Bioremediation of urban soils polluted with non-conventional petroleum in the Canadian context

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Master´s degree in Ecosystem Restoration

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Signed this 3rd of September, 2015 at Quebec (Canada).

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Signed this 4th of September, 2015 at Madrid (Spain).

BIOREMEDIATION OF URBAN SOILS POLLUTED WITH NON-CONVENTIONAL PETROLEUM IN THE CANADIAN CONTEXT

Master´s degree in Ecosystem Restoration

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Abstract:

The increase of transport of non-conventional crude petroleum in Canada is associated with risks of spills. Accidental spills such as in Lac-Megantic revealed a lack of understanding of its biodegradation during soil treatment. The present work studied the feasibility of bioremediation methods in affected urban soils by non-conventional petroleum. Soil with similar composition to the affected soils in Lac-Megantic was sampled in Quebec City and presented a high content in heavy metals (3 mg Cd kg^{-1} soil). Natural attenuation and biostimulation were applied to representative soil samples (polluted in laboratory with Alberta´s Tar Sands Petroleum) at 20ºC and 5ºC for 31 days. Total petroleum hydrocarbons (TPH) determined the extent of degradation. Respirometric analyses and plate-counting of colony-forming units (CFU) monitored microbial activity. Results showed 20% TPH removal for both methods at 20ºC. At 5ºC biostimulation had 12% and natural attenuation 2%. For the studied experimental conditions the following strategies are recommended in restoration of soil ecosystems (1) no treatment of biostimulation at temperate temperatures and (2) amendment of N and P fertilizers to improve microorganisms degradation at temperatures < 10ºC.

KEYWORDS: urban soils; non-conventional crude petroleum; bioremediation; natural attenuation; biostimulation.

Resumen:

El aumento del transporte de petróleo crudo no convencional en Canadá tiene un elevado riesgo de vertidos. Vertidos accidentales como el sucedido en Lac-Megantic revelaron lagunas de conocimiento respecto a su biodegradación en la recuperación de suelos contaminados. Por este motivo se estudia en este trabajo la viabilidad de métodos de biorremediación en suelos urbanos contaminados por petróleo no convencional. El suelo fue muestreado en la ciudad de Quebec, con composición similar a los suelos afectados en Lac-Megantic y presentando un alto contenido en metales pesados (3 mg Cd kg^{-1} suelo). La atenuación natural y la bioestimulación fueron aplicadas a muestras representativas de suelos contaminados en laboratorio con petróleo de arenas bituminosas de Alberta y expuestas a 20ºC y 5ºC durante 31 días. El nivel de degradación se determinó por el contenido en hidrocarburos totales de petróleo (TPH) en las muestras de suelo. La actividad microbiana fue monitoreada con ensayos de respiración y con el conteo de unidades formadoras de colonias (UFC). Los resultados obtenidos mostraron una eliminación del 20% de TPH para ambas técnicas a 20ºC y, del 12% para la bioestimulación y del 2% para la atenuación natural a 5ºC. Del presente estudio se recomiendan las siguientes estrategias para la restauración de ecosistemas edáficos ante este tipo de escenario: (1) no aplicar bioestimulación en temperaturas moderadas y, (2) adicionar enmiendas de fertilizantes N y P para aumentar la degradación de los microorganismos si las temperaturas son <10ºC.

PALABRAS CLAVE: suelos urbanos; petróleo crudo no convencional; biorremediación; atenuación natural; bioestimulación.

Résumé:

L´augmentation du transport de pétrole brut non conventionnel au Canada est associée à un risque élevé de fuites. Des fuites accidentelles comme celui survenu à Lac-Mégantic ont mis en évidence le manque de compréhension de sa biodégradation dans l´environnement. Le présent document étudie la faisabilité de méthodes de bioremédiation dans les sols urbains pollués avec du pétrole brut non conventionnel. Le sol a été pris à la ville de Québec avec une composition similaire aux sols affectés à Lac-Mégantic et avec une concentration élevée de métaux lourds (Cd 3 mg kg^{-1} sol). L´atténuation naturelle et la biostimulation sont appliquées en échantillons représentatives de sol (pollués en laboratoire avec pétrole brut de sables bitumineuses d´Alberta) à 20ºC et à 5ºC pendant 31 jours. La mesure des hydrocarbures pétroliers totaux (TPH) permet de déterminer le niveau de dégradation. L´activité microbienne a été mesurée avec des analyses respirométriques et par le comptage sur plaque de pétri des unités formatrices de colonies (UFC). Les résultats montrent une réduction de TPH de 20% pour les deux méthodes à 20ºC, et de 12% pour la biostimulation et 2% pour l´atténuation naturelle à 5ºC. À partir de ces résultats, les stratégies suivantes sont recommandées pour la restauration des écosystèmes de sols urbains dans un scénario similaire : (1) ne pas appliquer de traitements à des températures modérées et, (2) l´utilisation d´amendements N et P afin d´augmenter la dégradation par microorganismes si les températures sont inférieures à 10ºC.

MOTS-CLÉS: sols urbains; pétrole brut non conventionnel; bioremédiation; atténuation naturelle; biostimulation.

1. Introduction

1.1 Non-conventional crude oils in Canada

Non-conventional crudes oils (petroleum not extracted by oil wells) are leading the Canadian crude production (1.98 million barrels per day of oil sands production against 1.38 million barrels per day of conventional oil in 2013) (CAPP, 2014). The majority of non-conventional crudes correspond to oil sands such as Alberta´s Tar Sands Oil. In addition, other non-conventional crudes are broadly transported through Canada for exportation (e.g. Bakken petrol shale oil from North Dakota in USA).

As a result, several miles of pipelines are being projected (e.g. the Keystone XL Pipeline from Alberta to Texas, the Northern Gateway from Alberta to British Columbia or the Energy East Pipeline from Alberta to Quebec and New Brunswick) along with the increasing traffic on railways and maritime transportation. In fact, it is not strange observing railcars transporting crude petroleum even in big Canadian cities (Figure 1).

Fig. 1 Wagons transporting crude petroleum in Montreal (Quebec, Canada) on May 22nd, 2015.

1.2 Associated risks with the transportation of crude

Spills and accidents may occur as a consequence of the increase in crude transportation in Canada.

Spills may occur during petroleum products transportation or storage (Yang et al. 2009). Some reported cases were the pipeline spill in Ellesmere Island (Nunavut, Canadá) (Whyte et al. 2001), or fuel tank spills on the Canadian tundra (Mohn & Stewart, 2000) and on sub-arctic soils of Fairbanks (Alaska, US) (Walworth et al. 2001).

Accidents may also be expected. The variety of accidents in the period 1990 to 1999 involved maritime transport (47.4%), pipelines (37.6%), storage tanks, refineries and other fixed buildings (13.6%), and platform, wells and mobile units (1.4%) (Burgherr, 2007). Indeed, the most important petroleum accident in Canada was the train wreck of Lac-Megantic in the Province of Quebec (Galvez-Cloutier et al. 2014).

On July 5th, 2013, a convoy composed of 72 wagons transporting Bakken crude petroleum (non-conventional petroleum imported from North Dakota, US) and owned by *Montreal, Maine, and Atlantic Railways (MMA)* stopped in Nantes (Québec), after which a fire was declared. Despite the fire being extinguished, the train moved downwards the slope of 1.2% between Nantes and Lac-Megantic, where the speed produced the train wreck on July 6th. As a consequence, four wagons exploded at the beginning and several wagons would follow the same trend due to the excessive temperature (Galvez-Cloutier et al. 2014).

The consequences were the destruction of Lac-Megantic´s downtown, 47 casualties, the pollution of water bodies (Lake Megantic and Chaudière River) and soil by the leak of 5978m^3 of Bakken crude along with the emission of incomplete combustion products (Galvez-Cloutier et al. 2014).

The Lac-Megantic episode revealed a number of deficiencies in transport (TSB, 2014), accident management and even in the selection and speed of rehabilitation measures (Galvez-Cloutier et al. 2014).

Although train transport of petroleum products is more polluting and dangerous than transport by pipeline (Galvez-Cloutier et al. 2014 and TSB, 2014), pipelines may also have accidents such as the one in Kalamazoo River (Michigan, USA) (NTSB, 2014). In July, 2010, a six-foot pipeline tear resulted in diluted Alberta´s Tar Sands Oil (non-conventional petroleum called dilbit) flowing into Talmadge Creek (a tributary of the Kalamazoo River). One of the largest inland spills in the USA required closing 56 Km of the Kalamazoo River for clean-up (NTSB, 2014).

All in all, vulnerabilities are inherently bound to the rise in the transport of non-conventional crude petroleum. Spills may pollute huge extensions of soils with negative effects in neighboring ecosystems, which incur expensive restoration costs (1 billion US dollars in Kalamazoo River and >200 million Canadian dollars in Lac-Megantic) (Galvez-Cloutier et al. 2014 and NTSB, 2014).

1.3 Effects of crude petroleum on ecosystems

Soil pollution by hydrocarbons may disrupt ecosystems dynamics. Crude oil contains toxic compounds that may inhibit microbial biomass and hydrolase activities associated with N, P or C cycles (El-Tarabily 2002 and Labud et al. 2007). In addition, hydrocarbon pollution reduces soil water adsorption and retention owing to the augmentation of the mean aggregate size, high concentrations of salt or the increase in water stress (Li et al. 1997).

Toxicity may also reduce diversity of plant comunities, benthic invertebrates and soil and water microorganisms (Nyman, 1999).

These effects in dynamics and the induced toxicity may reduce the success of restoration in hydrocarbon polluted ecosystems due to interferences with microorganisms (Nyman, 1999; El-Tarabily, 2002 and Labud et al. 2007) or with plant growth (Li et al. 1997 and Labud et al. 2007), compromising the ecosystem´s ability to regenerate after perturbations.

1.4 Bioremediation of cold soils polluted with hydrocarbons

Remediation is one of the preferred approaches to restore soils impacted by hydrocarbons. It comprises physical, chemical and bioremediation methods. Physical-chemical methods (e.g. excavation or chemical addition) tend to be aggressive to soil ecosystems and present operational issues which increase costs in restoration. On the other hand, bioremediation methods (based on biological activity) can be economically appealing, easy to apply, effective and less destructive for ecosystems.

However, climatic conditions in North latitude countries limit restoration of polluted soils with hydrocarbons. Indeed, factors such as scarce availability of nutrients, low temperatures, soil moisture, pH and freeze-thaw processes can extend the time of restoration of bioremediation methods to decades instead of years (Mohn & Stewart, 2000; Aislabie et al. 2006 and Yang et al. 2009).

Focusing on Canada, low temperatures are the main limitation in the application of bioremediation and are the reason for using bioremediation methods only during summer (Chang & Ghoshal, 2014 and Whyte et al. 2001). Canada has mainly three big climatic regions: humid continental climate (Dfb), sub-Arctic climate (Dfc) and Arctic climate (ET) (Kottek et al. 2006 and MDDELCC, 2010). Both are characterized by annual average temperatures closer to the freezing point (Environment Canada, 2015).

Concerning the treatment of soil ecosystems in cold areas, it has been observed the biodegradation of many components of conventional petroleum or derived products in Arctic and Antarctic areas (Whyte et al. 2001 and Aislabie et al. 2006) as well as in alpine soils (Margesin & Schinner, 1997).

When considering the temperature of metabolic activity there are two broad groups of Autochthonous microorganisms able to degrade hydrocarbons (Aislabie et al. 2006): mesophilic and psychrotolerant microorganisms. In detail, biodegradation is carried out by mesophilic microorganisms at temperate temperatures (≤10ºC to 45-50ºC) as they are inactive at temperatures ≤8-10ºC. At lower temperatures, biodegradation is produced by psychrotolerants. The group of psychrotolerants has activity at ≤0ºC and is divided into two branches: psychrophilic (growth range ≤0ºC to 15-20ºC) and psychrotrophic (growth range ≤0ºC to 30-35ºC) (Whyte et al. 2001).

While most of studies on petroleum bioremediation focus on conventional oils, there is a gap of knowledge on how microorganisms or plants degrade non-conventional crude petroleum. Furthermore, there is no exact information of the composition of this variety of petroleum (Galvez-Cloutier et al. 2014). In such conditions, it is difficult to discern the behavior of hydrocarbons in soil and water (Environment Canada, 2013 and Galvez-Cloutier et al. 2014).

1.5 Description of the project

Based on the above facts, it is recommended to test biodegradation of hydrocarbons prior to the application of bioremediations methods in the field. Lab treatability studies can minimize costs on field work as they provide information regarding kinetics of hydrocarbon degradation.

The work reported here is a complete biotreatability study in a typical urban soil presenting a high risk of being affected by spills of non-conventional crude petroleum, using as context the Lac-Megantic´s scenario.

For such purpose, the common limiting factor of bioremediation in Canadian urban soil, which is temperature, was tested in laboratory. Experimental scenarios, including soil and non-conventional petroleum, were exposed (31 days) at two different temperatures to mimic summer conditions of the different Canadian climatic regions (humid continental climate (Dfb) and Arctic (ET) climate).

Then, two bioremediation methods were tested: natural attenuation and biostimulation. Their extent of degradation was examined by the evaluation of chemical and biological characteristics of soils. Microbiological growth potential was evaluated by the study of variation in the number of culturable colony-forming units (CFU).

Finally, according to the results, conclusions are drawn that take into consideration the restoration of soil ecosystems affected by spills or accidents associated to non-conventional petroleum in urban soils in the Canadian context.

1.6 Aims, objectives and tasks

Considering the facts previously pointed out, it is evident that the increase in transport of non-conventional petroleum may cause more episodes of soil pollution. For this reason, the following aims are addressed in the current study:

- To increase the efficiency of bioremediation methods in the restoration of polluted areas with non-conventional petroleum in North latitude countries.
- To improve the management of accidents to ensure a higher health of ecosystems.

These points motivated the following objectives of the current research based on scientific and engineering perspectives:

(1) To test the feasibility of natural attenuation and biostimulation in the reduction of Total Petroleum Hydrocarbons (TPH) in urban soils affected by non-conventional petroleum.

(2) To provide recommendations in the choice of bioremediation methods in case of spills.

In order to achieve these objectives, (I) chemical properties from urban soils were determined, and (II) the amount of TPH, the respiration rate and the abundance of microorganisms were monitored during lab scale remediation.

2. Materials and methods

Our study followed an experimental approach in laboratory with the use of small scale reactors.

2.1 Location and climate conditions of the sampling area

The sampling area was in Quebec City. Quebec City is the capital of the Province of Quebec in Canada and located ~300 km north of Montreal.

The sampling area was at coordinates of 46º46´40.18´´N and 71º18´21.26´´W, placed at 2910 Chemin de Saint-Foy by the highway 73 (Figure 2).

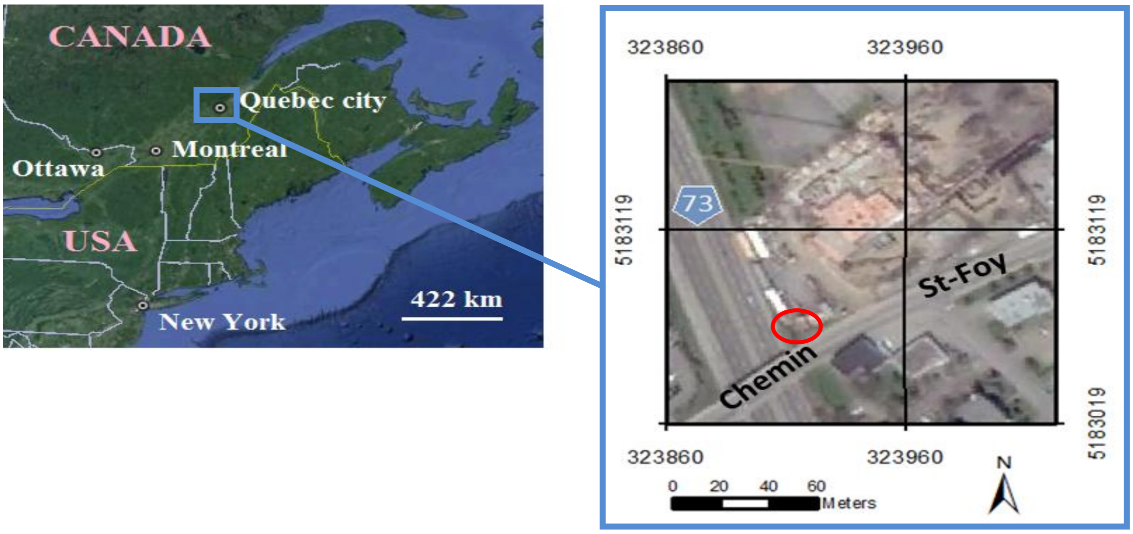

Fig. 2 Location of the sampling area at coordinates of 46º46´40.18´´N and 71º18´21.26´´W.

The climate of the sampling area is a humid continental climate (Dfb). Data obtained from meteorological station “Quebec/Jean Lesage International A” at coordinates 46º48´N and 71º23´ W (Figure 3) indicates an average temperature of 17.9ºC ± 1.4 for June, July and August with an average annual precipitation of 1189.7 mm (Environment Canada, 2015).

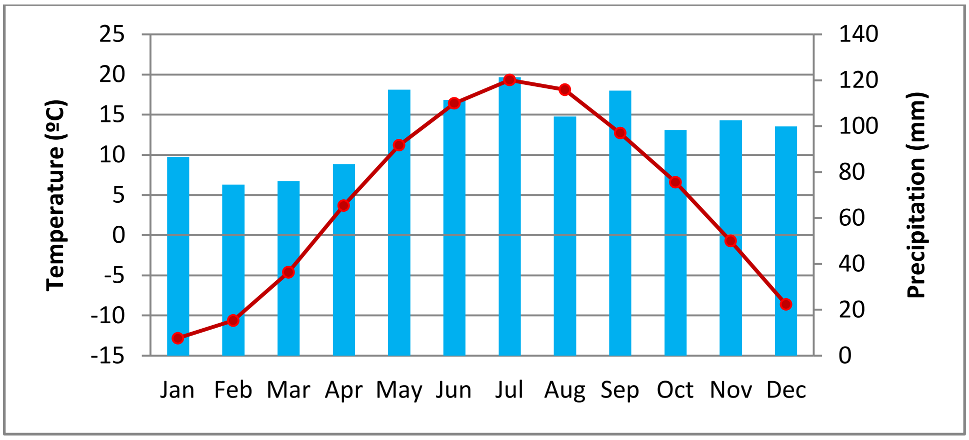

Fig. 3 Temperature and precipitation chart for 1981-2010 Canadian Climate Normals of station “Quebec/Jean Lesage International A” at coordinates 46º48´N 71º23´W (Environment Canada, 2015).

2.2 Soil sampling

Technosols (Figure 4), soils which contain >30% artefacts (strange materials and soils, made by humans from other materials and wastes) (IUSS Working Group WRB, 2014), are usually referred to as urban soils and are susceptible to petroleum leakages of pipelines or railway transportation in Canada. Such was the case of the urban soils polluted in Lac-Megantic´s accident (Golder Associés Lté, 2013). Lac-Megantic´s technosols were composed of a heterogeneous concentration of cracked stones, sand, gravel and debris (Golder Associés Lté, 2013).

Fig. 4 Profile of sampled technosols (FAO WRB).

Following a random sampling, the criteria was to find accessible soil with similar composition to the concerned soils in Lac-Megantic´s spill and non-affected by industrial activities or earth moving. This soil was used as control in the experiment.

For sampling, we firstly removed with shovel a layer of snow and ice of ~1m of thickness. Secondly, we capped 30-40cm of soil using Jackhammer and shovel (Figure 5).

Fig. 5 Removal of snow, capping and soil storing in plastic cubes in sampling.

The collected soil (~30kg) was stored in plastic cubes. We set aside several soil sub-samples in order to determine soil moisture on field. The soil was gently disaggregated, mixed, and sieved (<2mm) prior to characterization.

2.3 Petroleum characteristics

The petroleum employed in this study was “Tar Sands´ Synthetic Crude CNRL”, supplied by *Énergie Valero Inc*, which is a variety of diluted Alberta´s Tar Sands Oil such as the spilled petroleum in Kalamazoo River (NTSB, 2014). “Tar Sands´ Synthetic Crude CNRL” is a sort of non-conventional crude petroleum broadly transported in Canada via pipelines or railway. It had a density of 37º API (0.838 g/mL) with <0.18% total Sulfur and <0.06% total Nitrogen. Therefore, the employed petroleum is a light crude (API>25º) similar in density to conventional petroleum (Meyer et al. 2007).

2.4 Experimental design

Eight experimental scenarios, containing 500g of urban soil and 10000 mg kg^{-1} of non-conventional crude petroleum, were set up in duplicates including controls (Figure 6). Two types of controls were used: one containing only the same amount of added petroleum to the studied scenarios and another one with unpolluted soil. The soil of each scenario was placed in complete obscurity during the experiment to mimic field conditions.

Two bioremediation methods were tested in duplicate for 31 days, imitating biopiles on site:

1. Natural attenuation: soil´s ability to degrade hydrocarbons by indigenous microorganisms.

2. Biostimulation: addition of nutrients (N, P) to improve microorganism degradation. NH_4NO_3 and K_2HPO_4 was added (Sabaté et al. 2004) to adjust to a final equivalent C:N:P molar ration of 100:10:1 (Sabaté et al. 2004 and Gallego et al. 2011).

Experimental scenarios were deposited at average temperatures of 20ºC and 5ºC to mimic summers in humid continental climate (Dfb) and Arctic climate (ET).

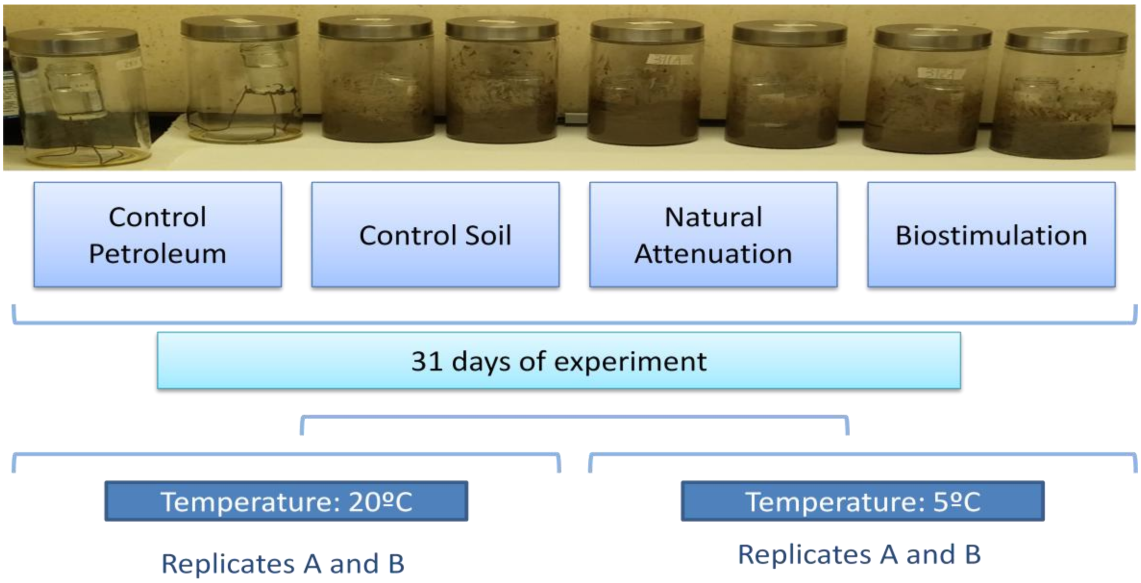

Fig. 6 Set up of experimental scenarios: Control Soil (Cs), Control Petroleum (Cp), and polluted soils with the treatments of Natural Attenuation (N) and Biostimulation (B).

Experimental temperatures were specified according to climatic data of Canadian stations. The average temperature of the Canadian humid continental climate (Dfb) for June, July and August is 17ºC ± 2.0 (Annexe I). On the other hand, the average temperature in Arctic climate (ET) in the most northerly Canadian stations for July and August is 4.6ºC ± 1.8 (Annexe I).

Fig. 7 Example of clayey compaction during homogenization.

The soil of experimental scenarios was previously dried at an average temperature of 25.0ºC. Soil was homogenized, sieved and again homogenized. It was sieved to <5mm instead of <2mm to avoid soil compaction processes (Figure 7).

Experimental scenarios were prepared in 1.5L precleaned (with acetone and hexane) wide neck glass bottles sealed with caps with Teflon liners (Whyte et al. 2001). Then, 500g of soil were deposited in a glass jar for each experimental scenario. Water holding capacity was adjusted to 60% for 72 hours prior to the experiment (Sabaté et al. 2004) by means of relative humidity (Kos & Leštan, 2004). An open glass flask with 25ml H_2O was placed inside each biopile to minimize the humidity loss.

Natural attenuation and biostimulation scenarios were polluted in day 0 of experiment with petroleum “Tar Sands´ Synthetic Crude CNRL”.

Petroleum pollution was adjusted to 10000 mg kg^{-1}. Such pollution was determined to reach average pollution in other case studies (Siegrist et al. 1994; Jørgensen et al. 2000; Whyte et al. 2001; Sabaté et al. 2004; Greco et al. 2010 and Gallego et al. 2011) and in Lac-Megantic´s spill (Golder Associés Lté, 2013) (Table 1).

Table 1 Study cases of polluted soils by hydrocarbons in bibliography. Lac-Megantic´s data is shown in Annexe II.

Case Study	TPH (mg/kg)	Pollutant
Gallego et al. 2011	5 000	Jet Fuel
Greco et al. 2010	>10 000	Light crude oil
Jorgensen et al. 2000	1 950	Mineral oil
Sabate et al. 2004	21 405	Mineral oil / Petroleum product
Siegrist et al. 1994	8 920	Diesel
Whyte et al. 2001	4 641	Diesel Fuel
Mean study cases	**8 653**	
Mean Lac-Megantic (Golder Associés Lté, 2013)	**13 525**	Non-conventional Petroleum
Median Lac-Megantic (Golder Associés Lté, 2013)	**8 600**	Non-conventional Petroleum

2.5 Monitoring

Analyses were conducted to determine the potential of biodegradation of the studied bioremediation methods. The cycle of analyses was 31 days as pointed out (Figure 8). The TPH and microbiological analyses were done in days 1 and 31. Respiration rate in the studied scenarios was monitored each 72 hours. The total amount of analyses was 26 analyses for TPH, 528 for respiration and 288 for microbiology.

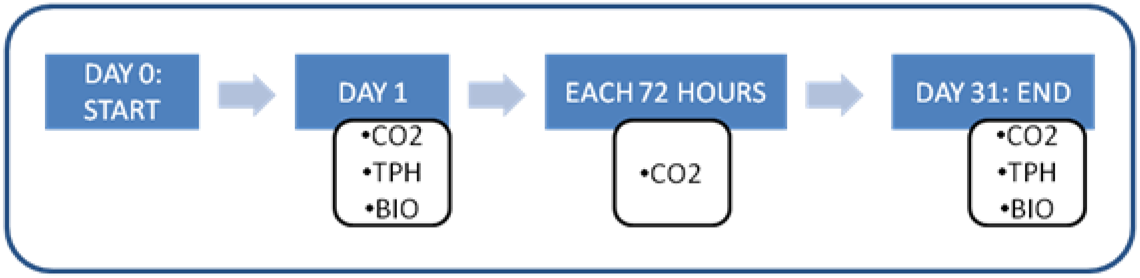

Fig. 8 Analyses done in the experiment: respiration (CO_2), Total Petroleum Hydrocarbon (TPH) and microbiological (BIO). Respiration (CO_2) was monitored by titration. TPH was measured by means of C10-C50 content in soil according to standard methodology in the Province of Québec (Canada) (CEAEQ, 2013). Microbiological analyses (BIO) were done by plate counting.

2.5.1 Respirometric Analysis

Respirometry analysis based on CO_2 production was employed to determine, indirectly, the degradation of hydrocarbons; the basis is that the produced CO_2 is linked with the aerobic degradation of organic compounds (organic matter and hydrocarbons) (Jørgensen et al. 2000 and Chang & Ghoshal, 2014).

For that purpose a CO_2 trap (including 50mL 0.2M NaOH) was set to each scenario (natural attenuation and biostimulation) and controls.

The quantity of produced CO_2 was measured in day 1, each 72 hours after the first day and in day 31. The frequency was determined taking into consideration that the CO_2 trap must be replaced periodically (Knoepp & Vose, 2002). During the replacement, glass jars were opened between 15 minutes and 60 minutes to ensure enough amount of Oxygen for degradation (ASTM, 2012).

$$\mathrm{CO_2 + 2\,NaOH(0.2M)} \Rightarrow \mathrm{Na_2CO_3}$$
$$\mathrm{Na_2CO_3 + BaCl_2(1M)} \Rightarrow \mathrm{2\,NaCl + BaCO_3 + NaOH}$$
$$\mathrm{NaOH\ (remaining) + HCl\ (0.1M)} \Rightarrow \mathrm{NaCl + H_2O}$$

Fig. 9 Chemical reactions during the determination of CO_2 produced by soil (Belloncle et al. 2012).

Each analysis was done in triplicate. The principle was that NaOH captures CO_2 producing carbonates (Figure 9). A solution aliquot of 5mL was sampled to add 1mL 1N $BaCl_2$ in order to remove such carbonates. The non-used NaOH was measured by titration with 0.1N HCl, using thymolphthalein as indicator; the color changes from blue to milk white in the end point (Knoepp & Vose 2002; Sabaté et al. 2004; Hopkins, 2007; Belloncle et al. 2012). Chemical reactions during CO_2 determination are shown in Figure 8. The amount of CO_2 was calculated according to Hopkins (2007), as follows:

$$\mathrm{CO_2\ (mol)} = 0.5\ \Big(\underbrace{([\mathrm{NaOH}]\ V_{\mathrm{NaOH}})}_{\text{NaOH at the beginning}}\ -\ \underbrace{([\mathrm{HCl}]\ V_{\mathrm{HCl}})}_{\text{NaOH remaining}} \Big)$$

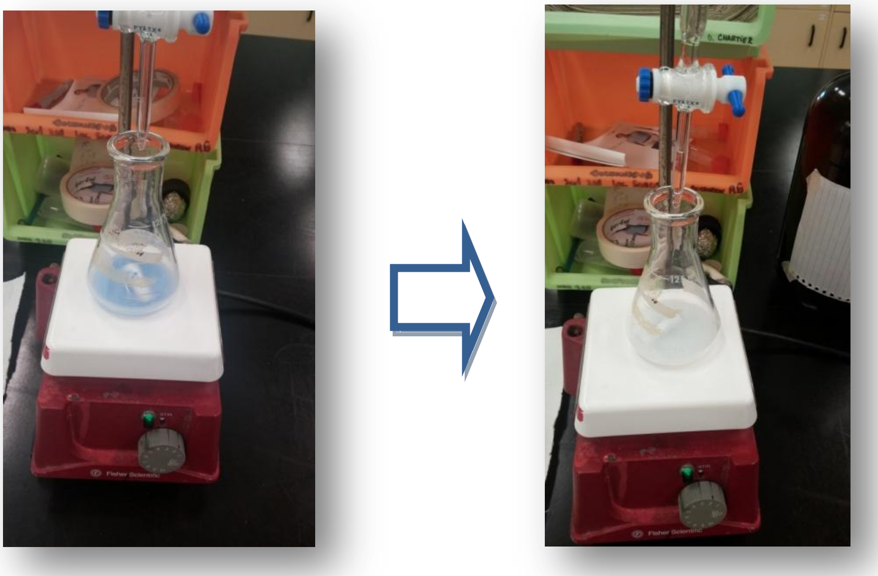

Fig. 10 Titration end point and calculation of CO2 in the trap (Hopkins, 2007).

2.5.2 Hydrocarbon determination

Hydrocarbon pollution involves a complex mixture of compounds. The parameter chosen to determine hydrocarbon in soils was TPH. The TPH could be defined as the sum of the heaviest fraction of extractable hydrocarbons (Rosales et al. 2014). The current study refers as TPH hydrocarbons on the carbon atom range of C10 to C50.

The TPH was determined in each experimental scenario with polluted soils in days 1 and 31 of the experiment. Results from the same scenario were employed to calculate the TPH degradation of each bioremediation method (natural attenuation and biostimulation) as defined in Figure 11.

$$\boxed{\text{Degradation Ratio} = 100 - \left[\left(\frac{\text{TPH}_{\text{DAY 31}}}{\text{TPH}_{\text{DAY 1}}}\right)100\right]}$$

Fig. 11 Definition of degradation ratio, employing the amount of TPH in soil in days 1 and 31.

Samples from each scenario were analyzed by *Maxxam laboratories* according to standard methodology in the Province of Québec (Canada) (CEAEQ, 2013). This method follows a soil extraction with hexane, assisted by ultrasonic bath and a vortex. Finally, soil extract is analyzed through gas chromatography with a flame ionization detector (GC-FID).

2.5.3 Microbiological analysis

Microbial activity may be lower on polluted soils with hydrocarbons than on unpolluted soils (El-Tarabily, 2002). Colony-forming units (CFU) of microorganisms may reduce according to toxicity. Furthermore, a correlation is usually found between TPH removal and the abundance of microorganisms (Aislabie et al. 2006).

For this reason, CFU was employed to find out the variation in abundance of viable microorganisms and their link with TPH degradation.

The method proposed here was oriented to identify the number of CFU of culturable indigenous microorganisms in days 1 and 31 of the experiment. This was applied to each type of scenario (control soil, natural attenuation and biostimulation). Three replicates were done for each analysis. Results were presented in CFU per gram of soil.

Population of viable indigenous microorganisms was monitored using plate-counting methodology, shown in Fig. 12.

Briefly, 1g of soil sample was treated with 10 mL of 0.1% w/v Na_2HPO_4 solution. The mixture was vortexed for 10 minutes. After standing for 2 minutes, serial dilutions in sterile water (autoclaved in MLS-3751, Sanyo Ltd.) were made from this solution.

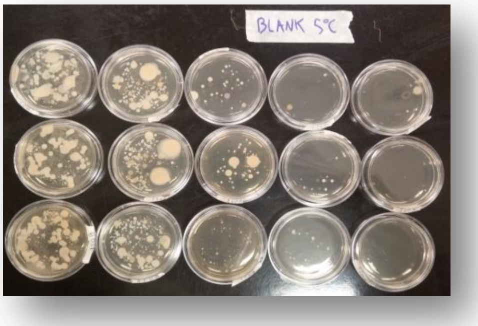

Fig. 12 Example of plate-counting.

The sterile water was composed of 0.9% w/v NaCl in de-ionized water. The growth media was a rich medium designed for most chemo-organotrophic bacteria (3% tryptic soy broth, 0.05% yeast extract, 1% agar) (Gallego et al. 2007).

The duration and temperature of incubation depended on the target branch of microorganisms. Mesophilic microorganisms from the experiment at 20ºC were incubated at 37ºC for 1 day (Gallego et al. 2007).

Psychrophilic and psychrotrophic organisms from experimental scenarios at 5ºC were incubated at the same temperature for 11 days.

2.5.4 Statistical analyses

Three statistics were used to identify significant differences: ANOVA, F and t-Student. One-factor ANOVA´s test was chosen to compare results between the studied scenarios.

Student´s t test was employed to compare one scenario in days 1 and 31. The variety of one-tail was applied to results of TPH, CFU and pH, considering their decrease with the treatments. The variety of two-tails was used only in the respiration data which was expected to increase due to the addition of carbon sources to microorganisms. The equality of means was tested with F-test prior to the application of t-Student.

Only significant differences were considered: p<0.05 for statistics ANOVA, test F and one-tail t-Student and p<0.025 for two-tails t-Student.

2.6 Soil analytical methods

Soil characterization was done in unpolluted soil before starting experiments, except pH which was also measured after 31 days of experiment. Physicochemical analyses determined soil moisture, pH, electrical conductivity, total organic carbon (TOC), available P, total element content, and particle-size distribution.

Soil moisture and total organic carbon (TOC) were measured in triplicate (Figure 13). The former were measured by drying 24 hours at 105ºC (Jørgensen et al. 2000 and Kos & Leštan 2004) and the latter by loss-on-ignition of grinded soil at 550ºC for 4 hours (Heiri et al. 2001 and Santisteban et al. 2004).

A) B)

Fig. 13 Determination of soil moisture (A) and organic matter (B).

Total carbon (TC), sulphur (TS) and nitrogen (TN) contents were determined in duplicate using a CNS LECO analyzer (Figure 14). Briefly, grinded samples are placed in an oven at >700ºC, combustion gases are directed to detectors and total elements quantified by infrared detector (in case of TC and TS) or thermal conductivity detection (in case of TN) (Kowalenko, 2001).

Fig. 14 CNS LECO Analyzer.

Soil pH was measured in four replicates prior to the experiment and in duplicate after its completion (including control soil) (Figure 15). pH determination was done in a suspension of soil and de-ionized water (1:2.5) with a glass electrode (ISRIC, 2002).

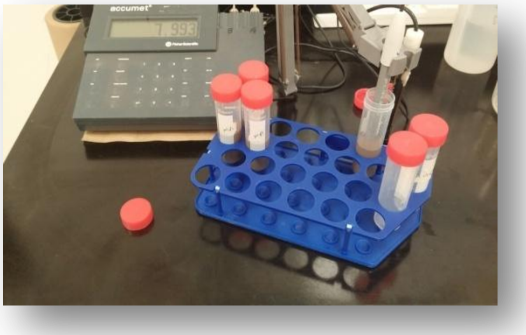

Fig. 15 Measuring the pH at the end of the experiment.

Electrical conductivity was determined in a suspension of soil and de-ionized water (1:5) in eleven replicates (ISRIC, 2002).

Total element contents were determined in triplicate by flame atomic absorption spectroscopy (FAAS) (AA240FS, Varian Inc.) after wet digestion of unpolluted grinded soil with nitric acid (HNO_3) and perchloric acid ($HClO_4$) (Figure 16) (Barnhisel & Bertsch, 1982).

Fig. 16 Digestion of soils with nitric and perchloric acid.

Available phosphorus was measured in triplicate in non-grinded soil samples. Briefly samples were extracted with $NaHCO_3$ and then phosphorous in supernatants was quantified by colorometry (Hach spectrometer) following the Olsen-method (Figure 17) (SA & SSSA, 1982 and ISRIC, 2002).

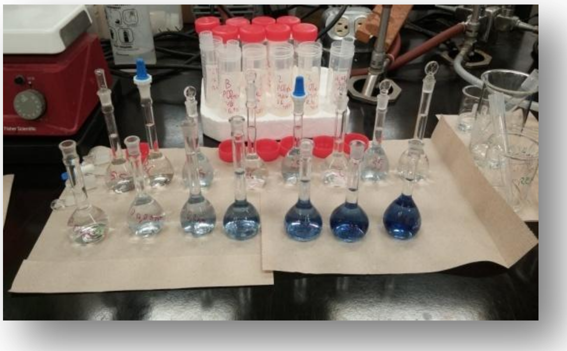

Fig. 17 Standards and soils extracts ready for the determination of P Olsen.

Particle-size distribution analysis was conducted in three phases. It started with the removal of organic matter using 30% H_2O_2 according to ISRIC (2002). Then, the fraction of coarse sand (0.5-2mm) was separated by sieving (ISRIC, 2002). Finer particles (<0.002-0.5mm) proportion was measured with Mastersizer Microplus (Malvern Instruments Ltd), based on laser diffraction principle.

All chemicals and reagents were analytic grade from *Fisher Scientific Inc.* (Canada) and *EMD Chemical Inc.* (USA). All glassware used was rinsed with nanopure water (Ultrapure Water System, Barnstead Nanopure).

2.7 Quality Control

In order to ensure the quality of measures, the following actions were taken: (1) for respiration monitoring, CO2 traps were filled with 0.2M NaOH which contained <0.065 m mol in 50 mL, (2) for microbiological analyses, sterility controls were done prior to analyses to avoid interferences, and (3) for TPH content in soil, analyses were subjected to *Maxxam laboratories* ´ quality controls according to standard methodology in the Province of Québec (Canada) (CEAEQ, 2013).

3. Results

3.1 Soil physicochemical characterization

The pH values from the initial soil were moderately alkaline according to USDA (2001) (Table 3). Soil pH KCl values were lower than pH H_2O, ~0.4units less. The characterization revealed moderately high levels of organic matter (OM) but very low contents of nutrients N and P according to the ratio C:N:P (Sabaté et al. 2004 and Gallego et al. 2011). The C/N values were too high and difficult to interpret given the anthropic nature of the OM in urban soils, which is often added (organic wastes, ashes) (Lehmann & Stahr, 2007).

The values of electrical conductivity (0.18ds m^{-1} (1 ds m^{-1} = 1000 µS cm^{-1})) corresponded to the class of non-saline soil. This level of salinity was correct for the growth of soil microorganisms (USDA, 2001).

A high content of cadmium (3.04 mg kg^{-1}soil) was detected when considering the average content in urban soils ranges about 0.3 to 0.5 mg kg^{-1} soil (Jennings & Petersen, 2006 and Ajmone-Marsan & Biasioli, 2010).

Table 2 Characterization of the initial soil. The chemical parameters were: soil humidity, pH (pH H_2O), potential acidity (pH KCl), Electrical conductivity (EC), available Phosphorous (P), Total organic carbon (TOC), Total inorganic carbon (TIC), Total nitrogen (TN) and ratio of organic Carbon, Nitrogen and available Phosphorous (C:N:P). The trace elements measured by Flame atomic absorption spectroscopy (FAAS) were Copper (Cu), Zinc (Zn), Lead (Pb), Nickel (Ni), Cadmium (Cd) and Chrome (Cr). SD = Standard Deviation.

		Mean		SD
Chemical parameters	**Humidity (%)**	**11,33**	±	**1,28**
	pH (H_2O)	**8,06**	±	**0,03**
	pH (KCl)	**7,61**	±	**0,03**
	EC (dS m^{-1})	**0,184**	±	**0,06**
	P (mg kg^{-1})	**11,89**	±	**0,57**
	TOC (%, w/w)	**3,33**	±	**0,58**
	TIC (%, w/w)	**1,10**	±	**0,58**
	TN (%, w/w)	**0,044**	±	**0,00**
	Ratio C:N:P	**75,76 : 1 : 0,03**		
Trace elements	**Cu (mg kg^{-1})**	**34,17**	±	**0,72**
	Zn (mg kg^{-1})	**65,35**	±	**1,60**
	Pb (mg kg^{-1})	**25,76**	±	**1,62**
	Ni (mg kg^{-1})	**24,23**	±	**3,97**
	Cd (mg kg^{-1})	**3,04**	±	**0,06**
	Cr (mg kg^{-1})	**12,17**	±	**0,71**

3.2 Degradation rate

The TPH degradation differed depending on temperature (Table 4). The TPH degradation was statistically observed at 20ºC, being closer to 20%. At this temperature, natural attenuation and biostimulation had a similar degradation rate.

At 5ºC the degradation rate was lower for both methods with statistical distinction from control soils. The TPH degradation was about 2% for natural attenuation and 12% for biostimulation. In contrast to the behavior at 20ºC, biostimulation was the method with higher degradation rate.

Table 3 Degradation of Total Petroleum Hydrocarbons (TPH) by natural attenuation and biostimulation at temperatures of 20ºC and 5ºC for one month. Results are statistically grouped with letters.
SEM= Standard Error of the Mean.

Mean TPH (mg kg^{-1})						TPH Degradation (%)
Temperature	**Type of scenario**	**Day 1**	**SEM**	**Day 31**	**SEM**	
20ºC	Control Soil	100	± 0	100	± 0	- $^{(b)}$
	Natural Attenuation	6300	± 100	5125	± 409	**18.7** $^{(a)}$
	Biostimulation	6850	± 150	5500	± 300	**19.7** $^{(a)}$
5ºC	Control Soil	135	± 5	115	± 7	- $^{(c)}$
	Natural Attenuation	6650	± 150	6500	± 200	**2.3** $^{(b)}$
	Biostimulation	7400	± 100	6500	± 200	**12.2** $^{(a)}$

3.3 Respirometric results

The trend over time of CO_2 released from soil showed an increase up to 4 days in all the experimental scenarios at both temperatures and up to 6 days in the case of natural attenuation at 5ºC (Figure 18). The increase period was followed by a decrease up to 10 days.

Between days 10 to 31 at 5ºC, the release of CO_2 from soil was constant in all the scenarios.

At 20ºC the CO_2 released from soils was steady in days 10 to 22 in scenarios of natural attenuation and controls. In the same period, CO_2 released from soils with biostimulation had an augmentation with another maximum at day 19. After day 22 at 20ºC, the release of CO_2 decreased slightly for all the experimental scenarios.

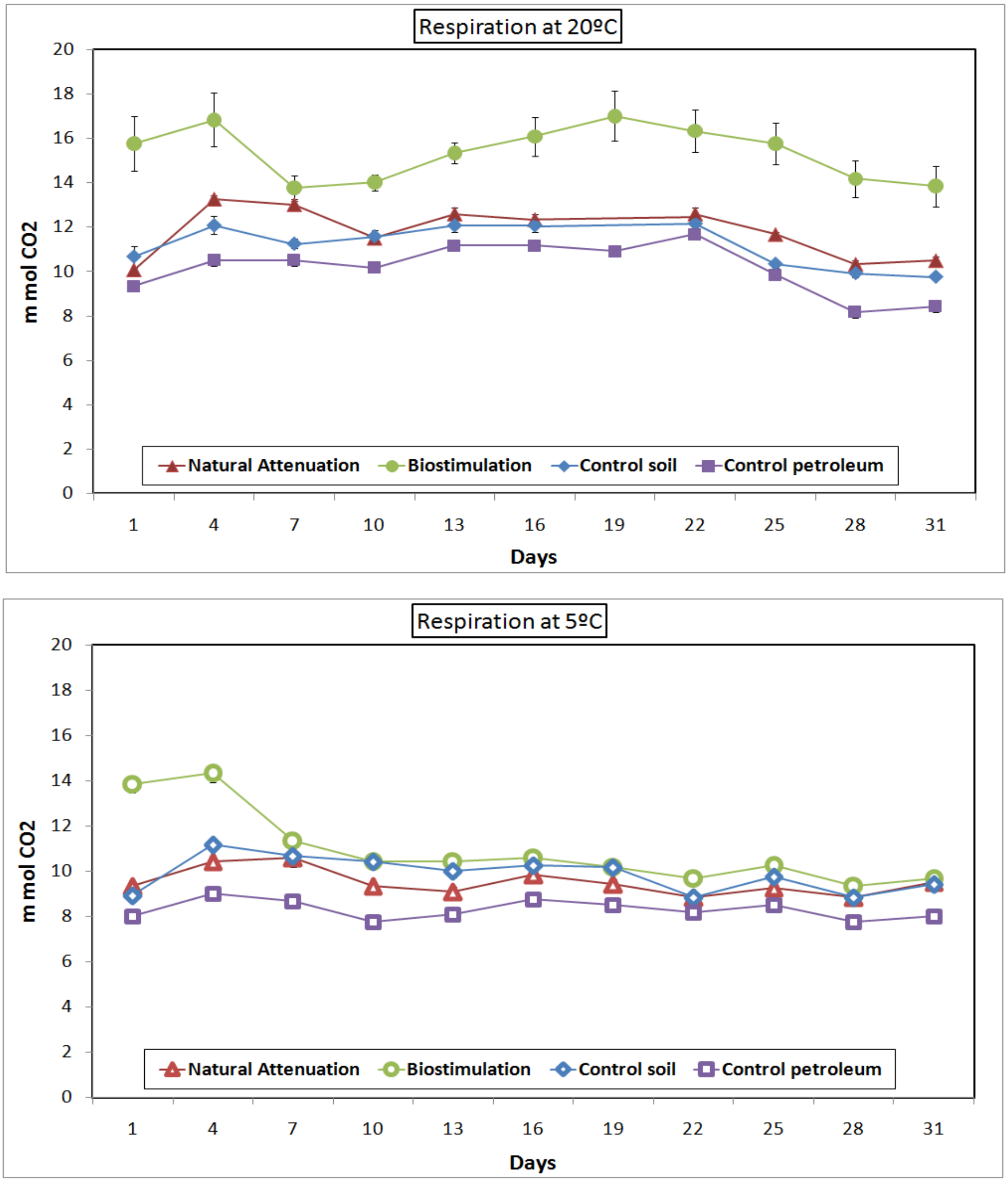

Fig. 18 Release of CO_2 in experimental scenarios (natural attenuation, biostimulation and controls) at 20ºC and 5ºC during the extent of the experiment.

Considering the cumulative release of CO_2 after 31 days of experiment, soils from scenarios at 20ºC released significantly more than at 5ºC (Table 5). Biostimulation technique released more CO_2 from soil ($p<0.05$) than natural attenuation at both temperatures.

At 20ºC, soil from biostimulation scenario released, significantly, 39.6% more CO_2 than control soils. Soils from scenarios of natural attenuation had the same release as controls.

At 5ºC, soils from biostimulation technique released 15.1% CO_2 greater than control soils. The released CO_2 from soils with natural attenuation was similar to control soils. At this temperature, soil controls released 15.9% CO_2 more than petroleum controls except in days 1, 22 and 25.

Table 4 Cumulative CO_2 released in experimental scenarios after 31 days of experiment. Results are statically grouped with letters. SEM= Standard Error of the Mean.

Experimental Scenario	10^-2 m mol at 20ºC	SEM	10^-2 m mol at 5ºC	SEM
Natural Attenuation	**127.8** (b)	**± 2.17**	**104.4** (b)	**± 3.58**
Biostimulation	**168.8** (a)	**± 20.83**	**124.2** (a)	**± 2.17**
Control Soil	**120.9** (b)	**± 3.92**	**107.9** (b)	**± 3.25**
Control Petroleum	**112.5** (c)	**± 1.5**	**91.2** (c)	**± 2.5**

3.4 Affection to microbiological populations

Abundance of culturable mesophilic microorganisms was significantly reduced one day after the addition of petroleum, with higher impact on soils with biostimulation technique (Figure 19). In day 31 of experiment, the number of CFU mesophiles was similar in all the studied scenarios.

During days 1 to 31, abundance in soils of scenarios with natural attenuation did not vary, statistically increased in scenarios with biostimulation, and decreased in control soils.

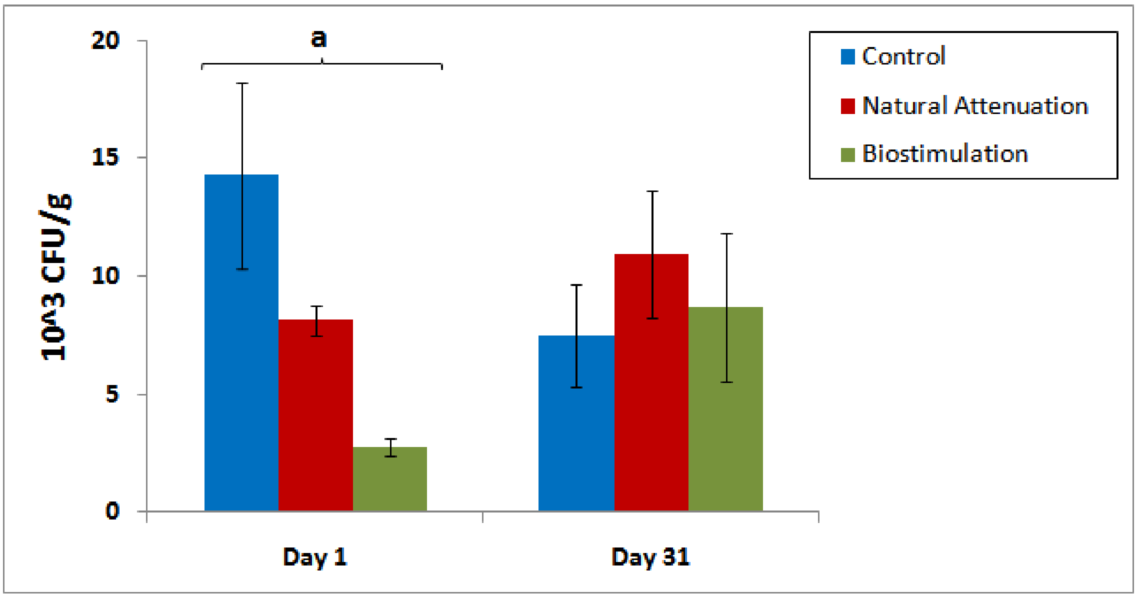

Fig. 19 Number of total viable colony forming-units (CFU) of mesophiles per gram of soil in experimental scenarios at 20ºC. (A) Significance between experimental scenarios.

Contrary to the response of mesophilic populations, psychrotolerants were by far more affected (Figure 20). Biostimulation and natural attenuation had a significant reduction in days 1 and 31 respecting control soils.

Considering the same kind of scenario, viable CFU of psychrotolerants were substantially diminished in one month of experiment for all the experimental scenarios.

In addition, psychrotolerants abundance decreased in controls as occurred with mesophiles in the given experimental conditions.

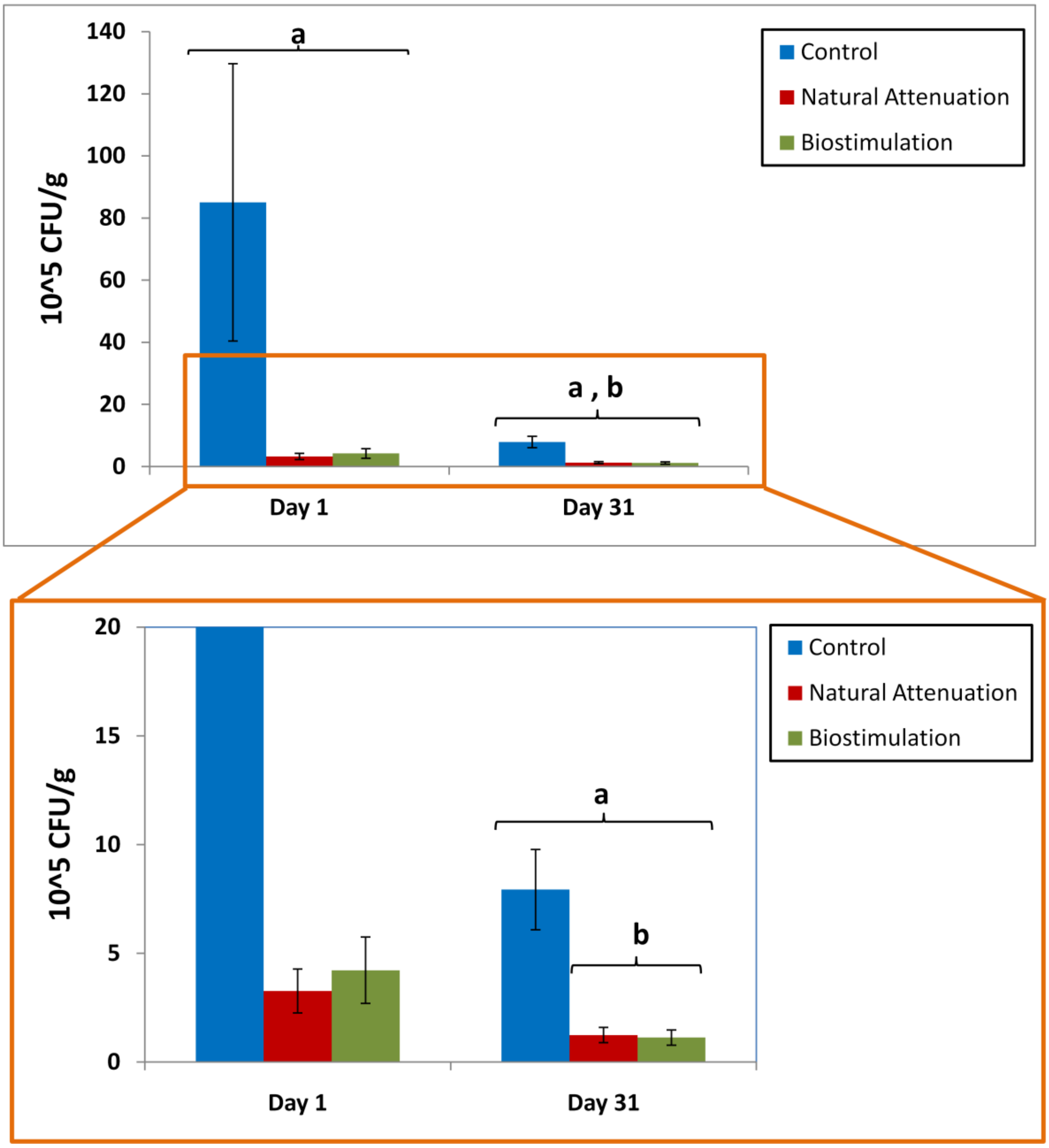

Fig. 20 Abundance of psychrotolerants (psychrophiles and psychrotrophs) in experimental scenarios at 5ºC. Results expressed on total culturable colony forming-units (CFU) per gram of soil. Levels of significance: (A) between scenarios in the same day and (B) the same scenario between days 1 to 31.

3.5 Changes in pH

The measured pH in soil varied after 31 days of experiment with respect to values from the initial soil (Figure 21). Soil values of natural attenuation and biostimulation decreased towards neutrality at the end of the experiment in comparison with observed values in the control. Such decrease was significant in studied temperatures except in the case of natural attenuation at 20ºC, which was not significantly different from the controls.

Control soils had an increase at the end of the experiment in the given experimental conditions respect from the pH of initial soil.

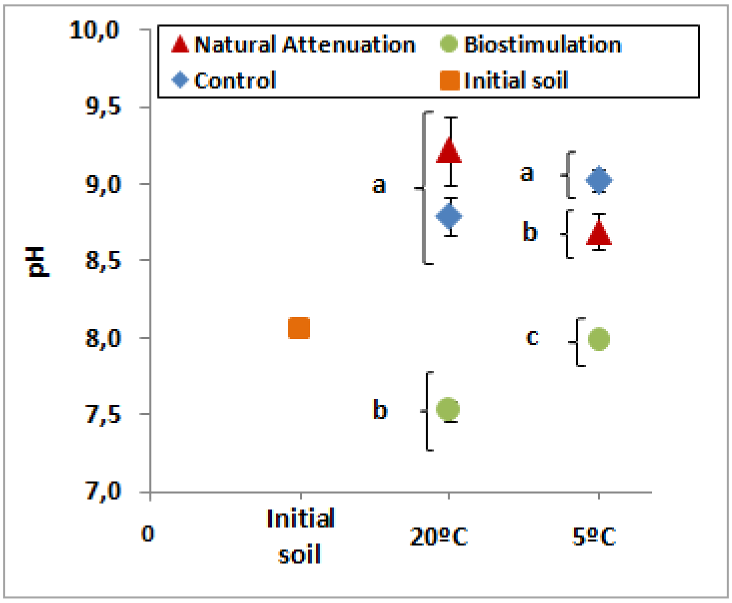

Fig. 21 pH of replicates in the soil prior to the experiment and in experimental scenarios at 20ºC and at 5ºC after 31 days of treatment. Statistically similar data are grouped by letters.

4. Discussion

4.1 Biodegradation

In our experiment biodegradation of petroleum was observed despite soil characteristics that were not optimal in terms of nutrients and heavy metals content.

Soil characterization revealed that nutrients did not reach the desired ratio C:N:P of 100:10:1 (Sabaté et al. 2004 and Gallego et al. 2011).

Concerning the chemical parameters of the soil, the content of cadmium was 10 times greater than the average in urban soils as previously discussed. Cd could have been accumulated from batteries, tires, petrol, diesel fuel, lubricating oil, pigments, plastics or from plated or coated materials with Cd (Ajmone-Marsan & Biasioli 2010). It is important to note that pollution is determined by normative. For instance, the measured Cd did not comply with the limit values of 1 mg kg^{-1} for residential use in the UK (Ajmone-Marsan & Biasioli 2010) but it did according to the 5 mg kg^{-1} for the same use in the Province of Quebec (Ge et al. 2000 and Madrid et al. 2002) or to the 10 to 35 mg kg^{-1} in the rest of Canadian Provinces (Jennings & Petersen, 2006). Despite the excessive concentration of Cd on the studied urban soil, hydrocarbon biodegradation took place. Heavy metals could not be a limit for TPH biodegradation (Mohn & Stewart, 2000). Instead, it could be more important to have the right abundance of adapted microorganisms to local conditions. In fact, the minimum number of three orders of magnitude in forming-colony units was fulfilled whether for mesophiles or psychrotolerants (Gallego et al. 2011).

On the other hand, hydrocarbon biodegradation was observed in spite of the large pollution of non-conventional petroleum. Reported in the laboratory was that high concentrations of TPH did not prevent their degradation by microorganisms (Mohn & Stewart, 2000). Furthermore, soils with high levels of petroleum pollution (around 8100 mg kg^{-1}) are easier to treat than old residual concentrations in soils (500 mg kg^{-1}) (Walworth et al. 2001). Contrary to logical thinking, large episodes of fresh pollution contain compounds more biodegradable such as alkanes than recalcitrant hydrocarbons (e.g. aromatics), as occurred in weathered spills (Coulon et al. 2005 and Aislabie et al. 2006).

The TPH removal of the studied pollution showed a divergence in behavior depending on the temperature. The extent of degradation dropped from 20% in the first month at 20ºC to 12% at 5ºC. TPH removal may be lower at 5ºC due to the fact that petroleum is more viscous, less volatile and less bioavailable for microorganisms as long as temperature decreases (Coulon et al. 2005). Although degradation of hydrocarbons usually follows a degradation of first order (Margesin & Schinner, 1997 and Chang et al. 2010), the light increase in removal at 20ºC could be explained with the point that temperatures from 4ºC to 10ºC tend to have non-linear degradation (Chang et al. 2011 and Coulon et al. 2005).

Biostimulation was the only scenario which showed degradation at 5ºC. Indeed, biostimulation had a greater degradation rate than natural attenuation in other cases in cold environments (Margesin & Schinner, 1997; Mohn & Stewart, 2000 and Whyte et al. 2001). The standard practice in hydrocarbon remediation is the addition of nutrients so as to reach in soil the ratio C:N:P closer to microorganisms requirements. Amendments of nutrients should stimulate degradation during the duration of the treatment (Sabaté et al. 2004 and Gallego et al. 2011). However, in the current study biostimulation had the same degradation rate of natural attenuation at 20ºC.

In general terms, the lack of response in biostimulation is associated to a poor understanding of fertilizers. Some fertilizers can provide enough amount of N or P at the beginning but they may become more and more unavailable if their solubility is low (Bento et al. 2005). This option can be discarded given the fact NH_4NO_3 and K_2HPO_4 have proved to provide a right source of N and P (Sabaté et al. 2004) and that their effect was substantial at 5ºC. Then, the fact that the addition of macronutrients N and P did not accelerate degradation at 20ºC could be due to a limitation in micronutrients. In this sense, mesophilic microorganisms could be limited by trace substances contained in the petroleum (Nyman, 1999) or there could be a lack of aminoacids, vitamins and other organic molecules that are indispensable for these organisms (Yang et al. 2009).

4.2 Respiration

Respiration of soil is considered an indicator of microbial activity. In other studies, biostimulation had a notorious increase of respiration directly linked with degradation (Walworth et al. 2001; Siddiqui & Adams, 2002 and Sabaté et al. 2004).

Results should have showed the same respiration for both methods at 20ºC and an increase in biostimulation at 5ºC to fit with the degradation results. Far from this expectation, release in biostimulation was substantially higher at 20ºC and only significant in days 1 to 4 at 5ºC.

The lack of correlation of respiration with degradation might be associated to unspecific degradation of the added organic pollutant (Jørgensen et al. 2000). In addition, part of the respiration may proceed from soil organic matter (Nyman, 1999). Therefore, the higher respiration of bioremediation at 20ºC and the observed respiration of natural attenuation at 5ºC may have come from organic carbon in the soil.

On the other hand, hydrocarbon pollution often has a period immediately after spills where there is no an increase in released CO_2 with respect to unpolluted soil (Mohn & Stewart, 2000 and Siddiqui & Adams, 2002). Such lag phase can extend for several days depending on the petroleum product. Results showed that natural attenuation at 5ºC may be in the lag phase for 31 days of the experiment. However, natural attenuation at 20ºC had a lag time lesser than 4 days, and lesser than one day in the case of biostimulation at both temperatures. Therefore, the addition of nutrients and temperature may reduce lag time in episodes of pollution with non-conventional petroleum. In fact, some authors found that lag time was reduced with amendments of macronutrients such as N and P (Mohn & Stewart, 2000) and with bioaugmentation (addition of microorganisms) (Jørgensen et al. 2000). However, inoculation of autochthonous degraders might be employed rather than commercial strains in case the soil did not have enough microorganisms to remove TPH (Aislabie et al. 2006).

4.3 Microbial population

Hydrocarbon degraders are a heterogeneous group composed of bacterial and fungal species. Degraders are expected to be at least 10% total microorganisms in soil (DOE/PERF, 2002). Bacteria are the dominant degraders in bioremediation of petroleum products (Siddiqui & Adams, 2002).

In polluted soils, the abundance of mesophilic organisms were reduced at the start with a greater reduction in treated soils with biostimulation. After one month, natural attenuation and biostimulation methods enhanced microbial culturable populations to levels of unpolluted soils. Such an increase in the CFU of microorganisms may be joined to TPH removal (Coulon et al. 2005). In spite of the increase in abundance of culturable mesophiles, microorganisms diversity was probably reduced in polluted soils, with special effect in those treated with biostimulation (Bell et al. 2013). In fact, degrader populations could have been enhanced since disruptive petroleum effects might be reduced competitors of degraders such as bacterivores and other bacterial competitors (Bento et al. 2005).

Regarding psychrotolerant microorganisms, case studies in Arctic areas observed an increase in total viable CFU in biostimulation and a significant removal of TPH (Aislabie et al. 2006). Nevertheless, the abundance of psychrotolerants from the current study were discordant, being diminished in polluted soils in days 1 and 31. The decrease of psychrotolerants might be due to the toxicity of the non-conventional petroleum which would not follow the expected trend of less toxicity at 4ºC than at 20ºC. However, it is noteworthy that remediation methods tend not to reduce toxicity owing to subproducts originated in the partial degradation of aromatic compounds (Coulon et al. 2005).

4.4 pH

Another factor to take into account before conducting bioremediation is pH. It needs to be adjusted into optimal ranges to guarantee the action of microorganisms. The advised range is about 6.5 to 8 in temperate temperatures and higher than 8.8 in polar soils (Aislabie et al. 2006). According to the obtained results, the pH complied with the optimal ranges. Further, soil controls registered an increase in pH of one unit and polluted soils decreased towards the neutrality.

In bibliography, pH was reduced from 8.1 to 7.7 in soils affected by a break of a pipeline which transported crude petroleum. Such results were described in soils with recent pollution and also in bioremediated soils (Li et al. 1997).

The decrease towards neutrality could be explained by added nutrients. In addition to that fact, it is thought that acidification could be produced owing to remediation subproducts such as aliphatic acids derived from alkane degradation (Aislabie et al. 2006).

5. Conclusions

(1) Spills of non-conventional crude petroleum can be treated by bioremediation in affected urban soils. Natural attenuation and biostimulation proved to be low-budget, easily applied methods that can substitute for more aggressive methods to soil ecosystems such as capping and ex-situ treatment.

(2) Natural attenuation can be recommended to remediate polluted urban soils at temperate temperatures as it had the same degradation extent of biostimulation. In other words, in one month of experiments, soil microorganisms did not require fertilizers to perform degradation of total petroleum hydrocarbons (TPH) during summer temperatures in the Canadian humid continental climate (Dfb).

(3) Biostimulation (Fertilization) was the best strategy to manage polluted urban soils when the temperature dropped below 10ºC. Psychrotolerants (active microorganisms at these temperatures) showed a limitation of macronutrients N and P which was overcome with the use of simple mineral fertilizers (e.g. NH_4NO_3 and K_2HPO_4). It would be useful to study the effectiveness of other commercial fertilizers or of other common methods in the field such as bioventing.

(4) In spite of high concentrations of TPH and heavy metals (Cadmium), petroleum biodegradation was observed. This may not be inconvenient for restoration of soil ecosystems if the soil contains enough microorganisms adapted to local conditions.

(5) It would be interesting to work with the studied bioremediation methods in small experimental plots so as to extrapolate results to the restoration of polluted soils in the field.

(6) Further research is needed to understand whether non-conventional petroleum has disruptive effects on soil microorganisms, with more emphasis on psychrotolerants. Microbiological analyses and a set of toxicity assays should provide more information to determine if bioremediation methods require additional treatments in cold temperatures.

(7) Further studies are required to explain if microorganisms at >10ºC could be limited whether by trace substances contained in non-conventional petroleum or by a lack of indispensable molecules (e.g. vitamins or aminoacids).

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To conclude, I would like to dedicate my last words of acknowledgement to Luis Balaguer. He was a genius in Restoration Ecology, a day dreamer who believed in turning dreams into reality. Thanks for inspiring me to restore in each dimension of my life.

Una palabra de belleza, una bella palabra. En tus labios cualquier palabra es un placer de escuchar, de retener. Desde un tímido hola hasta el más fatídico adiós, que es la tempestad, la mentira y el miedo de perderte.

No importa. Son tuyas tus magníficas palabras. Magníficas, bellas porque ellas son musas que provienen de tu boca de diosa. El invierno comienza cuando la cierras, la primavera en el momento que está abierta.

Mas, mas, nadie te recuerda salvo mi mancillado cuerpo.

Abre los labios y las flores viven. Cierra la Luna y da la bienvenida a la desolación y al frío. Tengo miedo de escucharte silenciosa. Un sonido, una palabra, un verbo, son maravillas para mi ser, para el fondo de mi alma.

Pero, si tú sonríes, todo esto no deja más que ser un sueño.

Canción de un terrestre a la Tierra, Jesús Díaz-Sanz

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8. List of figures

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