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Qualitative and Quantitative Assessment of a Surfactin *biosurfactant* in the Bioaugmentation of Crude-oil Contaminated Soil in Garages in the Republic of Congo

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Abstract The objective of this research was to perform a survey of different garages in seven (7) districts of the Republic of Congo and to further study the influence of biosurfactant produced by isolate M28 (*Bacillus subtilis* based on *fib-E* gene identification and RNA 16S gene sequences) and a consortium of selected bacteria on soil remediation. A total of 140 garages were found in Brazzaville. The evaluated soil samples showed that total petroleum hydrocarbons (TPH) content varied from 165 to 206 g/kg of soil. Unfortunately, in the Republic of Congo, the regulatory intervention values for TPH have yet to be determined. Four experiments were conducted in order to improve bioaugmentation. First experiment: wild contaminated soil and bacteria: The second experiment included wild contaminated soil, bacteria, and a biosurfactant; the third experiment included wild contaminated soil, a consortium, and a biosurfactant. Surfactin, a biorsurfactant produced by M28, was used to enhance bioaugmentation of wild contaminated soil from a garage from Bacongo.

Keywords: *Bacillus, bioaugmentation, biosurfactants, garages*

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1. Introduction

The release of contaminants such as hydrocarbons and their by-products into the environment is one of the main causes of global pollution. Anthropogenic activities are related to the current increase in industrialization and civilization. The main sources include volcanic eruptions, leaking underground storage tanks, gas station services, wastes from chemical and petrochemical industries, vehicular exhausts, garages, etc. One of the most serious problems in Brazzaville resulting from garage activities is the inadequate handling of petroleum waste from vehicle exhausts. Studies leading to higher degradation rates of these potential contaminants are of interest, especially so when considering pollution risks to people living within the boundaries.

These garages generate a million tons of waste engine oil, which is then carelessly dumped into the environment [1]. Up to this date, no study has been conducted in the Republic of Congo to evaluate the impact of garages on the environment based on their working conditions. Little information is known about the health hazards associated with time spent in garages. Conditions of occupational and user exposure were evaluated during the survey. It is important to note that, unfortunately, in the Republic of Congo the regulatory intervention values for total petroleum hydrocarbon (TPH) is yet to be determined. The oily wastes are often expensive to destroy, and contaminated areas required expensive remediation processes to reduce the dispersion of contaminants. Physical, chemical and biological processes are employed for remediation. Physical and chemical methods are neither environment friendly nor cost-effective. In past years, bioremediation, a biological method, has emerged as an effective and environmentally approachable treatment for contaminated soils. Most of those compounds are removed from the environment through the consumption by microbes. The release of biosurfactants is one of the strategies used by microorganisms to influence the uptake of PAHs and

hydrophobic compounds in general [2]. Surfactants of biological or chemical origins have been used to improve bioavailability of water-immiscible compounds.

Among the surfactants that can enhance hydrocarbon degradation are lipopeptides and lipoproteins, glycolipids, phospholipids and polymeric surfactants [3]. Studies have shown the effectiveness of these surfactants in liberating hydrocarbons from oily surfaces [4]. *Bacillus* strains are important sources for the production of lipopeptides [5]. Biosurfactants are amphiphilic compounds that have both hydrophobic and hydrophilic moieties in their chemical structure and can effectively reduce both intersurface and surface tensions and are highly useful in emulsification processes. Biosurfactants form micelles to enhance the process of hydrocarbon emulsification in the aqueous phase and critically enhance their bioavailability for microbial utilization and biodegradation.

Enhanced bioremediation aims to stimulate the rate of this process with two complementary approaches: bioaugmentation and biostimulation. Bioaugmentation is the introduction of exogenous specific competent strains or consortia to contaminated sites to boost the biodegradation rates, while in biostimulation, the growth of indigenous hydrocarbon degraders is stimulated by the addition of appropriate conditions, nutrients, or other growth-limiting factors.

The main aim of this study was to determine the biodegradable capacity of biosurfactant-producing isolate **M28** on contaminated soil. In order to achieve this aim, objectives include: 1. generating a map of all garages in 7 districts (Bacongo, Makélékélé, Poto-Poto, Moungali, Ouenzé, Talangai, and Mfilou) of the Republic of Congo; 2. Determination of physical and chemical parameters of soil samples coming from some of the garages mentioned above; and 3. assessment of the biosurfactant produced by isolate M28's potential for bioaugmentation of contaminated soils.

Although the bioaugmentation experiments were not conducted on all soil samples, this study is very important as it may help to predict biodegradation in garages where similar conditions are observed.

2. Material and Methods

2.1. Garages Survey

Garages were surveyed in 7 districts of the Republic of Congo. Sites were located using GPS coordinates. The survey was conducted in September 2020. In this survey, certain working conditions were also determined, including: whether or not there is a fence, whether or not there is a dwelling, the exterior floor covering, the type of pollutants used, the oil-derived waste recovery system, and the life-time of the garage.

2.2. Soil Sampling

Considering the number of garages surveyed, physical parameters as well as total petroleum hydrocarbon (TPH) were calculated from one garage of each district [6]. Garages selected were located at Bacongo (4°30’10’71.3”S 15°’24’94.7’’ E) Makélékélé (4°27’97.2’’S 15°23’91.8 E) Poto-poto (4°24’14.1’’S 15°26’89.3 E) Moungali (4°25’85.2 S, 15°28’83.4)E Ouenze (4°24’01.3 S, 15°28’06.6 E) Talangai (4°21’12.3 S 15°29’12.1 E) Mfilou (4°24’65.2S, 15°29’24.2 S).

2.3. Physicochemical Parameters of Soil Samples

Apart from the TPH measured, the pH is determined according to the standard (ISO-10390, 2005) by measuring a suspension of soil in distilled water according to the ratio of 1/5 (w/v). The pH is measured with a water quality meter (EZ9908) type pH meter. The electrical conductivity is determined according to the standard (ISO-11265, 1994) or NF X 31-113 by measuring a suspension of soil in distilled water according to the ratio of 1/5 (w/v). The CE is determined according to the standard (ISO-11265, 1994) or NF X 31-113 by measuring a suspension of soil in distilled water according to the ratio of 1/5 (mass/volume).

2.4. Microbiological Assay

Isolates M28, M29, ST70, and ST55 used in this study were previously characterised [7]. These bacterial isolates are Gram positive, spore-forming bacteria, catalase +, oxidase +, and able to swarm. Enzymatic activities were tested in the previous study.

2.5. Production of Biosurfactant-like Molecules

Two tests, including the emulsification index and the oil displacement method used for the screening of biosurfactant producers, showed the screened *Bacillus* species as effective biosurfactant producers. Considering the extracellular selection and aiming to confirm the isolates’ potential to produce biosurfactants, they were cultured again. The cell-free supernatant was used to determine E24. The biosurfactant extraction was performed as described by [7] with a few changes as sucrose 10g.l^{-1} was added to the production medium. Once obtained, the biosurfactant was weighed and a partial characterisation was conducted.

2.6. Genomic DNA Extraction

Bacterial genomic DNA of isolates was extracted with the NucleoSpin Microbial DNA (Macherey-NAGEL) kit according to the manufacturer’s instructions. Isolates were grown in 5 mL Luria Broth for 24h at 37°C. The culture was centrifuged, and the cell pellet was resuspended in 120 µl buffer (10 mM Tris, pH 7.5, 1 mM EDTA) before the addition of lysozyme at 37°C for 1 h. Then, 80 µl 10% sodiumdodecyl sulfate was added, and the sample was incubated at 50°C for 1h and then extracted with the same kit. PCR products mixed with loading buffer were subjected to electrophoresis on a 1% agarose gel (w/v). The 10 kb 2-Log (BIOKE) was used as a molecular weight marker.

2.7. Detection of Surfactin Genes

The PCR amplification were performed using primers of a gene chosen from the literature [8] and enlisted in Table 1. The PCR master mix (25 µL) consisted of deoxynucleoside triphosphate (0.2 mM), primer (1.32 µM), DNA polymerase (0.5 U), buffer (5 µL), and 5uL DNA template.

The following cycle conditions were used: initial activation at 95°C for 4 min; 35 cycles of 94°C for 1 min, followed by annealing for 30 s at different temperatures depending on the primers used, and extension step of 1 min at 70°C, and final extension step of 5 min at 70°C. The experiment included negative control-mixture without added DNA. A total of 2 of each amplification reaction was analysed by electrophoresis using a 1% agarose gel followed by ethidium bromide staining and ultraviolet visualization.

Table 1. The primers used for screening of involved in Surfactin biosynthesis by PCR method [8]

Gene	Sequence	PCR product size
sfp	F-5'ATGAAGATTTACGGAATTTA 3' R- 5' TTATAAAAGCTCTTCGTACG 3'	675

2.8. Bioaugmentation by *Bacillus* Isolates

Based on the pollution level of the garage, the wild contaminated soil used in this study was obtained in Bacongo (4° 17′ 14.3″ S15° 15′ 33.7″ E).The experiment was conducted as described by [7] with a few modifications. Here, four tests were designed as follows: The first test consisted of testing the ability of isolate M28 to degrade hydrocarbons of a wild contaminated soil; the second one was to test the ability of the isolate M28 supplemented with the biosurfactant-like molecule produced by the same isolate to degrade hydrocarbons; and lastly, to verify the effect of the biosurfactant and the consortia (M28, M29, ST70, and ST55) on the biodegradation of hydrocarbons. Physical parameter changes (pH, conductivity, and TPH content) were also determined during a period of 30 days.

2.9. Statistical Analysis

Statistical analysis results are presented as mean value ± standard deviation (SD). GraphPad Prism 7 was used for data analysis.

3. Results and Discussion

3.1. Survey of Garages

To our knowledge, this is the first study conducted in the Republic of Congo to determine the number of garages in Brazzaville, the working conditions, and the potential health risks that these garages pose to people living in the surrounding area. A total of 140 garages were enumerated in Brazzaville (Figure 1). Of the 140 garages, only 50 are registered with the ministry of commerce, while the others work illegally.

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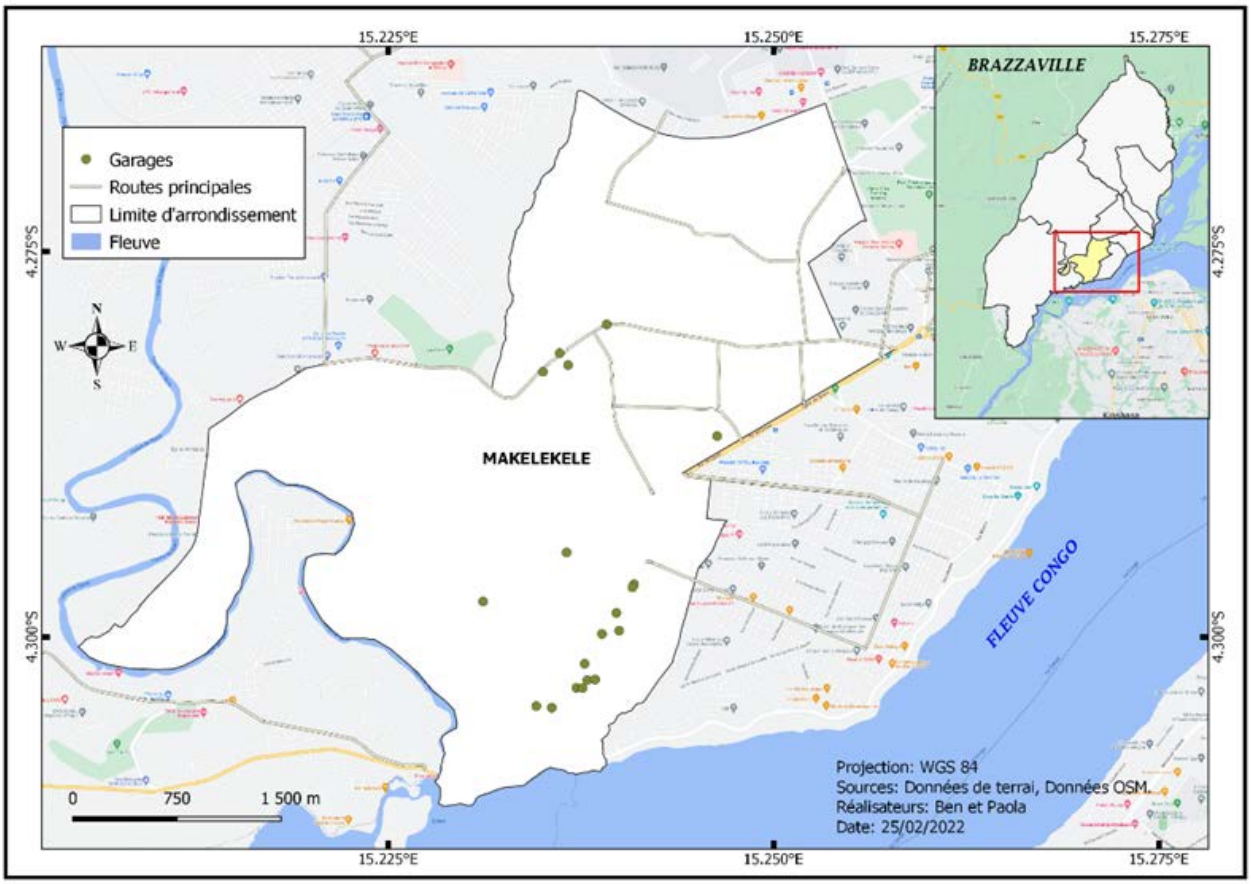

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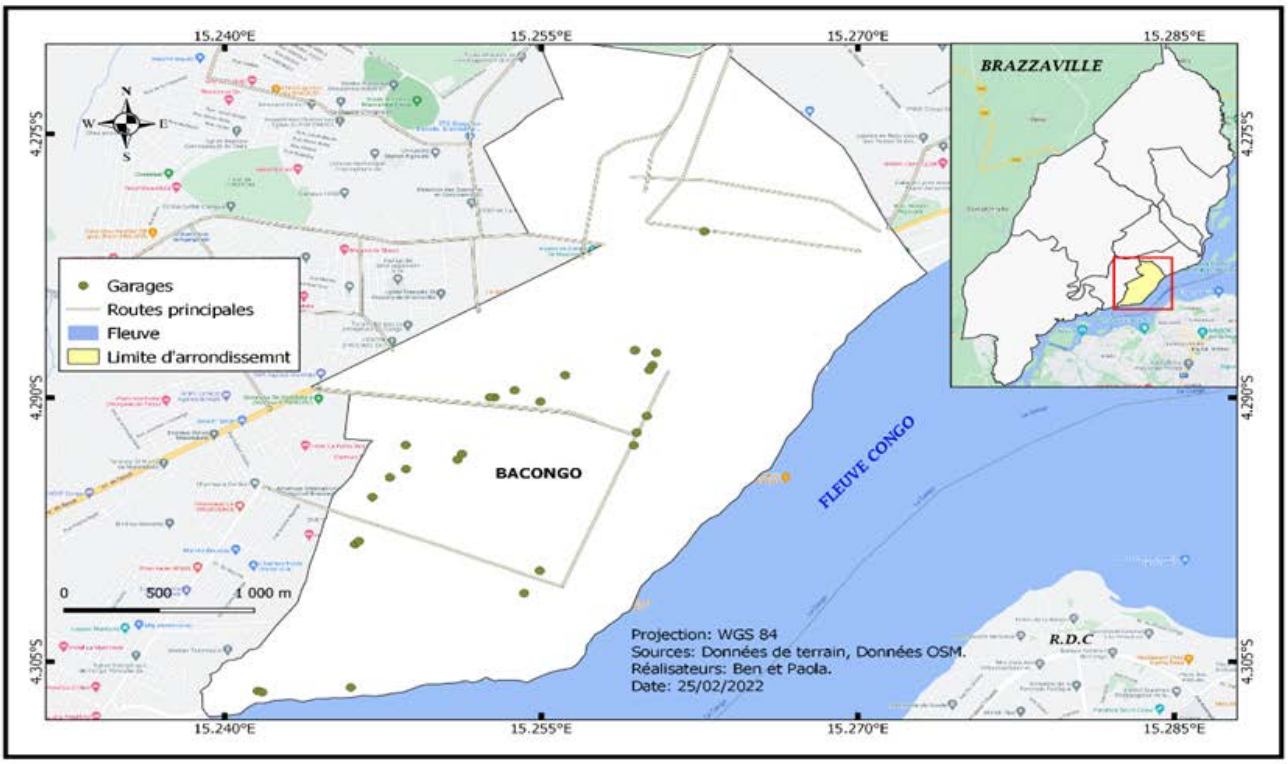
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Sources: Données de terrain, Données OSM.
Réalisateurs: Ben et Paola.
Date: 25/02/2022

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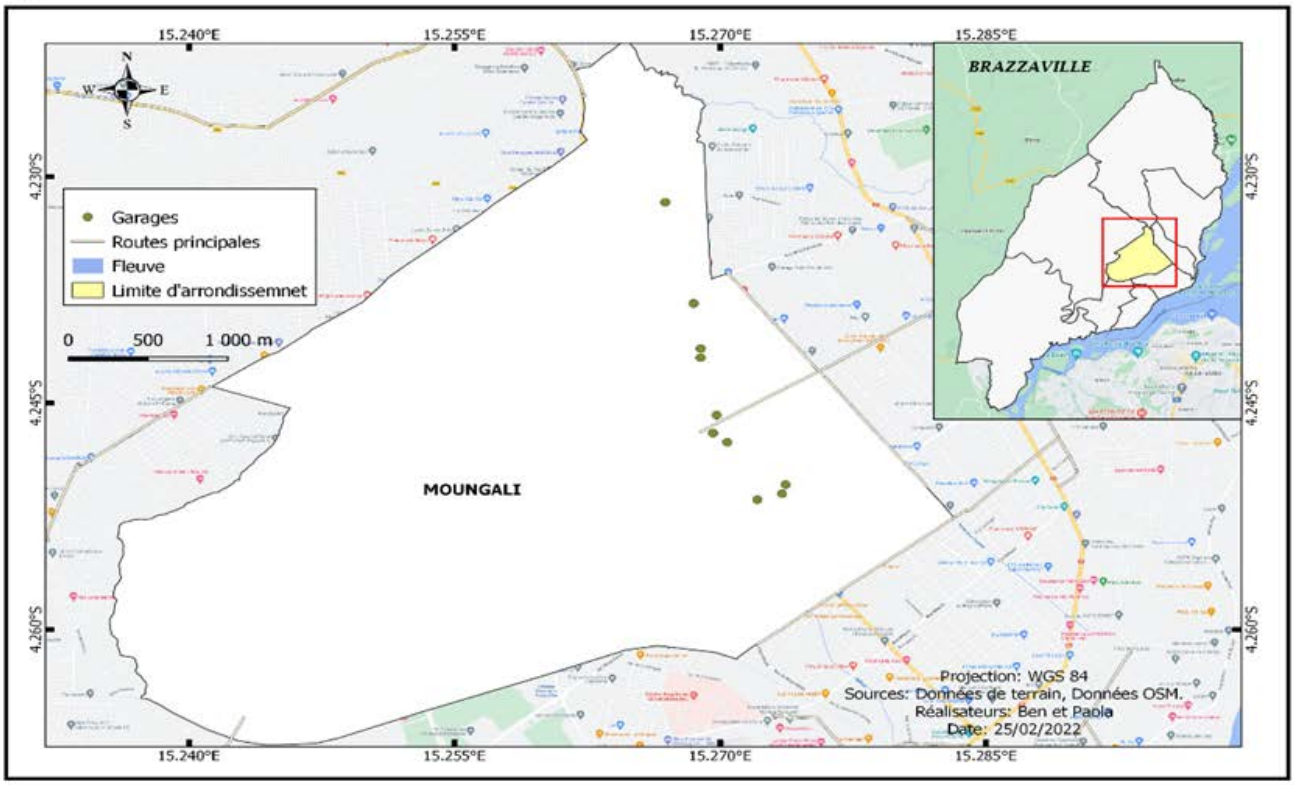
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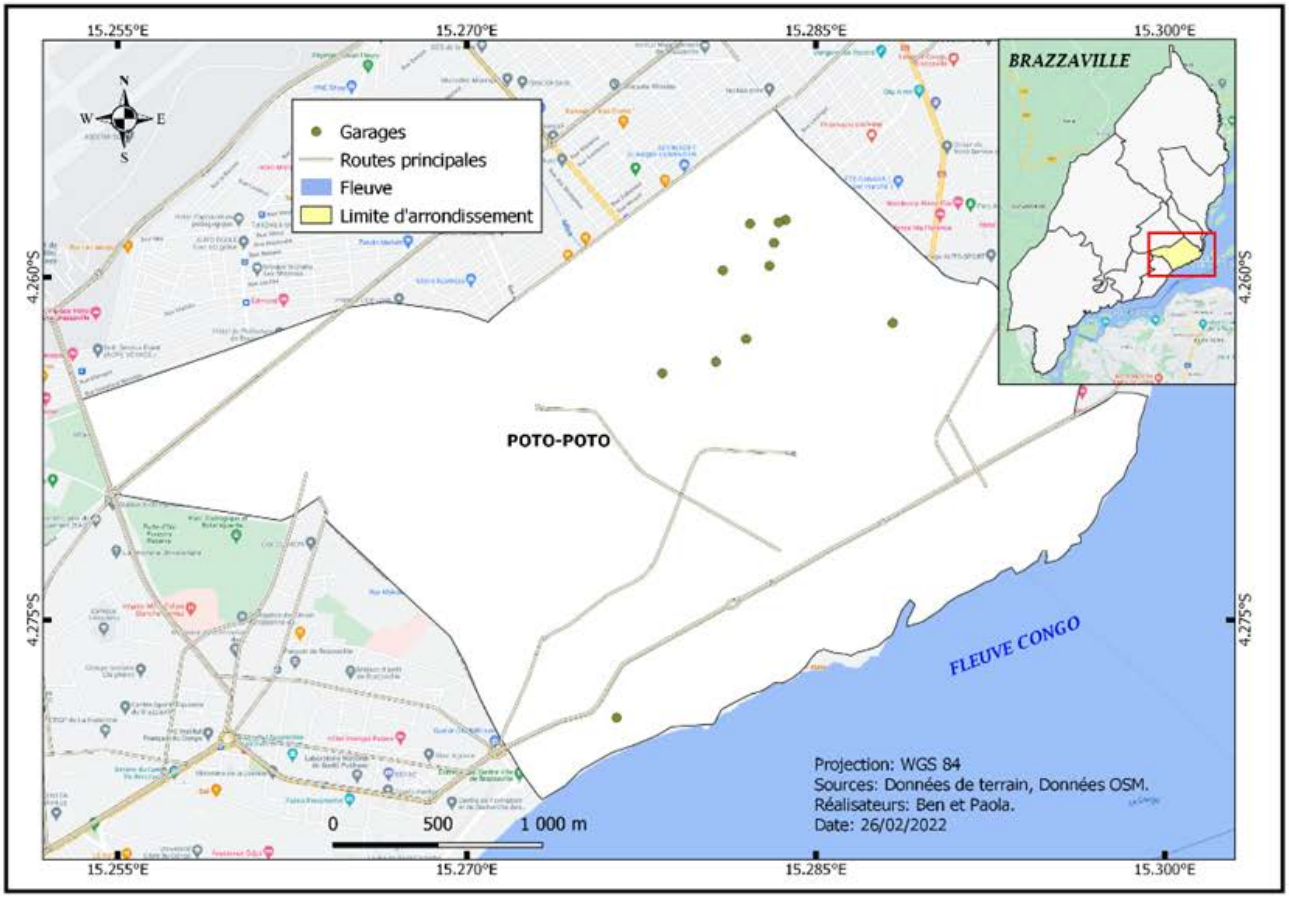
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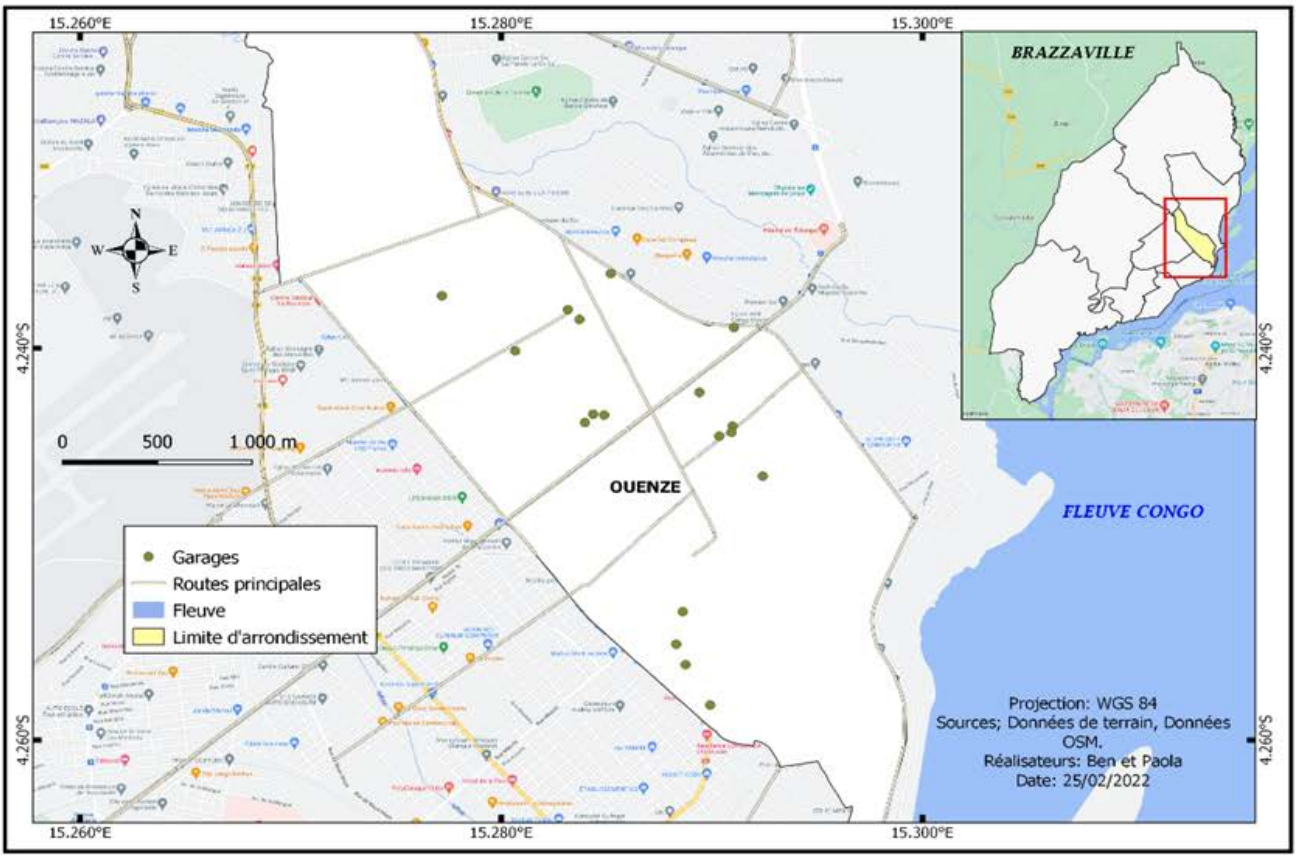
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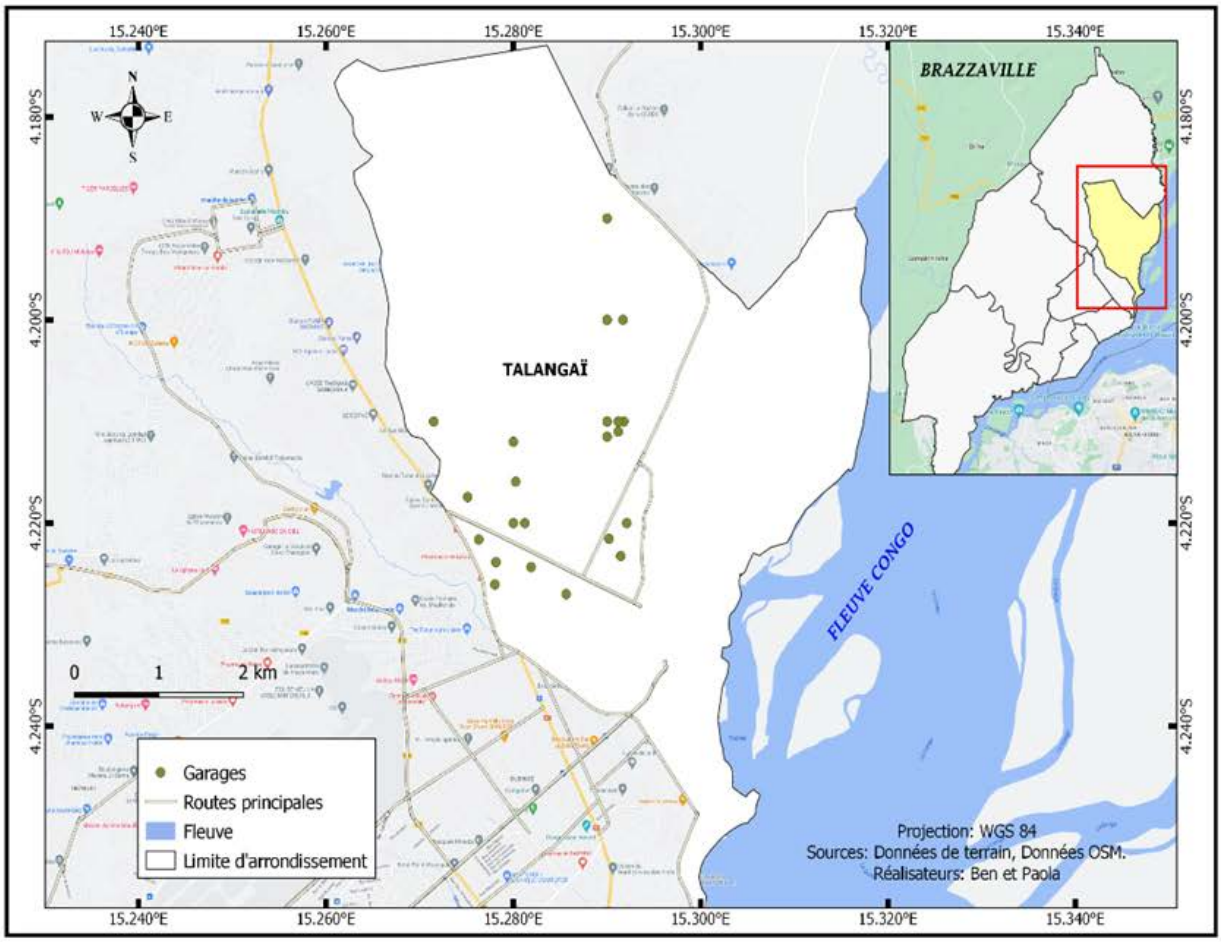
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Sources: Données de terrain, Données OSM.
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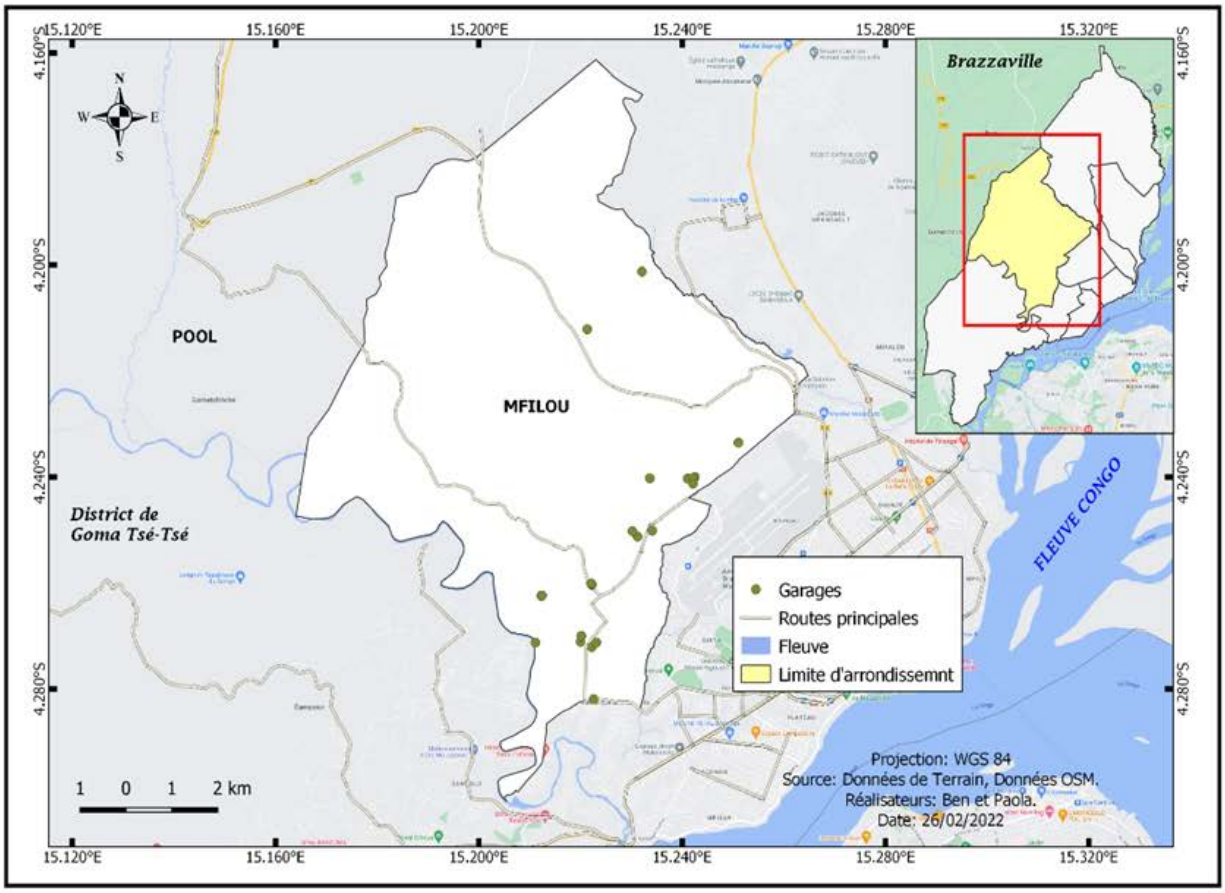
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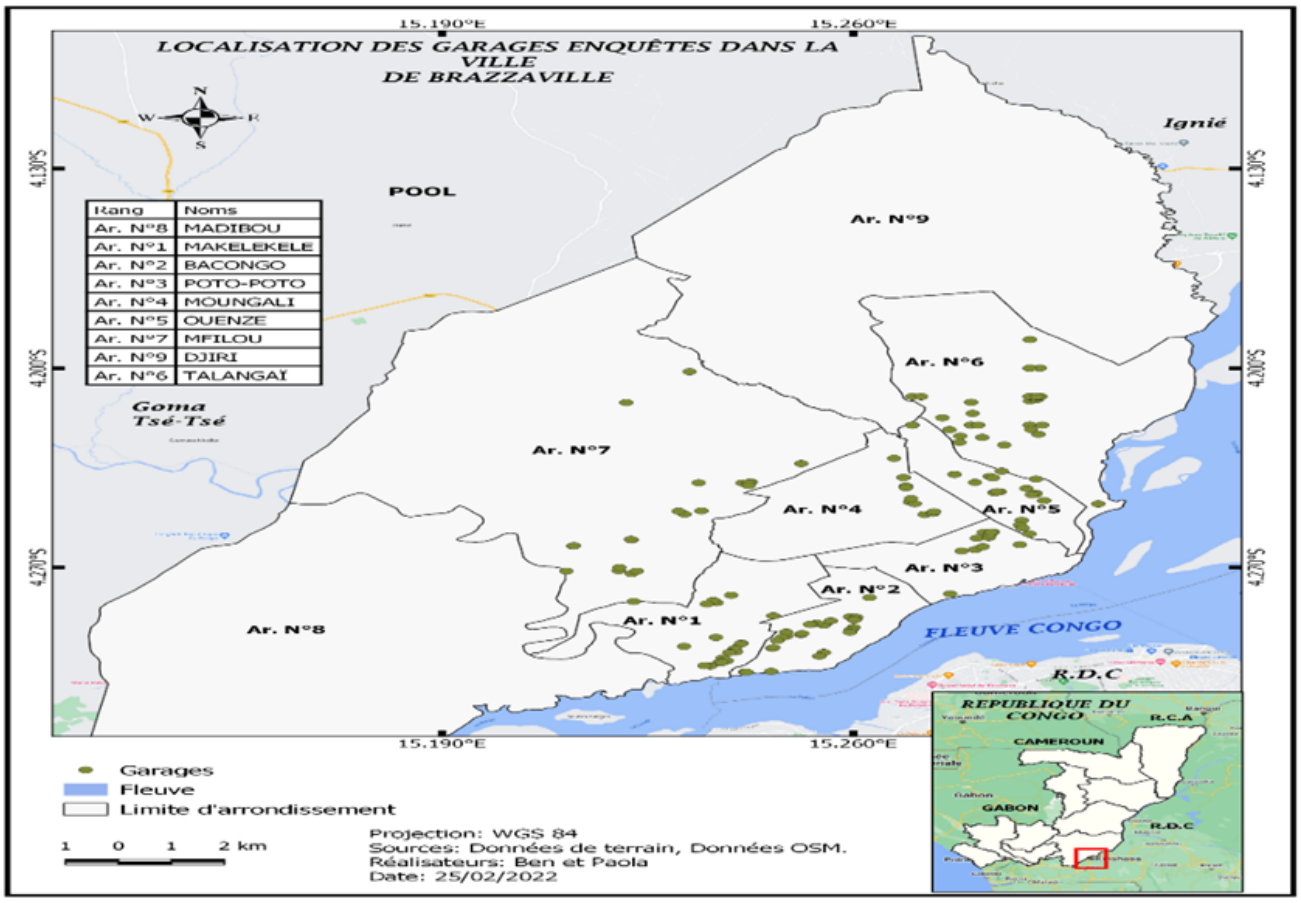


Figure 1. Geographic map of garages found in 7 districts. (a)- Makélékélé, (b)- Bacongo, (c)-Poto-Poto, (d)- Moungali, (e)- Talangai, (f)-Mfilou, (g)- all districts) of Brazzaville

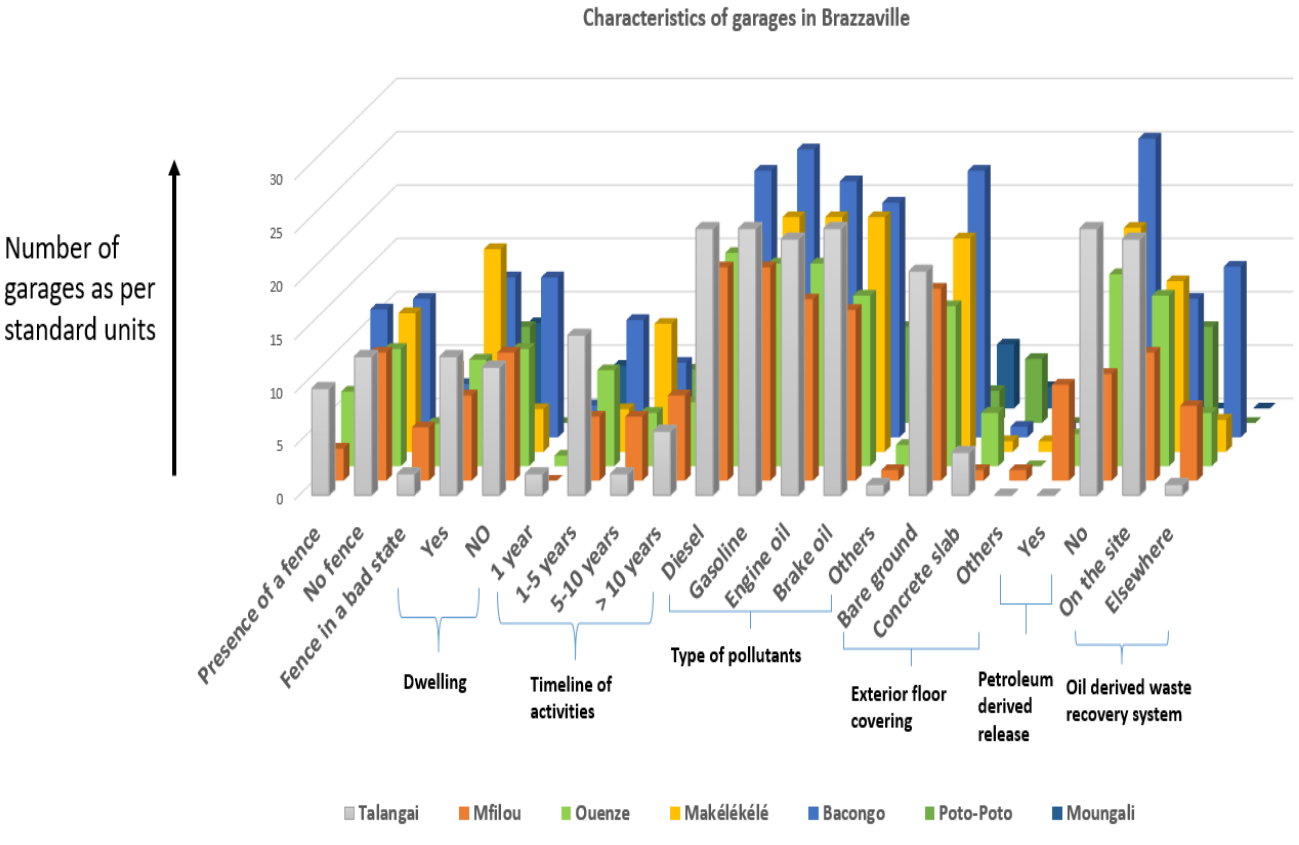


Figure 2. Characteristics of garages in Brazzaville

The survey has shown that Bacongo, the first district of Brazzaville, has the most garages in Brazzaville, followed by Talangai, Makélékélé, Ouenze, Mfilou, Poto-Poto and finally Moungali as stated in Figure 2. Most of these garages have existed for a period of over 10 years. 60% of these garages have no fence, 70 % have houses in the garages, and 55 % have existed for over 10 years. The survey also revealed that 78% of garages release oil-derived products onto the bare ground, and 87 % do not possess an oil derived waste recovery system. These results are completely different from the ones from the study conducted in France, where garages happened to be in very good condition and did not have a huge impact on people's health as compared to the ones in Brazzaville.

3.2. Soil Parameters

The physiochemical parameters of the soil samples before treatment are evaluated and recorded in Table 2.

Table 2. Physiochemical parameters of soil samples before treatment

Garages	pH	Electrical conductivity (µs/cm)	TPH content (g/Kg) of soil
Makélékélé	7.10	329	195.8
Bacongo	7.22	201	206
Moungali	7.66	192	177.20
Poto-Poto	7.2	127	165

The selected garage in district 2, Bacongo, recorded the highest TPH content. All the soils tested were neutral, pH varies from 7.10 to 7.66. These pH values are favourable for the degradation of hydrocarbons by bacteria. These results are similar to the ones obtained by [9]. As for electrical conductivity, these results show that most soils from garages are poor in trace elements. This explains why bioavailability of nutrients is difficult, making bioremedition inefficient.

3.3. Biosurfactant Production

The biosurfactant-producing isolate M28, isolated from wild contaminated soil, was subjected to an emulsification index. It was found that this isolate is able to emulsify gasoline (95%) and diesel (90%). The emulsification test is a straightforward quantitative method to prospect biosurfactant-producing microorganisms [10]. The biosurfactant obtained is brownish (Figure 3).

3.4. Genomic Extraction

DNA sample isolated from *Bacillus* isolate M28 showed molecular weight above 10000 kb and was used for detection of surfactin genes by PCR.

3.5. Identification of Biosurfactant Genes

The primers were designed from the *B. subtilis* sfp gene fragments to amplify 675 bases from the CDS region of the gene. The PCR results revealed that amplification of the surfactin gene was positive with sfp gene at around 675 bp. Surfactin is produced as a result of nonribosomal biosynthesis catalysed by a large multienzyme complex consisting of four modular building blocks, called surfactin synthetase (Figure 4). The sfp gene in *Bacillus* species encodes phosphopantetheinyl transferase, which is required for the nonribosomal biosynthesis of surfactin [11]. The sfp gene is an essential component of peptide synthesis systems and also plays a significant role in the regulation of surfactin biosynthesis gene expression. The sfp gene converts the inactive protein, which transforms surfactin synthetase into an active form [12]. Therefore, sfp plays an essential role in the production of biosurfactants.

Figure 3. A- Biosurfactant like molecule produced by M28, B- Emulsifaction index of M28

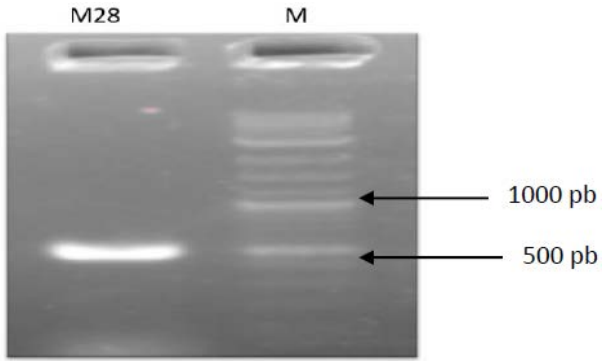
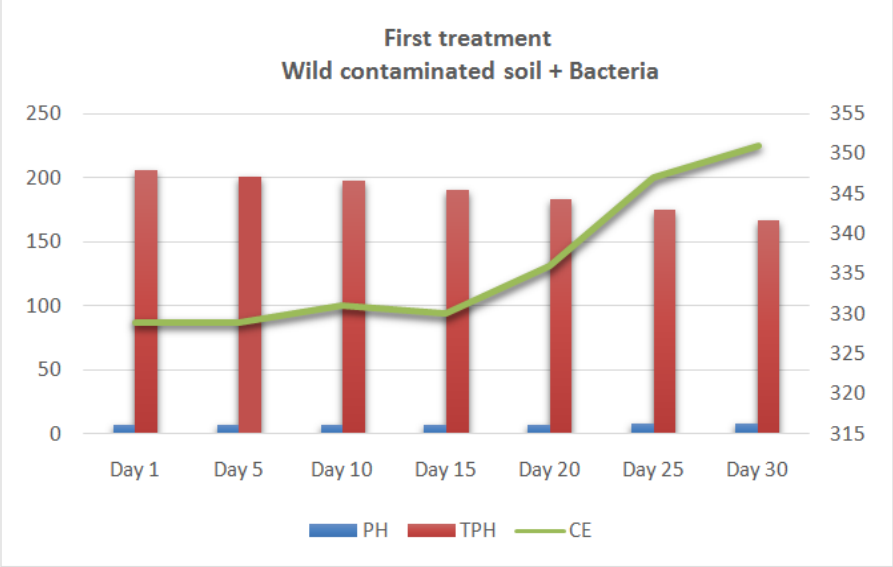


Figure 4. Agarose gel electrophoresis of PCR product for the *sfp* gene encoding the production of surfactin biosurfactant; M28 referring to the reference isolate M28, M: DNA molecular marker 2-log (BIOKE)

3.6. Bioaugmentation by *Bacillus* Isolates

The remediation of crude oil contaminated soil is an international problem. The main component of petroleum pollutants in soil is polycyclic aromatic hydrocarbons. Their solubility is very low in soil aqueous solution resulting in poor availability. Indigenous microbes associated with contaminated sites are a key source for degrading hydrocarbon from contaminated soils [13]. This study revealed that isolate M28 is able to reduce the TPH content from a wild contaminated soil at a rate of 18.93% in 30 days. Previous studies have shown that indigenous bacteria are very efficient in degrading hydrocarbons from contaminated soils [14]. Under the same conditions, the biodegradation rate reached 50.97% with the addition of biosurfactant produced by the same isolate M28. These results are in accordance with the ones obtained by Zhang, Wu & Ren (2020) as certain strains reached a degradation level of 41% after 40 days incubation. Wild contaminated soils of garages are usually polluted with diesel, gasoline and other types of pollutants. Studies have reported the addition of biosurfactants increase oil degradation efficiency in diesel oil contaminated soils [15]. The age of the soil contamination may be an important factor which influence the efficiency of bioremediation. The majority of studies read in the literature focus the bioremediation processes on soils that freshly contaminated with pollutants [16] while we worked with a soil contaminated for a period over 10 years.

(a)



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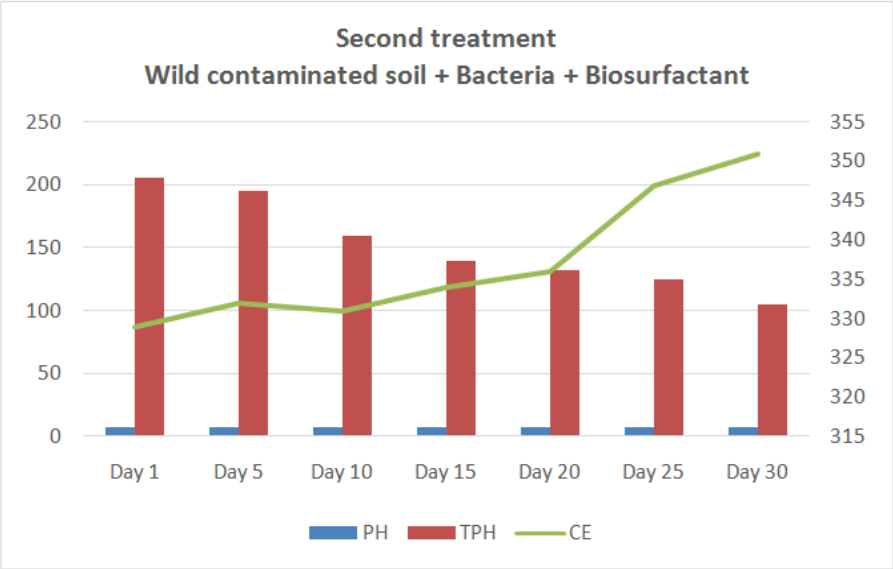

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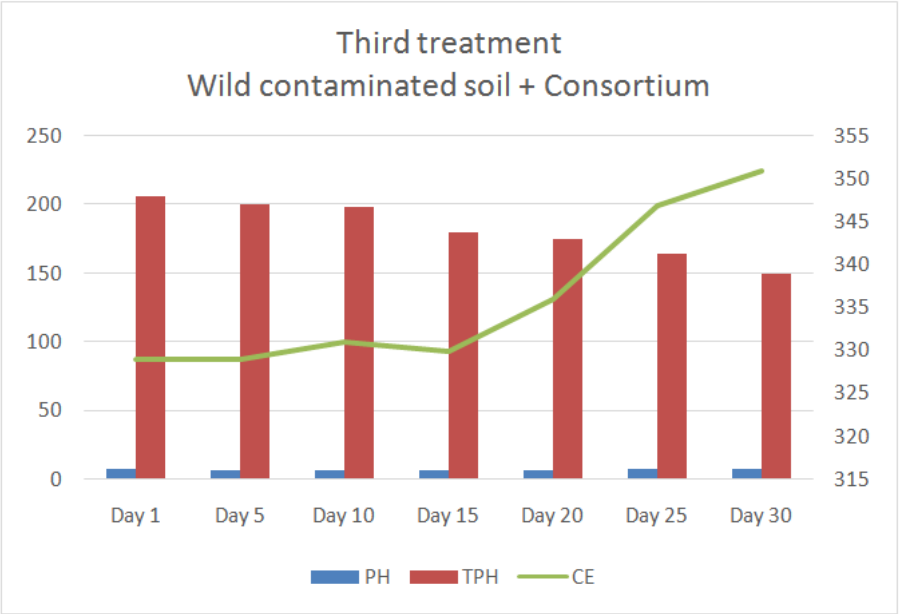

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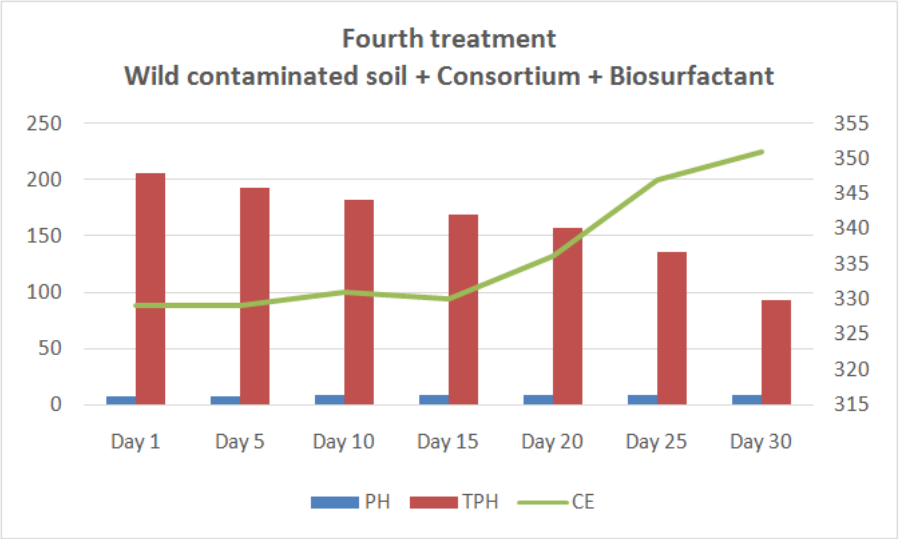

Figure 5. Four Treatments of wild contaminated soils a: Soil + Bacteria, b: soil + Bacteria + biosurfactant, c: soil + Consortium d: Soil + Consortium + biosurfactant

Based on the results obtained in the laboratory (Figure 5), we can say the concentration of biosurfactant seems to also have an important effect on bioaugmentation. In this study, the crude extract of biosurfactant used greatly contributed to the enhanced increase in microbial activity of isolate M28. Too much of the biosurfactant could have also hindered the process, as Elenga Wilson *et al* (2021) showed that these surfactants have very important antimicrobial activities.

In the present study, the biosurfactant produced by M28 stimulated the degradation activity and shortened the time required for hydrocarbons degradation. In the third and fourth treatment, one can notice that remediation was higher than that of M28 done alone. The choice of the consortium was based on the excellent emulsification activities of the isolates individually, ranging from 80 to 100%. Thompson, Van Der Gast, Ciric, & Singer (2005) stated that the choice of a consortium is very important as many principles should be taken into consideration. Based on the results obtained upon bioaugmentation with the consortium, one can state that there was no competition between isolates, hence the bioaugmentation rate is higher. The effect of mixed bacteria is better than that of a single bacterium. After 30 days, the content of petroleum hydrocarbons decreased to 93 g/kg, and the removal rate of petroleum hydrocarbons reached 65.1%. These results are very promising as many studies have observed these kinds of results after weeks [14].

It should be noted that the pH value before treatment of the wild contaminated soil was neutral at 7.22. After inoculation of the bacteria, there is an increase in the pH values of the garages, tending towards basicity. The increase in pH shows the presence of carbonates in the environment [17]. Furthermore, many studies [18] discovered that hydrocarbon degradation is faster under basic conditions.

The conductivity observed, on the other hand, before treatment is low. These low conductivity values show that

the soils sampled are not rich in mineral elements. However, they are qualified as ferallitic soils and correspond to the work of [19] who showed that ferallitic soils have a low mineral reserve. These values slightly increased after the addition of microbes and biosurfactant.

4. Conclusions

The TPH content obtained in garages in Brazzaville is way above the international standards, showing the health hazards that these garages pose to people living in or around garages. The results obtained during the study suggest an understandable approach towards the treatment of wild contaminated soils from garages. Based on the results, it can be established that the introduction of a hydrocarbon degrading consortium notably improved the degradation efficiency as compared to a single type of bacteria. Surfactin in the form of crude extract increased hydrocarbon bioavailability. The addition of the biosurfactant was very significant as it enhanced bioaugmentation of wild contaminated soils and shortened the time required for natural biodegradation.

Data Availability

The experimental data used to support the findings is included within the article.

Conflicts of Interest

The authors declare no conflict of interest or personal relationship that could have appeared to influence the work of this paper.

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