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In vitro degradation of benzene by the use of ubiquitous bacteria

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Abstract

This study focuses on specifying a number of ubiquitous non-fastidious bacteria isolated from urine hospitalized patients for their abilities to degrade benzene. Using the MALDI-Tof technique (Bruker Daltonics), these opportunist bacteria have been identified. The bacteria were inoculated and incubated in sterile water contaminated with pure benzene (99.98% purity) for 63 days, at room temperature with continuous oxygenation. Analysis by Gas Chromatography/Mass Spectrometry (GC/MS) HP6890/HP 5973 MS (Agilent Technologies) allowed us to determine the concentration of benzene and its derivatives. The results showed that not only the strains were able to completely degrade benzene in a single derivative: cyclohexane after less than 30 days. But also reveals variations in cyclohexane concentrations from one strain to another. The 2 strains belonging to the family Moraxellaceae S1670 and S1671 degrade benzene faster with concentrations 0.0475 µg/µl and 0.0727 µg/µl respectively, While both strains S5 and S476 of the Enterobacteriaceae family, had consumed totally and more easily cyclohexane with the lowest concentrations 0.0316 µg/µl and 0.0449 µg/µl respectively, this research confirmed that benzene and cyclohexane were completely consumed without producing other identifiable intermediate metabolites.

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Introduction

Human activity (industrial, agricultural, fishing, tourism etc.) is one of the major environmental aggressions. According to the commitments from the Blue Book of the Oceans Round Table, 80% of the pollution comes from terrestrials activities, and 20% from maritime activities. However, the most conventional the oldest and the most mediatized of marine pollution remains indisputably hydrocarbon pollution (ATSDR, 2007).

Hydrocarbons whether total or aromatic, monocyclic or polycyclic, contain a very large number of organic compounds. The most toxic are mono/polycyclic Aromatic Hydrocarbons, as they are classified as carcinogenic, mutagenic and reprotoxic. In addition to their ubiquitous nature, their high toxicity justifies their classification as Persistent Organic Pollutants (POP) and their inclusion as priority substances on the lists of the European Commission, the United States Environmental Protection Agency and the World Health Organization (Marchand *et al.*, 1996, Khodaei K *et al.*, 2017).

Different solutions have been proposed to treat environments contaminated with mono/polycyclic hydrocarbons, Physical and chemical processes such as stripping, flotation, thermal desorption, solvent extraction, ultrasound, electrochemical treatment (Fritsche and Hofrichter, 2008), have proven their limit do to their high expenses and the difficulties for their applications.

Current researches are focusing on elimination of aromatic hydrocarbons by bioprocesses; the interest of these new techniques mainly lies in their non-polluting aspect and the absence of chemical sub-products (Lan, 2009). Bacteria which degrade Hydrocarbon are not a new invention; they have existed since millions of years. They are omnipresent in seawater on a global scale, but only in small quantities and feed on traces of hydrocarbons naturally present in the environment. Given the number of anthropogenic pollution, the challenge for these bacteria would consist to degrade large volumes of aromatic hydrocarbons at a given site.

Despite their potential ecological importance, little knowledge about the biological processes of these bacteria.

We are interested in the biodegradation of monoaromatic hydrocarbons such as benzene, toluene, ethylbenzene, as they are considered as the most common monoaromatic pollutants. Except that, benzene is the most robust in almost all field studies, as a carcinogen and therefore the most toxic (Farhadian M *et al.*, 2006; Farhadian M *et al.*, 2008) It is within this context that our work studied the biodegradation of benzene by pure locally isolated strains already having the capacity to degrade aromatic hydrocarbons, so that the quantitative and qualitative monitoring of benzene and the growth of our strains would be Synchronous, for the purpose of large exploitation on a global scale.

Our objectives in this study were to identify and to target aero-anaerobic facultative bacteria that are ubiquitous and tenacious, capable of degrading high concentrations of benzene under unfavorable conditions. To this end, an experimental approach was developed consisting of isolating 10 bacterial strains and injecting them into sterile and contaminated sea water with benzene.

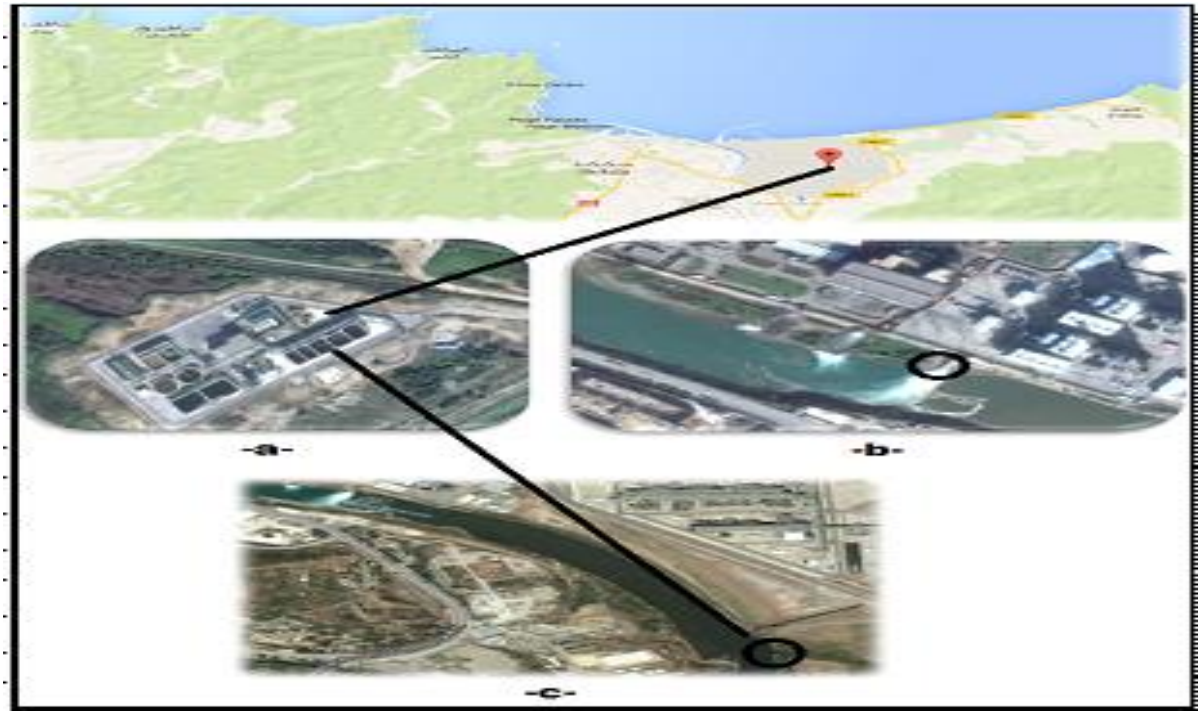
Materials and methods

Description of the field site

To preserve the hostile conditions of seawater polluted by industrial discharges, water samples were taken from the junction point of three discharge sites: effluent treatment plant 1100 (Effluent Treatment Plant 2) -b- Oued Zeramna -c- Oued Saf-saf, from the industrial zone of Skikda (Fig. 1).

Bacterial strains used

Microorganisms such as bacteria, fungi and microalgae play a key role in the elimination of mono-aromatic hydrocarbons *in situ*. However, as bacteria are faster and easier to handle (Table 2), their use is preferred (Roger and Jacq, 2000; Prenafeta-Boldu *et al.,* 2002; Schulze and Tiehm, 2004; Nikolova and Nenov, 2005).



a-[ETP2 1101: 36°52'00.4"N 6°57'57.7"E ,b- [Ouadzeramna36°52'41.2"N 6°56'08.9"E],
c- [Ouadsafsaf 36°87'23.4"N 6°94'25.7"E.

Fig. 1. Location of study and sampling sites (Google map2015).

Given the difficulties to cultivate bacteria found naturally in aquatic and telluric environments contaminated by aromatic hydrocarbons, we have used ubiquitous species isolated from human pathological samples (Urine) having specificity to degrade aromatic hydrocarbons. The use of bacteria isolated from human samples for purely environmental purposes is attempted for the first time.

Based on bibliographic data, their common properties as well as the fact that they belong to the same genus in varieties of different environments, we selected about 10 N_{os} bacteria which were isolated from different urine specimens at the laboratory of the Military University Regional Hospital of Constantine (Table 3). Whose 6 belongs to the Enterobacteriaceae family, 2 to the Moraxellaceae family, 1 to the Pseudomonadaceae family and one to the Enterococaceae.

Proteomic biochemical characteristics

Family of Enterobacteriaceae

It is a very large family that represents more than half of laboratory isolates, is mostly hosts of the gastrointestinal tract, but some such as *Serratia* are met predominantly in the external environments (Avril JL *et al*., 1992).

Family of Moraxellaceae

The family *Moraxellaceae* is a member of the order *Pseudomonadales*, currently countaning the genus *Acinetobacter, Moraxella* and *Psychrobacter*. Many of these microorganisms are part of this family have questionable identities, generating difficulties in the interpretation of publications (Shuai Li *et al*., 2017).

Acinotobacter are ubiquitous bacteria and are part of the normal flora of humans and animals (Avril JL *et al*., 1992).

Family of Pseudomonadaceae

This family currently includes 04 genuses: *Pseudomonas* and *Xanthomonas*, most of them are phyto-pathogenic, they are also found in the context of hospital-acquired infections and *Frateuria* and *Zoogloea* exclusively saprophyte (Avril JL *et al*., 1992).

Family of Enterococcaceae

They are commensals of gastrointestinal flora, there are about thirty species: *Enterococcus faecalis* and *Enterococcus faecium* are the most frequently isolated in pathological situation (Avril JL *et al.,* 1992).

The isolated bacterial species were identified by the Api®20 E galleries. Confirmation was made by Maldi-Tof mass spectrometry (Bruker, biotyper 2.0).

MALDI-TOF: Matrix Assisted Laser Desorption/Ionization-Time Of Flight

MALDI-Tof is a technique that was introduced as an ionization method for a wide range of biological molecules in 1998 by Hillen Kamp and Karas and has since become a generalized analytical tool for analyzing proteins (Hillenkamp and Karas, 1991).

The sample (young culture) to be analyzed by MALDI-TOF is prepared by mixing the sample with an excess solution of an organic absorbent compound called a matrix. When the matrix crystallizes during drying, the sample trapped in the co-crystallized matrix also is bombarded with a laser beam. Desorption and ionization with the laser beam generates mono-proton ions from analytes in the sample. The protonated ions are then accelerated to a fixed potential and the latter are separated from each other on the basis of the mass/charge ratio (m/z). The loaded analytes are then detected and measured using time-of-flight (TOF) analyzers (Neelja *et al*., 2015).

During the MALDI-TOF analysis, the m/z ratio of an ion is measured by determining the time it takes to travel the length of the flight tube (Yates, 1998) and to produce a mass spectrum. The resulting mass spectrum is a kind of unique and specific fingerprint of the analyzed microorganism protein composition (Protein Microorganism Fingerprint), which can be compared to a spectral database (*Descy et al*., 2010).
The identification of bacteria by MALDI-TOF MS is made by comparing the MS spectrum (PMF) of an unknown microbial isolates in comparison to the MS spectrum of the known microbial isolates contained in the database.

For the identification of germs at the species level, a typical mass range of m/z of 2-20 kDa is used, which mainly represents ribosomal proteins with some maintenance proteins. Ribosomal proteins are the most abundant, accounting for about 60-70% of the dry weight of a microbial cell. The mass range of 2-20 kDa is used to identify a particular microorganism by associating its PMF with the PMF of the ribosomal proteins contained in a large open database (Drancourt M *et al*., 2010) Thus, the identity of a microorganism can be established by gender and in many cases by species (Fagerquist *et al*., 2010).

Implementation of experiments on the biodegradation of benzene in seawater In Vitro

Preliminary tests

Seawater samples collected were sterilized and then divided into 11 sterile vials, one of which was negative control (T_0). Each vial contains 1000 ml of sterilized sea water by autoclaving, to which was added pure benzene (99.98% purity as shown in Table 04). The flasks were well stirred to obtain a representative sample, thus the initial benzene concentration was 250 mg/l. Each vial was subsequently inoculated with bacteria from our list (Table 3) at a rate of about $8x10^{10}$ vial bacteria.

The inoculated bacteria were cultured on ordinary agar medium and the enrichment was carried out on BHIB broth to facilitate bacterial multiplication (proteose peptone 10.0 g, veal brain's infusion 12.5 g, infusion of heart of beef 5.0 g, glucose 2.0 g, chloride of sodium 5.0g, sodium hydrogenphosphate 2.5g, pH = 7.4).

The vials were hermetically sealed with rubber stoppers to avoid contamination and also to minimize the evaporation of benzene and incubated for 63 days at 25 ° C with continuous oxygenation using an aquarium motors. Daily cultures were carried out to monitor the purity of the species. After 4 weeks, the first sample was taken, than a second sample was taken after 9 weeks in order to evaluate the biodegradation of the benzene.

Physico-chemical techniques

Analysis of benzene by GC/MS

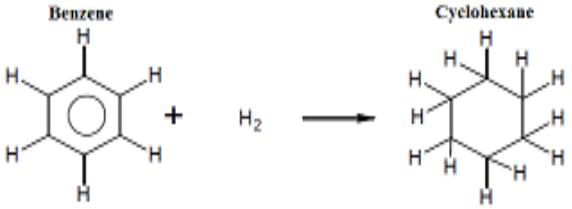
The association of gas chromatography with mass spectrometry is an analytical technique of choice. Due to the high sensitivity of this technique and the quality of the results obtained, it's used to identify many components of complex mixtures such as monocyclic aromatic hydrocarbons (Yassaa *et al*., 1999). The analysis of benzene and its degradation products was carried out by Gas Chromatography (GC) using an HP6890 (Agilent Technologies) equipped with a column MDN12 (30 mx0.25 mm, 25 µm d stationary phase thickness, Supleco) in which passes ultra-pure helium (Helium purity: 6.0), the vector gas, at 0.5 ml/min. the GC is coupled to an HP 5973 Mass Spectrometry (MS) (Agilent Technologies) mass detector operating with 70eV ionization energy and a 2000 volt voltage. The instrument was set to Total-Ion-Chromatogram (TIC) mode to detect the intensities of all mass spectral peaks belonging to the same scan, including background noise and sample components.

Analysis of benzene

To extract the organic matter, 1 ml from the original sample T_0 (negative control) as well as 1 ml of each sample was mixed with 0.5 ml of diethyl ether. The whole is carefully shaked for 15 min in order to reach the extraction equilibrium, 1 µl of the supernatant was injected into the column with a flow rate of 0.5 ml/min. The oven temperature was increased from 45 °C. to 150 ° C. according to a gradient of 10 ° C/min of pitch and then maintained for 10 min at 150 ° C. This program has been optimized for the separation of monocyclic aromatic hydrocarbons (Yassaa *et al*., 1999). The monocyclic aromatic hydrocarbons were indicated between 2 and 17.5 min and detected by the mass spectrometer in order to be identified. The concentration of benzene was calculated by injecting 0.5 µl of pure benzene (purity of 99.98%), based on a calibration curve obtained from a standard mixture (supplied by Agilent).

Analysis of Cyclohexane by GC / MS

Among the benzene derivatives, cyclohexane (Table 5). That is obtained by passing a gas stream rich in hydrogen in benzene containing a catalyst of hydrogenation; each molecule of benzene fixes three molecules of hydrogen. Thus cyclohexane is obtained.



Cyclohexane standards were prepared S1, S2, S3, S4, S5, S6 of different concentrations 7.785x10-1, 7.785x10-4, 7.785x10-, 7.785x10-5, 7.785x10-3, 7.785 x10-2 respectively.

Results

MALDI-OF analysis

For the majority of our bacteria, we were able to identify the species with a credible score (Table 6).the identification by Maldi Tof (Bruker Daltonics) had allowed the confirmation of the results obtained by the Api®20 galleries.

Table 1. Geographical coordinates of the discharge sites of the industrial zone, Wilaya of Skikda-Algeria.

Site's name	Geographical location
a-Effluent Treatment Plant 2 (ETP2 1101)	36°52'00.4"N 6°57'57.7"E
b-Zeramnaouad	36°52'41.2"N 6°56'08.9"E
c-Saf-safouad	°87'23.4"N 6°94'25.7"E

The two species S1670 and S1664 showed a highly probable identification score: 2.397 and 2.367 respectively. While 7species which are S1671, S1620, S5, S1663, S1687, S2 and S476 indicate secure genus identification whose score is between [2.277-2.058]. However, strain S1850 has the lowest score of 1.976.

Results of the Gas Chromatography and Mass Spectrometry (GC / MS)

Expression of results before treatment

Analysis of T_0 by GC/MS confirmed the presence of benzene, even before starting the experiment, with a concentration of 0.097 µg/µl (Fig. 3).

Expression of results after 4 weeks

By analyzing our samples one by one after 4 weeks of incubation, the results obtained indicate the almost total biotransformation of benzene to cyclohexane, under normal conditions, by all the strains, at different concentrations.

Table 2. Some microorganisms involved in the degradation of monoaromatic hydrocarbons (Be: Benzene, To: Toluene, Eb: Ethylbenzene, Xy: Xylenes, oXy: o-xylene, pXy: p-xylene, mXy: m-xylene).

Bacteria	Hydrocarbons used	References
Rhodococcus rhodochrous	Be, To, Eb, Xy	Deeb and Alvarez-Cohen (1999)
Rhodococcus sp. *RR1, RR2*	Be, To, Eb, mXy, pXy	Deeb and Alvarez-Cohen (2000)
Ralstonia picketii PKO1	To	Parales *et al.* (2000)
Rhodococcus sp. *strain DK17*	Be, To, Eb, oXy	Farhadian*a et al.* (2007)
Pseudomonas sp. *ATCC 55595*	Be, pXy	Collins and Daugulis (1999)
Burkholderiacepacia G4	To	Parales*a et al.* (2000)
Pseudomonas aeruginosa	Be	Kim *et al.* (2003)
Pseudomonas fluorescens	Be, To, Eb, oXy	Shim *et al.* (2002,2005)
Pseudomonas putida F1	Be, To, Eb	Parales*a et al.* (2000)
Pseudomonas putidasouche mt-2	To, mXy, pXy	Morascha *et al.* (2002)
Achromobacter xylosoxidans	Be, To, Eb, Xy	Nielsen *et al.* (2006)
Blastochloris sulfoviridis ToP1	To	Van Hamme *et al.* (2003)
Desulfobacterium cetonicum	To	Shim *et al.* (2002,2005)

Table 3. The bacteria used in the trial.

Reference	Species
S05	*Citrobacter freundii*
S476	*Citrobacter youngae*
S1620	*Klebsiella pneumoniae*
S1664	*Klebsiella pneumoniae*
S02	*Klebsiella oxytoca*
S1687	*Serratia marcescens*
S1670	*Acinetobacter baumannii*
S1671	*Acinetobacter baumannii*
S1850	*Pseudomonas aeruginosa*
S1663	*Enterococcus faecalis*

The highest concentrations of cyclohexane are 0.0964 µg/µl, 0.0864 µg/µl and 0.0846 µg/µl assigned to the bacteria S1663 (*Enterococcus faecalis*), S476 (*Citrobacter youngae*) and S1664 (*Klebsiella pneumoniae*), respectively. However, S1670 (*Acinetobacter baumannii*), S1620 (*Klebsiella pneumoniae*), and S1671 (*Acinetobacter baumannii*) the cyclohexane concentrations were lowest respectively 0.0475 µg/µl, 0.0599 µg/µl and 0.0727 µg/µl (Table 8).For the remaining their concentrations fluctuate between 0.0817 µg/µl and 0.0835 µg/µl.

Expression of results after 9 weeks

After 9 weeks of incubation, a decrease in cyclohexane concentrations was observed in all strains with varying proportions. It is S05 (*Citrobacter freundii*) which consumes cyclohexane more rapidly than the others with a concentration of 0.0316 µg/µl, while S1664 (*Klebsiella pneumoniae*) is the slowest with a concentration of 0.0842 µg/µl.

Discussion

Monoaromatic hydrocarbons in particular benzene are compounds mutagenic and reprotoxic. Benzene is considered the most recalcitrant which makes it very difficult to oxidize both chemically and biologically.

This compound has been shown to serve as carbon and energy sources for aerobic bacteria growing with nitrate, manganese, ferric iron, sulfate, or oxygen as the sole electron acceptor (Chakraborty and Coates, 2004).

Table 4.characteristics of added Benzene in our samples.

Parameter / Product	Tank number	Non-aromatic products	Benzene
Benzene	38	0.02 %	99.98 %

Table 5. Description and identification of the cyclohexane used.

Product identification	
Product form	Substance
Trade name	Cyclohexane for synthesis
Chemical name	Cyclohexane
Index number	200-661-000
CE number	203-806-2
CAS number	111-82-7
REACH registration number	BA 110-82-7-0109-011
Raw formula	C_6H_{12}
Molar mass	84.16 g/mol

This has been confirmed by the results of Hunkeler *et al*, the added oxidants were almost completely consumed during the last year of engineered in situ bioremediation, and dissolved Fe(II), Mn(II) and CH4 were detected (Table 9). The availability of oxidants probably still limited the total rate of biodegradation in the source area during the final period of engineered in situ bioremediation. However, the total flux of dissolved petroleum hydrocarbons decreased from 5 mol/day at the beginning to 0.2 mol/day at the end of the study (Hunkeler *et al.*, 2002).

Which corroborate with Scow and Hicks research thus Kao *et al,* The biodegradation is naturally enhanced by providing nutrients, electron acceptors and microorganisms degrading aromatic hydrocarbons (Scow and Hicks, 2005). Kao and Prosser have adopted a mass flux approach to calculate contaminant mass reduction and field-scale decay rate at a gasoline spill site. The mass flux calculation shows that up to 87% of the dissolved total benzene, toluene, ethylbenzene, and xylene (BTEX) isomers removal was observed via natural attenuation at this site. Results reveal that natural biodegradation was the major cause of the BTEX mass reduction among the natural attenuation processes, and approximately 88% of the BTEX removal was due to the natural biodegradation process. The calculated total BTEX first-order attenuation and

biodegradation rates were 0.036 and 0.025% per day, respectively. The results reveal that natural biodegradation was the main cause of mass reduction of BTEX in natural attenuation processes, and that about 88% of BTEX removal was due to the natural biodegradation process. The first-order attenuation and biodegradation rates calculated for the total BTEX were 0.036 and 0.025% per day, respectively (Kao and Prosser, 2001).

Table 6. The scores of each species identified by MALDI-TOF (or even the meanings of the scores in Table 7 above).

Analyte name	Analyte ID	Organism (best match)	Score value	Organism (second best match)	Score value
A7 (++) (C)	S5	*Citrobacter freundii*	2.261	*Citrobacter freundii*	2.199
A8 (++) (B)	S476	*Citrobacter youngae*	2.058	*Citrobacter freundii*	2.018
A10 (+++) (A)	S1670	*Acinetobacter baumannii*	2.367	*Acinetobacter baumannii*	2.221
D3 (+++) (C)	S1664	*Klebsiella pneumoniae*	2.397	*Klebsiella pneumoniae*	2.283
D4 (++) (C)	S1620	*Klebsiella pneumoniae*	2.270	*Klebsiella pneumoniae*	2.226
D5 (++) (A)	S1663	*Enterococcus feacalis*	2.158	*Enterococcus feacalis*	2.157
D6 (++) (C)	S2	*Klebsiell oxytoca*	2.151	*Raoultella omithinolytica*	1.933
D7 (++) (A)	S1671	*Acinetobacter baumannii*	2.277	*Acinetobacter baumannii*	2.15
D10 (++) (A)	S1687	*Serratia marcescens*	2.157	*Serratia marcescens*	2.022
A9 (+) (B)	S1850	*Pseudomonas aeruginosa*	1.976	*Pseudomonas aeruginosa*	1.976

Table 7. Meaning of the scores obtained.

Range	Description	Symbols	Color
2.300…3.000	Highly probable species identification	(+++)	Green
2.000…2.299	Secure genus identification probable species identification	(++)	Green
1.700…1.999	Probable genus identification	(+)	Yellow
0.000…1.699	Not reliable identification	(-)	Red

In this study, the degradation *in vitro* of benzene using ubiquitous strains under ordinary conditions was found to be more than effective. Indeed, the added benzene was completely eliminated by all our strains after less than 30 days with the appearance of a single compound in our water, which is cyclohexane.

Table 8. Concentrations of cyclohexane after 4 weeks and 9 weeks of incubation.

Time / Stains	After 04 weeks (µg/µl)	After 09 Weeks (µg/µl)
S 1850	0.0821	0.0632
S 05	0.0812	0.0316
S 476	0.0864	0.0449
S 02	0.0817	0.0531
S 1687	0.0835	0.0746
S 1664	0.0846	0.0824
S 1671	0.0727	0.0705
S 1663	0.0964	0.0446
S 1670	0.0475	0.0457
S 1620	0.0599	0.0483

It is a hydrogenation of benzene, with different concentrations, which can be explained by the fact that each of our bacteria degrades not only benzene but also derivatives derived from benzene.

While knowing that hydrogenation of benzene can only be carried out under conditions specific of temperature, pressure and particular asset catalyst.

Table 9. Rate of supply (*F*) and rates of consumption (*ΔF*) of species involved in microbial processes in the subsurface (Hunkeler *et al.*, 2002).

Compounds / Years	O_2		NO_3^-		$Mn_{(II)}$	$Fe_{(II)}$	SO_4^{2-}		CH_4	PHC
	F	*ΔF*	*F*	*ΔF*	*ΔF*	*ΔF*	*F*	*ΔF*	*ΔF*	*ΔF*
1993	32	-26	89	-80	1.0	5.6	17	-5.6	19	0.5
1994	17	-15	24	-21	0.8	8.1	12	-9.2	19	-
1995	16	11	21	-10	0.1	2.4	10	-2.6	7.8	0.2
1996	21	-14	22	-7	0.4	3.5	10	-1.5	7.8	0.2
1997	20	-13	22	-7	0.3	3.9	10	-2.2	5.8	-

All rates are in mol/day. - : not determined. O_2: oxygen. NO_3^-: Nitrate. $Mn_{(II)}$: Manganese. $Fe_{(II)}$: ferserique. SO_4^{2-}: Sulfate. PHC: Petroleum Hydrocarbons.

In this study, benzene is completely converted to cyclohexane under normal conditions, it is also noted that the concentration of cyclohexane decreases without the formation of other toxic secondary products.

Indeed, strains S1670 (*Acinetobacter baumannii*), S1671 (*Acinetobacter baumannii*) and S1620 (*Klebsiella pneumoniae*) degrade benzene faster than cyclohexane according to the results obtained. After 4weeks, cyclohexane concentrations were 0.0475 µg/µl, 0.0727 µg/µl and 0.0599 µg/µl respectively. Whereas after 63 days the concentrations decreased slightly as follows 0.0457 µg/µl, 0.0705 µg/µl and 0.0483 µg/µl.

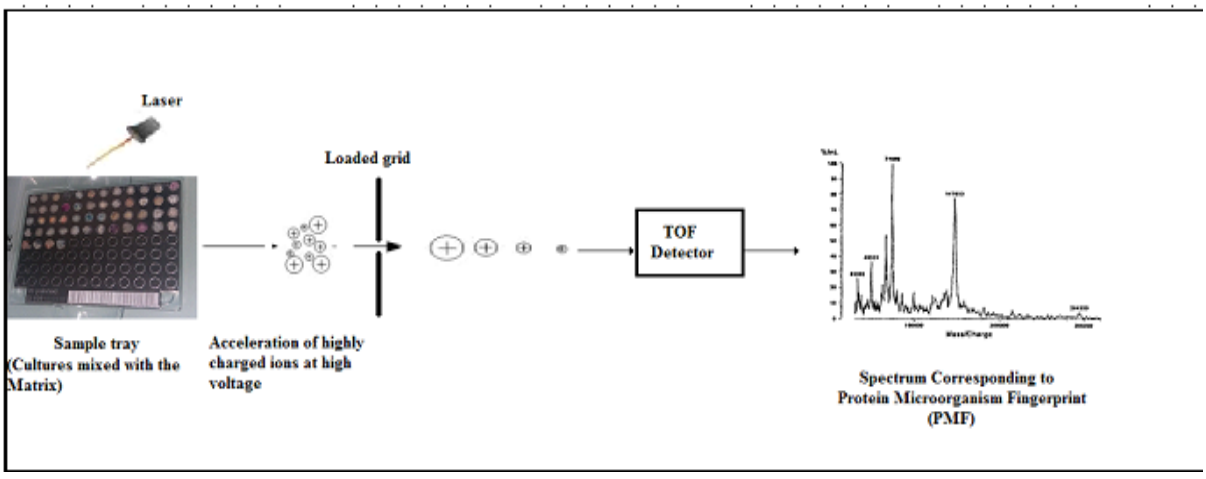


Fig. 2. Diagram showing the principle of MALDI-TOF MS.

According to researches of Reinhard, the rates of benzene's biodegradation through bacteria were negligible (Reinhard *et al.*, 2005), while the experiments of Anderson and Lovely have proved the degradation of benzene within 25 a 35 days (Anderson and Lovley, 2000).

The S1663 strain (*Enterococcus faecalis*) degrades benzene much less rapidly than cyclohexane, as shown by concentrations 0.0964 µg/µl (after 30 days) and 0.0446 µg/µl (after 63 days).

The same behavior is observed with S05 (*Citrobacter freundii*) and S476 (*Citrobacter youngae*), which had found difficulties to degrade benzene, but degrade more readily cyclohexane. Grbic-Gralic and Vogel found that total benzene's biodegradation occurs only in the absence of any other carbon substrate, and that when other monoaromatic hydrocarbons are present with higher amounts than benzene, degradation of benzene did not happen.

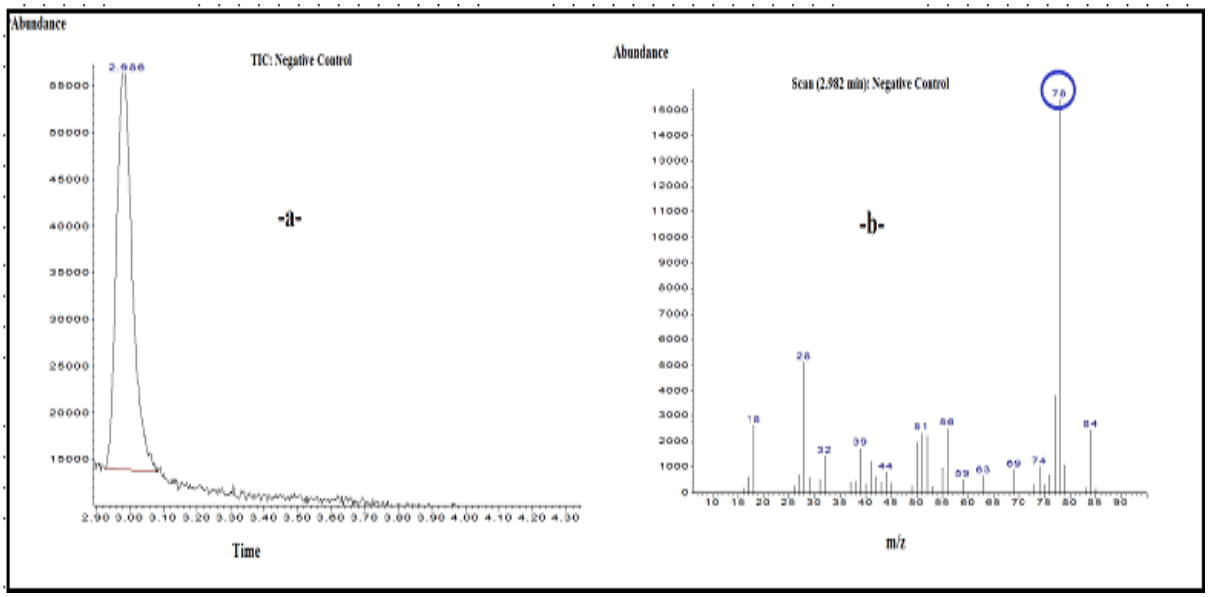


Fig. 3.GC/MS spectrum of negative control at T0: theoretical retention time 2.98 min- m/z =78. The signature m/z of the benzene is surrounded in bleu.-a-Gas Chromatography -b-Mass Spectrometry**.**

This shows that bacteria degrade benzene only as a last resort, because benzene is the most resistant pollutant in almost all field studies, as a carcinogen and therefore the most toxic (Grbic-Gralic and Vogel, 1987).

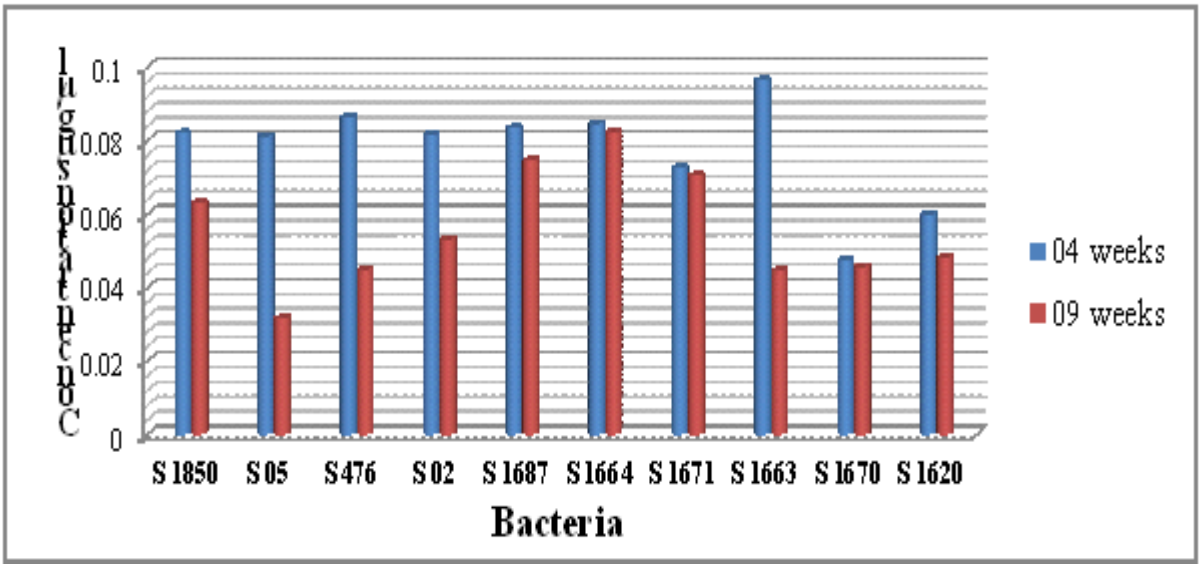


Fig. 4. Kinetics of biodegradation of benzene by the culture of different bacterial species. (S1850: *Pseudomonas aeruginosa*, S05: *Citrobacter freundii*, S476: *Citrobacter youngae*, S02: *Klebsiella oxytoca*, S1687: *Serratia marcescens*, S1664: *Klebsiella pneumonia*, S1671: *Acinetobacter baumannii*, S1663: *Enterococcus faecalis*, S1670: *Acinetobacter baumannii*, S1620: *Klebsiella pneumoniae*).

For the remains of the bacteria of this study, S02 (*Klebsiella oxytoca*), S1687 (*Serratia marcescens*), S1664 (*Klebsiella pneumonia*) and S1850 (*Pseudomonas aeruginosa*) they proceed to a proportionately considerable elimination of cyclohexane, after having completely eliminated the benzene.

Conclusion

This study has allowed evaluating the performance of aerobic degradation of benzene through 10 non-fastidious bacteria in the aqueous phase. The results of the study reveal the absolute elimination of benzene with the appearance of a single derivative cyclohexane in all bacteria. The most important is the decreasing of cyclohexane concentrations as a function of time, without the formation of secondary component. This indicates the reliability of the biological treatment without the necessity of using other types of processes (physic-chemical techniques).

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