RESEARCH

Biodegradation in vitro *of diesel bilge waters using a microbial native consortium isolated from Córdoba, Colombia*

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ABSTRACT

The aim of this investigation was to evaluate the biodegradation of diesel bilge water using a microbial consortium native to the region of Cordoba, Colombia. Bacteria were isolated from a natural petroleum source, using mineral minimal (0.5 g/L KH_2PO_4; 1.4 g/L Na_2HPO_4; 0.6 g/L NH_4NO_3; 0.1 $MgSO_4 \cdot 7H_2O$; 0.02 g/L $CaCl_2 \cdot 2H_2O$; 0.03 g/L $MnSO_4 \cdot H_2O$; pH adjusted to 7.0) under conditions of bacterial growth (pH 7.0, 28 ± 2° C and shaking at 150 rpm). Microorganisms were tested for contaminant tolerance using different concentration of diesel bilge water: 3.0, 5.0, 7.5, 10.0, 12.5 and 15 %. Subsequently, a bioassay was performed in microcosms for 28 days to assess the biodegradation of diesel linters water to the highest concentration water tolerance found, *i.e.*, 7.5 %. The bacterial consortium was grown up to 10^6 c.f.u./mL, the ratio of carbon/nitrogen adjusted to 100/5 and the biodegradation products were determined by gas chromatography, at the beginning and the end of the assay. There was found that some components were completely degraded (1-Himidazol-4-carbazamida), others around 70 % (1, 2, 3-triazol 4-carbohidrazida) and the rest at smaller rates. Among the species isolated with standard procedures and identified with the API 20 NE and API 20 E commercial kits were: *Achromobacter denitrificans*, *Sphingomonas paucimobilis* and *Pseudomonas putida*. *Rhizobium radiobacter* was also reported, as a new contribution to the list of bacterial species displaying biorremediation capacity.

Keywords: bioremediation, biodegradation, microbial consortium, bilge diesel, oil service station

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RESUMEN

Biodegradación *in vitro* **de aguas borras de diesel mediante el uso de un consorcio microbiano nativo aislado en Córdoba, Colombia.** El objetivo de la presente investigación fue evaluar la biodegradación de los componentes de aguas borras de diesel, utilizando un consorcio microbiano nativo de la región de Córdoba, Colombia . Se aislaron bacterias a partir de un pozo de petróleo natural con el uso de medio mínimo mineral (KH_2PO_4 0.5 g/L; Na_2HPO_4 1.4 g/L; NH_4NO_3 0.6 g/L; $MgSO_4 \cdot 7H_2O$ 0.1 g/L; $CaCl_2 \cdot 2H_2O$ 0.02 g/L; $MnSO_4 \cdot H_2O$ 0.03 g/L) y bajo condiciones de crecimiento bacteriano (pH 7.0, 28 ± 2° C y agitación a 150 rpm). Los microorganismos fueron sometidos a prueba de tolerancia a diferentes concentraciones de agua de borras: 3.0; 5.0; 7.5; 10.0; 12.5 y 15 % v/v. la mayor tolerancia de los microorganismos se encontró a la concentración de 7.5 % de agua de borras. Posteriormente se realizó un bioensayo en microcosmos durante 28 días, para evaluar la biodegradación de aguas borras a la concentración de mayor tolerancia. El consorcio bacteriano se creció hasta 10^6 u.f.c./mL, se ajustó la relación de carbono/nitrógeno (100/5) y se determinó la biodegradación de los compuestos por cromatografía de gases al inicio y al final del bioensayo. Algunos componentes se degradaron completamente (*e.g.*, 1-H imidazol-4-carbazamida), otros alrededor del 70 % (*e.g.*, 1, 2, 3-triazol- 4-carbohidrazida) y la gran mayoría en porcentajes menores. Dentro de las especies aisladas e identificadas con los kit API 20 NE y API 20 E se encontraron *Achromobacter denitrificans*, *Sphingomonas paucimobilis* y *Pseudomonas putida*, y de forma novedosa *Rhizobium radiobacter*.

Palabras clave: bioremediación, biodegradación, consorcio microbiano, sentina de diesel, estaciones de servicios

*I*ntroduction

Diesel, one of the main products derived from oil refining, is a complex mix of paraffins, olefins and aromatic hydrocarbons, among other compounds. It also contains small amounts of substances such as sulfides, nitrogen, metals and oxygen [1]. Common maintenance operations carried out in diesel storage tanks at service stations generate significant amounts of aqueous residues named diesel linters waters or diesel bilge waters, which are dangerous wastes causing environmental pollution in terrestrial and water ecosystems [2].

Diesel bilge waters have been reported as composed of an aliphatic fraction of n-alkanes, from the homologous series of C_9 to C_{27}, isoprenoid pristane and phytane; a fraction of a complex mix of branch-aliphatic and cyclic hydrocarbons and aromatic hydrocarbons, which are very resistant to biodegradation, and polycyclic aromatic hydrocarbons comprising acenaphthylene, fluorene, phenanthrene, 3-methyl phenanthrene, 2-methyl phenanthrene, 9-methyl phenanthrene, 1-methyl phenanthrene, 2,7-dimethyl phenanthrene, and trace amounts of anthracene, fluoranthene and pyrene [3]. All these compounds are significant sources of severe environmental damage.

The need for eliminating these pollutants has fostered the emergence of bioremediation strategies for waste biodegradation, which have been regarded as effective, safe and economically affordable [1]. In this sense, bioremediation presents itself as a feasible al-

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ternative, by using certain microbial species to transform or degrade compounds of complex chemical structure into simpler ones, in certain cases achieving a full biodegradation cycles down to carbon and water [5, 6]. Several bacteria genera has been identified of having great potential for biodegradation: *Pseudomonas* spp., *Alcaligenes* spp., *Bacillus* spp., *Mycobacterium* spp., *Rhodococcus* spp., *Corynebacterium* spp., *Moraxella* spp., *Micromonospora* spp., *Achromobacter* spp., *Cupriavidus* spp., *Rhodanobacter* spp., among others [5-7]. As a rule, bacterial communities tend to degrade the aliphatic hydrocarbons better than the aromatic ones [9].

Moreover, bioremediation research has brought positive results in the recovery of oil-contaminated soils, by accelerating the natural biodegradation processes [9, 10]. Several studies have evidenced that 26-61 % of the oil can be removed from soil by using native bacteria [9]. Similarly, 96 % of total oil hydrocarbons were successfully removed [11], as well as 89 % of polycyclic aromatic hydrocarbons [12].

Therefore, bioremediation can be considered an environmental preservation strategy, since it helps on minimizing the environmental impact of oils and its derivatives. This has led to its inclusion in the strategies for sustainable development, as part of action plans for environmental preservation programs, to comply with legal requirements such as the Decree Law 3930 of 2010 in Colombia [13].

However, the adaptation of the native microbial community to the ecosystem subjected to decontamination, throughout its resistance to the toxic environment of the oil residues, is essential to maximize its biodegradation capacity. Hence, this work was aimed to evaluate the capacity of a native microbial consortium isolated from the soil of an oil well in Montería city, Colombia, for the biodegradation of oil residues in diesel linters waters.

Materials and methods

Microbial consortium

The microorganisms used were isolated from the soil of an oil well located in San Sebastián, at Lorica municipality in Cordoba, in the northern region of Colombia. Several samples were taken at a 20-cm depth and further quartered to select a representative sample. Microbial populations were isolated in 250-mL flasks by adding 10 g of the sample and 10 mL of mineral oil (0.5 g/L KH_2PO_4; 1.4 g/L Na_2HPO_4; 0.6 g/L NH_4NO_3; 0.1 $MgSO_4 \cdot 7H_2O$; 0.02 g/L $CaCl_2 \cdot 2H_2O$; 0.03 g/L $MnSO_4 \cdot H_2O$; pH adjusted to 7.0). The system was hermetically sealed to avoid the loss of volatile hydrocarbons and was incubated under constant agitation of 150 rpm, at 28 ± 2° C for 96 h [1]. The microbial consortium was multiplied up to 10^6 cfu/mL, and used for subsequent tests.

Identification of the native microbial consortium

Consortium microorganisms were identified by macroscopic and microscopic observations, the last also using specific staining and biochemical tests as specified in the Bergey's manual [14]. The commercial kits API 20 NE and API 20 E (Biomerieux, Lyon, France) were also used (to identify gram-negative bacillus, either oxidase positive or negative, respectively.

Microbial tolerance test to diesel bilge waters

Different concentrations of diesel bilge water were tested: 1.0, 3.0, 5.0, 7.5, 10.0, 12.5 and 15 % v/v. Briefly, 200-mL flasks were prepared, containing 100 mL of mineral medium at each contaminant concentration and 10 mL of microbial consortium inoculums (10^6 c.f.u./mL). Flasks were incubated under constant agitation at 150 rpm, at , pH 7.0 and 28 ± 2 ºC, for 96 h [1]. The assay was run in triplicate.

Microcosms preparation

Considering the highest linters water concentration tolerated by the microbial consortium, microcosms were prepared by adding bilge water and mineral medium as previously described. The nitrogen concentration was adjusted at 100:5 C:N ratio, this parameter established at the highest tolerated concentration of the contaminant, and under the abovementioned operational parameters. The pH, total carbon, total phosphorous and total nitrogen contents were determined following procedures SM 4500-H^+, SM 4500-P B.4, 4500-P E and SM 4500-N_{org}B, and 4500-NH_3 C of the Standard Methods for the Examination of Water and Wastewater [15].

Bilge water degradation assessment

Microcosms samples were collected and preserved with hydrochloric acid at pH ≤ 2 and stored in a cold room (4 ± 2 ºC) until use. The assay was run by extracting the hydrocarbon compounds with hexane and further assessing their concentration by gas chromatography coupled to mass spectrometry. For this purpose, a DB-TPH column, 30 m × 0.32 mm in diameter, 0.25 µm packing film thickness (Part 123-1632; Agilent) was used. The injector and the detector were kept at 250 ºC and 340 ºC, respectively. Hydrogen was used as mobile phase, at a constant flow rate of 2 mL/min. The percentage of degradation was assessed for each experimental sample at the end of the operation process (28 days, approximately). Assays were run in triplicate.

Statistical analysis

Variance and regression analyses were run to establish the statistically significant differences between total hydrocarbon biodegradation conditions and the different concentrations of diesel bilge water.

Results and discussion

Properties of the native microbial consortium

In this work, the isolated microorganisms corresponded to bacilli and cocci. Macroscopically, bacilli formed colonies of creamy consistency and beige, yellow or coffee-like in color. Cocci varied in consistency and were yellow-colored. Microscopically, there were Gram-positive and Gram-negative bacilli and Gram-positive cocci.

Identification of the native microbial consortium

The bacterial species were identified by procedures as described in the Bergey's manual and with the aid of the API 20 NE and E kits for species identification (specificity percentage): *Achromobacter denitrificans* (98.8 %), *Sphingomonas paucimobilis* (99.7 %),

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Pseudomonas putida (97 %), *Brevundimonas vesicularis* (97.5 %), *Acinetobacter baumanii* (97.8 %), *Rhizobium radiobacter* (98.5 %), *Comamonas testosteroni* (98.5 %) and *Chryseobacterium indologenes* (98.3 %).

As far as we know, except for *Rhizobium radiobacter*, the other bacterial species found were previously reported as biodegrading hydrocarbons. In fact, they were originally isolated from soils contaminated with polycyclic aromatic hydrocarbons, and its hydrophobicity and adhesion were evaluated as measures of the adherence to *n*-octanes and their retention in sandy soils [5].

Among *Rhizobium* spp., *Rhizobium tropici* is a remarkable example, being used for degrading polycyclic aromatic hydrocarbons [16]. *Pseudomonas* spp. are ubiquitous gram-negative bacteria, belonging of the gamma subclass of proteobacteria [17], which have been found in sites contaminated with oil- and oil-derivatives sites and displaying high metabolic activities for hydrocarbons. Moreover, they produce biosurfactants such as rhamnolipids, which are effectively involved in the removal of oils and their related products [18, 19]

Another species identified belong to the *Acinetobacter genus* of gram-negative bacilli, including environmentally relevant species, such as: *A. baumanii*, *A. calcoaceticus* and *A. baumanii*, which efficiently remove alkanes fractions [20].

In the case of the *Sphingomonas* genus, they are bacilliform gram-negative bacteria, some of them used for bioremediation, as the case of *S. yanoikuyae* and *S. paucimobilis* which efficiently degrade polycyclic aromatic hydrocarbons as sole carbon and energy source, and display catechol 2,3-dioxygenase, phenanthrene and anthracene metabolic activity [21].

And lastly, *C. testoteroni* was isolated and identified, a soil bacterium metabolizing 3,4-benzopyrene as sole carbon and energy source, but not salicylate [19].

In summary, it is evident that bacteria comprise a significant microbial group for bioremediation, with a wide spectrum of genera and species of versatile metabolic capacities [5]. Bioremediation processes performed under controlled conditions effectively reduce contaminants loads. It is necessary to consider other environmental and soil-related parameters, such as the amount and availability of nutrients, the composition of the native microbiota, temperature and pH, among others [22, 23]

Contaminants tolerance test

Data obtained from tolerance tests at different contaminant concentrations are shown in table 1, as mean values of the microbial population resistant to bilge water. The highest microbial population with the highest tolerance was found at 7.5 % v/v contaminant concentration, in spite of similar microorganisms growth values in the 3-10 % v/v concentration range.

Tolerance testing is relevant to identify which microorganisms are able to adapt to the toxic contaminant environment, enough to exert their biodegrading activity. Previous research indicates that optimal degradation rates are determined by the type of microorganism used, its enzymatic factors as well as its intrinsic degradation capacity [5].

Table 1. Tolerance to diesel bilge water contaminants of bacterial populations isolated from contaminated soils of an oil well in Colombia*

Bilge water concentration (% v/v)	Microbial population (c.f.u./mL)
1.0	1.4×10^7
3.0	1.8×10^8
5.0	2.5×10^8
7.5[a]	9.7×10^8
10.0	3.9×10^8
12.5	3.0×10^6
15.0	6.0×10^5

* Values are reported as the mean tolerance to diesel bilge water contaminants concentrations (% v/v) in mineral oil. See text for composition details. Tests were run in triplicates.
[a] Major bacterial population.

Assessment of biodegradation of diesel bilge waters

The highest degradation of diesel bilge waters was obtained at 7.5 % v/v as shown in table 2, thus, corroborating the selection criterion used based on microbial tolerance. Previous research showed that microbial tolerance to the contaminant is determinant for the efficiency of the biodegradation process [17]. The variance analysis of our results from total hydrocarbon degradation in diesel bilge water demonstrated that contaminant concentration significantly affected that process ($p < 0.05$). The lowest biodegradation percentage was achieved at 10 and 12 % v/v of bilge water, suggesting that total hydrocarbons content at those levels of the contaminant could be toxic for the microbial consortium. These findings are in agreement with other research indicating that the degradation capacity of the bacterial community is affected by high levels of contaminants. Toxicity factors and type of compounds present were associated to the suppression of genes responsible for the enzymatic activity mediating the metabolic processing of contaminants and their intermediary metabolites [1, 13, 24].

Table 3 shows the hydrocarbon compounds being highly degraded by the microbial consortium for 28 days, presented as the area under the curve in gas chromatography experiments. The native microbial population differentially degraded some compounds, with complete removal of 1H-imidazole-4-carboxamide, 2 hexanedioic acid and benzoic acid and the rest of compounds being degraded at approximately 70 %. Total hydrocarbons gas chromatograms are shown in the figure, showing showing the components that

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Table 2. Degradation of diesel bilge water contaminants by bacterial populations isolated from contaminated soils of an oil well in Colombia*

Experiment	Bilge water concentration (% v/v)	Components concentration (mg/L) Start (C_S)	End (C_E)	Biodegradation (%)
1	1.0	1889	1337	29.22
2	3.0	3150	2140	32.06
3	5.0	4956	1898	61.70
4	7.5	7710	610	92.09
5	10.0	10185	4258	58.19
6	12.5	13011	9223	29.11

* Highest degradation percentage. Biodegradation was calculated by the formula: Biodegradation (%) = $(C_S - C_E)/C_S \times 100$.

change by the biodegradation activity of the microbial consortium at ths start and the end of the assay.

The bilge water components are varied and degraded at differential rates. Furthermore, hydrocarbons removal could be also influenced and improved by the synergic interaction among the microbial species found in the native microbial consortium. Such interactions has been reported, either by one species removing the toxic metabolites that activates the biodegradation process by the decontamination-relevant microbial species or by the sequential cooperative degradation of contaminants and its intermediaries by two distinct bacterial populations [24].

Experiments for diesel removal by using a mineral medium at 1 % (v/v) and processing for 50 days achieved 64.1 % of maximum degradation values for an *Acromobacter anthropi* isolate and 90 % for a microbial consortium [25]. Minimal values of 20 % total hydrocarbons removal were attained after 50 days for *Pseudomonas fluorescens*. Similarly, 57 to 65 % total hydrocarbon biodegradation of 1 % v/v diesel bilges and linters water was reported [26], and in a batch-operated reactor in four successive cycles (72 h/cycle) for bilge water at 10 % v/v achieving 53.3, 96.2, 76.2 and 75 % degradation, respectively [27].

Other studies have focused on the capacity of a bacterial consortium formed by *Stenotrophomonas acidaminiphila*, *Bacillus megaterium*, *Bacillis cibi*, *Bacillus cereus* and *Pseudomonas aeruginosa* to biodegrade aliphatic and aromatic hydrocarbons generated by the petrochemical industry. This bacterial consortium demonstrated an excelent degradation capacity for 40 days, reducing in 90.7 and 51.8 % the aliphatic and aromatic hydrocarbons fractions, respectively [18].

The pH in diesel linters or bilge waters treated with autochthonous microorganisms have seen to decrease throughout the biodegradation process, also accompanied by the increase in turbidity and the dispersion of the oily phase. Such changes are normally attributed to the production of biosurfactants, bioemulsifiers, or both by the microorganisms implicated. Some biosurfactants display specific performances, mostly influence by pH [5]. Moreover, total hydrocarbons in bilge waters were biodegraded up to 57-65 % after six-day incubation by using a microbial consortium secreting a bioemulsifier [26].

Particularly, microorganisms isolated from soils contaminated with oil for long periods could be used to treat the diesel bilge waters generated in service stations' storage tanks. The efficiency for biodegrading the total hydrocarbons fraction in this type of contaminant depends mostly on the bioaugmentation processes (with native microbial consortia which have been previously tolerated such contaminant concentrations), and biostimulation (with nutrients to support the microbial metabolic requirements for biodegradation). Moreover, studies indicate that microorganisms adapt to and degrade contaminants based on two main mechanisms: cometabolism and cooperation [5, 28].

The native microbiota isolated from oil well soils display distinct degradation activity on diesel bilge water.

Table 3. Gas chromatography determination of total hydrocarbons in diesel bilge waters by a microbial consortium isolated from an oil well soil in Cordoba, Colombia*

Bilge water components	Components concentration (mg/L) Start	End	Biodegradation (%)
1, 2, 3 triazol - 4 carbohydrazide	8.19	2.46	69.96
Diethyl phtalate	14.05	3.99	71.60
Dibutyl phtalate	18.36	4.97	72.93
5 bromo - 8 (4- hydroxybenzylidene)	9.77	3.08	68.47
Benzoic acid	8.18	0	100
1H-imidazole-4-carboxamide	6.42	0	100
1, 2 benzenedicarboxylic acid	16.90	5.08	69.94
2 hexanedioic acid	3.13	0	100
Phthalic acid	3.49	1.03	70.49

* Results from a biodegradation at start and end of assay. The experiment was run by incubating for 28 days the microbial consortium (10^6 c.f.u./mL) in flasks containing mineral minimal medium plus diesel bilge water at 7.5 % v/v under constant agitation of 150 rpm, at 28 ± 2 °C, pH 7.0. The carbon/nitrogen ratio was adjusted to 100/5, as determined for the optimal activity of the consortium at the contaminant concentration used. Results are presented as the area under the curve (cm^2) from gas chromatograms.

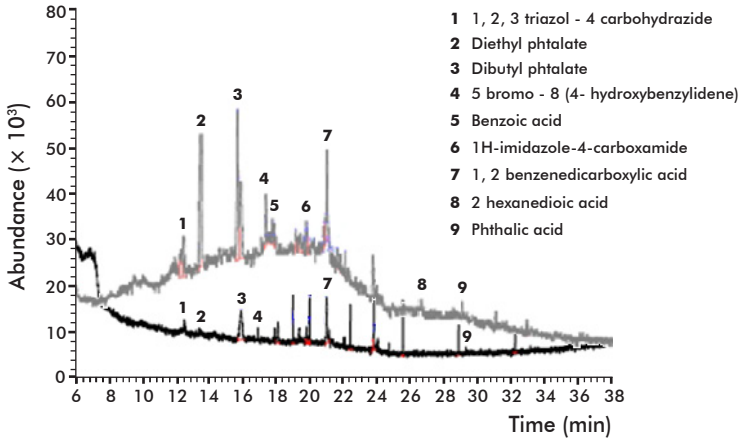

Figure. Comparative analysis of diesel bilge water components before and after the biodegradation process with a native bacterial consortium isolated from Cordoba, Colombia. The assay was run by extracting the hydrocarbon compounds with hexane and further assessing their concentration by gas chromatography coupled to mass spectrometry for compound characterization. Chromatograms of a representative sample are shown, as determined at the start (gray) and the end (black) of the assay. Compounds 5, 6 and 8 were completely biodegraded.

The number of compounds normally found, together with their complexity and heterogeneity, are factors undermining the efficiency of the biodegradation process. High weight hydrocarbons, highly branched or carrying numerous aromatic rings are very difficult to degrade, by interfering with the successful start of the enzymatic degradation process by the microbial community.

Among oil derivatives, polycyclic aromatic hydrocarbons are the most toxic and resistant to traditional bioremediation processes. Conversely, the structurally simpler ones are easy to degrade. Either the case, those oil derivatives actually degraded by native bacteria comprise some molecules carrying a benzene ring. Those compounds can be further decomposed down to benzene, which is largely recognized as a potent carcinogen and harmful for human and animal health. This makes of bioremediation bacteria a remarkable tool to face such a threat, particularly native bacterial strains with biodegradation potential for environmental preservation and restoration approaches.

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Conclusions

The isolated microbial consortium was able to degrade the diesel bilge waters components found in the soil of an oil well of a service station from Córdoba, Colombia. It comprised bacterial species reported with biodegrading activity for oil and its derivatives, except for *Rhizobium radiobacter*. This last is a new contribution to the list of bacterial species displaying biorremediation capacity for diesel bilge water decontamination. The bacterial consortium completely removed the 1H-imidazole-4-carboxamide, 2 hexanedioic acid and benzoic acid present in the bilge water.

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