Hydrocarbons removal from real marine sediments: analysis of degradation pathways and microbial community development during bioslurry treatment

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ABSTRACT

In this study, real marine sediments polluted by petroleum compounds were treated by means of a bioslurry pilot scale reactor. The treatment performance was evaluated by measuring the removal of total petroleum hydrocarbon (TPH), coupled to further analyses required to understand the mechanisms involved in the biodegradation process. The maximum TPH-removal efficiency reached 86% at the end of experiments. Moreover, high throughput 16S RNA gene sequencing was used to describe the microbiome composition in sediment prior to, and after, bioslurry treatment, in order to identify the taxa mostly entailed in the TPH removal process. The raw sediment was mostly colonized by members of *Sulfurimonas* genus; after bioslurry treatment, it was noticed a shift in the of the microbial community composition, with *Proteobacteria* phylum dominating the remediation environment (high increase in terms of growth for *Hydrogenophaga* and *Sphingorhabdus* genera) along with the *Phaeodactylibacter* genus (*Bacteroidetes*). Furthermore, the assessment of gaseous emissions from the system allowed to quantify the volatile hydrocarbon component and, consequently, to obtain a more accurate evaluation of TPH-removal pathway by the bioslurry system. Finally, phytotoxicity tests on sediment samples highlighted an increase of the treated sample quality status compared to the untreated one.

KEYWORDS: microbial community; hydrocarbon pollution; bioremediation; contaminated marine sediment; slurry reactor.

1. INTRODUCTION

Contaminated marine sediments poses a serious threat to ecosystems and human health, representing a potential reservoir for the release of toxic compounds as they are often subject to anthropogenic inputs, especially in industrial areas close to the coastaline **(Rocchetti et al., 2012; Maletić et al., 2018; Avona et al., 2022**). Sediment contamination is mainly due to the discharge of urban or industrial waste streams entailing different environmental issues (**Labianca et al., 2022**). Petroleum hydrocarbons (HCs) represent a class of contaminants of particular concern, since some of them are mutagenic, carcinogenic, teratogenic and might also induce sterility in marine organisms (**Zhou et al., 2014**). Moreover, HCs can move for long distances in water due to hydrophobicity and persistency, and can accumulate within sediments for long time periods thus becoming a potential release reservoir and representing an environmental threat for aquatic organisms and a public health risk **(McGenity et al., 2012; Maletić et al., 2018**). In this light, the remediation of polluted sediments has become an imperative need and several remediation techniques have been developed in the last years, with the aim to remove HCs from sediments. They can be classified either by nature (thermal, physicochemical, chemical or biological treatment) or by place (*in-situ* or *ex-situ*). Generally, chemical and thermal treatments are more expensive compared to biological treatments as well as less eco-friendly (**Pino-Herrera et al. 2017; Yan et al., 2021**).

As already mentioned, the remediation of polluted sediments can also be accomplished through biological processes, which are based on pollutant degradation by microbial populations able to exploit the organic pollutants as a source of carbon and energy **(Varjani, 2017; Varjani and Upasani, 2017; Usman et al., 2018**). Several literature studies highlighted the advantages of degradation pathways related to microbial activity, thus arousing the interest on biological processes **(Huesemann et al., 2002; Perelo, 2010**). Bacteria revealed to be the prevailing class of microorganisms responsible of hydrocarbon removal via aerobic oxidation (**Zhao and Wong, 2009; Zhao et al., 2011**). In this context, the most abundant taxa found in marine sediments belongs to *Proteobacteria* and *Bacteroidetes* phyla (**Iannelli et al., 2012; Chiellini et al., 2013)**. Previous studies highlighted an increase of *Alphaproteobacteria* and *Gammaproteobacteria* during biodegradation of oily products (**Lamendella et al., 2014)**.

Moreover, the autochthons bacteria associated to these classes showed a sort of adaptation during bioremediation. Specifically, dynamics in indigenous microbial population during bioremediation revealed a shift in the bacterial community composition (**Fuentes et al., 2014; Smith et al., 2015; Fuentes et al., 2016**). This aspect relies on several factors, including the environmental compartment

(eg. soil or marine sediment, freshwater, or seawater), sediment properties (e.g. texture, pH, organic matter, nutrients, temperature), the features of contamination (age and concentration), the indigenous microbial community, and the bioremediation treatment selected **(Kaplan and Kitts, 2004; Hamamura et al., 2013; Fuentes et al., 2014; Fuentes et al., 2016**). Particularly, the bioremediation technology strongly affects the microbial population dynamics, especially for *Proteobacteria* phylum **(Fuentes et al., 2014, Fuentes et al., 2016**).

Bioslurry reactors represent an interesting *ex-situ* technology for the remediation, under controlled conditions, of soils and/or sediments polluted by hydrocarbons. In order to improve the mass transfer rate as well as the contact between microorganisms, oxygen, nutrients and contaminants, the raw matrix is mixed with water in the typical range of 10 to 60% w/v, to get a slurry suspension (**Gan et al., 2009; Pino-Herrera et al., 2017; Lumia et al., 2020; Avona et al., 2022**). In this way, the performance of biodegradation process might be enhanced.

Bioslurry reactors are commonly operated under batch conditions thus entailing higher process rates **(Prasanna et al., 2008; Venkata Mohan et al., 2008**). The used reactors are generally characterized by completely mixed conditions, in order to keep homogeneity of the suspension over time, and equipped with aeration devices to ensure aerobic conditions **(Robles-González et al, 2008**).

Nevertheless, literature studies highlighted the need to consider volatilization among the possible paths for contaminant removal; indeed, a proper mass balance of hydrocarbons, usually expressed as total petroleum hydrocarbon (TPH), inside the system is required to exclude an overestimation of the biodegradation pathway (**Pino-Herrera et al., 2017**). To evaluate the effectiveness of the treatment, it is also useful to perform phytotoxicity tests on the sediment matrix (**Shen et al., 2016**); for matrices contaminated by hydrocarbon compounds, *Lepidium sativum* seeds are considered effective bioindicators, thanks also to the short germination period required **(Maila and Cloete, 2002; Hosokawa et al., 2009**). However, to authors' best knowledge, very few studies on bioslurry technology have been reported in literature so far and even fewer refer to the treatment of real contaminated sediments. For the latter, the available studies usually report the results of laboratory-scale experiences and rarely the results of experiments at pilot plant scale (with volumes greater than 1 litre) (**Pino-Herrera et al. 2017**). In general, there is still a lack of knowledge about the mechanisms governing the process and basic aspects such as microbial diversity, role of bioaugmentation, removal performance during the treatment of real sediment, potential and actual reuse of decontaminated sediments. Basing on the above discussion, and after the preliminary results achieved by authors in a forms study (**Avona et al., 2022**) the aim of the present paper is elucidate the mechanisms involved in the treatment of real marine contaminated sediments through a bioslurry reactor, highlighting the

removal pathways as well as the behavior of the biological community involved in the process. In detail, the paper shows the results achieved during an experimental study carried out on a lab scale bioslurry reactor fed with real TPH-contaminated marine sediments sampled from the Augusta Bay (Sicily, southern Italy). The systems performance has been assessed in terms of TPH removal. Given the site-specific characteristics of the sediments used, a detailed characterization of the microbial community was carried out using the high throughput 16S RNA gene sequencing. Furthermore, the percentage of volatile solids in the sediment, an indicator of bacterial growth, was evaluated. Another aspect examined was the potential toxic effect of sediments towards the biotic compartment, at the beginning and at the end of treatment, by means of phytotoxicity tests. Finally, the contribution in terms of TPH removal due to volatilization was assessed to evaluate the hydrocarbon mass balance in the system. This work provides useful information in view of further research activity aimed at assessing the potential feasibility of this treatment at full scale.

2. MATERIALS AND METHODS

2.1 Sampling and Area of investigation

The marine sediment samples used for the experiments were collected from the northern part of Augusta Bay, near some petrochemical facilities (**Lumia et al., 2020a**). The industrial area of Augusta-Priolo is one of the largest petrochemical areas in Italy and in Europe, characterized by the presence of several industrial plants whose polluting impact pours into the Rada of Augusta (often referred to as "Rada" only), causing considerable contamination of marine sediments. The Rada falls on the eastern coast of Sicily, within the homonymous bay and it develops between Capo Santa Croce and Punta Magnisi, for a length of about 8 km and a distance from the coastline of about 4 km, reaching a surface extension of about 23.5 km^2 and a depth close to 15 m. In the past, part of the natural inlet has been separated from the open sea by the construction of a breakwater, aimed at forming a large port basin communicating with the sea through two narrow mouthpieces (east and south), where the maximum depth is about 30 m b.s.l.

The sediment sampling involved three different locations within the Rada. All samples were mixed to evaluate a statistically suitable matrix as well as to obtain a sufficient volume for the development of the experimental analyses and the start-up of the pilot plant. In particular, basing on a previous campaign (**Lumia et al., 2020a**), the sediment samples were characterized by a medium-high hydrocarbons concentration; the TPH fractions revealed that C_{20}-C_{30} was the fraction mainly adsorbed on sediments (67%) followed by C_{12}-C_{18} (25%), whereas the heaviest one (C_{32}-C_{40})

represented the 8% of the total. The chemical-physical characterization revealed a high organic content (4.2%), moisture content (~44%), and sulphides (~61 mg kg^{-1}). The textural and mineralogical analyses indicated the presence of silt and calcite ($CaCO_3$). The observed salinity was also high (4.4% as pore water salinity concentration). The observed contamination mainly derived from accidental leakage from tanker or pipes as well as from discharge of slop wastewater deriving from washing of oil tankers with seawater.

In a previous experimental study, the sediment sample was subject to microcosm tests followed by a preliminary bioslurry experiment. The main aim was to define the operational conditions of the process and the pre-dimensioning of pilot plant (water-sediment ratio for slurry, potential bioaugmentation and inoculum for start-up, biostimulation need, etc. The results of these preliminary activities can be found in literature (**Avona et al., 2022**).

2.1 Bioslurry reactor and operation

The experimental set-up consisted of a closed cylindrical reactor realized in Polyvinyl chloride with a glass reinforced plastic (GRP) bottom with an overall volume of 30 L (Ø: 31 cm; H: 40 cm), according to what reported in Avona et al. (2022). The reactor was characterized by three baffles (H: 32 cm, W: 5.7 cm, S: 0.3 cm) disposed at 120° and fixed at 4.5 cm from the bottom, based on a former hydrodynamic study, acting as vortex breaker (Supplementary Material). The mixing system was realized with a mechanical stirrer, while the aeration system consisted of three porous cylindrical diffusers placed on the bottom between the baffles and connected to a MEDO LA-45C membrane blower, with a nominal power of 47 W and an airflow rate of 45 L min^{-1} at a pressure of 0.11 bar. The reactor was provided with slurry sampling sockets and closed at the top with a blind flange equipped with a gas sampling section. A schematic layout of the pilot plant is shown in **Figure 1**. For further details and the panoramic view of the experimental set-up, the reader is addressed to literature (**Avona et al., 2022**).

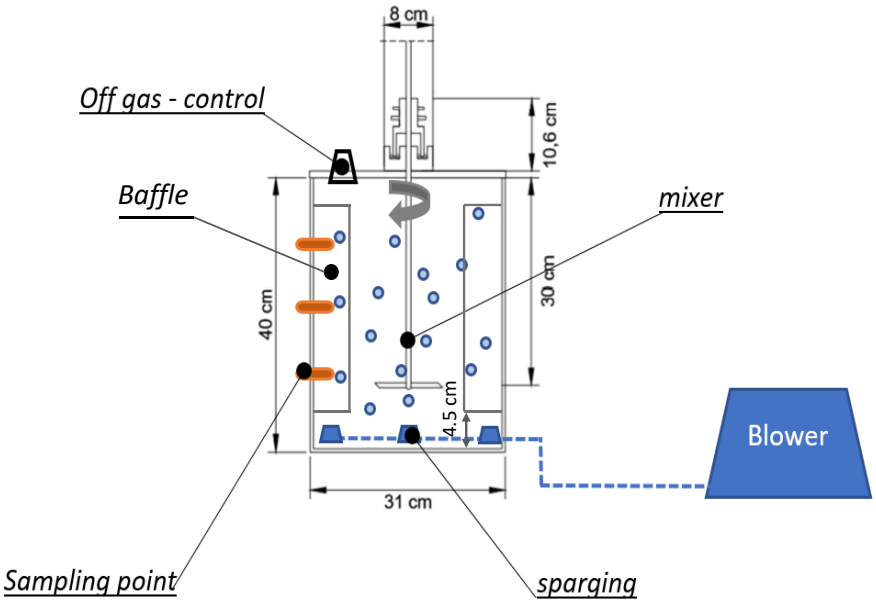

Figure 1 Schematic diagram of the Stirred Tank Reactor for bioslurry application

As above described, the study was conducted using a composite sample of real sediment collected from different points of the Augusta bay, previously characterized and subject to a preliminary evaluation (**Falciglia et al., 2020; Lumia et al., 2020a-b**). In detail, basing on the results observed in the previous experimental period (**Avona et al., 2022**), the sediment sample was achieved by mixing the highly polluted sediments used in the previous period with less polluted ones (even sampled from the "Rada"). The experiment duration was 102 days with a solid-liquid ratio of 10% as weight by volume (w/v) and a stirring speed of 675 ± 25 rpm. As inoculum, 2 L of slurry from the previous experimental campaign (**Avona et al., 2022**) were mixed with the initial sample to speed-up the start-up of the biological process, by shortening the acclimation period and avoiding bioaugmentation with allochthonous bacteria. The initial TPH concentration in the raw sediment sample was set at 1,001 mgTPH $kgDW^{-1}$. The main features of the experimental set-up are summarized in **Table 1**.

Table 1 Main features of the pilot scale configuration.

Slurry volume	V	26	L
Dry weight	M_{DW}	2.6	kg_{DW}
Moisture	θ_w	43.4	%
Solid-liquid ratio	w/w	10	%
TPH concentration	C_{TPH}	1001	$mg_{TPH}\ kg_{DW}^{-1}$
Duration	t	102	Days

2.2 Analytical methods

TPH concentration in the sediment and liquid phases was evaluated using the EPA 8010C and 3510C methods by GC–FID (Agilent 6890), respectively. The solid to liquid hydrocarbons extraction was carried out through EPA 3545A method "Pressurized Fluid Exaction (PFE)" and the "Speed Extractor E-916". Analysis of TPH was carried out in duplicate, and the standard error was computed according to (**Avona et al., 2022**).

The composition of the bacterial community was defined by the high throughput analysis of the V1-V3 region of the 16S rRNA gene. The methodology used for nucleic acid extraction, amplification, genomic library preparation, sequencing on the Illumina MiSeq platform, and bioinformatic analysis, are reported in **Tonanzi et al. (2021)**. Untreated (NT) and after bioslurry (BS) sediment samples were analyzed. Furthermore, the determination of Volatile Solids (VS) on the collected sediment samples was performed according to Standard Methods (APHA, 1998) and then expressed as percentage of total solids (TS).

The germination index (GI) of the liquid supernatant was assessed by using *Lepidium sativum* seeds, after settling of slurry samples (**Avona et al., 2022**). The analyses were conducted on raw and treated sediment samples. In detail, three different configurations have been considered for each sample: undiluted sample, diluted with 50% and 75% distilled water. For each configuration, 1 ml of matrix – water extract (1:10 w/v) was dosed to 10 seeds into a Petri dish that was subsequently incubated at 27°C for 72 h in the dark. Moreover, a control test was realized, with incubation of *Lepidium* seeds with deionized water. Subsequently, for each test, the germinated seeds (G) and the root length (L) were measured and compared with the control. Each test was carried out in triplicate.

The mass flow rate of volatilized hydrocarbons was estimated by multiplying the gaseous flow rate leaving the reactor by the related hydrocarbon concentration. The gas flow rate was estimated by measuring the flux velocity at the outlet by a hot wire anemometer, while the hydrocarbon concentration was measured with a portable GAS-TEC® MK III flame ionization detector (FID). The contaminant mass volatilized was determined as the time integral of the flow rate by weight. Finally, pH and dissolved oxygen were monitored with a WTW type multimeter by means of dedicated probes. These parameters remained almost constant throughout experiments, with average values of 8.5 and 9 $mg_{OD}\ L^{-1}$, respectively.

3. RESULTS AND DISCUSSIONS

3.1 Summary of the results achieved in the preliminary experimental campaign

Four different microcosm configurations were studied in the former study: in particular, Test 0 was the blank control to verify the bio-degradative potential of the autochthonous biomass in marine sediments. In Test 1 N and P were added in a 4:1 ratio, to assess the effect of a "biostimulation" process. Tests 2 and Test 3 were aimed at evaluating the effect of allochthonous biomass inoculum; bacteria acclimated to salt and hydrocarbons were added to Test 2 and Test 3, respectively (**Campo et al., 2018; Corsino et al., 2018a-b; Campo and Di Bella, 2019)**. The initial TPH concentration in the microcosm tests was close to 450 mgTPH kg_{SS}^{-1}. The main results of the microcosm tests are summarized in Table **2**.

Table 2 Main results achieved in the microcosm tests.

	Residual TPH [mg TPH kg_{SS}^{-1}]		**Removal efficiency [%]**		**Removal rate [mgTPH kg_{SS}^{-1} d^{-1}]**	
	Day 15	Day 24	Day 15	Day 24	Day 15	Day 24
Test 0	403	330	10	26	2.93	8.11
Test 1	354	315	21	30	6.20	4.33
Test 2	366	352	18	21	5.40	1.56
Test 3	298	223	33	50	9.93	8.33

From the results reported in Table 2, it can be observed that the lowest concentrations at the end experiments were obtained in Test 3 thanks to the inoculation of halotolerant biomass, while similar but lower values are found in the other configurations (removal performance of about 26%, 30%, and 21% respectively). Concerning the removal kinetics, for all tests the substrate removal rate slow down after the 15^{th} day, excepting Test 0, which rate increased maybe for a slower adaptation of the autochthonous biomass, mainly emphasized by the lack of nutrients (**Avona et al., 2022**).

Concerning the bioslurry period, the achieved results highlighted a TPH decrease in the solid phase during the first experimental days, corresponding to a moderate TPH increase in the liquid phase, maybe due to a leaching effect. Subsequently, the TPH concentration in the liquid phase showed a constant value while it significantly decreased in the solid phase reaching an overall removal efficiency of 40% with a maximum removal rate of 220 mg TPH kg^{-1} d^{-1} at the end of experiments, thus suggesting the potential of sediment remediation through a bioslurry process. Despite promising, the residual TPH concentration was higher compared to regulation limits, therefore suggesting the opportunity to implement this treatment for lower contamination of the raw sediments. For further details, the reader is addressed to literature (**Avona et al., 2022**).

3.2 TPH removal from contaminated sediment

First, it is useful to underline that the TPH measurement allows evaluating the recalcitrant organic pollution of contaminated marine sediments. Indeed, the global measurement enables to assess the presence of different hydrocarbon groups as a whole. TPHs define a wide group of chemical substances that includes aliphatic, aromatic and polyaromatic hydrocarbons, contained in petroleum residues and other substances. **Figure 2** reports the TPH concentration on the sediment and liquid phase during experiments, which enabled to evaluate the biodegradation ability of the system.

A rapid TPH removal in the solid phase was observed in the first 21 days of operation. The kinetics were slower from day 40 until the end of the experiments. Data quality and reliability were proven by the very low values of Standard Deviation (15.98 mg_{TPH} kg_{SS}^{-1}) and Standard Error (11.30) respectively. The trend of TPH concentration in the liquid phase remained almost stationary for the entire duration of experiments, with concentrations of 1.50 ± 0.16 mg TPH L^{-1}; the data quality was assured by the very low values of the Deviation Standard and Standard Error, equal to 0.16 mgL^{-1} and 0.113, respectively. This trend is likely due to an equilibrium reached between pollutant release from the solid matrix (desorption) and the contextual consumption by the bacterial population in the liquid phase since the beginning of experiments. This result could be favored by the biomass acclimation achieved in the previous experimental campaign with similar operating conditions (**Avona et al., 2022**), from which the initial inoculum was taken. The system reached stationary conditions from day 80 onward, as suggested by the constant trend of TPH concentration in the solid phase. The overall removal efficiency achieved at the end of the experiment was 86%. The maximum removal rate of 36 mgTPH kg^{-1} d^{-1} was achieved in the first 14 days of experiments. This is mainly due to the metabolic consumption of the more easily biodegradable compounds by the bacterial community.

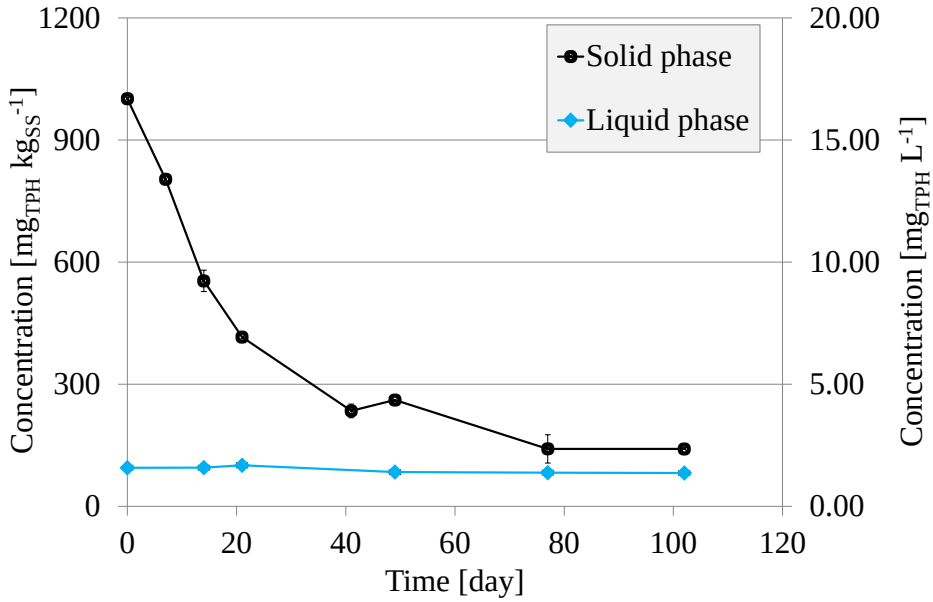

Figure 2 TPH residual concentrations in sediment and liquid phase.

The results obtained appeared very interesting; indeed, as previously pointed out, there are very few studies reported in the literature about the application of the bioslurry treatment for real contaminated marine sediments. Moreover, the degradation efficiencies obtained in previous experiments on contaminated sediments are quite lower than those obtained in the present work. In particular, **Table 3** shows the contamination efficiencies and main operational features of recent studies, comparable with this work on the basis of: 1) treatment with aerobic bioslurry; 2) sediments contaminated by petroleum hydrocarbons, aromatic and non-aromatic hydrocarbons; 3) initial hydrocarbons concentration in sediments higher than the regulatory limit.

Table 3 Removal efficiency and main operational condition in bioslurry for sediment remediation

Percentage of sediment in the slurry (% w/v)	Maximum Hydrocarbon removal efficiency (%)	Remarks and Operational condition	References
3-5	55% at the end of incubation (measured in terms of TOC removal)	• Aeration (6.04 e–4 m/s) • Bioaugmentation enriched bacterial culture • Volume of flask < 1L • Biostimulated with lactose • 40-day operation (6 weeks) • No aeration control	El-Gendy et al., 2009
10	70%	• Aeration (6.04 e^{-4} m/s) • No bioaugmentation • Volume of flask < 1L • Biostimulated with lactose • 4 different phase duration (from start-up to biostimulation)	Giordano et al., 2005
10	38% (without bio-stimulation – Phase 1) 80% (with antibiotic-stimulation – Phase 2)	• Bioaugmentation with activated sludge • 4000-ml reactor. • Aeration 3 l/min.	Lumia et al. 2020

		• 30-day operation for each phase. • Volatilization was considered and quantified.	
5-15	5-20%	• Native microflora degradation • 300-ml flasks. • unmixed system • 7-day operation.	Jee et al., 1998
5-15	29-41%	• Native microflora degradation • 300-ml flasks. • Aeration 0.015 l/min. • 7-day operation. • Volatilization was considered and quantified.	Jee et al., 1998
20	40%	• Natural soil microflora. • Abiotic removal not considered. • 100-ml flask. • Open system (no aeration). • 35-d operation.	Beolchini et al., 2010
10	86%	• Bioaugmentation with activated sludge • 30000-ml reactor. • Aeration 25 l/min. • 100-day operation. • Volatilization was considered and quantified.	This work

The few comparable literature references, mostly carried-out at batch scale (reactor volumes usually less than 1 L) or with artificial contamination, usually did not exceed 40% removal, rarely reached 70%, only due to the aid of particular techniques of microbial biostimulation.

In contrast, the results of bioslurry treatment on soils showed removal efficiencies up to 90% but are not comparable with the application on sediments. Few authors achieved efficiencies of 90% for the hydrocarbon removal from sediments but only under optimized conditions for maximizing performance. In particular, **Chikere et al. (2012)** and **Launen et al. (2014)**, achieved performance of 90% or higher only with artificially contaminated sediments and with initial concentrations of TPH in the sediment of 100 mg L^{-1}, significantly lower than the regulation limits.

Obviously, data reported in **Table 3** do not allow a complete and equal comparison of the results. In particular, the system performance achieved in the this work highlighted that after only 20 days of experiment the residual TPH concentration in the sediments was lower than the regulatory limits (750 mg_{TPH} kg_{DW}^{-1}). The performances further improved after the following 20 days, On the other hand, the significant removal efficiency at the end of the experimental campaign (over 85%), is significantly higher than those reported in the literature: however, in other works similar performances have been observed after almost 3 months of continuous treatment. This duration is however excessive for a biological treatment, and the data is intended as a mere extension of the potential of the treatment. Probably an "effective" duration of the biological pathway can be estimated between 30 and 40 days.

Finally, with the aim to define the real impact of bacterial degradation compared to the volatilization of hydrocarbons potentially promoted by the air flow rate, the evaluation of the actual biodegradation

ability of the system must also evaluate the comparison between biodegradation and volatilization. In particular, the Mass Flow Rate (MFR) in the off gas was monitored by measuring the flow rate of gaseous emission and its TPH concentration in the specific sampling section (see **Figure 1**). As shown in **Figure 3a**, the MFR was quite high during the first two experimental days and subsequently decreased, reaching zero values from the 56th day until the end of the experimental observation. The volatilized mass of hydrocarbons was determined as the integral of the weight flow for each time interval considered, consisting of the time elapsed between two subsequent days of slurry sampling. The data obtained were processed with TPH measurements in liquid and solid phase to achieve a mass balance for the system. The change in the volume of slurry inside the reactor (batch operation) due to the sampling of 1 liter of slurry for each sampling was considered for this count. The mass of volatilized contaminant was equal to 158 mg at the end of the 102 days of experimentation, compared to 2,344 mg removed overall. The results of the mass balance were processed to determine the impact of the volatilization path on the hydrocarbons removal (**Figure 3b**) and its contribution to the overall removal efficiency (**Figure 3c**).

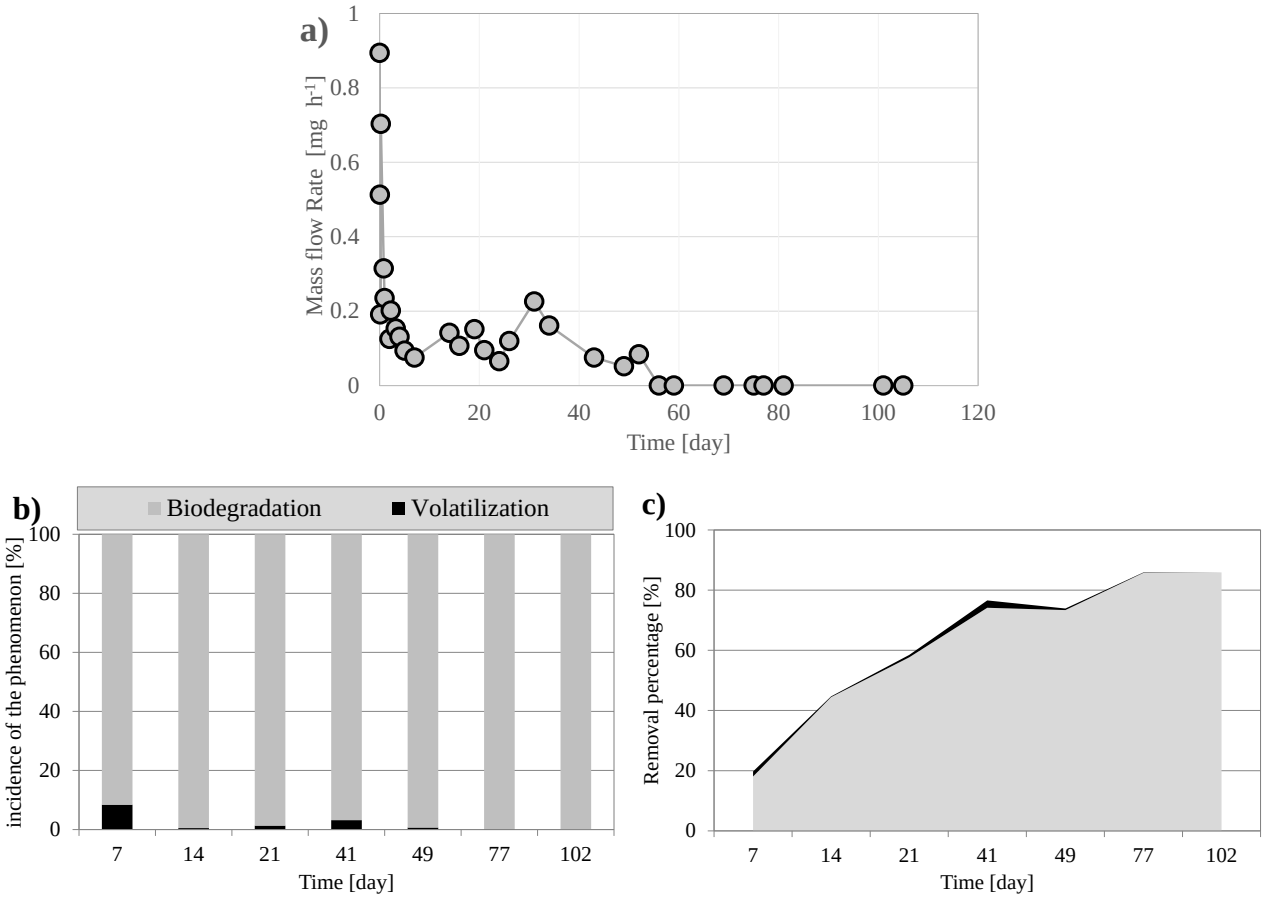

Figure 3 Weight flow (a), incidence of biodegradation and volatilisation (b), removal efficiency (c).

306 The volatilization effect was greater during the start-up phase of the plant, equal to 8.4% of the mass
307 removed, and decreased to less than 1% from the forty-ninth day onwards (**Figure 3b).** In general,
308 the volatilization pathway contributed marginally to the overall removal of TPHs, which is mainly
309 attributable to biological degradation (**Figure 4c**). Nevertheless, it is worth noting that volatilization
310 of the lighter part of the contaminants likely occurred immediately after sediment contamination.

311

312 **3.2 Biomass characterization**

313 The trend profile of Volatile Solids (VS) was monitored throughout the experimental campaign to
314 draw information about biomass growth tendency (**Figure 4**).

315 An increase in VS percentage was observed in sediment samples during the first days of experiments,
316 corresponding to a simultaneous sharp reduction of TPH concentration. The system reached
317 stationary conditions from day 49 onwards, in accordance with what observed for the TPH
318 concentration.

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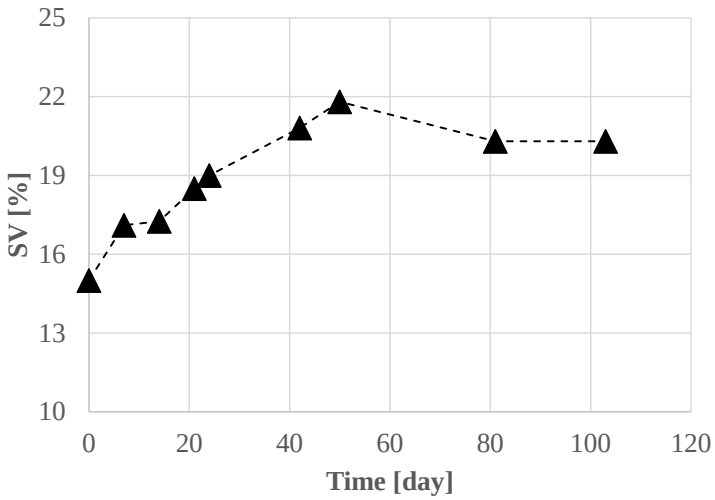

320

321 **Figure 4** Percentage of Volatile Solids.

322

323 High throughput analysis of the bacterial 16S rRNA gene was performed in order to describe the
324 microbial dynamics in the untreated sediment (NT) and after treatment in a bioslurry reactor (BS)
325 (**Figure 5 and 6**).

326 *Bacteroidetes, Epsilonbacteraeota, Firmicutes,* and *Proteobacteria* represented the major phyla in
327 the sediment at time zero (NT), covering up to 97% of the total ASVs retrieved in the sample (Figure

5). Conversely, ASVs affiliated with *Actinobacteria*, *Bacteroidetes*, *Chloroflexi* and *Proteobacteria* were predominant in the sample after treatment in a bioslurry reactor (BS) (up to 94.5% of the total AVSs; Figure 5).

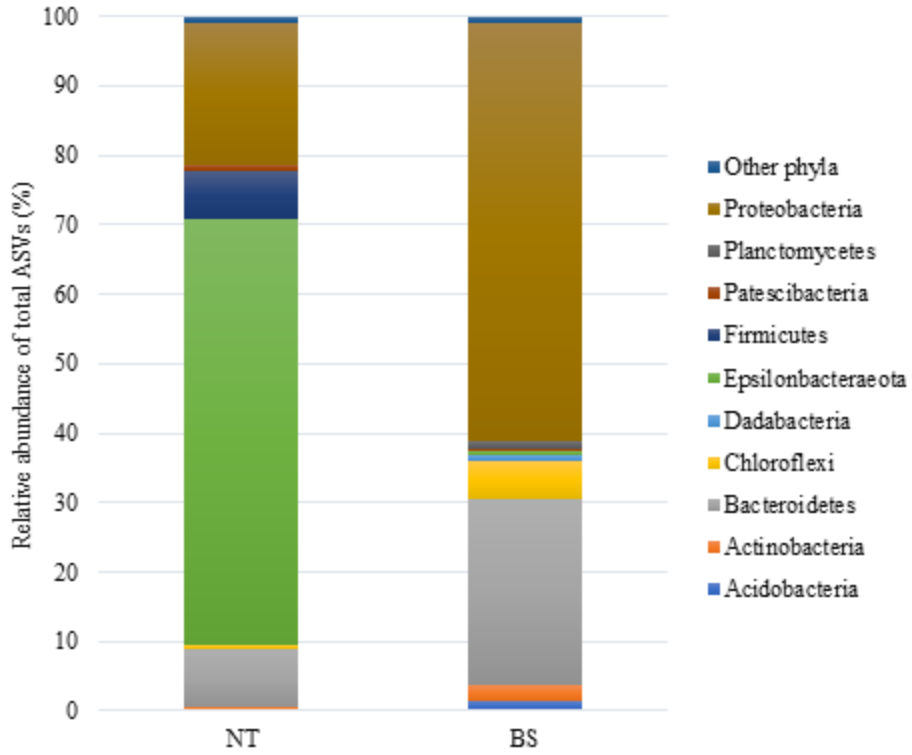

Figure 5 Bacterial microbial composition at phylum level estimated by high-throughput 16S rRNA gene sequencing in the sediment at time zero (NT) and after treatment in a bioslurry reactor

The major increase in ASVs at phylum level after the treatment was observed for *Actinobacteria*, *Bacteroidetes*, *Chloroflexi* and *Proteobacteria* (Figure 5).

A microbial shift was observed into the *Bacteroidetes* phylum. In particular, the *Phaeodactylibacter* genus (*Saprospiraceae* family) showed a significant increase over time zero and a relative abundance in the treated sample of 2.5% of the total ASVs (Figure 6). **Doyle et al. (2018)** found a parallel development of this taxa to the degradation of hydrocarbon compounds in the mesocosms of hydrocarbon-contaminated coastal seawater. Additionally, sequences affiliated with *Rhodothermaceae* family (*Bacteroidetes*) was present with an abundance of 8.2% in the treated sediment sample. This taxonomic group is also well related to the biodegradation of petroleum compounds. In particular, **Ribicic et al. (2018)** found a correspondence between the removal of PAHs and branched alkanes in seawater and the development of the *Rhodothermaceae* family.

Chloroflexi phylum was dominated by ASVs affiliated to the *Anaerolineaceae* family. The latter group represented 4.8% of the total ASVs and is commonly found in marine sediments. Studies proved that some members of this family are heterotrophic microorganisms with anaerobic metabolism and can play an important role in the degradation of hydrocarbons, such as long-chain n-alkanes **(Sherry et al., 2013; Sutton et al., 2013; Liang et al., 2016; Wang et al., 2019**). Their presence after treatment can be attributable to anaerobic niches in the stagnant areas of the reactor. In fact, previous studies highlighted the co-presence of aerobic and anaerobic groups belonging to the *Anaerolineaceae* family due to the formation of limited anaerobic zones even in the most superficial layers of the soil **(Liu et al., 2019).**

A marked increase in BS sample with respect to NT was also observed for sequences associated with *Alphaproteobacteria* and *Gammaproteobacteria* (*Proteobacteria* phylum), in particular for the *Betaproteobacteriales* and *Sphingomonadales* orders respectively. Several studies proved that both these groups contain microorganisms potentially active in hydrocarbon degradation **(Martin et al., 2012; McGenity et al., 2012a; Rojo, 2009).** An important increase was observed in *Burkholderiaceae* family (*Betaproteobacteriales*), as well as in the ASVs associated with *Sphingomonadaceae* family (*Sphingomonadales*).

Analysing the microbial population at genus level into *Proteobacteria* phylum also highlighted an increase in the total sequences especially for the genera *Hydrogenophaga*, *Sphingorhabdus*, and *IS-44* (4.0%, 10.5% and 5.2% of the total ASVs, respectively. **Figure 6**). In particular, the genus *Hydrogenophaga* has been identified as a potential degrader of petroleum compounds (**Prince, Gramain et al., 2010**) and a wide range of hydrocarbon contaminants, including PAHs **(Aburto and Peimbert, 2011; McGenity et al., 2012b**). Furthermore, **Hou et al., 2015** found a positive correlation between the genus *Hydrogenophaga* and the removal of hydrocarbon fractions of C_{21}-C_{34} oil. Members of the genus *Sphingorhabdus* have been isolated from coastal sediments which were contaminated with crude oil and previous studies proved that several species belonging to this taxa possess genes involved in the catalysing of aliphatic and aromatic hydrocarbons (**Jeong et al., 2016**).

Comparing the sediment at time zero (NT) and after bioslurry treatment (BS), a drastic decrease in ASVs affiliated to the phylum *Epsilonbacteraeota* was observed (from 61.2% to 0.5% of the total ASVs; Figure 5). The analysis showed that the typical ambient genera such as *Sulfurimonas* and *Sulfurovum*, decrease in the treated sediment from about 55.8% to 0.17%, and from 5.4 to 0.36% of the total sequences, respectively (**Figure 6**).

As expected, the treatment of the sediment promoted also the disappearance of anaerobic genera like *Magnetovibrio* (*Proteobacteria*) and *Fusibacter* (*Firmicutes*) which represented 3% and 4.6% of the total ASVs in the sediment at time zero **(Ravot et al., 1995; Bazylinski et al., 2013**).

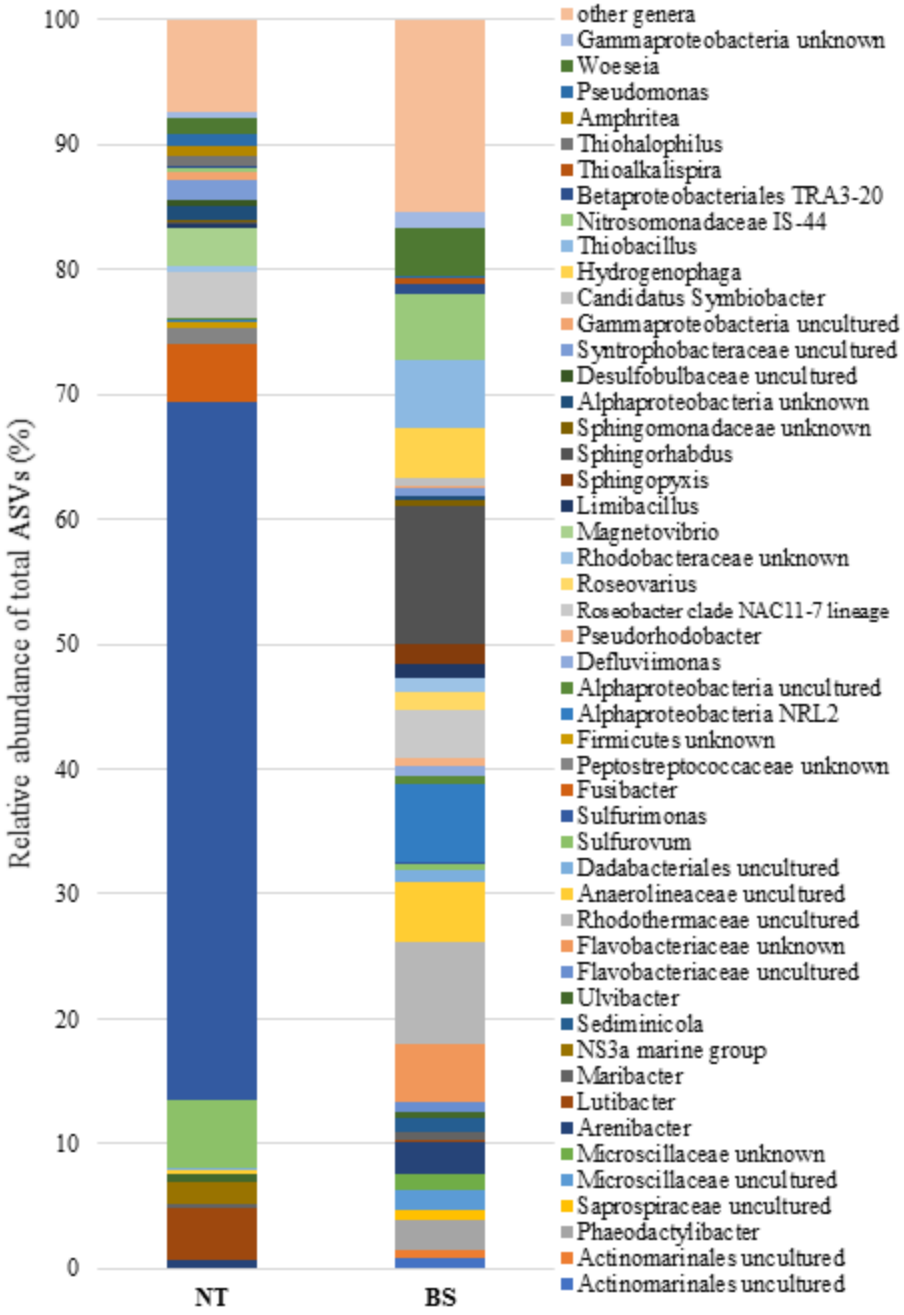

Figure 6 . Bacterial microbial composition at genus level estimated by high-throughput 16 S rRNA gene sequencing in the sediment at time zero (NT) and after treatment in a bioslurry reactor (BS).

3.3 Sediment phytotoxicity

Figure 7 shows the results of phytotoxicity tests in terms of root system elongation (a) and germination index (b).

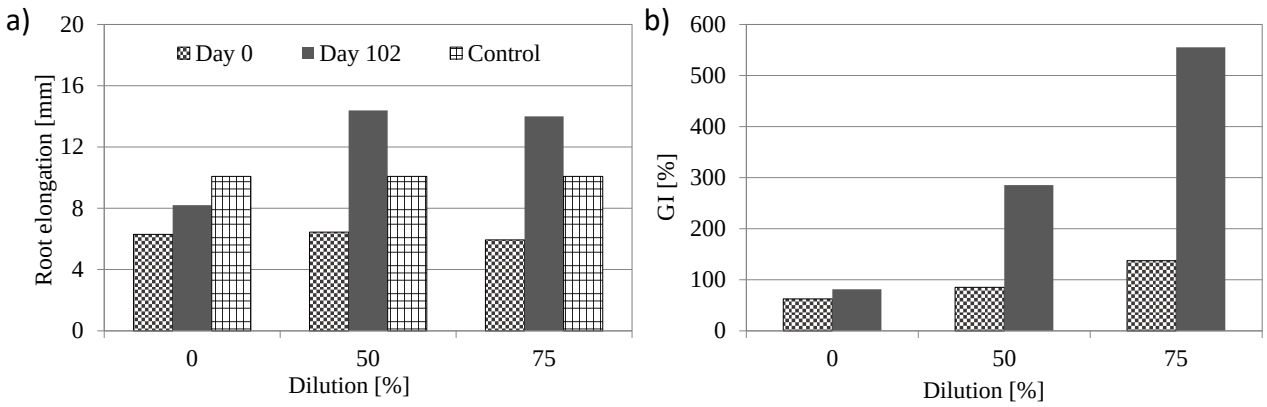

Figure 7 Average root system length (a); germination index (b).

As expected, the configurations showing the best features in terms of phytotoxic qualities were those characterized by dilution ratios of 50% and 75%, both for the untreated sediment sample (day 0) and for the treated sediment (day 102). Additionally, the root length was approximately 14 mm for the treated sediment sample (day 102) at both 50% and 75% dilution ratios, compared to that of raw sediment sample equal to 6 mm at the same dilution (**Figure 7a**). All configurations showed a quality improvement after bioslurry treatment (102 days), and the germination index (GI) ranged between 81% and 555%, compared to 62% and 137% for the untreated sediment (**Figure 7b**).

4. CONCLUSIONS

The treatment of contaminated marine sediments, from Augusta's Bay, by means of bioslurry reactors showed excellent performance for the removal of petroleum hydrocarbons. Volatilization contributed only marginally to the removal of the contaminant, despite the mixing and aeration conditions adopted. This result might be due to the age of the contamination and the resulting TPH composition. The main mechanism was biodegradation, as suggested by the increase in volatile solids and the shift in the dynamics of the microbial community after treatment. The taxonomic groups that best suited the process conditions were identified. Some groups originally present in the sediment with low relative abundance have developed predominantly. In particular, ASVs associated to the genera *Hydrogenophaga*, *Sphingorhabdus*, and *Phaeodactylibacter* had the largest increases. Scientific literature has confirmed their potential for degradation of hydrocarbons in particular matrices such as marine sediments. Finally, the phytotoxicity tests showed that after bioslurry treatment, the residual sediment improved its quality features compared to the original state. This is important for a possible reuse of the decontaminated matrix as an alternative to disposal.

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