Journal of Environmental Chemistry and Ecotoxicology Vol. 3(9), pp. 229-235, 2 September, 2011
Available online http://www.academicjournals.org/jece
ISSN-2141-226X ©2011 Academic Journals

Full Length Research Paper

Study of multi-resistance to heavy metals, antibiotics and some hydrocarbons of bacterial strains isolated from an estuary basin

K. Lyamlouli, K. Kharbouch, A. Moutaouakkil and M. Blaghen*

Laboratory of Microbiology, Pharmacology, Biotechnology and Environment, Faculty of Sciences, Université Hassan II Ain-CHock, route Eliadida, B. P. 5366, Casablanca, Morocco.

Accepted 14 June, 2011

Microorganisms contained in estuary water samples (Deltebre, Spain) have been the subject of several tests of resistance against various chemicals, such as heavy metals, hydrocarbons, and antibiotics. Isolates were plated (cultivated) on Trypticase Soy Agar plates and purified for further screening. The strains were extremely resistant to heavy metals, with peculiarly, high average minimal inhibitory concentration (18700 µmol/l for arsenic and 10600 µmol/l for lead), and they also showed that they were able to grow in the presence of significant concentrations of sodium chloride (more than 50 g/l), and an interesting resistance to hydrocarbons, and antibiotics. Results showed that the most resistant strains to all the tested pollutants belong to *Pseudomonas putida* and *Stenotrophomonas maltophilia*. The kinetics of growth in the presence of certain heavy metals (Arsenic (9600 µmol/l), Cobalt (1200 µmol/l), and Lead (4600 µmol/l)), showed that the isolates had a great ability to multiply in presence of such growth inhibitors, even in high concentrations. The study of growth of the isolated strains in the presence of aromatic hydrocarbons (Benzene (4 mmol/l), Toluene (4 mmol/l), Naphthalene (6 mmol/l)) as the sole carbon source was also carried out. Isolates showed a significant sensitivity in the presence of high concentrations of hydrocarbons however, the proliferation was surprisingly fast in the presence of naphthalene. The isolated strains have shown that they can be of considerable significance, regarding the remediation of some heavy metals and aromatic compounds in heavily polluted sites.

Key words: Heavy metals resistant bacteria, antibiotics resistance, hydrocarbons resistance, salt tolerance, bioremediation, seawater.

INTRODUCTION

Industrialization and human activities in general, shoulder a great responsibility for turning our environment into dumping sites for waste materials. As a result many water resources have been rendered unwholesome and hazardous to man and various ecosystems (Bakare et al., 2003). Many chemical substances are water soluble and therefore easy to gain access to various water systems forming a threat for the fauna and flora in these systems. Pollutants can be transported by water at all stages of the water cycle. The fauna and flora are also affected by the accumulation of pollutants in the tissues of plants and other terrestrial and aquatic animals, and more generally along the food chain, needless to say that it is seriously prejudicial to the natural balance of various ecosystems. In consequence, human welfare will be affected directly.

Estuaries are large areas where mass exchanges are done between drainage basins and the sea, and thus they have been greatly the focus of scientific research (Muxika et al., 2005; Sarkar et al., 2007; White and Wolanski, 2008; Wolanski et al., 2008; Wolf et al., 2009). They are among the most exposed areas to different types of pollution, especially pollution due to oil spills (Anupama and Padma, 2009), whether it is a direct or indirect contribution due to industrialization and urbanization. This is why living beings that inhabit these environments are generally exposed to multi-elemental pollution (hydrocarbons, antibiotics, dyes and heavy

*Corresponding author. E-mail: karim-2l@hotmail.fr. Tel: +(212) 22 23 06 80/84. Fax: +(212) 22 23 06 74.

metals); For example, a large number of textile dyes are sold each year, with approximately 2% released directly in various water sources and 10% lost during the coloring process (Pearce et al., 2003). Color is one of the most obvious indicators of water pollution, and discharge of highly colored synthetic dye effluents can cause damage to the receiving water bodies (Nigam et al., 1996).

In view of the fact that several authors have attested of the strong antibiotics, hydrocarbons and heavy metals resistance concerning bacteria isolated from a natural environment (Cohen, 1992; Levy, 1998; Levy and Marshall, 2004), the aim of our study was to contribute to the research and the isolation of bacteria with halophilic properties, in a relatively sensitive natural environment, and study the tolerance in the presence of several types of pollutants.

METHODS

Sampling sites

The description of sampling sites is represented in Appendixes A, B and C. Deltebre is located between Barcelona and Valencia, near the town of Tortosa in the south of the Tarragona province (southern part of Catalonia). This place is very special because it is part of a delta, where the Ebre (Spain's main river) flows into the Mediterranean Sea (hence the name Deltebre).

The sampling was carried out, more specifically, in the old abandoned Deltebre saline; characterized primarily by their high content of sodium chloride (NaCl) and their high biological activity (swamp).

Samples processing and bacteria isolation

Seawater and sediment samples were collected aseptically from an estuary basin in Spain. The samples were put on sterile tubes and conserved at 4°C.

The collected samples were diluted with sterile Distilled water, sown in TSA (Trypticase Soy Agar, Bio-born) and incubated at 37°C for 48 h; then a pure culture was obtained by successive isolation of colonies in the same media. Bacterial identification was done by biochemical analysis according to the standardized micromethod API 20E and 20 NE (Biomeriaux) (Filali et al., 2000).

Minimal inhibitory concentration (MICs)

Heavy metals

The liquid medium (Nutrient Broth), non-amended (controls) or amended by adding the metal element at different concentrations from stock solutions, was inoculated with uniform volume (100 µl) of cell suspensions of preculture of one night of each strain diluted to 1%, the minimal inhibitory concentration (MIC) is defined as the lowest concentration that causes no visible growth (Jennifer, 2001). For more accuracy, the MIC was determined using a Shimadzu UV-1800 spectrophotometer, set at a wavelength of 600 nm.

The resistance to heavy metals was tested using a concentration range, from 18.75 to 19.200 µmol/l.

The chosen dilution factor was half; nevertheless, to obtain results as accurate as possible, other concentrations were tested. According to the first obtained MIC value, we tested concentrations slightly lower, without using the dilution factor. For example, if the first obtained MIC was 19200 µmol/l, the following concentration to be tested will be 17200 µmol/l, then 1520 0µmol/l, so on. Metals used includes: $AgNO_3$, $HgCl_2$, $CdCl_2$, $Pb(NO_3)_2$, $CoCl_2$, $CuSO_4$, KH_2AsO_4, $Ni(NO_3)_2$, $FeCl_3$, $Bi(NO_3)_3$, $ZnCl_2$ and $BaCl_2$.

Following the obtained results, our interest has focused on both the bacteria showing the highest resistance degree, in the presence of the tested heavy metals, in order to study the growth in the presence of antibiotics, hydrocarbons, and dyes.

Hydrocarbons and sodium chloride

The MIC determination was also carried out for the sodium chloride. Tubes containing 5 ml of nutrient broth with different concentrations of NaCl were inoculated with uniform volume (100 µl) of preculture of one night of each isolated strain. The tested concentrations were 5, 6, 8, 10, 15, 20, 25, 30, 40 and 50 g/l. Incubation was done at 37°C for 24 and 72 h. The MIC concerning Naphthalene, one of the most studied aromatic hydrocarbons due to its relatively high solubility in water and especially the ease of isolating, bacteria involved in its biodegradation (Mrozik et al., 2003), was also determined by using the same method already mentioned.

A minimum medium of the following composition: ($MgSO_4$ (0.1 g/l); KH_2PO_4 (1.36 g/l); $(NH4)_2SO_4$ (0.6 g/l); $CaCl_2$ (0.0 2 g/l); $MnSO_4$ (1.1 mg/l); $CuSO_4$ (0.2 mg/l); $ZnSO_4$ (0.2 mg/l); $FeSO_4$ (0.14 mg/l); NaCl (0.5 g/l), was used instead of Nutrient Broth. The same study was carried out concerning, Toluene and Benzene. The following concentrations (in mmol/l), for each hydrocarbon, were tested: Naphthalene: 0.25, 0.5, 1, 2, 3, 4, 7, 8, 14.5, 15, 16 mmol/l. Toluene and benzene: 0.5, 1, 1.75, 3, 3.5, 5, 6.5, 7, 7.5, 8, 14 mmol/l.

Antibiotics and dyes

The resistance evaluation (assessment) of the isolated strains, to various antibiotics was carried out on liquid medium (Mueller Hinton). Tubes containing 5 ml of liquid medium and various antibiotics at different concentrations (between 0.0625 and 16 mmol/l using a dilution factor of 1/2) were inoculated with 100µl of preculture of each isolated strain.

Tested antibiotics were: Oxacillin, Ceftriaxone, Ceporine, Erythromycin, Ampicillin, Fosfomycin, Rifampycin, Carbenicillin and Amphotorecin. To assess the ability of the isolated strains regarding the degradation and the discoloration of synthetic dyes, bacterial growth was followed in the presence of; one anthraquinonic (Cibacron blue) and two azoic ones (Azorubin and Blue trypan). Isolated strains were sowed into plates containing 15 g/l of agar supplemented with 100 mg/l of each tested dyes. Incubation was done at 37°C for 4 days.

Growth rate of the isolates

The growth rate of each isolated strain in the presence of heavy metals was also determined. A set of Erlenmeyer flasks containing 100 ml of nutrient broth and different concentrations of tested heavy metals, was inoculated with 1 ml of preculture of each strain.

A control for each isolate was carried out under the same conditions, without heavy metals addition.

Erlenmeyer flasks were incubated in a shaker incubator at 37°C and at 70 U/min. Bacterial growth was followed (attended) by measuring absorbance at 600 nm during 48 h. Only three metals were tested: lead, cobalt and arsenic.

The growth of the bacterial strains in the presence of aromatic hydrocarbons as sole carbon source, has also undergone extensive tests, we proceed by inoculation of Erlenmeyer flask containing 100 ml of minimal medium with the following composition: ($MgSO_4$ -

(0.1 g/l); KH_2PO_4 (1.36 g/l); $(NH4)_2SO_4$ (0.6 g/l); $CaCl_2$ (0.02 g/l); $MnSO_4$ (1.1 mg/l); $CuSO_4$ (0.2 mg/l); $ZnSO_4$ (0.2 mg/l); $FeSO_4$ (0.14 mg/l); NaCl (0.5 g/l) in the presence of the following hydrocarbons concentrations:

S1: benzene (4 mmol/l), toluene (4 mmol/l), naphthalene (6 mmol/l).
S2: benzene (4 mmol/l), toluene (4 mmol/l) naphthalene (6 mmol/l).

A control blank containing the minimum medium supplied with 2% of glucose was prepared; the inoculated flasks were incubated at 37°C and agitated on a rotary shaker (150 rev/min) for 10-15 days. Absorbance was measured at 600nm.

RESULTS AND DISCUSSION

Bacterial diversity

Following the bacteriological analysis and microscopic observation, a total of 28 strains could be isolated. Bacteria that were the most tolerant in the presence of heavy metals (S1 and S2) were selected to undergo a battery of tests (resistance to antibiotics and hydrocarbons, and salt tolerance). API 20 analysis has been able to reveal the presence of two different strains: *Pseudomonas putida* (S1) and S*tenotrop*homonas *maltophilia* (S2).

Minimal inhibitory concentration of heavy metals, hydrocarbons and antibiotics

The obtained minimal inhibitory concentrations concerning heavy metals are represented in Table 1. The results, supported by the different MIC values, indicate that the strains are highly resistant regarding the tested heavy metals, compared to those listed in the literature (Blaghen et al., 1993; Seralathan et al., 2006), with respectively an average MIC of 18700 ± SD µmol/l and 10600 ± SD µmol/l for Arsenic and Lead, and 200 ± SD µmol/l for mercury. Naphthalene, benzene and toluene MIC are also reported in Table 1.

According to the obtained results, strains resistance toward hydrocarbons seems to be low. However, we have registered an average MIC of 15 ± SD mmol/l regarding naphthalene for S1. The obtained MICs regarding NaCl showed that all the strains are able to grow in the presence of large quantities of this element. We have obtained a value of 15 ± SD g/l for S2, and for S1 this value has exceeded 50 g/l. The MIC concerning antibiotics on liquid medium (Mueller Hinton) are represented in Table 1. All the strains were resistant to the selected antibiotics; the first signs of tolerance were clearly visible after only 18 h of incubation, the results were not surprising, since *Pseudomonas putida* and *Stenotrophomonas maltophilia* are known to exhibit a high resistance to various types of antibiotics. Microbial resistance to antibiotics is generally associated with a reduced penetration of these substances in the cell. In most cases, genes assembled in plasmids protect bacteria against antibiotics, however, there are bacteria resistant to antibiotics and do not contain any plasmids, in this case the resistance depends more on mobile genetic elements called transposons. There are four mechanisms of resistance specified by plasmids: inactivation, impermeability, bypasses and altered target site; all occur in aquatic environments (Mudry, 2002).

Several studies reported that there would be some association between resistance to heavy metals and antibiotics, which was demonstrated by the analysis results. In fact, under conditions of metal stress, resistance to these two types of compound would help the microorganisms to adapt faster by the spread of resistance factors than by mutation and natural selection (Edward et al., 2009).

Dyes effect regarding strains growth, on solid medium after 3 days of incubation, showed that there wasn't any discoloration or bacterial multiplication; we have concluded that all the strains have no discoloration activity, neither degradation ability regarding the tested dyes.

Growth studies of the isolated bacteria

The growth curves of the strains in the presence of different concentrations of lead, arsenic and cobalt are shown in Figures 1 and 2. Figures 1 and 2 also showed that bacterial growth for S2 seems not much influenced and we assisted to a short lag phase, indicating that there is no effect on the growth properties. In presence of 9600 µmol/l of arsenic, we obtained a delayed lag phase for S1.

As reported by some authors, the results may suggest that the isolated bacterial strains are likely to be involved in the redox cycling of arsenic (Inskeep et al., 2007; Quéméneur et al., 2008). The oxidation of arsenite can either produce usable energy, or simply be a step in an eventual process of detoxification, nevertheless further study is necessary, in order to affirm this hypothesis.

The study of the isolated strains capacity to utilize aromatic hydrocarbons as an energy source was also done; the results are represented in Figures 3 and 4. Bacteria with aromatic hydrocarbons degradation properties are generally isolated from soil samples, most of them belong to the genus *Pseudomonas*. The conducted study concerning the ability of the bacterial strains to grow in the presence of the selected hydrocarbons as sole carbon source showed that the strains could grow promptly in the presence of naphthalene, which was evident, referring to the obtained short lag phase concerning *Pseudomonas putida* (S1).

This relatively fast growth may be due to the use of naphthalene as a source of energy, implying a possible biodegradation of this elements, several studies have indicated that the bacteria involved in this degradation

Table 1. Minimal inhibitory concentration of NaCl, and the tested heavy metals, antibiotics, and hydrocarbons.

Compounds	Bacterial strains	
	S1	S2
As^{2+}	18700 ± 1000	18200 ± 1154,701
Pb^{2+}	10600 ± 1154,701	9100 ± 1000
Cu^{2+}	2300 ± 200	4800 ± 0
Co^{2+}	2400 ± 0	2400 ± 0
Ag^{2+}	625 ± 28,867	575 ± 50
Hg^{2+}	112,5 ± 25	200 ± 212,5
Cd	17,812 ± 1,875	150 ± 0
Ni^{2+}	2400 ± 0	562,5 ± 25
Cr^{2+}	287,5 ± 25	600 ± 0
Fe^{2+}	2400 ± 0	2400 ± 0
Ba^{2+}	4800 ± 0	4800 ± 0
Ceporine	7,875 ± 0,25	4 ± 0
Ceftriaxone	**	3,75 ± 0,5
Ampicillin	7,375 ± 0,478	O,625 ± 0,25
Oxacillin	10,25 ± 0,5	3,875 ± 0,25
Amphotorecin	**	4 ± 0
Erythromycin	11,625 ± 0,216	4 ± 0
Fosfomycin	3,75 ± 0,5	4 ± 0
Rifampycin	0,14 ± 0,07	0,156 ± 0,0625
Carbenicillin	3,25 ± 0,5	2 ± 0,408
Naphthalene(mmol/l)	15 ± 0,866	7,66 ± 0,577
Benzene (mmol/l)	3,5±0	1,5 ± 0,433
Toluene (mmol/l)	7,33±0,763	1,75 ± 0
NaCl (g/l)	More than 50	15 ± 0

The results are Means ± SD of quadruplicate tests concerning heavy metals and antibiotics, and triplicate tests regarding NaCl and hydrocarbons.
** The bacteria are sensitive to that antibiotic.

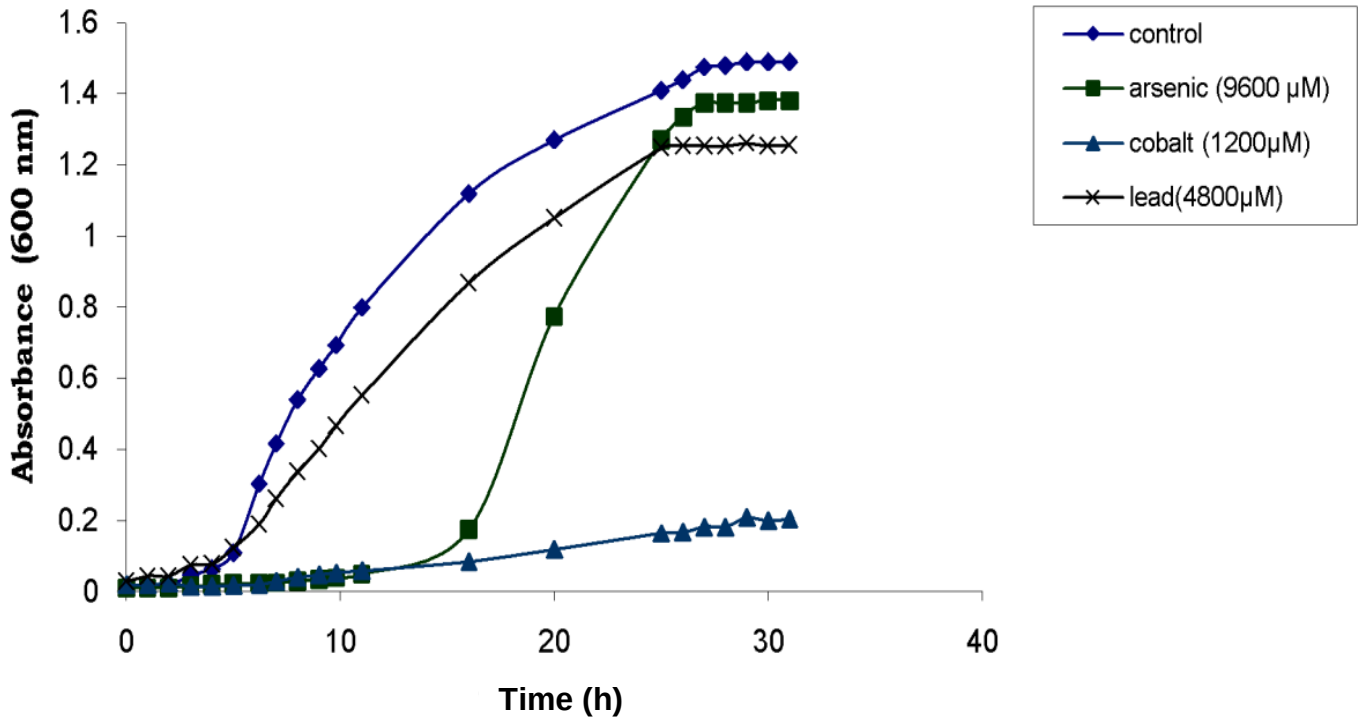

Figure 1. Arsenic, lead and cobalt effect on (S_1) growth, Incubation was carried out in aerobic conditions in a shaker incubator at 37°C, and at 70 U/min. Results are Means ± SD of triplicate tests.

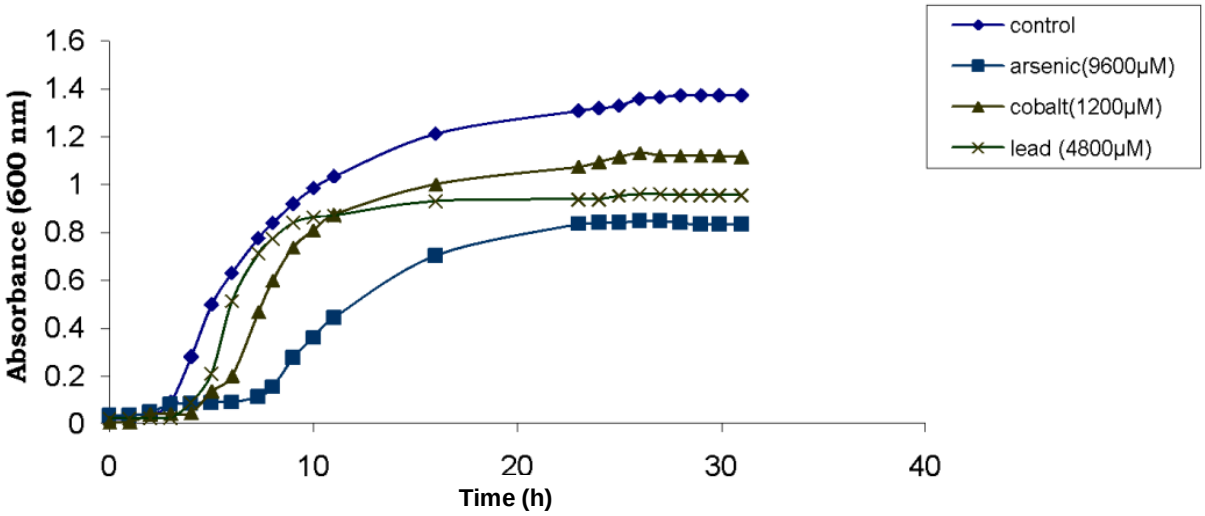

Figure 2. Arsenic, lead and cobalt effect on (S_2) growth Incubation was carried out in aerobic conditions in a shaker incubator at 37°C, and at 70 U/min. Results are Means ± SD of triplicate tests.

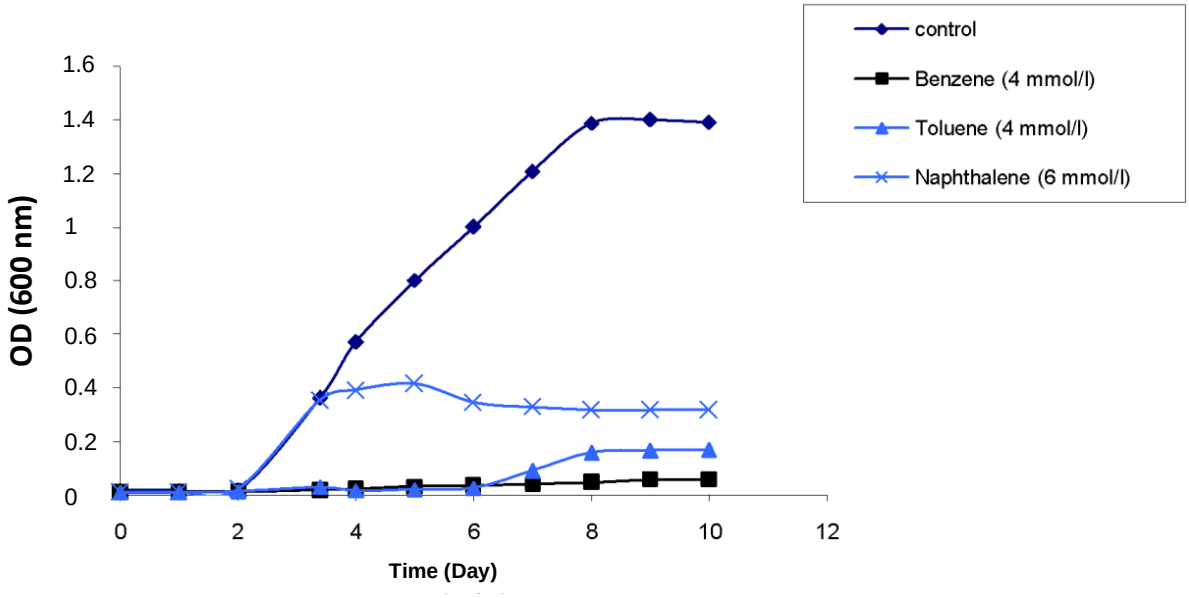

Figure 3. Growth of (S1) in the presence of hydrocarbons (naphthalene, toluene and benzene) as sole carbon source. Bacterial strain was incubated in 100 ml of minimum media containing the tested hydrocarbon, in a shaker incubator at 37°C, and at 150 Rev/min. Results are Means ± SD of triplicate tests. Results are Means ± SD of triplicate tests.

process, oxidize initially naphthalene by incorporating of both molecular oxygen into the aromatic molecule to form cis-1,2-dihydroxy-1,2-dihydronaphthalene.

In the case of *Pseudomonas putida* the Naphthalene dioxygenases acts as as multicomponent enzyme systems which are responsible for naphthalene cis-dihydrodiol formation (Mrozik et al., 2003).

In the presence of toluene, a low growth rate was reported concerning S1 after 6 days of incubation. In order to treat wastewater, several studies have been carried out, using a variety of bacteria, among them *Pseudomonas putida* is certainly the most popular, however it appears that the degradation potential is limited by the concentration, which in our case may explain the relatively slow growth rate, in fact beyond certain values, bacterial growth tends to drop, due to the toxicity caused by the high concentration of substrate (Bordel et al., 2007).

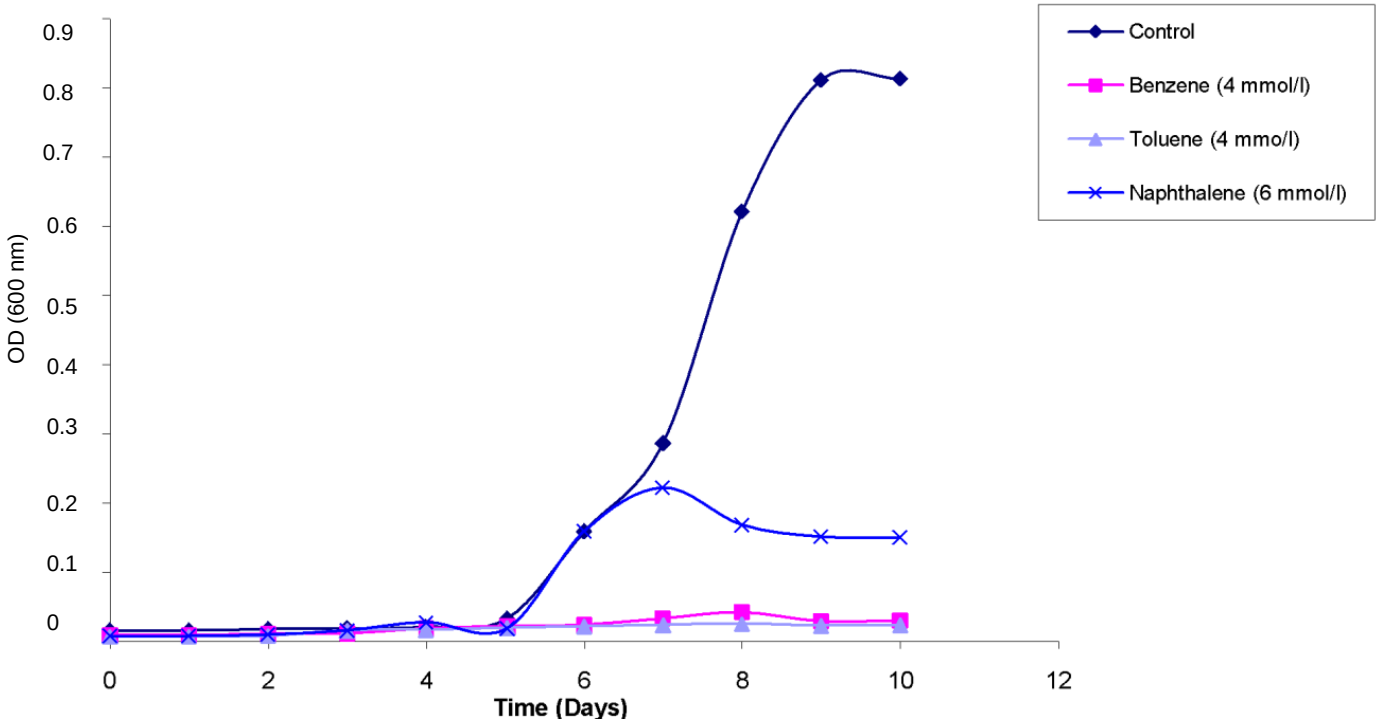

Figure 4. Growth of (S2) in the presence of hydrocarbons (naphthalene, toluene and benzene) as sole carbon source. Bacterial strain was incubated in 100 ml of minimum media containing the tested hydrocarbon, in a shaker incubator at 37°C, and at 150 Rev/min. Results are Means ± SD of triplicate tests.

Conclusion

From estuary basin water, the assorted strains have manifested by the experiments results that they were able to grow in the presence of high concentrations of heavy metals and NaCl, but also have showed an interesting degrees of tolerance against antibiotics, and some selected hydrocarbons.

The identification of these strains revealed the presence of *Pseudomonas putida* (S1) and *Stenotrophomonas maltophilia* (S2). Water is a precious commodity, more and scarcer, vital for the survival of humanity, and more generally for all species that inhabit our planet, its conservation is a duty that must be the top priority. The situation is critical, however, we should by no means deny, that efforts are being made constantly in order to propose methods and techniques more and more effective to fight against water pollution by various pollutants.

Our researches are included in this framework, with the aim of nurturing the current literature. The ability of the isolated strains to grow in the presence of heavy metals, could prove to be extremely useful in the treatment of waste water, where microorganisms are directly involved in the decomposition of organic matter, since in this biological process the heavy metals have often an inhibitory effect (Filali et al., 2000), thus the applied treatments can be optimized in order to incur a finer yield.

REFERENCES

Anupama M, Padma S (2009). Isolation of hydrocarbon degrading bacteria from soils contaminated with crude oil spills. Ind. J. Exp. Biol., 47: 760-765.

Bakare AA, Lateef A, Amuda OS, Afolabi RO (2003). The aquatic toxycity and characterization of chemical and microbiological constituents of water samples from Oba River, Odooba, Nigeria. Asia j. Microbiol. Biotechnol. Environ., Sci., 5: 11-17.

Blaghen M, Lett MC, Vidon DJM (1993). Mercuric reductase activity in a mercury resistant strain of yersinia enterocolitica. FEMS. Microbiol. Lett., 19: 93-96.

Bordel S, Muñoz R, Díaz LF, Villaverde S (2007). New insights on toluene biodegradation by *Pseudomonas putida* F1: Influence of pollutant concentration and excreted metabolites. Appl. Microbiol. Biot., 74:857-866

Cohen ML (1992). Epidemiology of drug resistance: implications for a post-antimicrobial era. Science, 257: 1050–1055.

Edward RC, Selvam GS, Kiyoshi O (2009). Isolation, identification and characterization of heavy metal resistant bacteria from sewage. International Joint Symposium on Geodisaster Prevention and Geoenvironment in Asia: JS-Fukuoka, pp. 205-211

Filali BK, Taoufik J, Zeroual Y, Dzairi FZ, Talbi M, Blaghen M (2000). Waste water bacterial isolates resistant to heavy metals and antibiotics. J. Currr. Microbiol., 4: 151- 156.

Inskeep WP, Macur RE, Hamamura N, Warelow TP, Ward SA, Santini JM (2007). Detection, diversity and expression of aerobic bacterial arsenite oxidase genes. Environ. Microbiol., 9:934-943.

Jennifer M (2001). Determination of minimum inhibitory concentrations. J. Antimicrobial. Chem., 48: Suppl. S1, 5-16.

Levy SB (1998). Multidrug resistance – a sign of the times. New Engl. J. Med., 338:1376–1378

Levy SB, Marshall B (2004). Antibacterial resistance worldwide: Causes, challenges and responses. Nat. Med., 10: S122–S129.

Mrozik A, Piotrowska-Seget Z, Labuzek S (2003). Bacterial Degradation

and Bioremediation of Polycyclic Aromatic Hydrocarbons. Pol. J. Environ. Stud., 12(1): 15-25.
Mudry ZJ (2002). Antibiotic Resistance among Bacteria Inhabiting Surface and Subsurface Water Layers in Estuarine Lake Gardno, Pol. J. Environ. Stud., 11(4): 401-406
Muxika I, Borja A, Bonne W (2005). The suitability of the marine biotic index (AMBI) to new impact sources along European coasts. Ecol. Ind., 5: 19–31.
Nigam P, Banat IM, Singh D, Marchant R (1996). Microbial process for the decolourization of textile effluent containing azo, diazo and reactive dyes. Process Biochem., 31: 435-442.
Pearce CI, Lioyd JR, Guthrie JT (2003). The removal of colour from textile wastewater using whole bacterial cells: a review. Dyes Pigm., 58: 179-196.
Quéméneur M, Heinrich-Salmeron A, Muller D, Lièvremont D, Jauzein, M, Bertin, PN, Garrido, F and Joulian, C (2008). Diversity surveys and evolutionary relationships of aoxB genes in aerobic arsenite-oxidising bacteria. Appl. Environ. Microbiol., 74: 4567-4573.
Sarkar SK, Saha M, Takada H, Bhattacharya A, Mishra, P, Bhattacharya B (2007). Water quality management in the lower stretch of the river Ganges, east coast of India: an approach through environmental education. J. Clean. Prod., 15: 1559–1567.
Seralathan KK, Mahadevan S, Krishnamoorthy R (2006). Characterization of a mercury-reducing Bacillus cereus strain isolated from the Pulicat Lake sediments, south east coast of India. Arch. Microbiol., 185(3): 202-211.
White L, Wolanski E (2008). Flow separation and vertical motions in a tidal flow interacting with a shallow-water island. Estuar. Coast. Shelf Sci., 77(3): 457–466.
Wolanski E, Newton A, Rabalais N, Legrand C (2008). Coastal zone management. Encycl. Ecol., pp. 630–637.
Wolf B, Kiel, E, Hagge A, Krieg HJ, Feld, CK (2009). Using the salinity preferences of benthic macroinvertebrates to classify running waters in brackish marshes in Germany. Ecol. Indic., 9: 837–847.

Appendix A. Characteristics of the sampling site.

Continent	Europe
Country	Spain
Region	Catalonia
Locality	Deltebre
Latitude	40°43'18'' N
Longitude	0°43'23'' E
Altitude	6 m

Appendix B. Geographical localization of the sampling site.

Appendix C. Sampling site "Deltebre" shown in aerial photo.