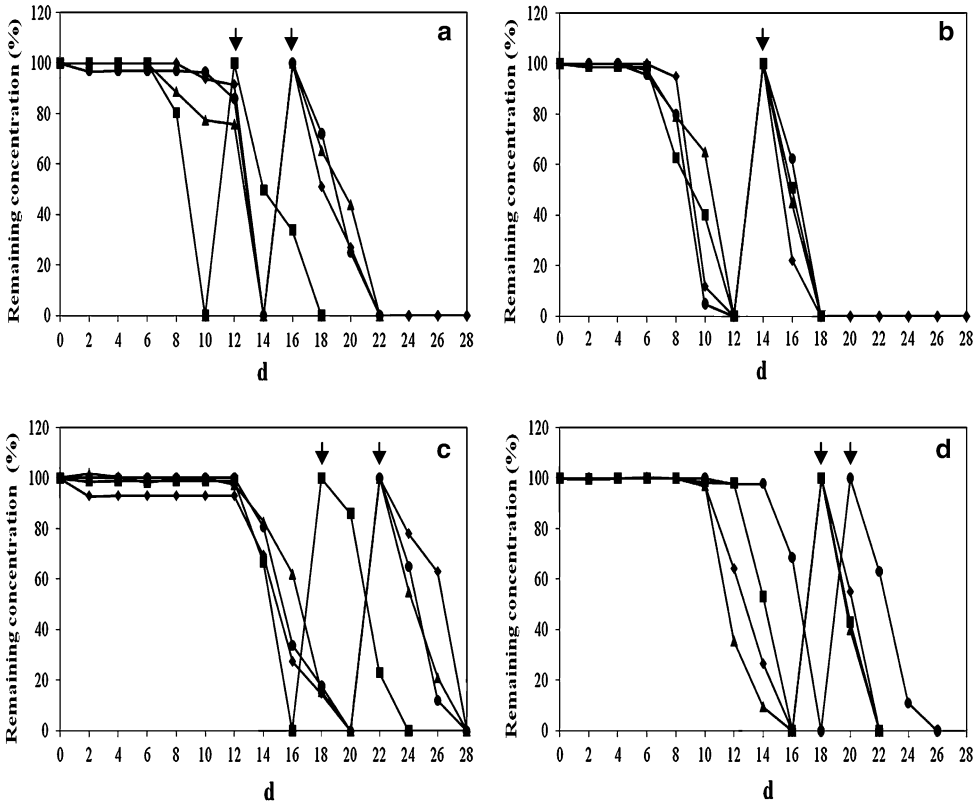

Fig. 1 Kinetics of 246TBP degradation (**a**, **c**) and 246TCP degradation (**b**, **d**) in microcosms incubated at 20°C (**a**, **b**) and at 4°C (**c**, **d**). (■) Lake Alto Reino; (◆) Lake Las Dos Torres; (▲) Lake Orilla Camino; (●) Lake Venus. The arrows indicate reinoculations with 246TBP or 246TCP

previously exposed to 246TCP, degraded chlorophenol (50 μg/ml) after 30 days of incubation. Furthermore, the bacterial communities isolated from unpolluted soils can degrade 2,4-dichlorophenoxiacetic acid, but this requires more than 10 days (Kamagata et al. 1997). In this work, at 20°C, degradation began after 6 days of incubation whereas, at 4°C, it was detected after 12 days. Once the degradation had begun, the time required for the total removal of the halophenols was approximately 4 days. This difference suggests that the degradation could be carried out by mesophilic bacteria since the adaptation period was shorter at 20°C than at 4°C. Hereby, a slower metabolic response could be explained by the lower incubation temperature. Nevertheless, a degradation process with psicrotolerant bacterial cells involved cannot be rejected since similar degradation rate was observed—but not calculated—at both incubation temperatures 4 or 20°C. The successive re-inoculations done with the halophenols revealed that the bacterial populations studied after the first assay did not present an adaptation period when they were inoculated with the halophenols being studied and, thus, 12 h after the halophenol was added, 10% of it had been removed. Nonetheless, differences in degradative profile was observed between microcosms added with 246TBP; on the other hand, microcosms ammended with 246TCP showed similar degradation rates (Fig. 2). The results obtained by HPLC confirmed that after 48 h incubation both 246TCP and 246TBP were completely degraded in all microcosms. A similar result was informed by Kharoune et al. (2002) in a bacterial consortioum able to degrade 246TCP. The authors indicated that the consortioum required a 15 day adaptation period to begin degradation, and after successive additions of chlorophenol, the degradation rate increased four times without requiring an adaptation period.

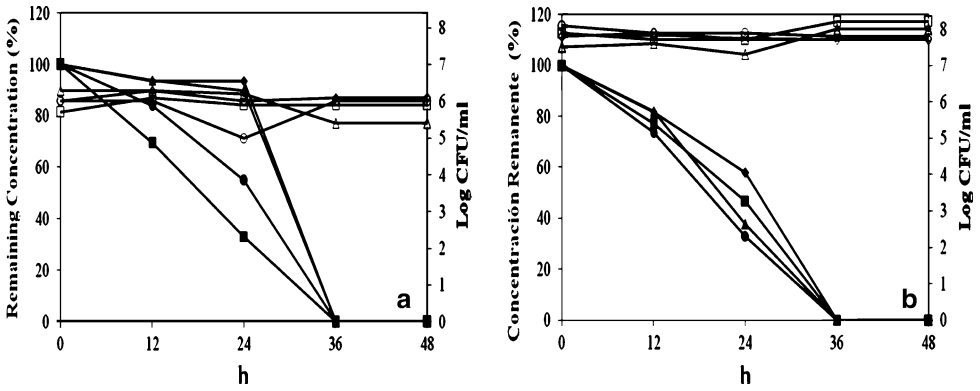

Fig. 2 Kinetics of degradation at 4°C in the microcosmos previously adapted to the presence of halophenols. **a** Kinetics of 246TBP degradation; **b** kinetics of 246TCP degradation. (■) Lake Alto Reino; (◆) Lake Las Dos Torres; (▲) Lake Orilla Camino; (●) Lake Venus. The bacterial counts are shown as empty shapes and the degradation as solid shapes

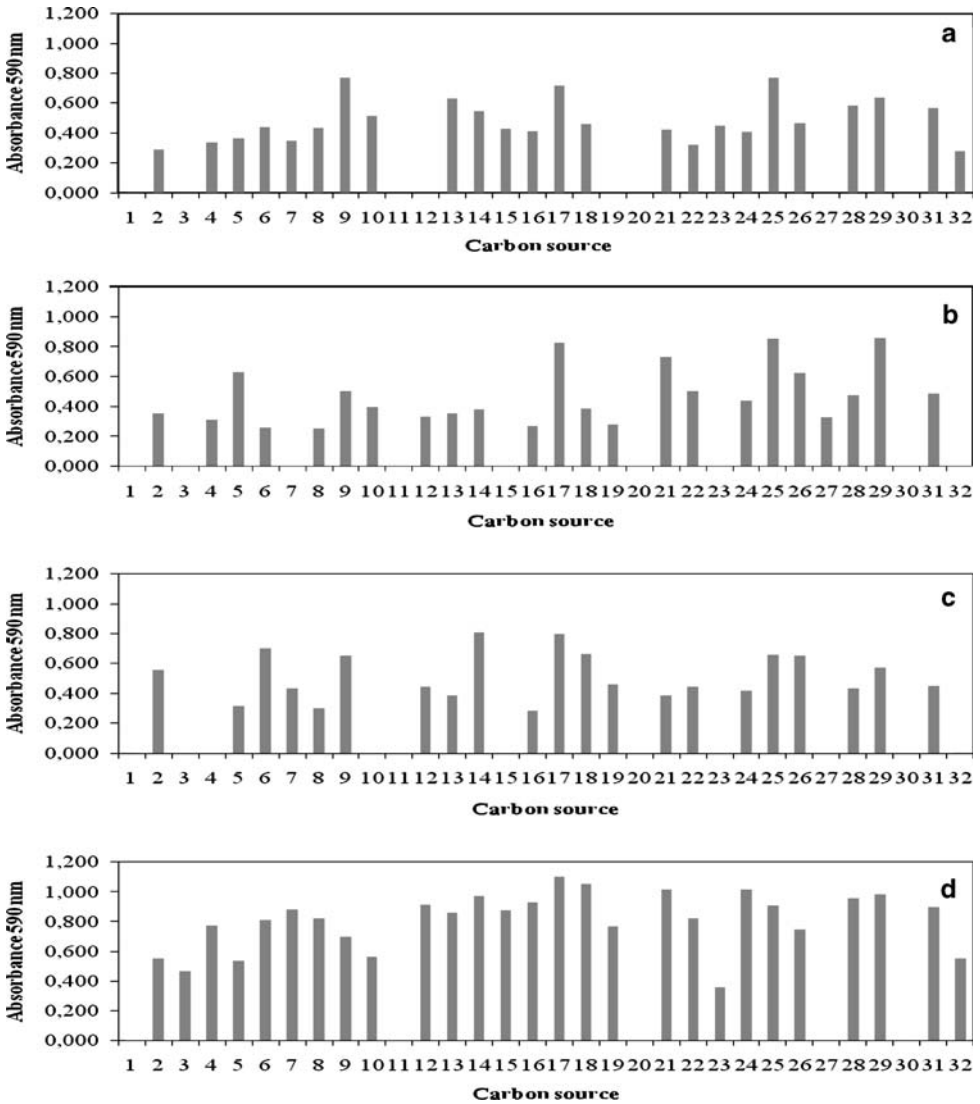

Fig. 3 Microbial activity of bacterial communities present in microcosms added with 246TBP **a** Orilla Camino; **b** Venus; **c** Alto Reino; **d** Las Dos Torres. (1) Water; (2) *β*-mehtyl-D-glucoside; (3) D-galactonic acid *γ*-lactone; (4) L-arginine; (5) pyruvic acid methyl ester; (6) D-xylose; (7) D-galacturonic acid; (8) L-asparagine; (9) Tween 40; (10) i-erythritol; (11) 2-hydroxy benzoic acid, (12) L-phenylalanine; (13) Tween 80; (14) D-mannitol; (15) 4-hydroxy benzoic acid; (16) L-serine; (17) α-cyclodextrin; (18) N-acetyl-D-glucosamine; (19) *γ*-hydroxybutyric acid; (20) L-threonine; (21) Glycogen; (22) D-glucosaminic acid; (23) Itaconic acid; (24) Glycyl-L-glutamic acid; (25) D-cellobiose; (26) Glucose-1-phosphate; (27) α-ketobutyric acid; (28) Phenylethyl-amine; (29) α-D-lactose; (30) D,L-α-glycerol phosphate; (31) D-malic acid; (32) Putrescine

It should be noted that, in the studied microcosms, the complete removal of the halophenols was achieved under conditions considered to be psychrophilic (4°C) and that these, once adapted, did not differ from the degradative activity of those incubated at 20°C. These results could be considered as an adaptation process since the degradation rates increased due to repeated exposure to halophenols. This observation has been reported in soils, estuaries and continental waters, and could be explained as the result of the selection of microorganisms capable of using the

halophenols by combinations of inducible enzymes (Aelion et al. 1987).

Metabolic profile of the microcosms

After the degradation of 246TBP, the metabolic profile of the microcosms prepared with the water samples from the studied lakes was determined according to the Biolog Ecoplate™ system. These results indicated differences between the carbon sources used by each community. Moreover, none of the microcosms used more than 89% of the carbon sources available through the Biolog Ecoplate™ system (Fig. 3). Choi and Dobbs (1999) reported the use of more than 95% of the substrates by bacterial communities present in continental and oceanic water samples. Although differences exist in terms of the type of substrate used, overall, the average metabolic responses shown by each microbial community were similar ($OD_{590\ nm}$) 0.461, 0.459, 0.556, and 0.402 for Orilla Camino, Venus, Alto Reino and Las Dos Torres, respectively). According to Gomez et al. (2006) microbial activity obtained in microcosm of patagonic lakes was nearly two fold lower than that obtained by them in soil communities. This difference could be due to the incubation temperature of the samples since the bacterial metabolism could be lower at 4°C than at the temperature assayed by Gomez et al. (2006). Though there are differences between the viable bacterial counts of the degrading microcosms, Garland and Mills (1991) indicated that color production in the BIOLOG community-level assay is caused by growth of bacteria within wells after inoculation rather than respiration of the inoculated community. Nevertheless, the results demonstrated that the bacterial communities present in microcosms are able to totally degrade both 246TBP and 246TCP. These results together with the different carbon sources oxidized by the degrading microsoms suggest that they could be considered as different communities.

Isolation of strains and degradation studies

A total of 14 Gram negative bacterial strains were isolated from the degrading microcosms. Only one isolated strain was identified at genus level and corresponded to *Sphingomonas* (84.4%). On the other hand, four of all isolated strains were identified at species level; *Stenotrophomonas maltophilia* (99.9%), *Crhyseobacterium indologenes* (97.9%), *Brevundimonas vesicularis* (98%) and *Burkholderia cepacia* (99.9%). None Gram positive bacterial strain was isolated from any degrading microcosms.

Although in microcosms 246TBP and 246TCP were degraded, none of the 14 bacterial strains isolated were able to degrade these compounds, when in pure or mixed culture, as the sole carbon source or supplemented with glucose. Thus, the degradation of 246TBP or 246TCP could be associated with the action of bacterial consortia in which two or more strains provide essential factors that allow them to degrade the halophenols, as proposed by Ronen et al. (2005). On the other hand, the results indicated that only 0.1% of bacterial cells were recovered in culture. According to this results, the possibility that bacteria with degradative abilities were not cultivable in the culture media used (R2A agar) cannot be rejected.

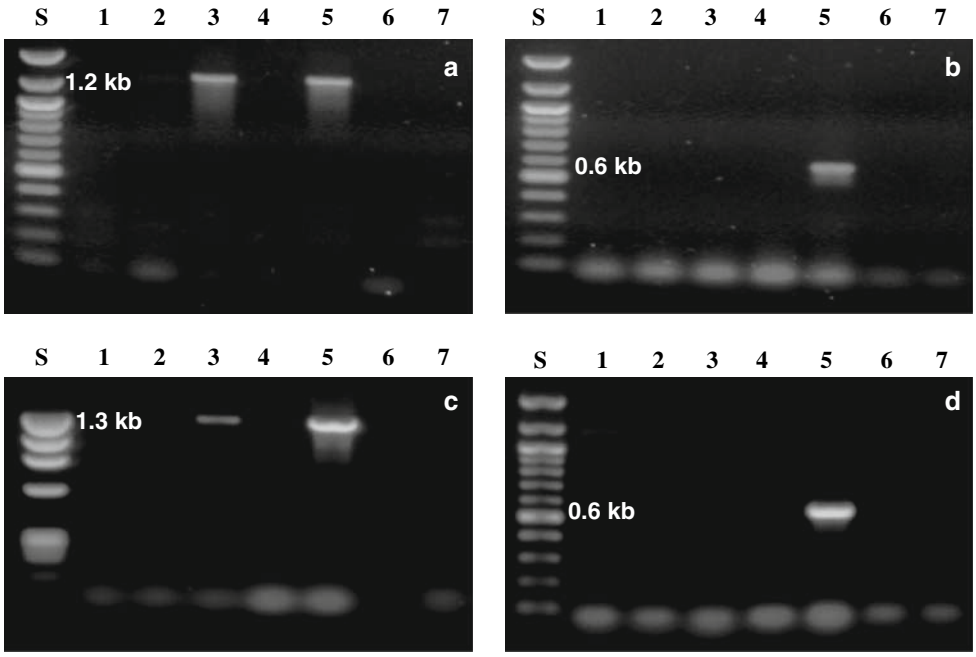

Fig. 4 Detection of the genes *tcp*A and *tcp*C with specific and degenerated primers in water samples. **a** and **c** amplification of *tcp*A with specific and degenerated primers, respectively. **c** and **d** amplification of *tcp*C with specific and degenerated primers, respectively. *Lane* (1) Lake Orilla Camino; (2) Lake Venus; (3) *Cupriavidus necator* JMP134; (4) negative control; (5) *Cupriavidus necator* PZK; (6) Lake Alto Reino; (7) Lake Las Dos Torres; (S) 1 kb (**c**) or 100 bp (**a**, **b**, **d**) DNA standards

Detection of *tcp*A and *tcp*C genes in microcosms

The *tcp*A and *tcp*C genes were not detected in the DNA obtained from the microcosms (Fig. 4). Similar results were found for *Sphingopyxis chilensis* S37, a bacterium that degrades 246TBP and 246TCP (Aranda et al. 2003) and also lacks *tcp*A or *tcp*C genes (Matus et al. 2003). This suggests that the degradation is carried out by enzymes codified in genes other than those described for the degradation of 246TCP in *C. necator* JMP134.

The ability to degrade 246TBP and 246TCP in the absence of *tcp*A and *tcp*C genes in the microcosms suggests a genetic diversity related to the degradation of halophenolic compounds. Aerobic heterotrophic bacteria have the ability to degrade both 246TBP and 246TCP, using enzymes codified by genes other than those described for 246TCP degradation by bacteria from environments contaminated by industrial activities. These genes should be studied in order to obtain their complete characterization.

Conclusions

The results demonstrate that in the Patagonic psychrophilic lakes, the proportion of bacteria recovery in culture is low. Nonetheless, the aerobic bacteria of these lakes without industrial or urban activity have the ability to degrade efficiently both 246TBP and 246TCP added as a sole carbon and energy source. However, we can not exclude that bacterial cells present in the microcosms lakes may have the ability to use either an endogenous carbon source as a polyhydroxyalanoate as describe Godoy et al. (2003) or organic material release by cellular lysis (Pinchuk et al. 2008). Thus, these bacterial communities could be participating in self-remediation processes of toxic compounds like halophenols incorporated into the environment by biological or industrial activities.

Acknowledgments The authors thank Dr. Carlos Smith for his critical review of this manuscript. This study was financed by the projects FONDECYT 1070497 and DIUC Patagonia No. 205.036. 029-ISP.

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