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Article *in* Orbital - The Electronic Journal of Chemistry · July 2016

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Orbital: The Electronic Journal of Chemistry
journal homepage: www.orbital.ufms.br
ISSN 1984-6428

| Vol 8 | | No. 4 | | Special Issue July 2016 |

Full Paper

Pesticide Degrading Bacteria in Aquatic Environment: Bioprospecting and Evaluation of Biotechnological Potential

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Article history: Received: 28 May 2015; revised: 11 January 2016; accepted: 27 February 2016. Available online: 07 July 2016.
DOI: http://dx.doi.org/10.17807/orbital.v8i4.748

Abstract: Pesticides play an important role in the increase of productivity in agro-industry and the extensive use of these substances cause environmental, economic and social damage in time. Microbial activity is an essential part in the dynamics and the destination of pesticides in the environment. This research focuses in prospecting and characterizing bacterial strains which are potentially able to degrade/tolerate Atrazine, Chlorpyrifos, Methyl parathion and Picloram. Bacteria were isolated from water samples collected according to the degree of salinity along the Pacotí River's estuary (Ceará), located in the semi-arid region of northeastern Brazil. A total of 49 bacterial strains were isolated, all of which tolerated/ downgraded concentrations up to 200mg/L of picloram, atrazine and methyl parathion. Tested in pesticide mixtures, the percentage and tolerance level showed that 73% grew in concentrations up to 200mg/L, 17,4% tolerated/ downgraded up to 150ml/L and the remainder only grew in concentrations under 100ml/L. The strains which had the best performance against pesticides, by points, were P1 (13Db e 14D); P2 (10E); P3 (2M, 9M, 10M, 12Mb, 14M, 17M 18Mp 19M e 20M). A high percentage of isolates (67%) expressed luminescence when exposed to the pesticides atrazine and methyl parathion in concentrations between 150 and 200ml/L.

Keywords: biodegradation; herbicide; estuary; semi-arid

1. INTRODUCTION

In the last decades, agriculture experienced a remarkable increase in productivity, surpassing the system expansion rates [13]. The use of new technology (irrigation, fertilizers, pesticides and mechanization) is a consequence of our present demands. The great challenge for this sector now is how to combine productivity gains in agriculture with the protection against adverse effects in ecosystems. Pesticides were developed as a tool to minimize losses caused by insects, fungi and invasive plants, among other agents [28]. Although pesticides are efficient in controlling pest proliferation, its unregulated and indiscriminate application might lead to adverse effects for humans, other living beings and ecosystems. The damage level depends on the degree of sensibility of the affected organisms and on the toxicity of the substance [16]. The persistence of the molecule on the environment and its bioaccumulation potential are also decisive [18].

In the environment, pesticide's toxic waste affects ecosystems and contaminate food. Soil is the most affected environmental matrix, and from there pollutants reach superficial waters through leaching and might reach groundwater through percolation. The transport of these substances to sources is also made by wind, especially when the pesticides are pulverized [25].

The behavior and accumulation of pesticides depends on their interaction potential and characteristics, along with a number of variables on the environmental matrices, including their microbiota. Once in the environment, pesticides might be transformed or degraded by microorganisms, individually or in consortia. This potential is the basis of bioremediation, a clean, viable and efficient technology that uses living microorganisms and/or their products to degrade or immobilize pollutants,

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minimizing their impact on the environment.

The degradation processes of pesticide substances in soil is being currently researched [5], but there is still little information about such processes in aquatic systems. Considering the fragility of semi-arid ecosystems, the introduction of pollutants in the water sources brings even more dramatic consequences to the region [14].

Prospecting is the first step for the selection of microorganisms that might be used in bioremediation processes against environmental pollutants. The pooling for applicable microorganisms in biodegradation might be more effective in environments where they have already been exposed to the recalcitrant molecules present in the medium [9].

Since the last decade Brazil has been one of the largest pesticide consumers in the world. There are 398 active ingredients and 1002 formulated products registered in the country, besides non-regulated products that continue to be used by farmers [12]. The Northeastern region ranks fourth in pesticide consumption; its use is concentrated in irrigated areas [3]. The main objective of this work was prospecting, isolating and characterizing bacterial strains able to degrade/tolerate the pesticides atrazine, chlorpyrifos, methyl parathion and picloram in the estuary of a river in the Brazilian northeastern region.

2. MATERIAL AND METHODS

Three water samples were collected, in different times of the day, in three previously chosen locations along the Pacotí River (in Eusébio, Ceará), following a crescent gradation of salinity. The georeferenced points are P1: 4º01'04.39''S; 38º31'05.59''W; P2: 03º50'02.49''S; 38º25'14.93''W; P3: 03º48'53.46''S; 38º24'37.51''W, corresponding to fresh water, brackish water and saline water, respectively (Figure 1).

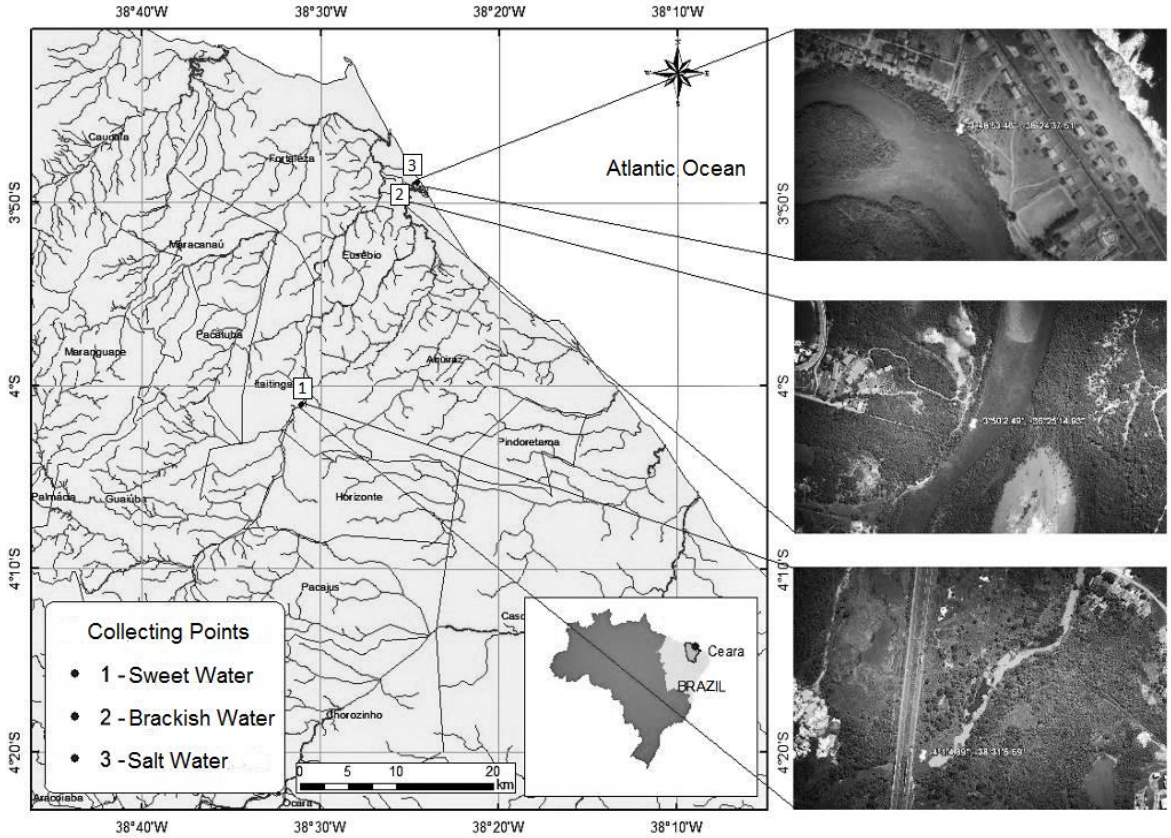

Figure 1. Geographic location of water sampling points along the Pacotí River.

The samples were collected in sterilized amber bottles and transported in isothermal boxes, under refrigeration, to the Laboratory of Environmental and Fishery Microbiology (LAMAP), in the Institute of Sea Science - LABOMAR/UFC, where they were processed and analyzed.

Sample processing and analysis

The water samples were diluted progressively until 10^{-5} using diluents with different NaCl concentrations as to adapt to the original salinity of the sample. For P1 samples: 0,85% NaCl saline solution, for P2 and P3: 1% NaCl saline solution. Inocula from each dilution were put in culture media for differential bacterial growth.

Bacterial quantification and characterization

The cultivable heterotrophic microorganisms, pesticide resistant or degrading, were counted by plating the diluted samples in Plate Count Agar (PCA), plus 50ml/L of a pesticide mixture (atrazine + methyl parathion + picloram + chlorpyrifos), through *Spread Plate* technique [10]. The physicochemical characteristics of the substances are shown on Table 1.

Table 1. Physico-chemical properties of the pesticides used on the research.

Pesticides	Atrazine	Methyl Parathion	Picloram	Chlorpyrifos
Pollutants category	Triazine Herbicide	Organophosphate Insecticide	Pyridinecarboxylic acid Herbicide	Organophosphate Insecticide
Chemical formula	$C_8H_{14}ClN_5$	$C_8H_{10}NO_5PS$	$C_6H_3Cl_3N_2O_2$	$C_9H_{11}Cl_3NO_3PS$
Molecular weight g/mol	215.69	263.21	241.46	350.59
Solubility (mg/L)	33	50	430	1.4
Half-life (days)	25	30	69.8	256
pH	-	-	7	6.7
Temperature (ºC)	25	28	10-30	-
Reference	[11]	[18]	[21]	[27]

Determining tolerance and/or efficiency of degradation

The bacterial strains underwent the efficiency test by Minimal Inhibitory Concentration (MIC). Broth dilution (Macrodilution) was performed using a *Bushell Haas* broth and the pesticides, individually and in mixtures, in concentrations of 50, 100, 150 and 200ml/L [7]. After incubation time (35ºC/48 hours) results were indicated by alterations in the turbidity of the medium, verified in a Micronal B542 spectrophotometer, in 625 mm absorbance.

From the tubes with higher concentration of bacterial growth, portions of 1 mL were extracted and plated using Peptone agar (*pour plate* technique). After inoculation, the plates were incubated for 48 hours in 30ºC. The lack of growth in bacterial colonies in the culture medium determined the minimal bactericidal concentration (MBC) of the tested substance [7].

3. RESULTS AND DISCUSSION

The microbial communities presented a high diversity of metabolic pathways in the environment and were able to transform a large variety of organic chemicals. Compared to chemical methods these microorganisms, which survive in the environment and use pesticides to obtain their energy, seem to be the best choice for bioremediation against those contaminants in the environmental matrices. In the quantitative analysis, the potentially pesticide degrading bacterial populations in the sampled water ranged from 53 x 10^3 to 53 x 10^4 UFC/ mL (P1), from 51 x 10^3 to 16 x 10^4 UFC/ mL (P2) and from 54 x 10^3 to 68 x 10^4 UFC/ mL (P3) (Table 2).

Table 2. Count of potentially pesticide (mix) degrading and/or resistant bacteria in the sampling points in different time intervals.

Time of sampling	Colony forming unity / mL of water		
	Fresh water (P1)	Brackish water (P2)	Saline water (P3)
9:20	84 X 10^3	15 X10^4	54 X10^3
13:20	53 X 10^3	16 X10^4	68 X10^3
17:20	14 X10^4	51 X10^3	54 X10^3

The highest bacterial count was verified in the P2 point and is probably related to a higher concentration of organic matter in this area. Changes in temperature, dissolved organic matter (OM) concentration, pH and salinity are common in estuary zones, and these parameters might affect the dynamics of pesticides. Variation in these factors is determinant to the process of substances' adsorption in aquatic environments [30], favoring the adaptation of microorganisms found in the medium. Estuaries are ideal environments to study the bacterial succession due to their higher stability of benthic organisms, which normally make up the microbiota [32]. The high count of these microorganisms in the water is related to the selective pressure exerted on the microbiota by exposing them to pesticide substances.

The isolation of degrading bacteria grown on selective culture media (60 bacterial strains, 20 from each point) accused the presence of bacteria in the viable but nonculturable (VBNC) state (20%), characterized by a primary growth in the selective cultivation media, but not being able to culture after isolation. The isolates displaying this characteristic came from all samples. Bacteria presenting this condition possess a set of adaptive mechanisms, which allow them to respond metabolically to pressure in the surrounding environment and still survive [8].

Study of bacterial isolates showed that most of them had characteristic cell walls composed of rod shaped gram-negatives (67%). Gram-positive bacteria made up 27% of the isolates. The remainder of the cultures (6%) was of gram-negatives, differently shaped bacteria that did not present individual growth - a feature of mixed cultures.

Bacteria possess characteristics that propitiate their adaptation to varying environmental conditions, such as rapid growth, metabolic versatility, genetic plasticity and fast adaptation to variations in the media [29]. This makes characterization of strains important for understanding the biodegradation of pesticides.

The higher the diversity and functional redundancy of the microbiota, the more resilient the ecosystem. The exposure of microorganisms to recalcitrant molecules, leads to a functional change in the system, associated with the substitution of dominant bacteria for those able to degrade the compounds [17]. In microbial consortia, cometabolism turns the mineralization of these compounds more efficient [20].

Individually, the isolates tolerated/ downgraded concentrations varying from 100 to 200mg/L of pesticide. picloram was the compound showing the most toxic effect on the tested bacteria, while chlorpyrifos was the substance more easily used by the bacterial isolates (Figure 2).

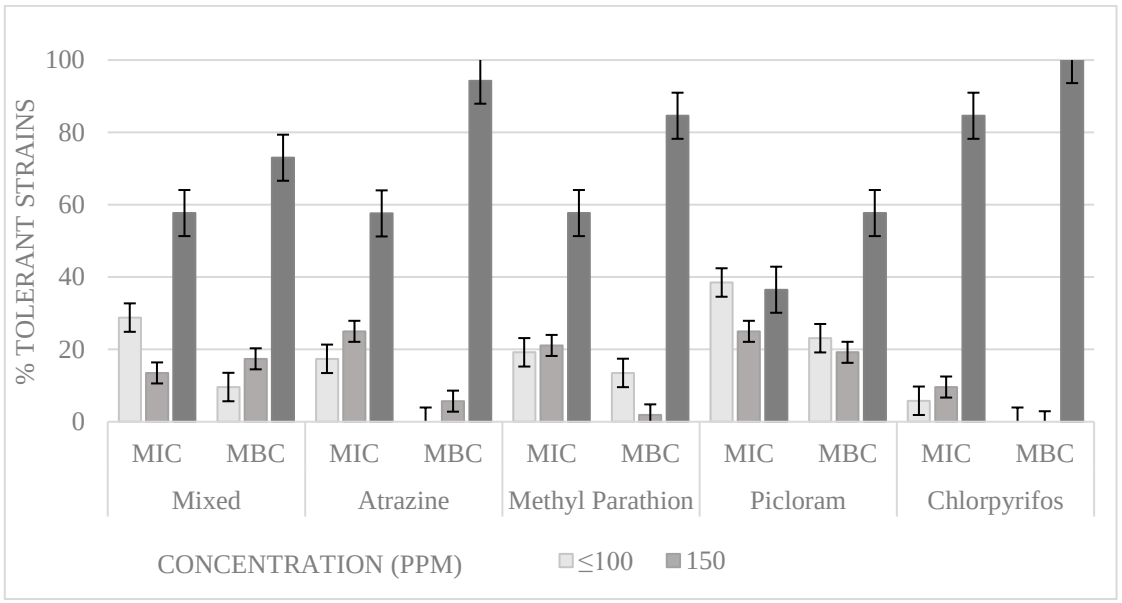

Figure 2. Minimal Inhibitory Concentrations (MIC) and Minimal Bactericidal Concentrations (MBC) of pesticides, individually and in mixture against bacteria isolated from the waters of Pacotí River (Ceará, Brazil).

Most of the bacterial isolates (73%) efficiently utilized the pesticides as an energy source, even in high

concentrations of the tested substances, isolated or in mixture. All strains consumed concentrations up to 200mg/L of chlorpyrifos and 57,7% of the isolates consumed concentrations up to 200mg/L of picloram, atrazine and methyl parathion. When tested in mixture, the percentage and tolerance levels were: 73% was capable of growing in concentrations up to 200mg/L, 17,4% tolerated/ downgraded up to 150mg/L and the remainder only grew in concentrations down from 100mg/L.

Although the degradation of atrazine is considered slow and partial, the microbiota started to degrade this pesticide as a result of repeated exposure. It was initially used as a substrate for growth and later the substance suffered mineralization [31]. Gram-positive bacteria are efficient in the initial phase of atrazine degradation, resulting in cyanuric acid. However, they are not capable of cleaving the triazine ring, which is done by gram-negatives bacteria, suggesting that the mineralization of the compound might be achieved through a bacterial consortium [24]. In a system containing lindane, methyl parathion and carbofuran in mixture, it was possible to reach a microbrial degradation efficiency up to the concentration of 50mg/L using a consortium (composed by three different species) [18]; in the present work, the bacterial isolates from Pacotí River presented a 5-times higher degradation/tolerance potential to M- Parathion.

In soils enriched with Picloram, relatively elevated doses resulted in an extension of the induction process and adaptation of the matrix macrobiota [21]. This might explain the lower relative concentrations (minimal and bactericidal) toxic to our isolates when individually exposed to Picloram. This herbicide, classified as a "restricted-use pesticide" [2], is a refractory compound of the alkylpyridine group, whose recalcitrance is due to the number of substitutes in the pyridine ring, being slowly or incompletely decomposed by microorganisms [19].

Bacterial strains that tolerated/downgraded concentrations of 3200 µg/mL of chlorpyrifos were isolated in the soil of an industrial area in India [11]. This concentration is quite superior to those tested in the present research, nevertheless, all of our isolated were able to withstand the highest tested concentration (200 mg/L). Researches show that bacteria and fungi are able to degrade chlorpyrifos, using it as a direct source of C, N and P. This degradation may also happen as co-metabolism, that is, the transformation of a target compound by microorganisms without the use of its elements as a source of C and energy. The 3,5,6-trichloro-2-pyridinol (TCP) is the main metabolic product detected in the water as a result of chlorpyrifos degradation. TCP is more persistent than the primary compound and that is one of the limiting factors for the mineralization process of chlorpyrifos in aquatic environments [6].

When a mixture of pesticides was used, thirty-three bacteria were able to degrade them, but not all of them could do so individually. Other sixteen strains were able to degrade the pesticides individually, although not in mixtures.

There are numerous studies about biodegradation of individual pesticides in controlled conditions using pure microbial cultures, but information on the dynamic of degradation in compound mixtures is still rare. Once in the environment, many pesticides with different chemical compositions are active pollutants. The development of an efficient biotechnological treatment in environments contaminated by multipesticides depends on the establishment of bio-systems that possess a broad degradation potential [18].

The inability to degrade the mixture by some of the bacteria (26,6%), was possibly due to the lack of an extra nitrogen source. Chrishina and Philip [18] assert that the addition of organic matter, in collaboration to the treatment systems, increases the concentration of bacterial cells, making the degradation of pesticides mixture easier. Other authors [17] determine that the presence of glucose in the culture medium was null, or even retarded the use of the target compounds by the bacteria.

Table 3 shows the individual profiles of the pesticide degrading bacteria. The parameter to measure performance was the intensity of the turbidity in the culture medium used to determine the MIC. The isolates that showed best performance, by points, against pesticides were: P1 (13D e 14D); P2 (19E); P3 (2M, 6M, 7M, 8M, 9M, 12M 13M 14M e 15M). Most of the isolates (67%) expressed luminescence when exposed to atrazine and methyl parathion in concentrations between 150 and 200mg/L.

Although Point 2 presented a higher count of bacteria capable of degrading different mixtures of pesticides, strains isolated from Point 3 were more efficient, because a higher number of isolates was able to use all pesticides (individually and mixed) as the only source of C, and in high concentrations. The results are valuable, but one has to consider that, under

natural conditions, performance is altered by a series of factors, physical, chemical and ecological as, for example, the adsorption potential of the compounds by the organic matter on the environment.

Table 3. Individual profiles of pesticide degradation from bacteria isolated on samples from Pacotí River (Ceará, Brazil).

Source point	Degradation profile	Bacterial isolates
P1	Mix, Atz, Mp, Pcl, Cpf	1D, 5D, 6D, 9D, 12D, 13D,14D
	Mix, Atz, Mp, Cpf	2D, 3D
	Atz, Mp, Cpf	10D
	Atz, Pcl, Cpf	4D, 7D
	Atz, Cpf	8D
	Cpf	11D
P2	Mix, Atz, Mp, Pcl, Cpf	13E, 14E, 18E, 19E
	Atz, Mp, Pcl, Cpf	17E
	Mix, Mp, Cpf	1E, 15E
	Mix, Atz, Cpf	5E
	Mix, Pcl, Cpf,	12E
	Cpf, Mp, Atz	2E, 4E, 6E, 7E, 8E
	Atz, Pcl, Cpf	10E, 11E
	Atz, Cpf	9E
	Mix, Cpf	16E
	Cpf	3E
P3	Mix, Atz, Mp, Pcl, Cpf	1M, 2M, 3M, 4M, 5M, 7M, 8M, 11M, 12M, 13M, 14M, 15M
	Mix, Atz, Mp, Pcl	10M
	Mix, Atz, Mp, Cpf	6M, 9M
	Atz, Mp, Cpf	16M

Mix: pesticide mixture, Atz: Atrazine, Mp: M-parathion; Pcl: Picloram; Cpf: Chlorpyrifos

Adsorbed chemical products become less accessible to the microorganisms and, consequently, limit their degradation potential [24, 26]. This might restrict the adaptation of the macrobiota to adsorbed contaminants in the estuary organic matter.

It was verified that in the presence of pesticides some of the isolates started to present luminescence, which may indicate a process regulated by the mechanism of quorum sensing (environmental stress to which they were exposed) inducted by the molecules of pesticide.

Bioluminescence furnishes an ideal system in which a physical reaction is produced instead of a chemical one, preventing the accumulation of compounds that may lead to toxicity or instability of the medium [22.]. M-parathion and Atrazine (150 and 200 mg/L concentrations) are the pesticides which had most effect on the expression of luminescence among the strains tested. This phenotype was verified among the strains from all water samples, most frequently among bacterial isolates from brackish water samples (P2).

Quorum sensing (or self-induction) as an important evolutionary acquisition, which allows an energy saving as it ensures that the luminescent bacteria do not synthesize their products until they have a sufficient concentration of microbial cells to luminescence is visible [15].

The bioluminescence provides an ideal system Bioreporter reaction that produces a physical rather than chemical and thus avoid the accumulation of compounds that can lead to toxicity or instability [22]. These microorganisms can be used as biondicador environmental contaminants.

Many biosensors were developed for the detection of organophosphate pesticides, they were based on the utilization of alkaline phosphatase, which catalyzes the dephosphorylation of a macrocyclic compound and liberates light [4], showing that the isolated strains have biotechnological potential.

4. CONCLUSION

Bioremediation techniques are a good

alternative in the decontamination of environments polluted by pesticides. However, numerous factors are necessary in order to reach a successful performance of this technology, such as a thorough knowledge of the ecology of degrading microorganisms, as well as the dynamics of such process; also the interaction between substances in the environmental matrix and with environmental matrix. There is some sort of a "pressure" inflicted on the waters from Pacotí River's estuary due to the abundance of the bacterial population able to degrade or tolerate pesticide substances, and such pressure is reflected on their functional diversity.

Salinity is a determining environmental factor in the abundance of bacteriadegrading of pesticides in the Pacotí River estuary environment.

The pesticide compounds act differently on microorganisms when exposed individually or in mixtures. For example, picloram was more toxic when tested alone than in a mixture with other compounds. As for the bacteria, high percentages of the isolates were able to degrade high concentrations of pesticide in mixture, but were not resistant to the compounds tested individually.

In our research, exposure to high concentrations of herbicides m-parathion and atrazine seemed to set off autoinduction of luminescence expression by the bacteria. This is a relevant fact for prospecting bioindicators of pesticide presence in environment. A deeper and more specific research is needed to further understand the mechanism.

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