PRELIMINARY INSIGHTS ABOUT THE TREATMENT OF CONTAMINATED MARINE SEDIMENTS BY MEANS OF BIOSLURRY REACTOR: PROCESS EVALUATION AND MICROBIOLOGICAL CHARACTERIZATION

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ABSTRACT

Contaminated marine sediments represent a critical threat towards human health and ecosystems, since they constitute a potential reservoir of toxic compounds release. In the present study, a bioslurry reactor was studied for the treatment of real marine sediments contaminated by petroleum hydrocarbons. The experimental campaign was divided in two periods: in the first period, microcosm trials were carried out to achieve useful indicators for biological hydrocarbon removal from sediments. The microcosm trials highlighted that the inoculum of halotolerant allochthonous bacteria provided the highest performance followed by autochthonous biomass. Based on the achieved results, in the second experimental period a bioslurry reactor was started up, based on a semisolid stirred tank reactor (STR) operated in batch mode. The process performances have been evaluated in terms of total petroleum hydrocarbon (TPH) removal, coupled with the characterization of microbial community through a Next Generation Sequencing (NGS) and phytotoxicity tests through the Germination Index (GI) with *Lepidium Sativum* seeds. The achieved results showed good hydrocarbons removal equal to 40% with a maximum removal rate of 220 $mg_{TPH}\ kg^{-1}\ d^{-1}$, but highlighting that high contaminant concentrations might affect negatively the overall removal performance. In general, the observed results were encouraging towards the feasibility of biological treatment of marine sediments contaminated by hydrocarbons. The microbiological analysis allowed the identification of taxa most involved in the degradation of TPH, highlighting after the treatment a shift in the microbial community from that of the raw sediment.

Keywords: bioremediation; bioaugmentation; hydrocarbon pollution; contaminated marine sediments; microbial community;

1. INTRODUCTION

Marine sediments constitute a complex environment, characterized by huge variability in the formation pathways, chemical-physical features, living organisms and types of contamination. They represent a potential reservoir of toxic compounds as they are often subject to anthropogenic inputs (Rocchetti et al., 2012; Maletić et al., 2018; Albarano et al., 2020). Sediment contamination is mainly due to urban or industrial waste discharges and among the most widespread pollutants, petroleum hydrocarbons (HCs), usually expressed as a total petroleum hydrocarbons (TPHs), represent a huge threat for ecosystems and human health (Maletić et al., 2018). In particular, petroleum hydrocarbons can be trapped in sediments for a long time due to their hydrophobic nature (Pino-Herrera et al., 2017), becoming an environmental concern and public health problem since they can be released from the sediments which might represent over time a primary source of contamination (McGenity et al., 2012; Maletić et al., 2018). For these reasons, the treatment of marine sediments contaminated by hydrocarbons represents an urgent need and has become an important topic of research (Pino-Herrera et al., 2017). Amongst the different alternatives, biodegradation by microbial populations capable of using hydrocarbons as a source of carbon and energy represent a promising technology for sediments remediation (Varjani, 2017; Varjani and Upasani, 2017; Usman et al., 2018). These strategies which are eco-friendly and more economic compared to thermal or chemical-physical processes, are gaining attention thanks to a number of studies on microbial behavior for the degradation of petroleum hydrocarbons (Huesemann et al., 2002; Perelo, 2010). In this context, bacteria have been recognized as the predominant microorganisms involved in hydrocarbons degradation under aerobic conditions (Zhao and Wong, 2009; Zhao et al., 2011), with *Proteobacteria* and *Bacteroidetes* phyla as the most abundant taxa in marine sediments (Iannelli et al., 2012; Chiellini et al., 2013). An increase in taxa belonging to *Alphaproteobacteria* and *Gammaproteobacteria* classes was observed during degradation of oil compounds (Lamendella et al., 2014), highlighting an adaptive behavior of indigenous bacteria

affiliated to these classes during bioremediation, with a shift in composition within these classes (Fuentes et al., 2014; Smith et al., 2015; Fuentes et al., 2016;). This change depends on a number of factors, including the environmental compartment (eg. soil or marine sediment, freshwater or seawater), sediment properties (eg. texture, pH, organic matter, nutrients, temperature), the level and age of contamination, the microbial community, and the selected bioremediation technology (Kaplan and Kitts, 2004; Hamamura et al., 2013; Fuentes et al., 2014; Fuentes et al., 2016). Among ex-situ biological treatments, bioslurry reactors are one of the best technology for the treatment of soils or sediments contaminated by hydrocarbons under controlled conditions. To promote the contaminant biodegradation, sediment is mixed with water to form a suspension, referred to as slurry, typically 10 to 60% expressed as weight by volume (w/v) (Gan et al., 2009; Pino-Herrera et al., 2017; Lumia et al., 2020). Indeed, in a slurry phase it is possible to enhance the mass transfer rates and the contact between pollutants, nutrients and microorganisms present. Generally, bioslurry reactors are operated in batch mode to facilitate the handling of slurries, thereby ensuring higher process yields (Prasanna et al., 2008; Venkata Mohan et al., 2008). Reactors are therefore equipped with mixing devices to maintain the characteristics of the suspension homogeneous over time and, frequently, with air diffusers to ensure the establishment of an aerobic metabolism (Robles-González et al, 2008). Despite the potential effectiveness of the treatment, to authors' best knowledge very few studies have been carried out on real marine sediments contaminated by hydrocarbons, often at very small scale and for limited duration (Beolchini et al., 2010; Chikere et al., 2011). Therefore, the aim of the present paper is to present the first results of an experimental study carried out on TPH-contaminated marine sediments dredged from the Augusta bay (Sicily, southern Italy), an Italian contaminated site of National interest. In detail, after sediments characterization, preliminary microcosm tests have been performed in order to assess the indicators useful to optimize the performance of biological treatment for the removal of hydrocarbons. After this preliminary phase, a bioslurry reactor conceived as a semisolid stirred tank reactor (STR) was designed and realized. The bioslurry reactor was then monitored in order to assess the

biodegradation performance in terms of TPH removal, microbial community composition and phytotoxicity of sediment samples during treatment.

2. MATERIALS AND METHODS

2.1 Microcosm tests

The experimental set-up consisted of different microcosm configurations aimed at assessing the most suitable one for the subsequent bioslurry reactor application. The preliminary microcosm study was carried out by using different raw sediment samples collected at Augusta harbor (Sicily, Italy). The details of sediments sampling and characterization has been reported elsewhere (Lumia et al., 2018). Briefly, physic-chemical characterization revealed a high organic matter (4.2%), moisture content (44%), and sulphides (61 mg kg^{-1}). The grain size and mineralogical analyses indicated the presence of silty and calcite ($CaCO_3$), (64.6%); while the particle size analysis indicated the presence of the silty fraction (77.4%), followed by sandy (19.6%) and clay (3%) fractions. Concerning TPH features, hydrocarbons were in the range C_{12}-C_{40}, mostly concentrated in the C_{16}-C_{28} classes (almost 79%). Hydrocarbons in sediments likely derived from petroleum compounds spillage from tanker or pipes, typical pollution phenomena in Augusta Bay, well as from accidental leakage of slop wastewater produced from the activity of oil tankers washing with seawater. The raw sediment samples have been mixed in order to obtain a homogeneous sample (namely "Sample 0"), which was used for the microcosm trials, carried out in glass vessels. A sub-sample of almost 120 g was withdrawn from Sample 0 and further divided into four portions of 30 ± 0.5 g by means of a laboratory analytical balance. These portions were placed into glass vessels filled with 970 g of tap water. Therefore, the slurry concentration in each vessel was equal to 30 g kg^{-1}. Furthermore, the different vessels, named to as Test 0, Test 1, Test 2 and Test 3 were differentiated according to what reported in Table 1.

115 **Table 1.** Main features of the investigated microcosms

Parameter	Units	Test 0	Test 1	Test 2	Test 3
Water	g	970	970	970	970
Sediment	g	30	30	30	30
NH_4Cl	g	-	40	40	40
K_2HPO_4	g	-	10	10	10
Halophilic biomass	mL	-	-	1	-
Halotolerant biomass	mL	-	-	-	1

116

117 Briefly, Test 0 was the "blank control" aimed at assessing the potential biodegradability of the
118 autochthonous biomass already present in the marine sediments. Test 1, characterized by the
119 spiking of N and P (proportion 4:1), was aimed at assessing the "biostimulation" of the eventual
120 autochthonous biomass. Test 2 and 3 were considered in order to evaluate the difference in the
121 biodegradation process due to the inoculum of allochthonous biomass ("bioaugmentation"); a
122 biomass already acclimated to high salinity was inoculated in Test 2, whilst in Test 3 it was
123 inoculated a bacterial consortium acclimated to salinity and hydrocarbons. Aerobic conditions were
124 ensured by and air blower connected with fine bubble porous diffusers placed at the bottom of each
125 vessel. The experimental campaign had a duration of 24 days and the microcosm's temperature was
126 kept at 20 ± 0.5°C by means of thermostatic incubator. After the initial characterization of "Sample
127 0", sampling on solid phase was carried at day 15 and at day 24, whilst sampling of the liquid phase
128 was carried out at the end of experiments (day 24). It is worth noting that, due to microcosm small
129 amount, no replicates were considered in order to avoid excessive disturbance of the system. The
130 procedure adopted for TPH measurement in both solid and liquid phase will be described in a
131 section below.

132

2.2 Bioslurry reactor

The experimental set-up consisted of a cylindrical reactor in PVC with a GRP bottom with a volume of 30 L (Ø: 31 cm; H: 40 cm). The reactor was equipped with three vertical baffles (H: 32 cm, W: 5.7 cm, S: 0.3 cm) fixed at 120° and at 4.5 cm from the bottom. Mixing was guaranteed by a mechanical stirrer (IKA RW 28 DIGITAL), equipped with a PBT (Pitched-Blade Turbine) impeller with 4 blades inclined at 45° with a diameter of 10 cm and placed at 3 cm from the bottom. The aeration system consisted of three cylindrical microbubbles porous diffusers placed at the bottom between the baffles and connected to a blower (MEDO LA-45C). The reactor was equipped with slurry sampling sockets. The above experimental set-up was chosen after a preliminary study (data not shown) that enabled to assess the best hydrodynamic configuration in terms of mutual position of diffusers and baffles, mixing speed and turbine depth. The bioreactor had an overall volume of 24 L and was characterized by a liquid-sediment ratio of 10% in weight and a stirring speed of 675 ± 25 rpm. Real contaminated sediment samples collected at Augusta harbor (Lumia et al., 2018) were mixed in order to have an initial hydrocarbon concentration close to 13,000 mgTPH kg_{SS}^{-1}, with the aim to assess the biodegradation potential of high polluted sediments. Based on the results achieved in the microcosm trials, the bioslurry reactor was inoculated with 200 mL of granular halotolerant biomass sampled from a pilot plant treating an industrial wastewater characterized by high salinity and hydrocarbon content (Campo and Di Bella, 2019). The biomass inoculum had total and volatile suspended solid concentrations (TSS and VSS) of 3.87 g L^{-1} and 2.33 g L^{-1}, respectively. The experimental campaign had a duration of 105 days. Figure 1 shows some details of the experimental set-up.

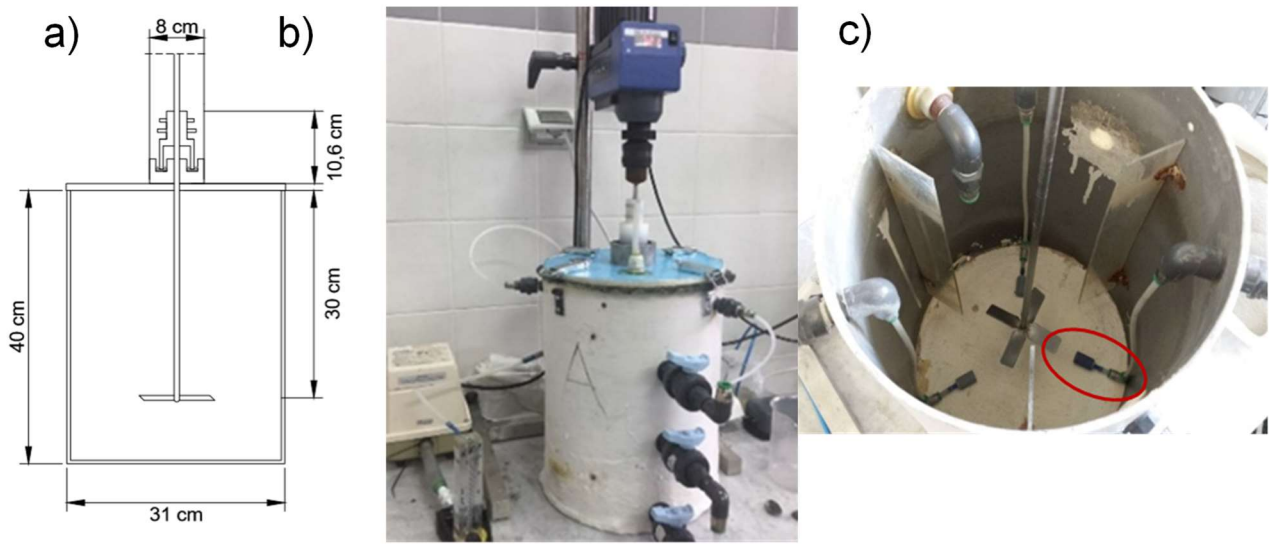

Figure 1. Schematic section (a), panoramic view (b) and internal details (c) of bioslurry STR reactor.

2.3 Analytical Methods

2.2.1 Total petroleum hydrocarbon measurement

The slurry samples were extracted in duplicate from the central sampling port (Figure 1b) of bioslurry reactor and then subject to 2 h settling for supernatant-solid separation. Four 50 mL samples were withdrawn from the settled solid and then dried at 27°C for two days prior to analysis. TPH concentrations in solid and liquid phase were evaluated using EPA 8010C and 3510C methods by GC–FID (Agilent 6890N), respectively. EPA 3545A method "Pressurized Fluid Exaction (PFE)" and the "Speed Extractor E-916" were used for the solid to liquid hydrocarbons extraction procedure. The TPH value was set as the mean value of the replicates, and the Standard Error (SE) was computed as the ratio between standard deviation and square root of total number of samples.

2.2.2 Microbial community analysis

The composition of the bacterial community was evaluated by the next generation sequencing (NGS) of the V1-V3 region of the 16S rRNA gene. The procedures adopted for nucleic acid

extraction, amplification, genomic library preparation and sequencing on the Illumina MiSeq platform have been carried out according to Matturro et al. (2017). Taxonomy has been assigned through an up-dated database (Silva Ribosomal RNA gene database) which provided the information of the bacterial consortium at phylum, class, order, family and genus, respectively. Sediment samples from inoculum (I), not treated slurry sediment (NT) and after bioslurry treatment (BS) were subject to microbial analysis in duplicate.

2.2.3 Germination index measurement

Lepidium sativum L. seeds were used to determine the germination index (GI) of the liquid phase generated following the sedimentation process of the slurry samples (Hoekstra et al., 2002; Doni et al., 2018). The analyses were carried out on the raw and treated sediment samples in duplicate. Three different dilution have been investigated: undiluted sample, diluted with distilled water at 50% and 75%. For each configuration 1 ml of matrix – water extract (1:10 w/v) was added to 10 seeds in Petri dish and incubated at 27 °C for 72 h in the dark. After that, the number of germinated seeds (G) and the relative root elongation (L) were measured and compared with the control obtained incubating the seeds with deionized water (G_C, L_C). Each test was carried out in triplicate. The GI (%) was calculated using the following equation: $GI\ (\%) = (G \cdot L)/(Gc \cdot Lc) \cdot 100$.

3. RESULTS AND DISCUSSIONS

3.1 Microcosm trials

3.3.1 TPH residual concentration

Figure 2 depicts the evolution of TPH concentration in the solid matrix (Figure 2a) during experiments while Figure 2b shows the TPH concentrations in the liquid phase at the end of

194 experiments (day 24), as well as the TPH removal efficiency (Figure 2c) for the different
195 microcosm trials.

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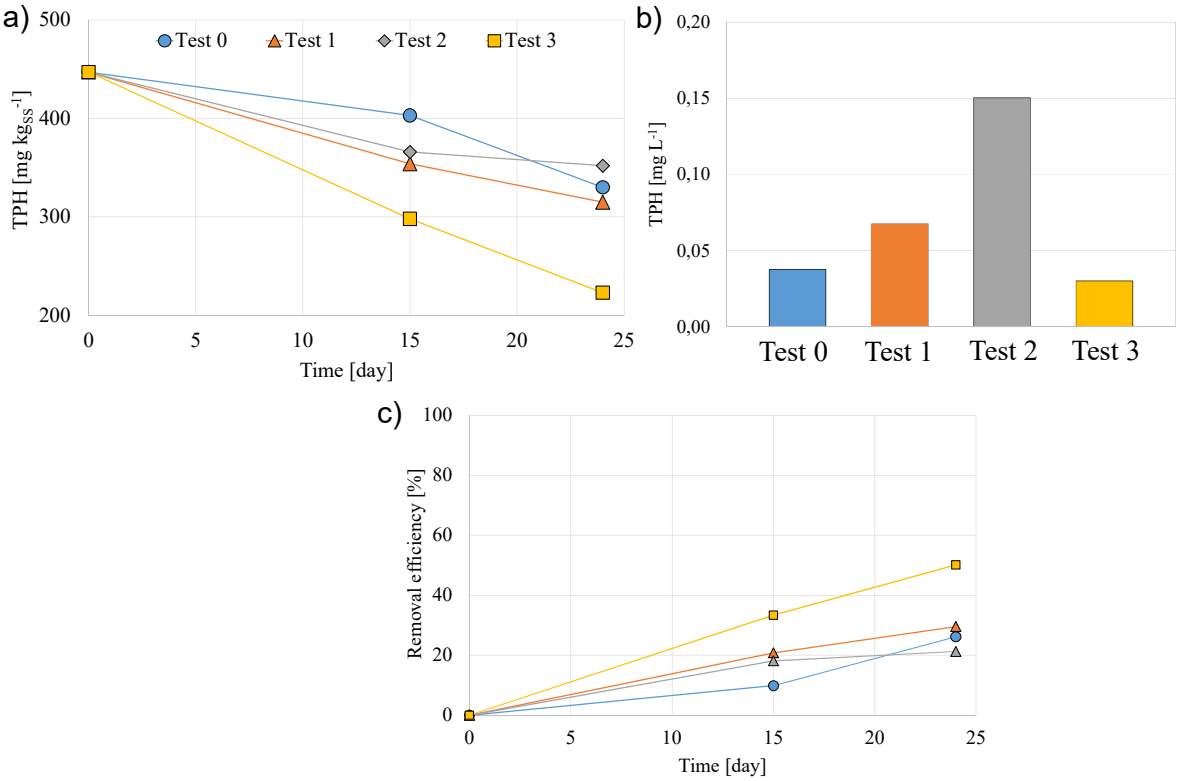

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198 **Figure 2.** TPH residual concentrations in sediment (a) and liquid phase (b), removal efficiency
199 referred to solid matrix (c).

200

201 Referring to the solid matrix, the lowest residual concentrations were found in Test 3, characterized
202 by the inoculum of halotolerant biomass, already acclimated to salinity and hydrocarbons. The
203 residual TPH concentration was similar in the other trials. Figure 2b shows the TPH concentration
204 in the supernatant of each trial at the end of experiments (after 24 days of treatment); from these
205 data, it is suggested that the liquid TPH concentration is an inverse indicator of the degradative
206 ability of the bacterial consortium in each trial. Indeed, microorganisms can degrade compounds in
207 the liquid phase; therefore, lower TPH concentrations in the phase liquid might be related to a

208 higher biodegradation activity, even if the contribution to volatilization must be taken into account
209 in further studies.

210 Concerning the removal rate in the solid matrix, by assuming a linear TPH decrease between
211 sampling, the highest values were obtained for the Test 3 inoculated with halotolerant biomass (9,33
212 and 8,33 mgTPH kg_{SS}^{-1} day^{-1} at day 15 and 24, respectively), in agreement with the above discussed
213 results. The autochthonous biomass (Test 0), deriving from an anaerobic environment, was subject
214 to an initial inhibiting effect due to the switch to aerobic conditions. After the acclimation period,
215 the autochthonous biomass showed a degradation rate similar to that of Test 3 (2,93 and 8,11
216 mgTPH kg_{SS}^{-1} day^{-1} at day 15 and 24, respectively). At the end of experiments, the lowest
217 degradation rate was observed in Test 2, inoculated with halophilic biomass, likely due to a
218 competition among the bacterial consortium (1,56 mgTPH kg_{SS}^{-1} day^{-1} at day 24). However, despite
219 the above assumptions could be realistic, a higher set of experimental data might have supported a
220 more rigorous data modelling and a more rigorous identification of the process kinetics. This aspect
221 will be deepened in future activities.

222

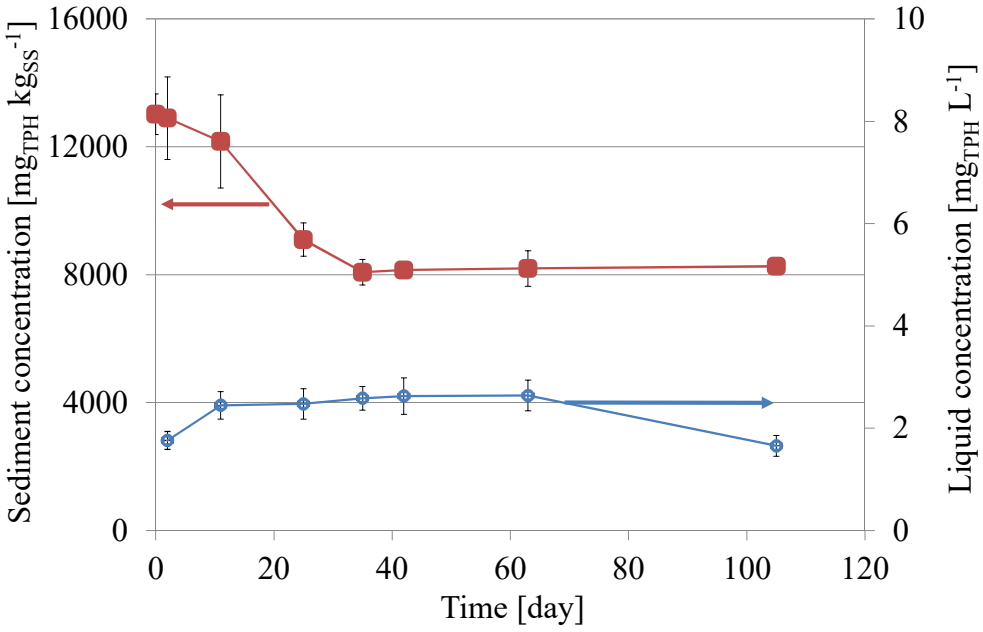
223 **3.2 Bioslurry reactor**

224 ***3.2.1 TPH removal performance***

225 Figure 3 shows the evolution of TPH concentration in the bioslurry reactor for both solid and liquid
226 phase. From the observation of data reported in Figure 3, it is worth noting an initial decrease of
227 TPH in the solid phase, corresponding to a slight increase of TPH in the liquid phase, likely due to
228 an initial leaching effect from the solid phase.

229 Afterwards, the TPH concentration in the liquid phase showed an almost constant value while it was
230 observed a significant decrease of TPH in the solid phase; such a behavior could be due to a sort of
231 balance among TPH desorption from the solid matrix followed by biological degradation in the

232 liquid phase. Until experimental day 40, the TPH biodegradation could be expressed through a 1st
233 order kinetic (k = 0.013 and R^2 = 0.98); while from experimental day 40 onwards the TPH
234 concentrations remained almost stable, suggesting an interruption of both biological and desorption
235 process, since also in the liquid phase the TPH concentration remained almost constant. The overall
236 removal efficiency was almost 40% with a maximum removal rate of 220 $mg_{TPH}\,kg^{-1}\,d^{-1}$. The
237 accuracy and reliability of the experimental data was confirmed by the low values of the standard
238 error, almost lower than 10% of the mean value. The majority of literature studies were carried out
239 on soils showing in general higher removal efficiencies compared to that achieved in the present
240 study, even if significantly different operational conditions make quite impossible a direct
241 comparison of different studies (Pino-Herrera et al., 2017). A previous study carried out on marine
242 sediment, highlighted a similar behavior in terms of removal efficiency during a 35 days treatment
243 (Beolchini et al., 2010). However, in the study of Beolchini and co-workers the initial contaminant
244 concentration was one order of magnitude lower that the present study. The results achieved in this
245 preliminary study suggest the potential feasibility of biological treatment for marine sediments
246 contaminated by hydrocarbons. However, the results achieved after day 40 could suggest an
247 inhibition effect likely because the remaining hydrocarbon classes were more recalcitrant and
248 characterized by high concentrations. Therefore, high TPH concentrations might negatively affect
249 the overall performance of the treatment, suggesting that treatment with bioslurry reactor might be a
250 good option with lower TPH concentrations, in order to have the chance to meet the standards
251 imposed by Regulations that were not met in the present study. In this light, future activities should
252 investigate the system behavior in presence of lower TPH concentrations.



253

254 **Figure 3.** Evolution of TPH concentration in the bioslurry reactor solid and liquid phase: the bars
255 indicating the standard error.

256

257 ***3.2.2 Microbial community characterization***

258 In the experimental study, the inoculum (I), sediment before treatment (NT) and after bioslurry
259 reactor treatment (BS) have been characterized in duplicate. As overall result, 240 genera afferent to
260 173 families and 150 orders with 62 classes and 33 phyla have been found, differently distributed in
261 the different samples. The following Figure 4 shows the data returned from the platform (genus
262 copies) in terms of relative abundance for the different taxonomic levels. From the taxonomic
263 analysis at phylum level (Figure 4a) the most represented taxa were constituted by *Proteobacteria*
264 (60%, 50% and 28%, for sample I, NT and BS, respectively). Numerous was the component
265 represented by *Bacteroidetes* for the inoculum (35%) and BS sample (17%); for the NT sample it
266 was much lower (7%), while taxa typical of anaerobic environment belonging to
267 *Epsilonbacteraeota* were more prevailing (30%). From Figure 4b, it is possible to notice that the
268 *Alphaproteobacteria* class was dominant for the inoculum (54.5%) and for the NT sample (28.5%).

In contrast, after bioslurry treatment, the above class showed a significant abundance reduction, down to 8%. The above results could be likely related to the features of mixing and aeration that might have altered the NT sample by promoting a shift in the microbial community and favoring the *Gammaproteobacteria* (Smith et al., 2015). At order level (Figure 4c) the *Alphaproteobacteria* and *Gammaproteobacteria* were characterized by different composition in the different samples investigated. The most abundant orders for *Alphaproteobacteria* were represented by *Sphingomonadales* (30%) and *Rhodobacterales* (20%) for the inoculum, *Rhodospirillales* (20%) and *Rhodobacterales* (2.5%) for NT, *Sphingomonadales* (1.7%) for BS. Concerning *Gammaproteobacteria* the most abundant orders were: *Alteromonadales* (3.8%) for the inoculum I, *Ectothiorhodospirales* (6.3%) and *Steroidobacterales* (4.9%) for NT sample, *Betaproteobacteriales* (14%) and *Steroidobacterales* (1.2%) for BS sample. In particular, the increase of *Betaproteobacteriales*, typical of sediments exposed to contamination by petroleum hydrocarbons, suggests their potential role in the biodegradation process (Head et al., 2006, Martin et al., 2012, Fuentes et al., 2016; Kucharzyk et al., 2018; Roy et al., 2018). Among the *Bacteroidetes* phylum, the most abundant class was *Bacteroidia* for all samples (Figure 4a). At order level, the *Flavobacteriales* were present in all samples, mostly for the inoculum and for NT samples. Taxa belonging to this order are common in marine ecosystems contaminated by TPH (Leahy e Colwell, 1990). At family level (Figure 4d) it was noticed an abundance increase of the unclassified taxa, equal to 54%; this result could be likely related to the high contamination level that, coupled to operational conditions could have inhibited the process favoring the growth of taxonomic group not present in the used database.

From the higher to the lower classification levels, it was observed that the inoculum did not influence the dynamic of the bacterial community composition at the end of the experimental period. For instance, referring to the genus *Muricauda* with an abundance of 16% in the inoculum (I) and not present in the NT sample, no genetic copies were found in the sediment after bioslurry

294 treatment (Figure 4e). This result could be related to competition between autochthonous and
295 allochthonous biomass. The former, already present in the raw sediment in a sort of quiescence,
296 might have overwhelmed the latter once the treatment process started. Afterwards, even the
297 autochthonous biomass was subject to a growth limitation, moving towards endogenous conditions,
298 which did not allow a clear identification of functional groups involved in the biodegradation
299 process for the lower taxa.

300 Concerning the reliability of the reported values, the Standard Deviation values were always lower
301 (< 2%), excepting genus *Magnetovibrio* found in the NT samples (5.86%). However, it is important
302 to stress that *Magnetovibrio*, belonging to the *Alpha-Proteobacteria* class, it is not functional to
303 hydrocarbon degradation (Bazylinski et al., 2013). Indeed, it was not found in the microbial
304 composition of treated samples (BS).

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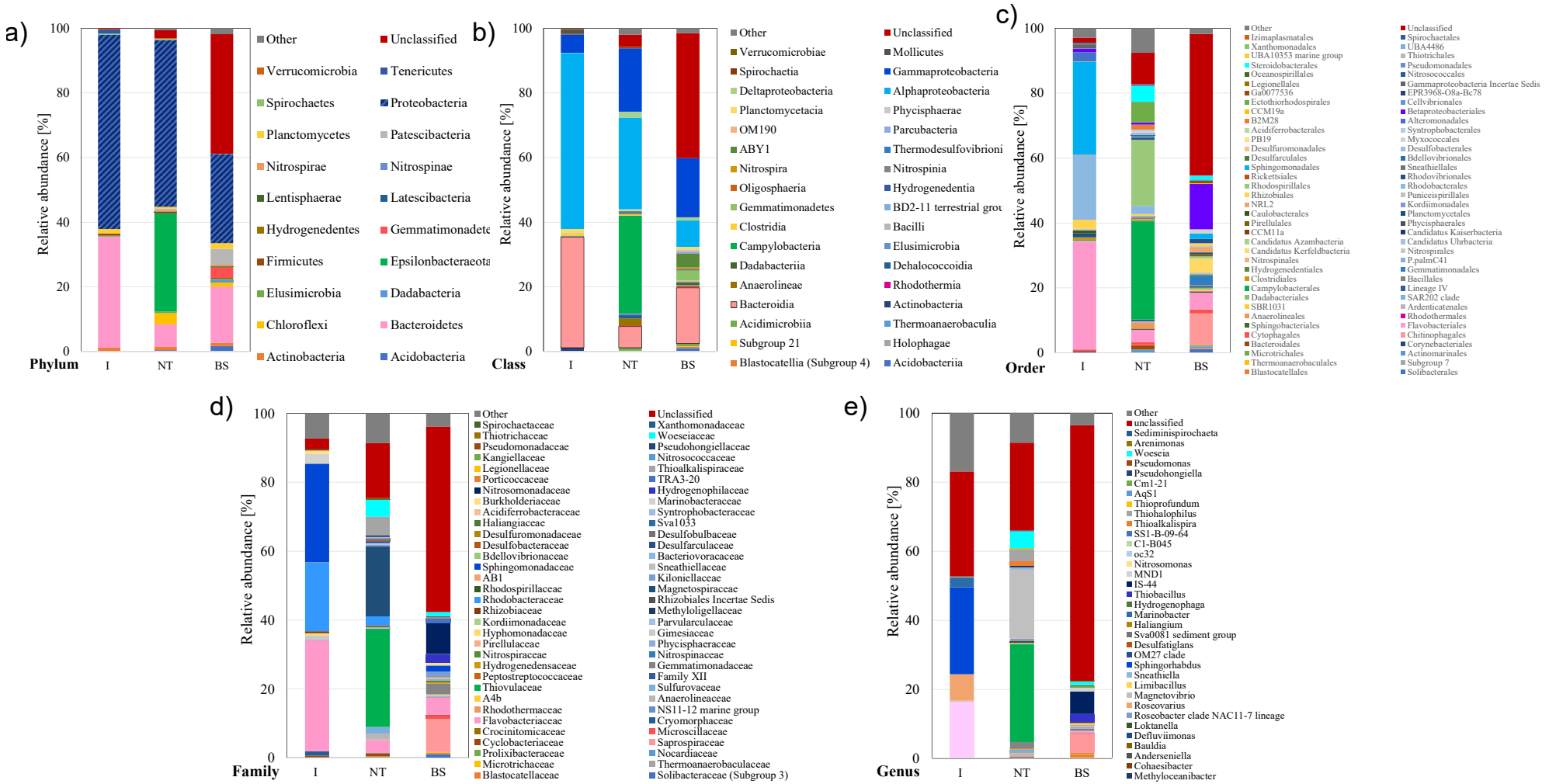

306

307 **Figure 4.** Relative abundance at phylum (a), class (b), order (c), family (d) and genus (e).

3.3 Sediment phytotoxicity

The phytotoxic features of the solid matrix, before and after bioslurry treatment, are shown in Figure 5, in terms of root elongation (Figure 5a) and germination index (Figure 5b).

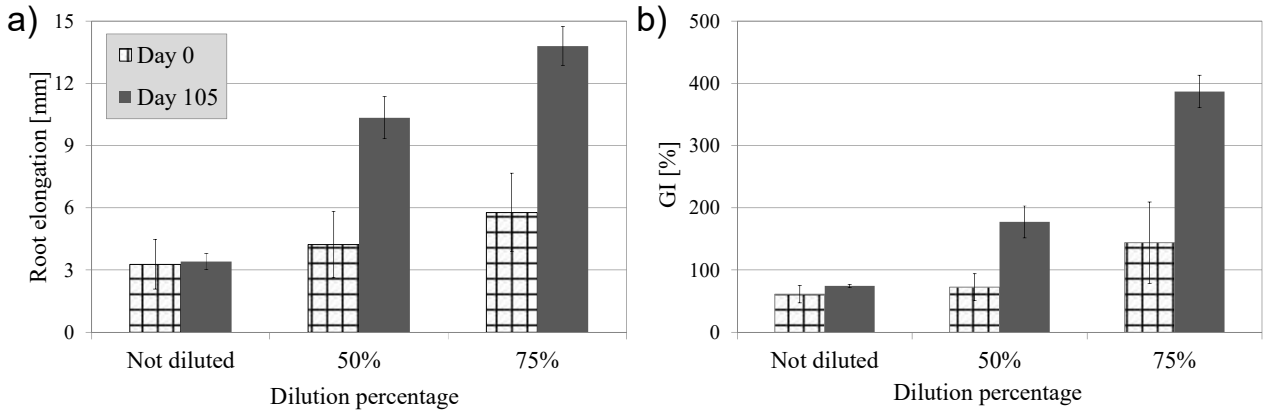

Figure 5. Average root system length (a); germination index (b), with bars representing the standard error.

From Figure 5 it is possible to observe a significant improvement of root elongation and GI for the configurations characterized by dilution ratios of 50% and 75%, for the treated sediment (day 105 compared to that of untreated sediment sample (day 0). In particular, the root elongation was approximately 14 mm for the treated sediment sample (day 105) with a dilution ratio of 75%, compared to the raw sediment sample at the same dilutions whose root length was less than 6 mm (Figure 5a). All configurations showed an improvement in quality for the treated sediment (105 days), with a germination index between 75% and 390%, compared to 61% and 144% for the untreated sediment (Figure 5b), highlighting the reduction of the toxic properties of the solid matrix after bioslurry treatment.

4. CONCLUSIONS

The results achieved in this preliminary study provided useful insights about the feasibility of biological aerobic treatment of marine sediments contaminated by hydrocarbons. In particular, the microcosm trials highlighted that the inoculum of halotolerant allochthonous biomass provided the highest performance in terms of hydrocarbon removal, even if the autochthonous biomass, after an acclimation period, revealed good activity in the aerobic hydrocarbon degradation. The results from the bioslurry reactor suggest that a high contaminant concentration may hinder the achievement of high removal efficiencies. In further experimental activities, lower hydrocarbon concentrations might be considered in order to achieve higher removal efficiencies. The assessment of gaseous emissions from the system could be useful to obtain a more accurate evaluation of TPH-removal pathways. The results of microbiological community highlighted that the autochthonous biomass likely overwhelmed the allochthonous one during treatment, thus suggesting that future investigations might be carried out without external inoculum. Finally, in light of potential reuse of the treated sediments, the results of the phytotoxicity tests showed that the treatment with bioslurry reactor provides a matrix with higher quality characteristics than the original state.

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