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INDIGENOUS HEAVY METAL MULTIRESISTANT MICROBIOTA OF LAS CATONAS STREAM

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Abstract. Las Catonas stream (Buenos Aires Metropolitan Area) receives a complex mixture of pollutants from point and diffuse sources because of the agricultural, industrial and urban land uses of its basin. Widespread detection of heavy metals exceeding aquatic life protection levels has occurred in monitoring reconnaissance studies in surface and pore water. As a result of the screening of Cu, Cd, Zn and Pb resistant/tolerant and culturable microbiota, B101N and 200H strains (*Pseudomonas fluorescens* or *putida*) were isolated and selected for further studies. They showed 65% Cd and 35% Zn extraction efficiency from aqueous phase. The potential use of these strains in wastewater treatment is currently investigated in order to contribute to decrease heavy metal pollution, a problem affecting every stream of Buenos Aires Metropolitan Area.

Keywords: bioremediation, environmental monitoring, heavy metal, multiresistant microbiota

1. Introduction

Rivers are open systems exposed to climatological and geochemical conditions affecting water quality. However, the most important spatial and temporal variations are introduced by anthropic activities. The high number of inhabitants and the location of main anthropogenic pollution sources increase the consequences of pollution in large urban areas. The leaching of chemicals through soil is a potential risk of groundwater pollution. This is of particular relevance in areas where public and domestic water supply is based on groundwater extraction.

Reconquista river, one of the most polluted watersheds in the Buenos Aires Metropolitan Area, presents progressive deterioration downriver as a consequence of the 82 streams it receives along its 55 km course. Las Catonas and Morón streams are the main affluents of this river (Figure 1). Morón stream determines a sharp change in Reconquista water quality (Topalian *et al.*, 1999), as it brings elevated amounts of organic matter, industrial and faecal pollution. The water quality of Las Catonas catchment, with a drainage area of approximately 180 km^2, is less known. It can be divided into two regions: the upper basin, where intensive floricultural and horticultural practices take place, and the lower basin, an urbanized area that

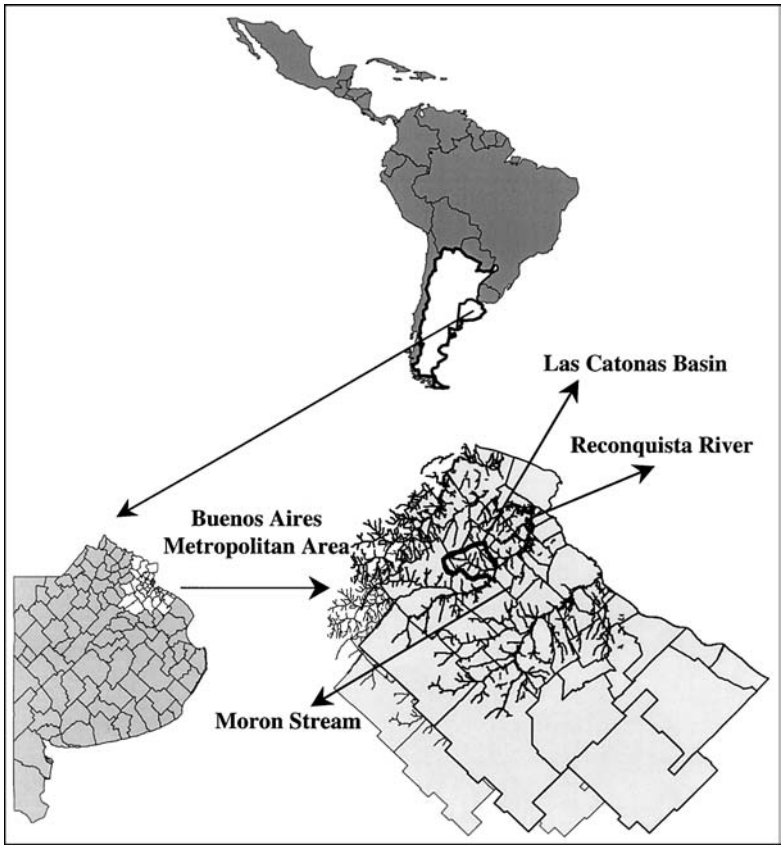

Figure 1. Geographic location of the watersheds in the Metropolitan Area in Buenos Aires.

in spite of a lower residential and industrial density than that of Morón stream, contributes to surface water pollution due to indiscriminate industrial and domestic waste disposal.

Microbiota develops different survival strategies in heavy metal polluted habitats (Bratina *et al*., 1998; Bruins *et al.*, 2000; Lovley, 2000; Lloyd and Lovley, 2001; Silver and Misra, 1988). The term "resistance" defines the ability to survive and grow through a heavy metal inducing mechanism encoded either in plasmids or chromosomes. On the other hand, the term "tolerance" indicates the presence of constitutive cell structures or biological excretion reactions that decrease the heavy metal local bioavailability; for example this can be achieved by metal complexion or precipitation. The isolation of resistant or tolerant microorganisms is important in order to identify their detoxifying mechanisms that can be used in bioremediation processes of metal contaminated sites (Gadd, 2000; Roane *et al.*, 2001; Sabry *et al.*, 1997; Sharma *et al.*, 2000; von Canstein *et al.*, 1999; Wang *et al.*, 1997).

The aim of our study is to enhance the knowledge of surface water quality in Las Catonas basin, monitoring nutrient, Cu, Cd, Pb and Zn levels and its sanitary

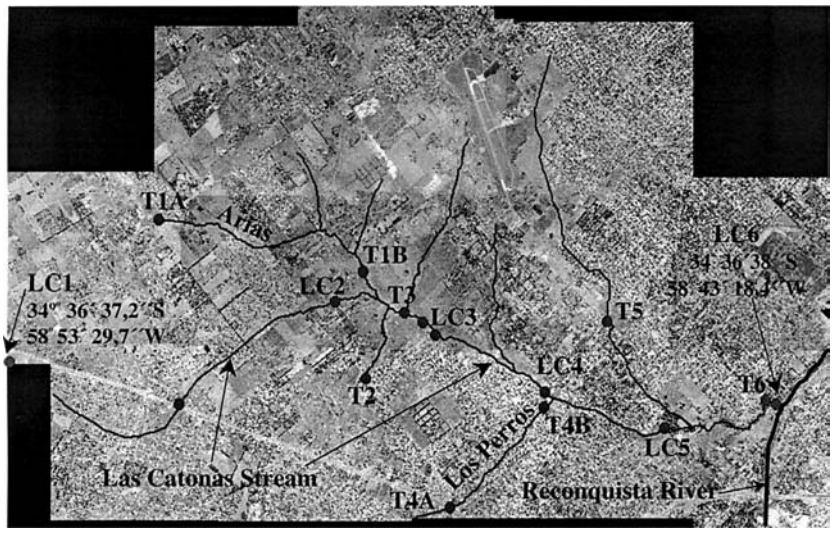

Figure 2. Las Catonas Basin sampling sites.

condition. Water samples of selected sites were used to isolate autochthonous metal multiresistant/tolerant microbiota for a potential use in bioremediation.

2. Materials and Methods

2.1. Sampling sites

Figure 2 shows Las Catonas basin and the sampling sites along Las Catonas (LC) stream and its tributaries (T), selected in order to evaluate physico-chemical and microbiological surface water quality. The domestic effluents from a housing area (Complejo Habitacional Las Catonas, 1500 units and 7000 inhabitants) are treated in a sewage treatment plant, located between sites LC4 (34 °36′36.7″S, 58 °46′ 22.8″W) and LC5 (34 °36′49.4″S, 58 °45′36.8″W). These sites present the highest industrial activity and population density. Metal distribution study between surface and pore water was performed in this area. Pore water samples for bacterial screening were taken only from site LC4, near the confluence with Los Perros (T4) stream, because at site LC5 the stream bed was covered with garbage.

2.2. Field studies and chemical analysis

Surface water samples were taken from 14 sampling sites (Figure 2) in October 2001 (spring in the southern hemisphere), March 2002 (summer) and October 2002. Previously, surface and pore water samples were taken at sites LC4 and LC5 (October 2000 and April 2001). Temperature, pH, conductivity and turbidity were

measured at each site, using a Horiba U-10 water quality analyser. Samples were transported to the laboratory under cool and dark conditions and filtered through 0.45 μm pore diameter membranes. Water samples were stored at 4 °C and nitrate, nitrite, ammonia, DQO and chloride analyses were performed within 24 h of collection according to APHA, AWWA, WEF *Standard Methods for the Examination of Water and Wastewater*. Streambed-sediment samples were taken from the 2 to 3 cm surface sediment horizon by using a grab sampler, in order to collect pore water from recently deposited sediments. Pore water for microbial screening was obtained by filtration through a glass fiber prefilter membrane (Millipore). Pore water for metal analysis was obtained by filtration after centrifugation of sediment samples. Surface and pore water samples for metal analysis were acidified to pH = 2 and stored at 4 °C.

Filtered surface and pore water samples were pre-treated with NaClO, a strong oxidant, in order to eliminate organic matter and release metals from their organic complexes. Total content for zinc, lead, cadmium and copper was determined by anodic stripping voltametry (APHA, AWWA, WEF *Standard Methods for the Examination of Water and Wastewater*, method 3130B).

2.3. MICROBIAL STUDIES

2.3.1. *Determination of Bacterial Indicators of Sanitary Condition*

For simultaneous detection of total and faecal coliforms the surface plate technique was used with Chromocult Coliform Agar (Merck). Duplicates of 0.1 mL of the respective sample dilution (10^0 to 10^{-3}) were spread on plates and incubated at 37 °C for 24 h. Non-faecal coliforms were detected as red colonies, resulting from salmon-galactoside cleavage (β-galactosidase activity) and faecal coliforms (presumptive *Escherichia coli*) as violet colonies, resulting from salmon galactoside (β-galactosidase activity) and X-glucuronide (β-glucuronidase activity) cleavages, and a positive indole production reaction performed with Kovac's reagent (Merck).

2.3.2. *Screening and Selection of Heavy Metal Multiresistant (Tolerant) Bacteria*

Metal multiresistant or tolerant culturable strains were screened from Las Catonas stream surface and pore waters, sampled at sites LC4 and LC5 (April 2001 and October 2000). Twenty-two multiresistant (tolerant) strains were isolated. For the microbial enumeration, 0.1 mL from each sample (or its 10-fold dilutions) were spread on Agar Plate Count (APC: casein peptone 5 g/L, yeast extract 2.5 g/L, D(+)-glucose 1 g/L, agar 14 g/L) and on metal supplemented APC. Six different supplemented plates were prepared with: (a) 0.5 mM Cu^{2+}, (b) 0.5 mM Cd^{2+}, (c) 0.5 mM Zn^{2+}, (d) 0.5 mM Pb^{2+}, (e) 0.5 mM Cu^{2+}, Zn^{2+} and Pb^{2+}, (f) 0.5 mM Cu^{2+}, Cd^{2+}, Pb^{2+} and Zn^{2+}. Incubation was performed at 30 °C, from 24 to 96 h. As a selection step, colonies grown in presence of the four heavy metals were also tested on plates prepared with APC supplemented with increasing concentrations

(1, 2, 4.8 and 9.6 mM) of a mixture of Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+}. The surviving strains were then tested on batch cultures. The heavy metal medium (HM) used for batch culture growth evaluation of the multiresistant (tolerant) isolated strains contained 2.5 g/L casein peptone, 1.25 g/L yeast extract, 0.5 g/L glucose, 0.5 mM Cu^{2+}, 0.5 mM Cd^{2+}, 0.5 mM Zn^{2+} and 0.25 mM Pb^{2+}. An amount of 150 mM KCl cell suspension of each isolated strain (with a similar turbidity to No. 1 Mc Farland Scale tube) was used as inoculum. Inoculum volume represented 10% of total final culture volume. Incubation was performed for 6 days at 30 °C, following growth rates by monitoring absorbance at 600 nm. The selected strains were biochemically characterised with API20E (BioMérieux).

2.3.3. *Heavy Metal Distribution in Batch Cultures*
Fifty milliliter HM culture medium was inoculated with 5 mL of a 4-day culture of the selected strains. Representative batch culture aliquots from the different growth phases were taken during 168 h incubation (200 rpm and 30 °C). Growth was tested by absorbance measurement at 600 nm. All samples were centrifuged ($1000 \times g$, 15 min), and the total Cu, Cd, Pb and Zn content in supernatants and pellets (re-suspended in 150 mM KCl) was analysed by ASV. Non-inoculated medium was tested as control.

2.3.4. *Subcellular Distribution of Cu, Cd, Zn and Pb*
Cell suspensions in 150 mM KCl obtained from centrifugation ($5000 \times g$, 15 min) of a 72 h late exponential culture in HM medium (30 °C, 200 rpm) were sonicated for 3 min (Ultrasonic Homogeniser 4710 Series, Cole Palmer). Fractioning scheme is shown in Figure 3.

3. Results and Discussion

3.1. Chemical analysis

Las Catonas can be described as a typical lowland stream: it originates in a shallow depression in the plain, the watershed shows mild slopes resulting in slow current speeds, there is a lack of arboreal vegetation and the primary water source is groundwater (Feijoo *et al.*, 1999). Both main characteristics, low cross-sectional area of these watercourses and low flow rates, enhance the vulnerability of the stream due to weather conditions and human activities. Las Catonas basin is exposed to a mixture of pollutants originated from agricultural, industrial and domestic land uses. Except for some country clubs in the upper basin, this area is inhabited by low to medium income population, mostly without urban services such as public water supplies or sewage treatment plants.

The results of the chemical analysis of surface water are shown in Table I. Large spatial and temporal variations of environmental pollutants are expected depending

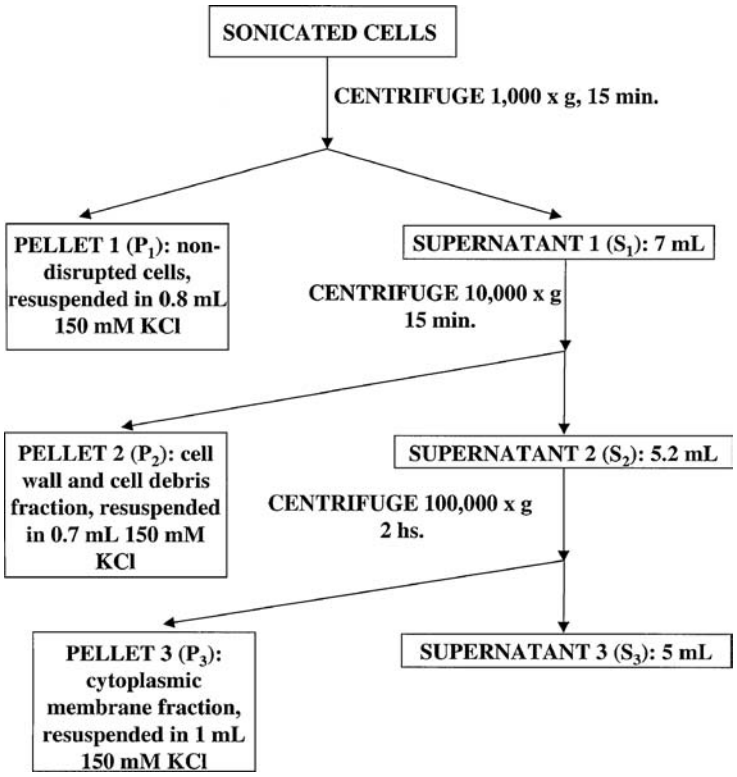

Figure 3. Differential centrifugation of multiresistant isolated strains.

not only on input rates and dilution, but also on changes in chemical composition, speciation and solubility within the stream. As a general trend, all tributaries present higher pollution levels than the main stream, increasing pollutant concentrations along Las Catonas stream. The most polluted ones are Los Perros (T4) and tributaries T2, T5 and T6. All these streams cross-urban areas, corresponding to low income population. The tributary T2, which receives the discharge from a food-processing plant, is the most polluted. T6 is not visible in Figure 2 because it flows in an underground channel. According to our results, it can be considered as a sewage disposal sink.

Las Catonas and its tributaries show higher concentrations of the analysed species when compared to Reconquista river (Topalian *et al.*, 1999) either in the upper or the middle stretch. Las Catonas drains into Reconquista river after sampling site LC6. However, estimating Las Catonas contribution to pollutant content in Reconquista river is not a straightforward task; factors like the temporal variability of constituents, the quality and scarcity of data and the cross-sectional representativeness of the sampling sites need to be taken into account.

TABLE I
Physico-chemical parameters of surface water in Las Catonas basin

	Sampling sites													
Parameter	LC1	T1A	T1B	LC2	T2	LC3	T3	T4A	T4B	LC4	LC5	T5	T6	LC6
pH	7.45	6.89	7.76	8.15	8.58	8.16	8.25	7.88	8.68	8.18	8.19	7.76	7.90	7.99
	7.42	7.68	8.14	8.30	8.57	8.72	8.76	8.44	8.24	8.30	8.35	7.64	8.09	8.04
	8.47	7.46	7.96	8.86	8.56	8.40	8.44	8.61	8.20	8.71	8.60	7.67	7.91	8.03
T (°C)	20.0	20.8	20.9	22.7	22.4	22.1	22.0	25.6	26.0	23.7	24.4	23.7	19.0	23.4
	20.7	21.6	22.8	23.1	23.1	25.4	24.7	25.2	27.0	27.7	26.5	24.5	21.8	24.0
	28.2	25.9	22.4	25.8	20.5	22.2	24.5	25.2	23.4	20.4	21.3	18.4	17.4	17.8
Conductivity	0.354	0.227	0.670	0.610	1.490	0.662	0.741	0.99	1.19	0.683	0.769	0.99	1.27	0.779
(mS/cm)	0.274	0.490	1.36	0.784	1.90	1.03	0.688	0.72	0.602	1.02	1.05	0.503	1.01	0.93
	0.464	0.333	0.92	0.741	0.681	0.758	0.723	0.876	1.23	0.775	0.97	1.00	1.26	1.06
Turbidity	17	38	37	31	281	71	114	66	60	34	61	25	12	27
	96	230	104	23	528	172	266	64	97	73	106	33	29	43
	151	73	64	29	192	33	120	46	44	36	54	33	23	17
Cl^- (mg/L)	11.3	7.4	35.6	20.4	254	25.0	16.0	33.4	59.8	25.2	31.1	43.8	61.8	30.9
	16.3	42.3	110.8	18.9	250.9	31.1	16.0	18.9	23.8	45.2	42.7	21.1	33.0	38.4
	10.8	<2	30.0	25.2	74.2	18.0	10.0	30.3	53.2	20.5	26.0	33.7	45.2	30.3
NH_4^+ (mgN/L)	0.11	<0.06	1.12	0.08	3.87	0.37	0.13	1.17	1.13	0.15	3.09	0.95	4.12	0.81
	0.06	0.09	6.40	0.17	1.47	1.23	0.10	0.66	1.57	0.50	9.37	0.75	0.15	1.70
	0.12	0.11	0.28	0.07	0.35	0.07	0.06	2.38	1.54	0.06	3.96	1.35	1.65	0.76
NO_2^-(mgN/L)	0.014	<0.01	0.232	0.047	0.805	0.142	0.047	0.129	0.392	0.140	0.136	0.456	0.188	0.252
	<0.01	<0.01	0.432	0.099	1.501	0.223	0.125	0.399	0.360	0.333	0.285	0.248	0.349	0.673
	Na	Na	Na	Na	Na	Na	Na	Na	Na	Na	Na	Na	Na	Na

(continued on next page.)

TABLE I
(continued)

Parameter	Sampling sites													
	LC1	T1A	T1B	LC2	T2	LC3	T3	T4A	T4B	LC4	LC5	T5	T6	LC6
NO_3^- (mgN/L)	2.78	3.37	3.25	5.45	4.09	5.42	6.08	2.32	6.40	5.56	4.10	7.15	10.80	5.32
	0.96	0.68	1.94	3.28	3.94	2.90	2.78	3.34	1.72	3.80	3.50	2.90	15.6	3.22
	0.53	2.12	4.41	2.66	3.13	3.40	3.46	2.23	6.64	4.91	3.94	6.46	13.41	6.81
DQO (ppm)	Na	Na	Na	Na	Na	Na	Na	Na	Na	Na	Na	Na	Na	Na
	76	119	64	<10	324	49	59	31	139	34	79	26	21	19
	67	92	36	<10	151	69	52	104	82	11	58	29	36	56
Cd^{2+} (μg/L)	<0.5	<0.5	0.11	<0.5	1.0	0.7	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	0.6
	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	6.1	4.6
	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Cu^{2+} (μg/L)	38	56	18	21	19	27	24	20	35	17	26	51	29	16
	33	17	2.6	28	16	10	19	16	18	15	15	19	22	14
	13	13	15	20	34	11	12	17	14	14	13	14	9.0	12
Pb^{2+} (μg/L)	24	34	13	41	30	53	26	23	75	109	213	75	72	103
	182	27	30	129	57	22	10	170	31	35	33	34	27	27
	17	23	18	44	37	26	17	28	22	58	31	33	27	32
Zn^{2+} (μg/L)	149	72	53	117	69	79	14	83	15	40	50	130	100	106
	36	30	30	40	60	21	16	20	17	91	47	27	49	40
	Na	3.9	6.8	4.9	4.3	6.3	55	4.5	5	13	4.8	4.4	17	5.0

Sampling schedules corresponding to Line 1: October 2001, Line 2: March 2002 and Line 3: October 2002. Na: not analyzed. LC: Las Catonas Stream. T: Tributary; T1: Arias Stream, T4: Los Perros Stream.

3.2. MICROBIAL ANALYSIS

3.2.1. *Bacterial Indicators of Sanitary Conditions*

Table II shows the total, non-faecal and faecal coliform enumerations of surface water at the selected sampling sites (March and October 2002). As a general trend, an increasing faecal coliform (presumptive *Escherichia coli*) bacteria number is observed downstream. In the central basin, corresponding to the most populated area, both total and faecal bacteria present concentration peaks. In most of the sampling sites, March results are higher than those of October, probably due to the enhanced surface runoff as a consequence of a heavy rainfall few days before the March sampling schedule (for example LC3, T2). In some sites the counts are practically invariant, as for example in site LC6, where the bacterial load (Table II) is so important that there is no great influence of the weather conditions. LC5 is located about 100 m downstream to the sewage treatment plant discharge. The maximal concentration of faecal coliforms at this site is in accordance with results obtained from plant effluent, evidencing the malfunctioning of the treatment plant.

High coliform bacteria loads point to bad sanitary conditions, associated with a potential health risk; this is of particular concern to people living in this area who are constantly exposed to these water courses, like children playing in or near the water. The sanitary condition of the streams within Las Catonas basin exceed regulatory levels for all uses, recreational (faecal coliforms: $<$200 MPN/100 mL), agricultural (total coliforms: $<$1000 MPN/100 mL) as well as to be used as water source for potabilization processes (total coliforms: $<$5000 MPN/100 mL, faecal coliforms: $<$1000 MPN/100 mL) (OSN, AGOSBA, SIHN, 1993).

3.2.2. *Screening and Selection of Heavy Metal Multiresistant (Tolerant) Bacteria*

Autochthonous metal resistant/tolerant microorganisms were isolated from surface and pore water samples in order to use the same indigenous microbiota for future water decontaminating treatments. The metals of choice included Zn, Pb and Cu for their abundance in Las Catonas stream water. Table III shows total metal concentrations in surface and pore water for samples obtained from sites LC4 and LC5, as well as data for the autochthonous culturable colonies in the presence of different metal additions. For the four analyzed metals, metal concentration in surface water is approximately one order lower than in pore water, being zinc the most concentrated. Despite the fact that Cd levels were lower, this metal was included in this study due to its high toxicity. There is a clear relationship between the origin of the sample and the metal resistant/tolerant growing colonies percentage. The autochthonous metal resistant/tolerant microorganisms obtained using pore water (8.4×10^5 CFU/mL) showed a higher culturable colony percentage than surface water (1.24×10^5 CFU/mL), for all the tested metals. In agreement with a higher concentration of heavy metals in pore than in surface water, a higher percentage of heavy metal resistant (tolerant) culturable population in pore water

TABLE II
Sanitary condition of Las Catonas basin streams by Coliform bacteria counting

	Non-faecal (CFU/mL)		Faecal (CFU/mL)		Total (CFU/mL)	
Sampling site	March 2002	October 2002	March 2002	October 2002	March 2002	October 2002
LC1	$2.1(\pm0.6)\times10^3$	$8.9(\pm1.3)\times10^2$	<10	<10	$2.1(\pm0.6)\times10^3$	$8.9(\pm1.3)\times10^2$
T1A	$1.7(\pm0.2)\times10^3$	$1.5(\pm0.2)\times10^3$	~10	~5	$1.7(\pm0.2)\times10^3$	$1.5(\pm0.2)\times10^3$
T1B	$2.4(\pm0.7)\times10^4$	$1.1(\pm0.2)\times10^3$	$1.7(\pm0.6)\times10^2$	<10	$2.4(\pm0.7)\times10^4$	$1.1(\pm0.2)\times10^3$
LC2	$1.1(\pm0.2)\times10^3$	$3.7(\pm0.9)\times10^2$	<10	<10	$1.1(\pm0.2)\times10^3$	$3.7(\pm0.9)\times10^2$
T2	$2.6(\pm1)\times10^2$	<10	<10	<10	$2.6(\pm1)\times10^2$	<10
LC3	$5.3(\pm1)\times10^4$	$4.7\ (\pm1)\times10^2$	$1.3(\pm0.5)\times10^2$	<10	$5.3(\pm1)\times10^4$	$4.7(\pm1)\times10^2$
T3	$4(\pm0.9)\times10^3$	$2(\pm0.1)\times10^3$	$40(\pm28)$	$25(\pm22)$	$4(\pm0.9)\times10^3$	$2(\pm0.1)\times10^3$
T4A	$2.9(\pm0.8)\times10^4$	$2.4(\pm0.7)\times10^4$	$3(\pm2)\times10^2$	$2.5(\pm2.2)\times10^2$	$3(\pm0.8)\times10^4$	$2.4(\pm0.7)\times10^4$
T4B	$2.5(\pm0.2)\times10^5$	$2.3(\pm0.7)\times10^4$	$4.5(\pm3)\times10^3$	$2(\pm1)\times10^2$	$2.5(\pm0.2)\times10^5$	$2.3(\pm0.7)\times10^4$
LC4	$1.5(\pm0.2)\times10^4$	$4.3(\pm0.9)\times10^3$	$1.3(\pm0.5)\times10^2$	$85(\pm41)$	$1.5(\pm0.2)\times10^4$	$4.4(\pm0.9)\times10^3$
LC5	$7.4(\pm1.2)\times10^4$	$1.1(\pm0.2)\times10^4$	$6.9(\pm1.2)\times10^3$	$1.5(\pm0.5)\times10^3$	$8.2(\pm1.3)\times10^4$	$1.2(\pm0.2)\times10^4$
T5	$7.1(\pm1.2)\times10^3$	$4.5(\pm0.9)\times10^3$	$55(\pm33)$	$90(\pm42)$	$7.2(\pm1.2)\times10^3$	$4.6(\pm0.9)\times10^3$
T6	$9.5(\pm1.4)\times10^4$	$3.8(\pm0.9)\times10^3$	$2(\pm1.8)\times10^3$	$90(\pm42)$	$9.7(\pm1.4)\times10^4$	$3.9(\pm0.9)\times10^3$
LC6	$6.2(\pm1.1)\times10^3$	$5(\pm1)\times10^3$	$1.6(\pm0.6)\times10^2$	$45(\pm30)$	$6.4(\pm1.1)\times10^3$	$5(\pm1)\times10^3$
LC STP		$6.2(\pm1.1)\times10^4$		$9(\pm4)\times10^3$		$7.1(\pm1.2)\times10^4$

LC STP: Las Catonas housing area sewage treatment plant.

TABLE III
Percent (%) of heavy metal resistant (tolerant) microbiota in Las Catonas stream

	October 2000		April 2001	
Metal (0.5 mM each in APC)	LC4 (surface water, 6.5×10^4 CFU/mL)	LC5 (surface water, 6.5×10^4 CFU/mL)	LC4 (surface water 1.24×10^5 CFU/mL)	LC4 (pore water 8.4×10^5 CFU/mL)
Cu	49	41	69	100
Zn	43	27	89	100
Pb	59	14	80	100
Cd	0.14	0.02	4.7	14
Cu, Zn and Pb	2.2	0.07	23	100
Cu, Zn, Pb and Cd	0.18	0.02	2.9	14

Total metal content in water samples were: October 2000—LC4 surface water: 375 ppb Zn.

was obtained. Screening results show that pore water samples contained a majority of culturable multiresistant microbial population due to the higher environmental selection pressure resulting from a higher heavy metal concentration in that phase. Every culturable strain exhibited resistance to Cu, Pb and Zn under the culture conditions. Cd presented the highest toxic effects as shown by the lowest number of resistant (tolerant) colonies obtained (Table III). Cd resistant (tolerant) isolates were also resistant (tolerant) to Cu, Zn and Pb. We may therefore conclude that Cu, Cd, Pb and Zn multiresistance (multitolerance) is clearly related to Cd.

After testing bacterial culturability for each of the 22 multiresistant (tolerant) isolated strain in APC supplemented with 1, 2, 4.8 and 9.6 mM in Cu, Pb, Cd and Zn, only seven strains were able to grow up to 2 mM in presence of the four heavy metals. Because of the higher growth rate and biomass obtained in batch cultures in presence of these metals, 200H and B101N strains were selected from the seven multiresistant (tolerant) isolated ones for total metal distribution studies.

Figures 4A and 5A show 200H and B101N batch culture growth pattern. Total Cu, Cd, Zn and Pb distribution between cells and culture supernatants from 200H and B101N strains are shown in Figures 4B and 5B, respectively. For both strains, interaction cell–Zn and cell–Cd were observed from exponential to stationary phase of growth. Zn was extracted from aqueous culture medium with 32% efficiency for 200H (Figure 4B) and 37% efficiency for B101N strain (Figure 5B). Cd efficiencies were 65% for 200H and 67% for B101N strain. 200H decreased Zn final supernatant concentration from 0.43 to 0.27 mM and Cd from 0.48 to 0.16 mM (Figure 4B). Meanwhile, total cellular Zn and Cd increased up to 0.235 and 0.25 mM, respectively. A similar pattern was observed for B101N (Figure 5B): supernatant Zn decreased from 0.45 to 0.31 mM and Cd from 0.54 to 0.187 mM while total cellular Zn and Cd increased to 0.32 and 0.38 mM, respectively. However, no apparent cell–Pb interactions were observed and no changes in copper concentration were detected at the different states of growth, either in 200H or B101N batch cultures.

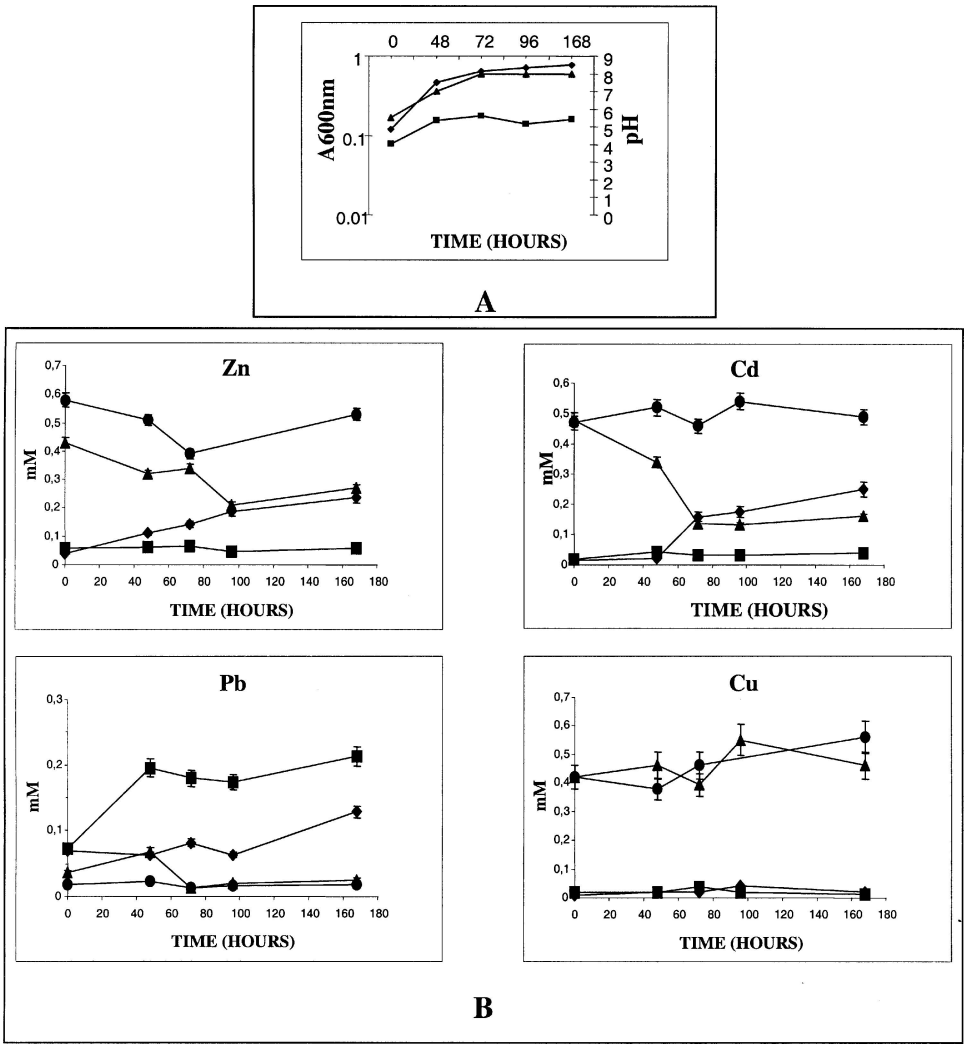

Figure 4. (A) 200H batch culture parameters: (♦) microbial growth ($A_{600\,nm}$); (■) non-inoculated culture medium (control); (▲) 200H culture pH. (B) Cu, Cd, Zn and Pb distribution in 200H batch culture: total Zn, Cd, Pb and Cu (mM) in 200H batch culture time course: (▲) 200H culture supernatants; (●) non-inoculated medium supernatants (control); (♦) 200H culture centrifugation pellets and (■) non-inoculated medium centrifugation pellets (control).

Although previous studies have reported efficient Cd removal in sea water (Sharma *et al.*, 2000; Wang *et al.*, 1997), coastal sediment and soil (Gadd, 2000), Cd and Zn removal in the presence of Cu and Pb in freshwater environments has never been reported before.

No changes in Cu concentration were detected during 200H and B101N strains batch cultures. It is most unlikely that cell–Cu interactions were absent or that the bioavailable Cu fraction was too small. Although the complexing capacity of

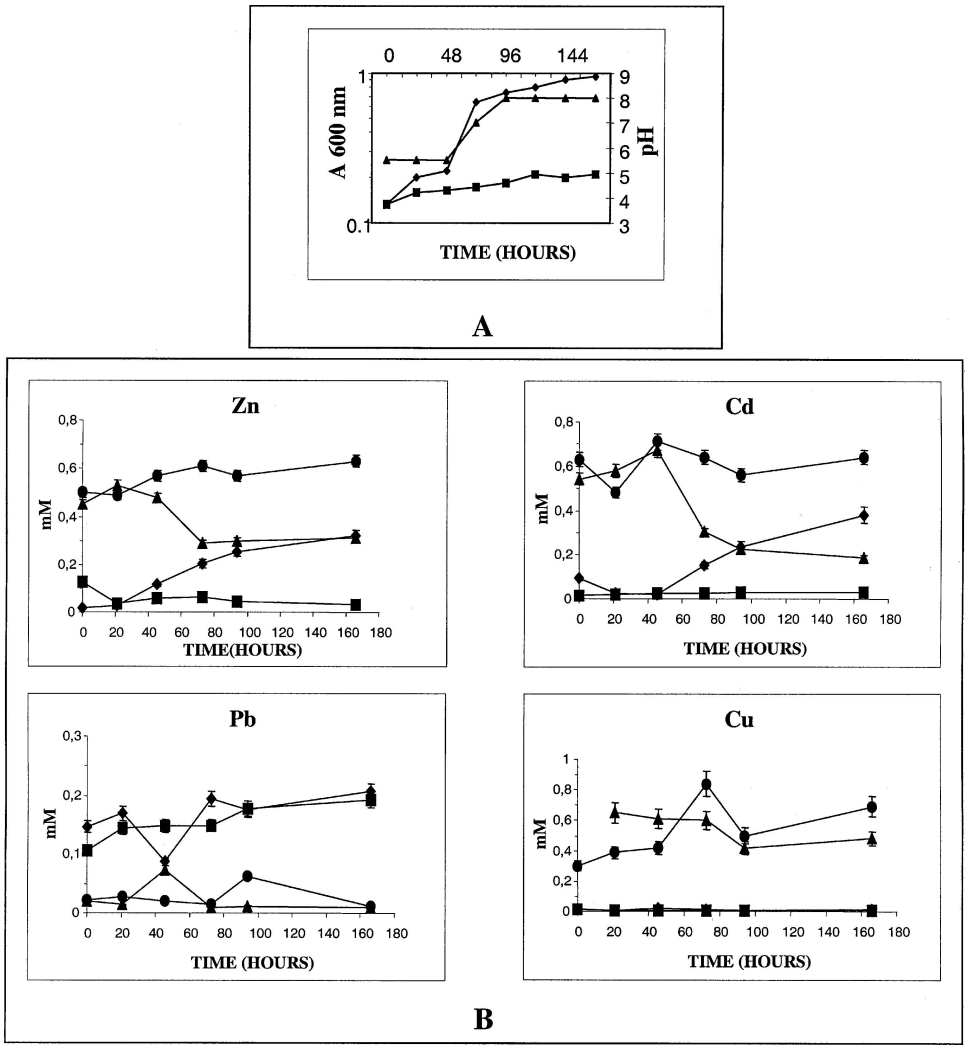

Figure 5. (A) B101N batch culture parameters: (♦) microbial growth ($A_{600\,nm}$); (■) non-inoculated culture medium (control); (▲) B101N culture pH. (B) Cu, Cd, Zn and Pb distribution in B101N batch culture: total Zn, Cd, Pb and Cu (mM) in B101N batch culture time course: (▲) B101N culture supernatants; (●) non-inoculated medium supernatants (control); (♦) B101N culture centrifugation pellets and (■) non-inoculated medium centrifugation pellets (control).

the culture media is not being underestimated, apparent cell indifference could be related to the excretion of biochelating compounds which moderate copper toxicity by a siderophore synthesis induction (Clarke *et al.*, 1987), preventing copper from entering the cell by conversion into a non-bioavailable form. In the case of 200H strain, a yellow-green fluorescence observed in presence of the four metals could probably be associated to siderophore pyoverdine, a pigment typically produced by the Fluorescent Subgroup of the genus *Pseudomonas* (Cox and Adams, 1985).

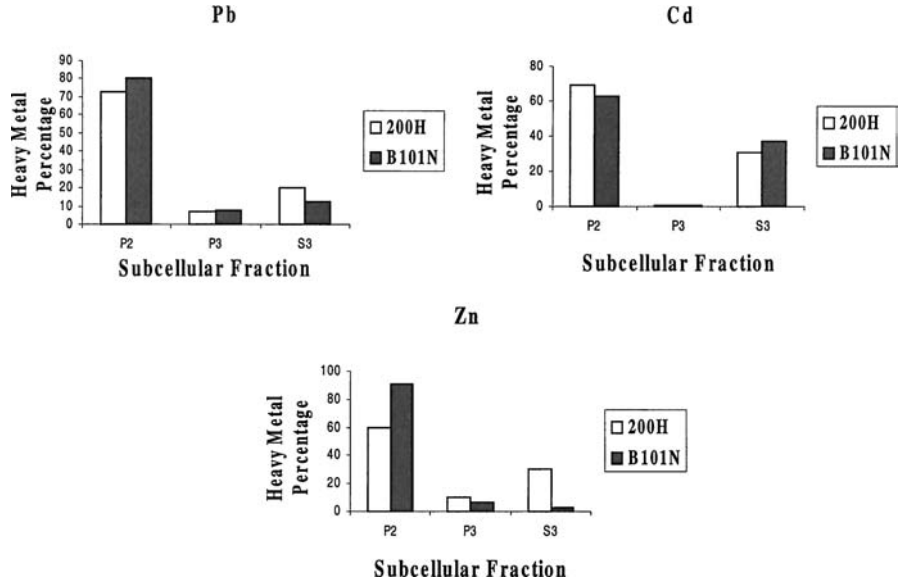

Figure 6. Subcellular distribution of Pb, Zn and Cd. Subcellular fractions: (P_2) cell walls and cell debris fractions; (P_3) cytoplasmic membrane fractions and (S_3) cytosolic fractions. Percentages were calculated from total heavy metal present in whole cell and each fraction analysis. Total Pb present in 3000 × *g* centrifugation culture pellets: 0.173 mM for 200H and 0.195 mM for B101N. Total Cd present in 3000 × *g* centrifugation culture pellets: 0.241 mM for 200H and 0.160 mM for B101N. Total Zn present in 3000 × *g* centrifugation culture pellets: 0.260 mM for 200H and 0.270 mM for B101N.

This was not observed in previous batch cultures in the absence of the four metals. Based on this, a new study about copper–pigment interactions, synthesis regulation and structural relations with pyoverdines molecules to compare it with previous reports (Clarke *et al.*, 1987; Cox and Adams, 1985; Inoue *et al.*, 2000; Visca *et al.*, 1992; Xiao and Kisaalita, 1995, 1998) is starting to be developed.

Strong interactions between Pb and culture medium components lead to a non-visible metal insolubilization only detected when quantifying Pb in non-inoculated medium centrifugation pellets (0.2 mM mean level, Figures 4 and 5). The remaining soluble Pb (10–50 μM) appeared to be non-bioavailable as no interactions were registered in batch cultures.

The subcellular fractioning results are shown in Figure 6. Total copper concentration on whole cell extracts was 6 μM for 200H strain and 4 μM for B101N strain, while the supernatants contained 0.51 mM each. This result, showing that Cu is only incorporated into cells for physiological requirements, is in agreement with further Cu analysis in subcellular fractions, which in all cases was under the detection limit.

The higher Pb percentage was detected on cell walls and cell debris fractions (P_2, Figure 6). However, detection of Pb in cytosolic fractions revealed the metal uptake and the presence of possible resistance mechanisms either in 200H or B101N strains.

A reformulation of the HM culture medium is now being considered to minimise Pb–nutrient interactions, according to other studies on Pb resistance (Konopka *et al.*, 1999a and 1999b).

Most Zn and Cd were detected on P_2 fractions, but a high concentration of Cd was also present in cytosolic fractions. There is clear evidence of resistance mechanisms for Pb, Zn and Cd present in 200H and B101N strains, since these three heavy metals were incorporated to cells. This resistance pathway seems to include outer cells structures, as major metal accumulation was detected in P_2 fractions (Figure 6) corresponding to cell walls and cell debris fractions. Two possible mechanisms would be involved in this heavy metal retention: cadmium and zinc sulfide precipitation and biosorption. Other authors (Gadd, 2000; Lloyd and Lovley, 2001; Wang *et al.*, 1997) previously reported sulfide precipitation by several Cd-resistant strains. In our case, the possible Cd, Zn and Pb biosorption phenomena could be of major interest, as described for many other bacterial species (Gadd, 2000), which result extremely useful as a tool for heavy metal extraction. Effluent Cd, Zn and Pb bioremediation process design is on development at present, using 200H and B101N cultures. Different techniques, which include suspension or immobilised-cell systems (Bréant *et al.*, 2002; Gadd, 2000; Lebeau *et al.*, 2002; McEldowney, 1994, 2000) are being considered in order to improve heavy metal removal efficiency.

API20E tests showed that 200H is a Fluorescent *Pseudomonas* (*Pseudomonas fluorescens* or *Pseudomonas putida*), and so is B101N (*Pseudomonas putida* or *Pseudomonas fluorescens*). Absence of growth at 42 °C suggests that 200H and B101N are not *Pseudomonas aeruginosa* strains.

4. Conclusions

Las Catonas stream and its tributaries are used as waste disposal sink, receiving a complex mixture of pollutants from surface runoff, tributary drainage and domestic sewage and industrial effluents. Isolation of multiresistant culturable strains from natural polluted environments was performed. Every culturable strain exhibited resistance to Cu, Pb and Zn under the culture conditions while Cd showed the highest toxic effects, meaning that Cu, Cd, Pb and Zn multiresistance (multitolerance) is clearly related to Cd. Selected 200H and B101N strains were able to remove Zn and Cd simultaneously from solution, and Pb was incorporated to the cells, suggesting the existence of a resistant mechanism. Successful removal of Cd and Zn from aqueous phase performed by the multiresistant 200H and B101N strains might be used as first tools in water bioremediation.

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