6

Change in Bacterial Diversity After Oil Spill in Argentina

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1. Introduction

During the past few decades, the impact and threat of oil pollution have resulted in extensive research. The anthropogenic origins of petroleum pollution (particularly via leak of coastal oil refineries) have raised interest among the scientific community in oil pollution distribution and its effects in the environment, mainly the marine environment. Tanker accidents are the major cause of oil pollution in marine environments. On December 26th, 2007, a tanker spilled approximately 100 m^3 of crude oil into the sea, which impacted on Caleta Córdova coast, Argentina. Being part of Comodoro Rivadavia city, Caleta Córdova is a small village, where the community's local economy is based on artisanal fishing. Over the course of two months, local oil companies collaborated to manually clean the coast and hydrocarbon controls were carried out to monitor the hydrocarbon concentration. The province environmental authorities prohibited the use of chemical products to disperse hydrocarbons and the use of any other chemical product, including nitrogen and phosphorus salts to help biodegradation. On the day of the spill, there was an extraordinary high tide (6.4 m), which resulted in the definition of three areas of impact: the high area, which was the most difficult to clean up; the middle area, which was cleaned up with minimal problem and from where the samples in this study were taken; and the low tide line area.

Petroleum hydrocarbon can be degraded by microorganisms such as bacteria, fungi, yeast, and microalgae (e.g.,Atlas, 1981) Bacteria are considered to represent the predominant agents of hydrocarbon degradation in the environment and hydrocarbon-degrading bacteria are ubiquitous (Pucci et al 2009a, b). Since crude oil is made of a mixture of compounds, and since individual microorganisms metabolize only a limited range of hydrocarbon substrates (Britton, 1984), biodegradation of crude oil requires a mixture of different bacterial groups or consortia functioning to degrade a wider range of hydrocarbons (Bordenave et al., 2007; Cagnon et al., 2011). Contaminated marine environments are inhabited by a range of selected microorganisms able to tolerate and remediate pollutants that impacted the environment, leading to the dominance of pollutant-tolerant bacteria. Hence, bacterial communities in contaminated sites are typically less diverse than those in nonstressed systems (Harayama et al., 2004). Another characteristic of marine environments is that the vast majority of bacteria (90–99%) are uncultivable (Amann et al., 1995); hence, the analysis of microbial communities that

contribute to in situ hydrocarbon biodegradation activities has been a challenge to microbiologists (Rollins & Colwell, 1986; Wilkinson 1988).

	Genus	%	Genus	%	Genus	%
Caleta Córdova						
	Acinetobacter	4.0	*Kurthia*	0.8	*Psychrobacter*	12.9
	Aeromonas	4.0	*Lysobacter*	0.8	*Rhodobacter*	2.4
	Arthrobacter	0.8	*Microbacterium*	1.6	*Rhodococcus*	0.8
	Bacillus	16.9	*Micrococcus*	1.6	*Shewanella*	5.6
	Brevibacillus	1.6	*Ochrobactrum*	0.8	*Sphingopyxis*	0.8
	Brevundimonas	1.6	*Paracoccus*	1.6	*Staphylococcus*	2.4
	Burkholderia	0.8	*Photobacterium*	1.6	*Vibrio*	7.3
	Halomonas	2.4	*Pseudoalteromonas*	21.0	*Zobellia*	0.8
	Kocuria	1.6	*Pseudomonas*	3.2		
Comodoro Rivadavia						
	Acinetobacter	8.12	*Microbacterium*	0.85	*Rhodobacter*	5.56
	Aeromonas	4.27	*Micrococcus*	1.71	*Rhodococcus*	1.28
	Bacillus	3.85	*Moraxella*	0.85	*Rhodovolum*	1.71
	Brevundimonas	2.99	*Ocrhobactrum*	4.27	*Roseomonas*	0.43
	Chryseomonas	0.85	*Paenibacillus*	0.43	*Salmonellas*	5.56
	Curtobacterium	0.85	*Pectobacterium*	0.43	*Shewanella*	1.28
	Enterobacter	0.85	*Photobacterium*	2.99	*Staphylococcus*	3.42
	Enterococcus	0.43	*Photorhabdus*	0.43	*Stenotrophomonas*	0.43
	Escherichia	3.42	*Proteus*	0.43	*Vibrios*	5.56
	Flavimonas	1.71	*Pseudoaltermona*	16.24	*Virgibacillus*	0.43
	Gluconobacter	1.71	*Pseudomona*	6.84	*Xanthobacter*	0.43
	Grimontia	0.85	*Pseudoxantomonas*	1.28	*Yersinia*	0.43
	Kluyveria	1.28	*Psychrobacter*	0.43	*Zobellia*	0.43
	Kokuria	3.85	*Rhizobium*	0.85		
Rada Tilly						
	Acinetobacter	1.7	*Grimontia*	0.6	*Psychrobacter*	1.1
	Aeromonas	7.4	*Kocuria*	4.0	*Rhodobacter*	4.5
	Arthrobacter	4.0	*Microbacterium*	1.1	*Rhodovulum*	0.6
	Bacillus	6.8	*Micrococcus*	11.4	*Roseomonas*	1.1
	Bradyrhizobium	1.7	*Neisseria-*	2.3	*Rothia*	0.6

	Genus	%	Genus	%	Genus	%
	Brevibacillus	2.3	*Nesterenkonia*	0.6	*Sphingomonas*	0.6
	Brevundimonas	3.4	*Ochrobactrum*	3.4	*Sporosarcina*	0.6
	Cellulomonas	0.6	*Paenibacillus*	1.7	*Staphylococcus*	10.2
	Chryseomonas	0.6	*Paracoccus*	0.6	*Stenotrophomonas*	0.6
	Corynebacterium	0.6	*Paucimonas*	0.6	*Vibrio*	1.7
	Dietzia	2.3	*Photobacterium*	1.7	*Virgibacillus*	0.6
	Escherichia	0.6	*Pseudoalteromonas*	11.9	*Zobellia*	2.3
	Flavimonas	1.1	*Pseudomonas*	2.8		
Caleta Olivia	**Genus**	**%**	**Genus**	**%**	**Genus**	**%**
	Acinetobacter	0.96	*Kocuria*	1.91	*Psychrobacter*	4.31
	Arthrobacter	0.48	*Lechevalieria*	0.48	*Rhodobacter*	3.35
	Bacillus	4.78	*Methylobacterium*	0.48	*Rhodovulum*	0.96
	Brevibacterium	1.44	'*Microbacterium*	3.35	*Salmonella*	0.48
	Brevundimonas	0.96	*Micrococcus*	3.35	*Shewanella*	2.39
	Clavibacter	0.48	*Neisseria*	1.44	*Sphingomonas*	3.83
	Corynebacterium	0.48	*Nocardioides*	0.48	*Staphylococcus*	4.31
	Curtobacterium	0.96	*Paenibacillus*	0.96	*Vibrio*	1.91
	Dietzi	0.48	*Photobacterium*	3.83	*Xanthobacter*	0.48
	Escherichia	0.48	*Pseudoalteromonas*	28.71	*Zobellia*	1.91
	Flavobacterium	0.48	*Pseudomonas*	19.14		

Table 1. Bacterial diversity taxas in four locations of San Jorge Gulf in 2006.

Since the pioneering work on marine bacteria by C.E. ZoBell, many bacterial strains have been isolated from the coastal and oceanic environments; these bacteria, including the genera *Alteromonas (*Beckman et al., 2008; Ivanova et al., 2004), *Aeromonas* (Stabili & Cavallo 2004), *Alcanovorax* (Head et al., 2006;Purkrtova et al., 2010), *Bacillus (*Oguntoyinbo, 2006), *Cycloclasticus* (Kasai et al., 2002), *Chromobacterium, Flavobacterium, Marinobacter spp., Shewanella (*Holt et al., 2005), *Microscilla (*Lennon, 2007), *Micrococcus* (Süss et al., 2004), *Rhodococcus* (Süss et al., 2004), *Photobacterium, Planacoccus, Pseudomonas, Pseudoalteromonas,* and *Vibrios*, among other genera, have been considered to be representative of marine bacteria. Many of them have the capacity to use different hydrocarbons (Head et al., 2006, Yu et al., 2005a, b).

Comodoro Rivadavia, known as Argentina's oil capital, is a city with an intense oil activity. However, and contrary to belief, there are no oil refineries in town, which means that the crude oil needs to be transported by ship on a daily basis; hence, the possibility of marine and coastal pollution is an ongoing risk. Caleta Córdova is located approximately 20 km north of Comodoro Rivadavia city in the San Jorge Gulf. The beaches of Caleta Córdova are characterized by being used for different purposes. In the south of Caleta Córdova, crude oil

is loaded into transport vessels by means of a buoy located about 4 km offshore. The local port is located in the town area of the beach, where activities related to fishing and shellfish harvesting are carried out. The northern area is used for recreation during the summer due to its large size and absence of industrial activity.

Bacterial diversity in four geographical points of San Jorge Gulf (caleta Olivia, Rada Tilly, Comodoro Rivadavia and Caleta Córdova) was studied before the oil spill (Table 1). The method used for the bacterial identification was fatty acids methyl ester (FAME).

The current study examined sediments collected from one spill located in the Caleta Córdova beach in Comodoro Rivadavia city, and assessed the temporal trends in distributions and concentrations of petroleum hydrocarbons from December 2007 to June 2008. The sediments were analyzed for total petroleum hydrocarbons (TPHs) and mineralization of crude oil and distilled oil. Bacterial counts were carried out and strain were isolated and identified. The analysis of the community growth on liquid medium was also included in the study.

2. Material and methods

2.1 Sampling

Sediment samples were collected 1 year before the spill and on six occasions during the study between December 2007 and June 2008 (on days 4, 20, 40, 60, 120, and 164 after the spill) to determine the temporal changes and spatial distribution of the petroleum in the sediment on the same coordinate. The samples of intertidal sediment were collected in Caleta Córdova from the coast, from the top layer (0–30 cm). The temperature at the sediment surface was around 14 ^{o}C. The fuel-oil sample containing 48.9% of aliphatic hydrocarbon, 35.9% of aromatic hydrocarbon, and 15.1% of resins and asphaltenes hydrocarbon, was obtained from the contaminated site after the spill.

2.2 Chemical analysis

Gas Chromatography (GC). Two grams of each individual sample were dissolved in 5 ml of pentane, phase separated, and percolated through 2 g of silicalgel. One mL of the eluate was carefully evaporated until dryness to determine the fuel oil content of the sample. The fractions were analyzed and quantified by gas chromatography utilizing Varian 3800 GC, equipped with a split/splitless injector, a flame ionization detector, and a capillary column VF-5ms (30 m, 0.25 mm, 0.25 µm). The injector and detector temperatures were maintained at 200 ^{o}C and 340 °C respectively. The Sample (1 µL) was injected in split mode and the column temperature was raised from 45 to 100°C at a rate of 5 °C/min and a second ramp from 100 to 275 °C at a rate of 8 °C/min with the final temperature of 275 °C, held for 5 minutes.

Hydrocarbons by Infrared determination. EPA Method 418.1. Two grams of each individual sample were dissolved in 10 mL of carbon tetrachloride, phase separated, and percolated through 2 g of silicalgel and the absorbance was measured at 2930 cm^{-1}.

Soxhlet determination. Hydrocarbon concentration was determined by Soxhlet extractor using trichlorinethane as the extraction solvent. The extracted hydrocarbons were quantified on a mass difference basis (Pucci & Pucci, 2003) and separated into class fractions by silica gel column chromatography. Aliphatic, aromatic and polar oil fractions were eluted with

250 mL of hexane, 150 mL of benzene and 150 mL of chloroform-methanol 1:1 respectively (Acuña et al., 2008).

2.3 Respiration

Basal respiration was measured by monitoring CO_2 evolution using Na OH to capture it. 5 g of sediment were poured into a brown bottle with 50 mL of sea water and 50 µL of mineral medium hydrocarbon degrading bacteria (HDB) (K_2HPO_4 100 g, $(NH_4)_2PO_4$ 200 g, distilled water 1000 mL, pH 7) and 0.1% of gasoil, diesel oil, kerosene, lubricant oil and crude oil and intrinsic microcosms without hydrocarbons and nutrient presence were used for rest of the values obtained on hydrocarbons mineralization. The NaOH was titrated by HCl 0.1N. (Bartha et al., 1972).

2.4 Enumeration and isolation of aerobic bacteria

The culturable bacteria in sediments were counted using standard plate dilution method. One gram (wet weight) of sediments was suspended in 9 mL of sterile seawater (pH 7.2) and vortexed for 1 min at low speed. Aliquots of 100 µL of undiluted samples, and 10^{-1} to 10^{-6} dilutions were spread on BBR, BRN and MBM-PGO agar plates (Pucci et al., 2009), incubated at 28 ^{o}C for up to 21 days. The media were BBR medium (g L^{-1}: Tripteine 0.5, yeast extract 0.5, K_2HPO_4 1; $(NH_4)_2SO_4$ 2; agar-agar 15, sterile marine water 700 mL, sterile water 300 mL, pH 7.2), BRN medium (g L^{-1}: Tripteine 5, yeast extract 1, K_2HPO_4 1; $(NH_4)_2SO_4$ 2; agar-agar 15, sterile marine water 700 mL, sterile water 300 mL, pH 7.2) and MBM-PG medium (20 mL of MM: NaCl 5 g, K_2PO_4H 0.5 g, $NH_4PO_4H_2$ 0.5 g, $(NH_4)_2SO_4$ 1 g, Mg SO_4 0.2 g, KNO_3 3 g, $FeSO_4$ 0.05 g, distilled water 1000 mL), which was distributed in a Petri plate and after solidification, 30 µL of a mixture 1:1 of petroleum-diesel oil were spread on the surface (Pucci & Pucci, 2003). Grown discrete colonies were isolated and purified.

2.5 Chemotaxonomic analysis

The diversity of cultured sediment bacteria was determined by fatty acids methyl ester (FAMEs) analysis of the samples taken after summer before the spill and 6 times after the spill. FAMEs analysis allows the characterization of individual bacterial colonies. Fatty acids are extracted and compared against a database, to identify isolated bacteria. From each culture plate containing between 30 and 300 colonies, individual colonies were randomly isolated and incubated on tryptic soy broth agar for 24h. The FAMEs were extracted and analyzed as described by MIDI (MIDI Newark, Del., USA).

2.6 Communities FAMEs analysis

10 g of sediments were incubated on the medium HDB (g L^{-1}: K_2HPO_4 1; $(NH)_4SO_4$)with 0.1% phenanthrene, 0.1% hexadecane and on BBR medium. After 10 days of incubation at 28^{o}C, the samples were centrifuged at 4000 rpm for 30 min. The FAMEs were extracted and analyzed as described by MIDI (MIDI Newark, Del., USA).

2.7 GC parameters

The MIDI microbial identification system (Microbial ID, Inc, Newark, NJ) was applied to separate fatty acid methyl ester using a gas chromatograph (HP 6890) equipped with a

split/splitless injector, a flame ionization detector, a capillary column Ultra 2 (25 m, 0.2 mm, 0.33 µm); an automatic sampler; an integrator; and a program which identifies the fatty acids (Microbial ID 6.0 version). The injector and detector temperatures were maintained at 250 ºC and 300 °C respectively. The Sample (2 µL) was injected in split mode and the column temperature was raised from 170 to 270 °C at a rate of 5 °C/min.

2.8 Statistical analysis

The mean and standard deviation of three replicates were calculated. The mean values were compared by ANOVA test BIOM (Applied Biostatistics Inc., 3 Heritage, Setauket, NY 117ll USA). Differences were considered significant when $P<0.05$. To identify possible similarity between FAMEs profiles, the data were subjected to analysis of variance using the PAST (Hammer & Harper 2005) and Sherlock (Microbial ID 6.0 version).

3. Results

3.1 Bacterial count

The bacterial count increased after the spill and the values did not show a significant difference ($P<0.05$) during the rest of the time (Table 2). The hydrocarbons under study on the rest of the beach were 2.2% but on the location subject to study, the TPH was only 0.22% before the incident and 4 days afterwards. On the rest of the beach, the TPH was 2.2% and there were three different areas of impact depending on the slope and the tide movement. However, the cleaning up of the oil took longer on the high area because the tide on December 26th was an extraordinary one of 6.05 meters; the ordinary tide is from 0.88 to 5.6 meters. The middle area was cleaned up in a shorter period of time (Iantanos et al., 2008).

Days	1 year before	4	20	40	60	102	164
BBR (cfu g^{-1})	3.2×10^2	1.1×10^4	9.9×10^3	2.63×10^4	1.06×10^6	2.0×10^3	5.9×10^4
BRN(cfu g^{-1})	1.7×10^2	1.36×10^6	1.24×10^5	1.87×10^5	6.0×10^4	1.2×10^4	4.7×10^3
MBM-PGO(cfu g^{-1})	4×10^1	7.5×10^5	1.29×10^6	1.79×10^5	2.12×10^6	7.2×10^4	1.74×10^5
Shannon_H	2.339	2.26	1.759	1.685	1.721	1.839	1.688
HC(%)	-	0.22	0.07	0.02	0.02	0.02	0.02
TPH –IR	-	1569.25	381.7	370	313.55	385	280.55
GC total	-	1712.150	551.510	200	187.180	154.346	153.717

Table 2. Heterotrophic (BRN), oligotrophic (BBR) and hydrocarbon degrading bacteria (MBM-PGO) counts determined 1 year before the spill and 6 times afterwards. Total hydrocarbons (TPH) determined by soxhlet (HC%), infra red (IR) and gas chromatographic (GC). (-) TPH not detected. Shannon H: Shannon diversity index.

3.2 Effects of bioremediation on oil degradation

Oil degradation was assessed by measuring carbon dioxide evolution and determining changes in oil sediment samples composition (Table 3). Carbon dioxide evolution was monitored intensively during the experience and afterwards, delta $mgCO_2$ days was done, which showed an important change in the values on day 4 and these values decreased with the time. The spill was crude oil, but the bacterial communities had a good performance in

the mineralization of all the hydrocarbons tested. In all of the three cases (gas oil, diesel and lubricant oil), on day 4, the values of mineralization rate doubled the previous values as determined before the spill; however, on day 164 the rate values were similar to those values determined 1 year before the incident. The bacterial communities were able to use poliaromatic hydrocarbon, which is the most problematic for the human health (Table 4). Degradation on the beach by the bacterial communities was studied by monitoring the disappearance of TPH measuring by three forms through the determination of their biodegradation percentages, and confirmed by GC chromatographic analysis of the residual crude oil on the six occasions (Table 4). Detailed chromatogram analysis showed visible degradation of n-alkanes ranging from n-C16 to n-C24 during the first 40 days and n-C5 to n-C16 were detected in a small quantity. Branched alkanes such as pristine and phytane experienced degradation during the first 40 days as well.

Hydrocarbon	**1 year before**	**4**	**20**	**40**	**60**	**102**	**164**
Gas oil	13.02	34.14	19.07	21.18	20.88	15.61	16.74
Kerosene	20.92	22.71	22.9	23.7	29.28	24.01	30.86
Diesel oil	14.91	31.01	24.97	29.24	25.75	22.43	19.81
Crude oil	18.62	22.48	24.57	19.59	26.58	20.52	18.59
Lubricant oil	14.22	30.62	31.66	27.05	19.19	28.6	11.68

Table 3. Delta Mineralization rate of hydrocarbons in mg CO_2 day.

Hydrocarbons ppm.	**4**	**20**	**40**	**60**	**102**	**164**
Naphthalene	1.213	0.5782	0.09	0	0	0
2-Metylnaphthalene	1.696	1.9188	1.1	0.322	0.197	0.187
Acenaphthalene	4.033	0.698	0.349	0	0	0
Fluorene	0.185	0	0	0	0	0
Pristane	0.126	0.234	0.125	0	0	0
Phytane	59.655	5.136	0.968	0	0	0
Phenanthrene	4,.14	1.38	0.37	0	0	0
Anthracene	0.617	0.685	0	0	0	0
Fluoranthrene	4.36	2.922	0.604	0	0	0
Pyrene	0.057	0	0	0	0	0
Benzo(k)fluorantreno	4.47	1.386	2.175	0	0	0
C6-C10	1.954	0.8878	0.8354	0.536	0.481	0.469
C11-C15	14.949	10.2774	3.726	2.282	1.461	1.292
C16-C20	259.714	103.638	9.49	0	0	0
C20-C25	468.095	210.45	85.028	0	0	0
C20-C25	468.095	210.45	85.028	0	0	0
C26-C29	46.156	29.738	21.383	0	0	0
PTH total	1383.543	334.406	133.163	3.463	2.952	3.35
PAH total	22.198	10.461	5.188	0.322	0.197	0.187
n-Alkanes total	917.557	411.1	126.883	3.141	2.214	2.074
Pristane/C17	0.004	0.019	0.034	0	0	0
Phytano/C18	2.346	0.393	0.851	0	0	0

Table 4. GC-Hydrocarbon (ppm) on the samples.

3.3 Communities

In the principal component analysis (PCA), Fig. 1a, the bacterial communities, which grew in alkaline peptone, proved to be, on day 164, near the bacterial community development analyzed in samples taken a year before the spill, specifically in the seasons of autumn and summer. On the other hand, in those samples taken 4, 20 and 40 days after the spill, the bacterial community experienced a change but it eventually returned to its original community state. Most fatty acids included the same proportion of *Vibrio* fatty acids (Hoffmann et al., 2010), whereas other fatty acids were in less proportion. There were cis fatty acids and absence of trans fatty acids; however, there was a major presence of unsaturated fatty acids. After 60 days, the concentration of hydrocarbons fell, and approximately 100 days after the spill, the bacterial communities proved to have similar characteristic to the pre- spill bacterial communities. No cyclo fatty acids were identified in these communities before or after the spill; values were 1.1 to 0.6% on day 4 and on day 164. On the contrary, methyl fatty acids were present only in the first three samples taken after the spill.

On BBR medium (Fig. 1b), in those cases where the beach samples contained Hydrocarbons, the sample points (days 4, 40 and 60) in the PCA were located in the left upper quadrant, but when the samples did not contain hydrocarbons, the sample points were located in the opposite quadrant, close to those samples taken before the spill, in autumn and summer. Similar to the case of the communities grown in alkaline peptone, no cyclo fatty acids were identified in these communities before the spill and afterwards; the values were 1.2% on day 4, but the value of methyl fatty acids was 1.9% which remained stable for the rest of the time. The percentage of hydroxy fatty acids gradually decreased and on day 164, their value was only 2% higher than their value 1 year before the spill. In addition, the cis and iso fatty acids increased until day 60, which indicates the presence of Gram positive bacteria.

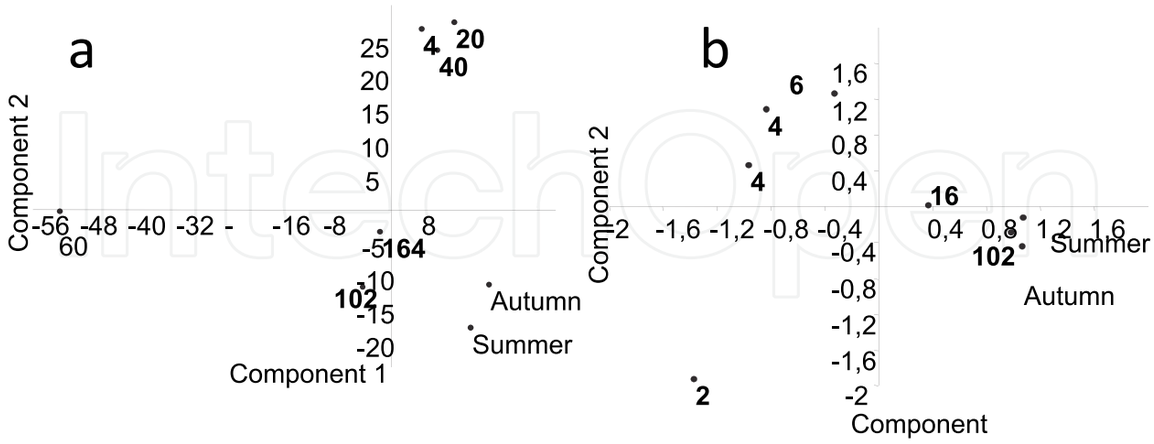

Fig. 1. Principal component analysis of: A) alkaline peptone medium and B) BBR medium. The summer and autumn points correspond to samples taken 1 year before the spill.

The medium with phenanthrene as carbon and energy source, showed that in the PCA graph, points 4, 20 and 40 are all located in the same quadrant whereas those samples taken

on day 102 have a tendency to be near the sample points referred to as autumn and summer (samples taken before the spill) (Fig. 2a). There was one exception with those samples taken on day 164 which presented a strange point with different fatty acids. Saturated fatty acids in pre-spill samples were around 40.5% in summer and 43.4% in autumn, after the spill this value changed to 73.2% and the value on day 164 was 60.5%. On the other hand, the value of iso fatty acids was 5.3% in summer and 8.45% in autumn after the spill whereas there was no significant difference in anteiso fatty acids (P>0.5). However, there was no presence of cyclo, trans and methyl fatty acids.

Hexadecane is usually used for degradation of bacterial communities and isolated strains; it does not present difficulty to be degraded and is less toxic. Principal component analysis showed a non-predictive performance (Fig. 2b). Anteiso, cyclo and methyl fatty acids were not present; and iso fatty acids were identified only when hydrocarbons were present on the beach. In addition, the percentage values of hydroxy fatty acids were around 6.8% to 15.3% on day 20 and the saturated fatty acids increased until day 60. The saturated fatty acids increased with the time and the hydroxy fatty acids did not show a pattern.

3.4 Bacteria identification

There are 34 genera and in total we found 39 different strains. Most of the species (25 out of 39) were found together in group A (*Aerococcus viridians, Bacillus atrophaeus, Bacillus pumilus Brevibacillus centrosporus, Curtobacterium flaccumfaciens, Flavobacterium ferrugineum, Halomonas aquamarina, Kocuria rosea, Kurthia gibsonii, Lysobacter enzymogenes enzymogenes, Micrococcus luteus, Microbacterium sp, Nocardia transvalensis, Pseudoalteromonas nigrifaciens, Pseudomonas aeruginosa, Psychrobacter immobilis, Rhodococcus, Rhodobacter, Roseomonas genomospecies, Shewanella putrefaciens, Streptoverticillium reticulum, Staphylococcus, Vibrio* sp., *Vibrio fischeri, Pseudoxanthomonas* sp.;). This group contained strains with the capacity to use hydrocarbon as *Rhodococcus, Pseudomonas, Micrococcus,* and *Kocuria* among other strains. This group is close to pre- spill samples and the samples taken on days 60, 102 and 164. Other small group was the group B with 9 species.

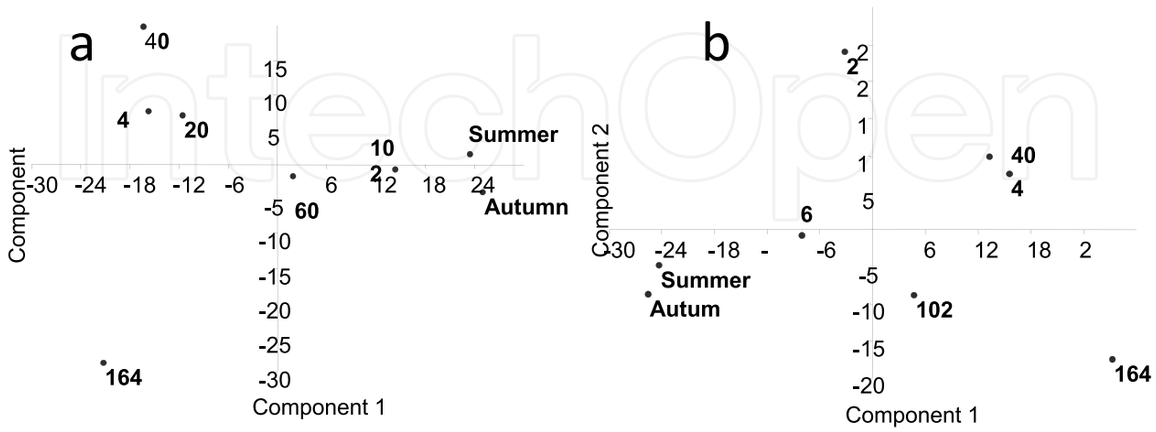

Fig. 2. Principal component analysis of a) phenanthrene and b) hexadacane as contaminants. The summer and autumn points correspond to samples taken 1 year before the spill.

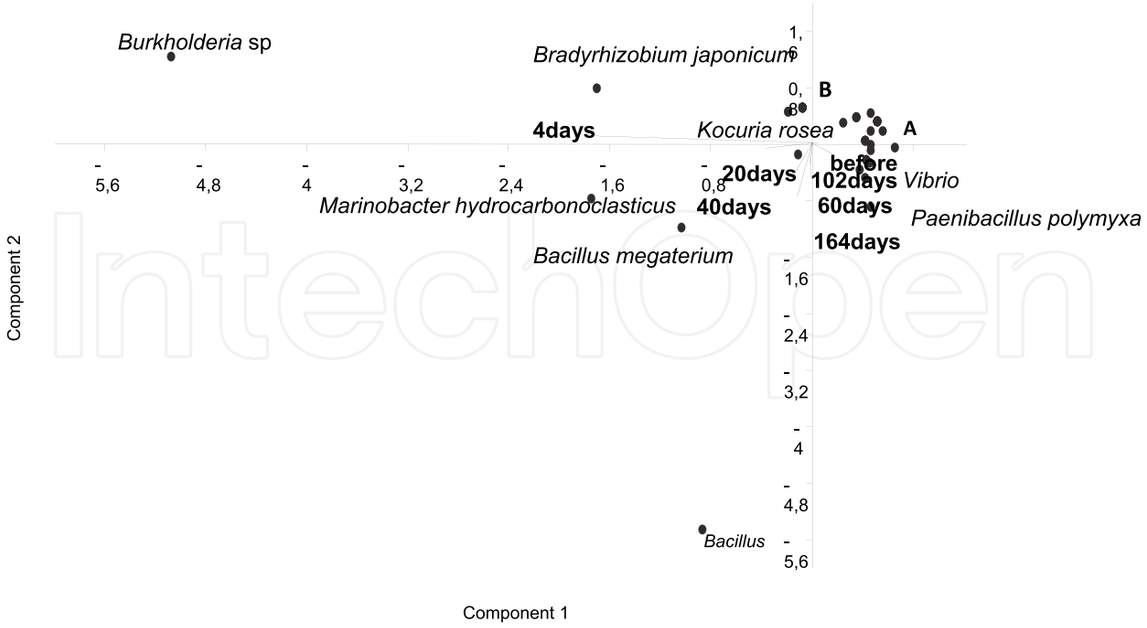

Fig. 3. Principal component analysis of bacteria and time. Group A Aerococcus viridians, Bacillus atrophaeus, Bacillus pumilus, Brevibacillus centrosporus, Curtobacterium flaccumfaciens, Flavobacterium ferrugineum, Halomonas aquamarina, Kocuria rosea, Kurthia gibsonii, Lysobacter enzymogenes enzymogenes, Micrococcus luteus, Microbacterium sp, Nocardia transvalensis, Pseudoalteromonas nigrifaciens, Pseudomonas aeruginosa, Psychrobacter immobilis, Rhodococcus, Rhodobacter, Roseomonas genomospecies, Shewanella putrefaciens, Streptoverticillium reticulum, Staphylococcus, Vibrio, Vibrio fischeri, Pseudoxanthomonas sp.

Shanon index gradually decreased, except on day 102, when it slightly increased (Table 2). Group B *Arthrobacter, Burkholderia cenocepacia, Brevundimonas vesicularis, Phyllobacterium myrsinacearum, Ochrobactrum anthropi, Pseudoalteromonas, Paucimonas lemoignei, Micrococcus sp, Zobellia uliginosa.*

4. Discussion

Oil degradation activities in natural environments are regulated by various physicochemical parameters such as water temperature, dissolved oxygen, nutrient and salt concentrations (Leahy, 1990). Seasonal variation in temperature can be a limiting factor for oil biodegradation in seawater (Minas 1999; Pucci et al., 2009a,b) and sediments (Shiaris 1989; Pucci et al., 2009a,b). The spill took place in Argentina during the summer and this was determinant in the fast decrease of the oil accumulated on the beach together with a good and fast job of a cleanup company. The variation on CO_2 accumulated changed significantly during summer and spring (Pucci et al., 2010) and this was always followed by the number of bacterial count.

The spill aimed at stimulating pollutant-degrading microorganisms to speed the recovery of contaminated ecosystems to a pre-pollution state in terms of biodiversity and ecosystem function. In this study, changes in the predominant bacterial populations occurred on day 4. Oiling and especially bioremediation led to a strong decrease in bacterial community

diversity on day 20, but a rapid recovery to near pre-oiling levels of diversity did not occur subsequently. Still, despite having a similar level of biodiversity, the component organisms contributing to that diversity were somewhat different from the original community, as revealed by FAMEs analysis, which agrees with Röling et al. (2002). Kaplan & Kitts (2004), reported an increase in diversity during a bioremediation of crude oil-contaminated soil. In the present study, although diversity decreased on the time in both treated and control plots, no significant differences were found. Similarly, in a recent study on bioremediation of creosote contaminated soil, we reported depletion of diversity during the maximum biodegradation period; diversity remained low until the end of the experiment (Viñas et al., 2005; Jimenez et al., 2007).

Caleta Córdova beach had a good microbiological population, which included hydrocarbon degrading bacteria. The presence of these bacteria can be attributed to old spills, which were not officially registered but remembered by elder people. The fast bacterial growth in summer, proved by a recount and mineralization rate, confirmed that there was an old spill in the zone. Hydrocarbon degraders may comprise less than 0.1% of the microbial community in unpolluted environments but can constitute up to 100% of the viable microorganisms in oil-polluted ecosystems (Atlas, 1981). Effective biodegradation requires an appropriate population of degraders that have adequate tolerance to environmental changes (Mosbech, 2002), and the environment should be conducive to potential active microorganisms. On the beach, the biodegradation could be a minor process, but the capacity to use hydrocarbon was showed in the mineralization microcosms. The highest values of mineralization rate were not found in crude oil, probably because of the presence of polar hydrocarbons in Patagonia oil. Diesel and lubricant oil are alkane hydrocarbons which are not toxic for the bacteria whereas gas oil has a toxic compound and its mineralization rate showed high values. The length of time that degraders become acclimated and enriched before the degradation provides an approximate clue of how rapidly the microbial populations in different cold environments respond, and thus helps in making cleanup decisions. It is also found that formerly polluted frozen soils are commonly enriched in hydrocarbon-utilizing organisms (Margesin & Schinner, 1997, 1999, 2001). However, the time for different microbial species to recover and degrade petroleum contaminants varies markedly with sites.

The capacity of bacteria, especially *Pseudomonas* and *Rhodococcus*, to metabolize aerobically oil or aliphatic hydrocarbons has been well known for a long time in Patagonia (Acuña et al., 2010; Pucci & Pucci, 2003). The n-alkanes are the most biodegradable of the petroleum hydrocarbons and are attacked by more microbial species than aromatic or naphthenic compounds. However, normal alkanes in the C5-C10 range are inhibitory to any hydrocarbon degraders at high concentrations; this is because, as solvents, they disrupt lipid membrane. On the other hand, the cycloalkanes (alicyclic hydrocarbons) are less degradable that their straight-chain parent, the alkanes, but more degradable than the polycyclic aromatics (PAHs) (Jeffrey, 1980; Bacosa et al., 2010). From some years, the biodegradation of alkanes correlated with in relation to denitrification, sulfate reduction, and metanogenesis (Aeckersberg et al., 1998; Rueter et al., 1994). Moreover, aliphatic hydrocarbons are not fermentable, and only in the presence of O_2, significant aliphatic hydrocarbon degradation occurs. The initial degradation of petroleum hydrocarbons often requires the action of oxygenase enzymes and, thus, it is

dependent on the presence of molecular oxygen (Brock, 1970; Atlas, 1991). Aerobic conditions are therefore necessary for the initial breakdown of petroleum hydrocarbons and in subsequent steps, nitrate or sulfate may serve as a terminal electron acceptor (Bartha, 1986), but oxygen is most commonly used. In the field of chemistry research after the 1989 Exxon Valdez oil spill accident in Alaska, Bragg et al. (1994) demonstrated that in situ oil biodegradation rates along oil polluted coasts were stimulated by fertilizer applications, suggesting that the rates depended mainly on the nitrogen concentration along the shoreline. The Maruyama et al. (2003) study also indicates that the application of inorganic nutrients (such as nitrate, ammonium, and phosphate) would have accelerated oil cleanup processes. Much more stimulation can be achieved by supplementation with organic nutrients such as yeast extracts. That was not possible in the case of Caleta Córdova spill because the environmental minister did not allow their use, but many bacteria in Patagonia soil had nitrogenases and the capacity to use hydrocarbons (Acuña et al., 2010); however, these genes were not searched during this study.

For this study, such a measure may have been of little value; in the plot, the bacterial community structure after 164 days was comparable to the pre-spill community structure. It has been suggested that restoration of the bacterial community structure to a state similar to that present prior to pollution could be used as a parameter for determination of the ecological end point of bioremediation (Stephen et al., 1999). Another explanation for this similarity would be the recovery of indigenous microbial populations present before the accident.

The use of diversity index in microbial ecology studies have been described previously by several authors (Hill et al., 2003; Hughes et al., 2001; Zrafi-Nouira et al., 2009; Pedrós-Alió, 2006). Our results are in agreement with Zrafi-Nouira et al. (2009) and Brakstad & Lødeng (2004), which indicate that the reduction of microbial diversity is observed early, from day 4 to day 164 after the spill. In fact, the crude oil spill can dramatically reduce bacterial diversity (Röling et al., 2002). Shannon diversity indexes shown in Table 2 seem to be low when compared to the results obtained in other studies, which is around 4 to 5 (Schloss & Handelsman 2006). Furthermore, lower values of the Shannon index were previously found in aged crude oil impact into the coast as indicated by Saul et al. (2005), Kaplan & Kitts (2004) and Popp the present study we found that bacterial diversity is gradually reduced during the biodegradation process, but Fig. 3 showed that the identified microorganisms, on day 164, were closer to the pre-spill community structure.

5. Conclusions

The four geographical points studied in the San Jorge Gulf share similar biodiversity and they can probably have a similar response to a crude oil spill impact. Previous to the spill, Caleta Córdova beach point had a bacterial population with the capacity to use hydrocarbons. After 60 days, there was not TPH and the communities returned to a state similar to that present before the spill. It can be concluded that the presence of hydrocarbonoclastics population is normal in the San Jorge Gulf coast and that it can rapidly respond to a natural disaster. The Shannon index was lower than that reported in other studies, and this needs further research especially of microorganisms that could grow in nitrogen absence, which is an important nutrient to biodegradation.

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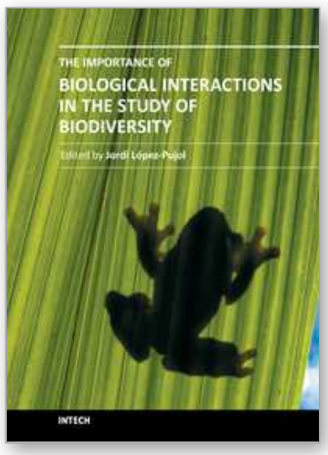

The Importance of Biological Interactions in the Study of Biodiversity

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The term biodiversity defines not only all the variety of life in the Earth but also their complex interactions. Under the current scenario of biodiversity loss, and in order to preserve it, it is essential to achieve a deep understanding on all the aspects related to the biological interactions, including their functioning and significance. This volume contains several contributions (nineteen in total) that illustrate the state of the art of the academic research in the field of biological interactions in its widest sense; that is, not only the interactions between living organisms are considered, but also those between living organisms and abiotic elements of the environment as well as those between living organisms and the humans.

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