Biodegradation potential of oil-degrading bacteria related to the genus *Thalassospira* isolated from polluted coastal area in Mediterranean sea

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ABSTRACT

Three bacterial species related to the genus *Thalassospira* (*T. lucentensis*, *T. xianhensis* and *T. profundimaris*), isolated from polluted sediment and seawater samples collected from Priolo Bay (eastern coast of Sicily, Ionian Sea), were analyzed for their biotechnological potential. For this purpose, the presence of specific catabolic genes associated to aliphatic and aromatic hydrocarbons metabolism, the production of biosurfactants and emulsification activity, the capability to degrade oil derived linear, branched, cyclic alkanes and polycyclic aromatic hydrocarbons (PAHs) were evaluated. Alkane hydroxylase gene (alkano-monoxygenase *alkb* and citocrome *P450*) were present in genome of all strains, confirming their hydrocarbons degrading capability. All strains of *Thalassospira* produced biosurfactants and showed emulsification activity. The two-dimensional gas chromatography analysis (GC×GC) showed that they were able to degrade oil fractions with the capacity ranging between 77-91%. The data obtained in this study demonstrated the biodegradation ability of *Thalassospira* and suggest that these strains play important role in marine contaminated ecosystems

Keywords:

Marine Oil Pollution„ *Thalassospira*, biodegradation of hydrocarbons, biosurfactants, emulsification activity, Comprehensive two-dimensional gas chromatography

1. Introduction

Bioremediation consist in the conversion of environmental pollutants in non-toxic compounds by microrganisms. During this transformation, organic and toxic compounds were convert into less complex and toxic metabolites, and with the mineralization process in inorganic compounds, H_2O and CO_2 (if the microorganisms are in aerobic condition) and CH_4 (if the microorganisms are in anaerobic condition) (Nikolopoulou and Kalogeraki, 2010).. The successes of bio-conversion depend on many factors, such as: pH, temperature, oxygen concentration, availability of nutrients and/or chemicals, type of pollutants, cellular transport properties, and chemical partitioning in growth medium (Singh and Ward, 2004). On the foregoing basis, bioremediation techniques have proved advantageous, and the study and the optimization of this eco-friendly technique can be a valid alternative to chemical and physical techniques for cleaning up of oil polluted marine environments (Alkatib et al., 2011). Oil derived hydrocarbon-degrading microorganisms are widely distributed in marine, freshwater and soil habitats (Chaillan et al., 2004; Yakimov et al., 2007,Catania et al., 2015, Catania et al, 2017, Djahnit et al., 2019). Despite the chemical stability, very low water solubility and bioavailability of hydrocarbon compounds, numerous bacterial species have been described as efficient break-downer of oil-pollutant as widely evidenced in literature (Cerniglia 1993; Sutherland et al. 1995; Kanaly and Harayama 2000; Peng et al. 2008; Haritash and Kaushik 2009, Catania et al., 2020a). Scientific interests and biotechnological applications about hydrocarbon-utilizing bacteria increased in recent years (Margesin and Schinner 2001; Le Borgne et al. 2008, Scaffaro et al., 2017, Catania et al., 2020b) and several studies on the biodegradation of crude oil and/or PAHs and other aromatic compounds have been performed (Kasai et al. 2002; Melcher et al. 2002; García et al. 2005; Li and Bai 2005; McKew et al. 2007, Catania et al., 2018). Bacterial degradation of PAHs was firstly described by Zhao et al. (2009), in a phenanthrene degrading consortium from a saline soil which was developed at 10% salinity

and included several strains belonging to different genera, among which *Thalassospira spp*. (Hassanshahian et al., 2010 and 2012). Members of *Thalassospira* genus are mesophilic, halophilic, Gram-negative, oxidase and catalase positive, aerobic and often facultative anaerobic α-Proteobacteria with the ability to utilize hydrocarbons or xenobiotics compound such as polyvinyl-alcohol as source of carbon and energy. (Baldani et al., 2014; Kiseleva et al., 2015). Recent studies revealed and demonstrated widely as the genus *Thalassospira* is correlated to hydrocarbons pollution in marine environment (Lopez Lopez, A. et al 2002, Chen, et al. 2007, Kodoma et. al. 2008, Zhaoet al. 2010, Thompson et al. 2017, Catania et al., 2018,),

Furthermore, numerous scientific evidences showed that some *Thalassospira* species were the dominant members in the hydrocarbon-degrading consortia, suggesting a central role in biodegradation and restoration of marine ecosistems.(Muangchida, et al. 2018).

However, the knowledge on some species of the genus *Thalassospira* is still limited, and the capabilities and mechanisms of degradation of contaminants are not well known. The main aim of this study was to identify the biodegradation potential and expand the knowledge on catabolic pathways involved in the degradation of hydrocarbons of three bacterial strains isolated from polluted coastal area, affiliated to species of the poorly investigated genus *Thalassospira,* . To achieve this goal, for the first time, the biotechnological potential) of the species. *T. lucentensis*, T. *xianhensis* and t. *profundimaris* were analyzed. In particular we evaluated: i) presence/absence of specific catabolic genes, ii) production of biosurfactants and iii) capability to degrade different oil fractions (linear, branched, cyclic alkanes and polycyclic aromatic hydrocarbons). The potential of hydrocarbons degradation was evaluate using a comprehensive two-dimensional gas chromatography method (GC×GC) with a dual detection, flame ionization detector (FID) and single quadrupole (Q MS). The use of this instrument has proven essential in order to achieve a detailed characterization and quantification of the different HC chemical families (Tranchida et al., 2009, Zoccali et al., 2015, 2018).

Materials and methods

2.1. Isolation of bacterial strains

Bacterial strains were isolated from enrichment cultures obtained from seawater and sediment samples collected in Priolo Bay (South-East Sicily, Italy, Mediterranean Sea). (Priolo Bay is a coastal marine basin located along the South-East coast of Sicily (Italy) in the southern part of Augusta Bay, that is one of the most industrialized and polluted areas in the Mediterranean Sea (Oliveri et al. 2016). In July 2012 seawater and surface sediment (0-5 cm upper seawater layer) samples were collected through sterile Plexiglas cores (20 cm long, 5 cm diameter), from three stations located along a northern transect (N1: N 37° 9′ 58,3″, E 15° 12′ 2.6″- depth 5 m; N2: N 37° 10′ 3,7″, E 15° 12′ 21.7″-depth 10 m; N3: N 37° 10′ 9.3″, E 15° 12′ 53,8″- depth 20 m) and three stations located along a southern transect (S4: N 37° 9′ 20.5″, E-15° 13′ 1.3″-depth 5 m; S5: N 37° 9′ 30.6″, E 15° 13′ 9.8″-depth 10 m; S6: N 37° 9′ 46.8″, E 15° 13′ 20.6″ - depth 20 m).

As previously reported (Catania et al., 2015), different sediments and seawater enrichment cultures sets were performed by supplementing the ONR7a medium (Dyksterhouse et al., 1995), with an *n*-alkanes mixture (C_{16}, C_{18} and C_{24}), PAHs (phenanthrene, pyrene, biphenyl and dibenzothiophene) or crude oil (Arabian Light Crude Oil). Phenotypically different colonies obtained from the plates were purified, and transferred to fresh medium with hydrocarbons as unique carbon sources (crude oil, n-alkanes mixture, PAHs). Plates without hydrocarbons were prepared to eliminate autotrophs and agar-utilizing bacteria. The procedure was repeated, and only isolates exhibiting pronounced growth on crude oil, *n*-alkanes mixture or PAHs were stored in stock media with glycerol at -20±1°C for further characterization (Hassanshahian et al., 2012). On the base of the literature data and 16S rDNA analysis, the more interesting isolates were selected for the following analysis. In particular two strains named AU5AG4 and AU3AA1, isolated from seawater samples in S5, N3 stations respectively,

and one strain named AU6SG1 isolated from sediment sample in the station S6 were selected and characterized.

2.2. Identification of the isolates

2.2.1. Molecular characterization of isolates (16S rDNA sequencing)

Total DNA extraction of bacterial strains obtained was performed through the MasterPure Complete DNA&RNA Purification Kit (Epicenter, Biotechnologies, Madison, WI) in accordance with manufacture's protocol. The 16S rDNA loci were amplified using the 27F (5'-AGAGTTTGATCCTGGCTCAG-3', Lane, 1991) primer and the 1492R (5' TACGGYTACCTTGTTACGACT-3', Lane, 1991) universal primer. Polymerase chain reaction (PCR) was carried out in 50 µL of reaction mixture containing 1x reaction buffer, 1x solution Q (both from QIAGEN), 1 µM of each primer, 200 µM dNTP (Gibco), 1 µL of template and 2.5 U of Qiagen *Taq* polymerase. The PCR conditions were as follows: 95 °C for 5 min (1 cycle); 94°C for 1 min, 50°C for 1 min and 72°C for 2 min (35 cycles); with a final extension step at 72°C for 10 min. PCR products were sequenced using Macrogen Service (Macrogen, Europe). The analysis of the sequences was performed as previously described by Yakimov et al. (2005). Sequences similarity of individual inserts was analyzed by the FASTA program Nucleotide Database Query, available through the EMBL-European Bioinformatics Institute. The phylogenetic affiliation of the sequenced strains was performed as described by Yakimov et al. (2006). 16S rDNA were deposited in GeneBank with Accession Number: KT348286, KT348281 and KT348287.

2.2.2. Biochemical identification

In addition to the morphological examination carried out in Catania et al. 2015 was perfomed: i) Oxidase/Catalase test; ii) cellular shape and mobility. These tests done according to the Bergey's manual for identification taxonomy (Holt et al. 1998).

2.2.3. Qualitative analysis of catabolic genes

Qualitative analysis (presence/absence) of catabolic genes [cytochrome (*P450),* alkane hydroxylase (*alkB)* and biphenyl dioxygenase iron sulfur protein (*bphA* gene) were carried out using different couples of primers. The presence/ absence of *P450* gene (~800 bp product) was assayed applying the primers P450F (5′TGTCGGTTGAAATGTTCATYGCNMTGGAYCC-3′) and P450R (5′TGCAGTTCGGCAAGGCGGTTDCCSRYRCAVCKRTG-3′)(Kubota *et al.*, 2005); while to assay the presence/ absence of alkane-monoxygenase gene (~550 bp product) was used degenerate primers alkBwf (5′-AAYACNGCNCAYGARCTNGGVCAYAA-3′ and alkBwr (5′-GCRTGRTGRTCHGARTGNCGYTG-3') (Kloos et al., 2006), and couple primers TS2S (5'-AAYAGAGCTCAYGARYTRGGTCAYAAG-3') and deg1RE (5'-GTGGAATTCGCRTGTCIGARTG-3') (Smits et al., 1999); the primers described by Ryslava et al. (2003) 352F (5'-TTCACCTGCASCTAYCACGGC -3') and 1178R (5'-ACCCAGTTYTCDCCRTCGTCCTGC-3') were used for the detection of *bphA* gene (~800 bp product). The PCR reaction mixtures contained 50 ng of DNA, 1× Qiagen reaction buffer, 1× solution Q (Qiagen), 0.4 mM P450 primers (or 0.8 mM for *alkB* primers and/ or 10 pmol for *bphA* primers), 200μM dNTPs (Gibco) e 2.5 U di Qiagen Taq Polymerase in a final volume of 50 ml; while the PCR reaction mixtures employed with the primers TS2S and deg1RE was carried out as described by Innis et al (1990). The PCR amplification of alkane-monoxygenase gene and P450 gene was carried out on a Mastercycler Gradient (Eppendorf) with the following conditions: initial denaturation 4 min at 94°C (1 cycle); 30 s at 94°C, 30 s at 52°C (for the degenerate primers alkBwf and alkBwr, it was 30 s at 55°C) and 1 min at 72°C (32 cycles);

and a final extension at 72°C for 10 min; while for the amplification of alkane-monoxygenase gene with the primers TS2S and deg1RE and *bphA* gene were used respectively the condition described by Smits et al. (1999) (1 cycle 4 min at 95°C; 25 cycles: 45 s at 95°C, 1 min at 40°C, 1 min at 72°C; 1 cycle 5 min at 72°C, indefinitely at 4°C) and Dudášová et al. (2014)(at 95 °C for 2 min; 30 cycles: 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and 30 s; and at 72 °C for 10 min). The gene amplicons obtained with PCR reaction were separated by electrophoresis on agarose (1.5%) gel (Whang et al.2010).

2.4.1. Starter culture and growth in laboratory conditions

The initial cultures were prepared by inoculating one loopful from a fresh single colony into 10 mL of ONR7a mineral medium based on seawater (Dyksterhouse et al., 1995) supplied with 5% of Sodium Acetate. After growing in a rotary shaker (New Brunswick C24KC, Edison NJ, USA; 150 ×g) at 25±1 °C for two days the culture was used to Biosurfactant Production, Emulsification Activity and Liquid Surface Tension, besides 500 µL of the seed-culture broth were washed (×2) with PBS 1× and ready for biodegradation experiment.

2.3. Biosurfactant Production, Emulsification Activity and Liquid Surface Tension

Biosurfactant production was screened by two distinct methods: *i*) The Drop collapse test and *ii*) Oil Spreading. In the Drop collapse test, 7 µl of (sterile) mineral oil was added to each well of a 48-well microtiter plate. Covered plates were equilibrated for 24 hours at room temperature before adding culture supernatant. 20 µl of the culture supernatant was added on the (sterile) oil surface in the microtiter well. Sodium dodecyl sulfate (SDS, Sigma-Aldrich, Milan) and sterile distilled water were used respectively as positive and negative control suspensions (Manjoubi et al., 2013). In the Oil Spreading, 20 ml of distilled water was added to an empty Petri dish followed by addition of 10 µl of crude oil to the surface of the water. An

aliquot of 10 microliters of bacterial culture were then added to the oil surface. The diameter of the clear zone around the bacterial suspension corresponds to the biosurfactant concentration (Youssef et al., 2004). Emulsification activity was measured with the Emulsification index (E_{24}). The E24 was determined in a clean flask by adding of hydrocarbon (benzene-C_6H_6 and diesel) in each bacterial broth cultures at the concentration of 1% (v/v). this mixture was vortexed for 2 min and leaving to stand for 24 h, the E_{24} index is given as percentage of height of emulsified layer (in millimeters) divided by total height of the liquid column (in millimeters) (Iqbal et al. 1995; Cappello et al., 2012; Hassanshahian, 2014). The Surface Tension of bacterial cultures was measured after 10 days of growth, by the Wilhelmy plate method, using a digital tensiometer (Gibertini, Italy), in accordance with the manufacturer's instructions. The surface tension was expressed in units of mN m^{-1}.

2.4. Biodegradation experiment

2.4.2. Experimental set-up of microcosm systems

The microcosm systems were performed in 250 mL sterilized Erlenmeyer flasks. Microcosms were incubated at 25±1°C for 15 days with shaking (100 ×*g*) and were realized in 100 mL of mineral ONR7a medium supplemented with 0.1% (v/v) -of sterile crude oil (Arabian Light Crude Oil; ENI Technology S.p.A.) and inoculating single bacterial strains. Crude oil used in the experimentation was introduced in microcosm systems after physical weathering (100 ×g, 25±1°C for 48 h), and supplemented with 0.1% (v/v) of heptamethylnonane ($C_{16}H_{34}$, Sigma-Aldrich, Milan) as internal spike for measure of bio-degradation rate. Abiotic microcosms untreated (without inoculation) were set as negative control. All experimentations were carried out in twice.

220 *2.4.3. Hydrocarbons extraction from microcosm system*

221 After 15 days of incubation the microcosms were analyzed for the residual hydrocarbons.
222 After acidification TERHCs from each microcosm system were extracted at room temperature
223 on a shaking table using dichloromethane (CH_2Cl_2, Sigma-Aldrich, Milan, Italy; 10% v/v).
224 Protocol used for extraction was 3510 EPA (Environmental Protection Agency).This procedure
225 was repeated three times, and the CH_2Cl_2 phase was combined and treated with sodium sulfate
226 anhydrous (Na_2SO_4, Sigma-Aldrich, Milan, Italy) in order to remove any residual water
227 (Ehrhardt et al., 1991; Wang et al., 1998; Dutta and Harayama, 2001; Denaro et al., 2005). The
228 extracts were concentrated by rotary evaporation (Rotavapor model R110; Büchi Labortechnik
229 AG, Switzerland) at room temperature (~30 °C), followed by evaporation under a stream of
230 nitrogen.

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232 *2.4.4. Cryogenic Modulation GC×GC-Q MS/FID Analyses*

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234 Quali-, quantitative analysis of hydrocarbons from microcosms were carried out by
235 Cryogenic Modulation (CM) GC×GC-Q MS/FID consisting of two GC-2010 gas
236 chromatographs (Shimadzu Corporation, Kyoto, Japan), a TQ8040 triple quadrupole mass
237 spectrometer (Shimadzu) and a flame ionization detector (FID). All Data were collected by the
238 GCMS Solution v.4.41 software (Shimadzu); the ChromSquare ver 2.3 software was used for
239 GC × GC data processing (Shimadzu). MS spectral matching was performed automatically by
240 using the ChromSquare ver 2.3 software and the Wiley Registry 11th Edition/NIST 2017 Mass
241 Spectral Library (Wiley, NIST). Moreover, the identification was performed by comparison
242 with linear retention index, and elution order. The first gas chromatograph (GC1) was equipped
243 with an AOC-20i auto-injector, and a split–splitless injector (350°C). The primary column (^{1}D)
244 was an SLB-35 ms [(silphenylene polymer, which can be considered equivalent in polarity to

poly(35%diphenyl/65% dimethylsiloxane)] with dimensions 30 m × 0.25 mm id × 0.25 µm d_f. A 1.0 m × 0.10 mm id uncoated column was used to create the modulator loop. The second dimension (^{2}D) column was an SLB-5 ms [(polymer which can be considered equivalent in polarity to poly(5%diphenyl/95% dimethylsiloxane)] with dimensions 1.0 m × 0.10 mm id × 0.10 µm d_f. All the columns used were provided by Merck Life Science (Merck KGaA, Darmstadt, Germany). The connections between the ^{1}D and ^{2}D columns, and the modulator loop, were made by using two SilTite mini unions (Trajan, Ringwood, Victoria, Australia). The outlet of the ^{1}D column was connected to a MXT "Y"-Union (Restek); the latter was then linked to a 0.4 m × 0.10 mm id (for FID analysis) and to a 1.0 m × 0.10 mm id uncoated column (for MS analysis). An average of 63% of the column effluent was directed to the FID with the objective of quantification, while the remaining fraction reached the MS for identification purposes. Helium was supplied at the GC1 inlet at a pressure of 289.5 kPa (constant average linear velocity mode); volume and mode of injection: 1 µL in the split mode (25:1). GC1 temperature program: 80–350°C (20 min) at 3°C min^{-1}; GC2 temperature program: 60-350 °C (13.3 min) at 3 °C min^{-1}. Modulation was performed by using a cryogenic fluid-free modulator; modulation period was 4 s (the heating step was performed at 400 °C, for 0.35 s). MS conditions: the temperature of the interface was 330°C; the ion source temperature was 280 °C, with analyte fragmentation induced by electron ionization (70 eV). A spectral generation frequency of 33 *Hz* was applied with the following mass range *m/z* 45-360. FID (360 °C) sampling frequency was 125 *Hz*.

2. Results

2.1 Taxonomic and Biochemical characterization of *Thalassospira* isolates

Three different bacterial strains HC-degrading were isolated by sediment and seawater samples in Priolo Bay as previously reported in Catania et al. (2015). The isolates were identified by 16S rDNA analysis. The strains AU5AG4, AU3AA1 isolated from seawater, and strain AU6SG1, isolated from sediment, were affiliated to the genus *Thalassospira* (Alpha-proteobacteria class), species *T. xianhensis* (KT348286), *T. lucentensis* (KT348281) and *T. profundimaris* (KT348287) respectively. The results of phylogenetic identification are shown in **Fig.1.** The three bacterial strains formed an independent branch related to other type strains HC degrading affiliated to other genera among Alpha proteobacteria. In particular, The sequences showed considerable evolutionary divergence from the sequences of *Aquaspirillum* sp., *Terasakiella* sp.. Colonies were positive for catalase and oxidase. Phenotypic and biochemical characteristic analyzed are shown in **Table 1**.

3.2. Detection of catabolic genes

Positive PCR amplification with alkBwf-alkBwr, TS2S- deg1RE, and/or P450F-P450R couple primers evidenced the presence of alkane-monoxygenase (*alkB*) and/or cytochrome *P450* genes, while the biphenyl dioxygenase (*bphA*) targeting primers didn't show any amplification product in the strains in study. The presence /absence (positive/ negative amplification) of these genes is an indirect index of potential capability of the strains in study of degradation of hydrocarbons, but in the same time, it isn't express efficiency in degradation process.

290 *3.3 Biosurfactant production and emulsification activity*

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292 The results obtained from the Surface Tension, drop collapse, oil spreading test and
293 Emulsification Activity (E24%) have been reported in the **Table2**. The *Thalassospira* spp.
294 strains are producers of biosurfactants and showed a good emulsification activity. Data obtained
295 with drop collapse test identified positive results for all isolates, but this result is in contrast
296 with data obtained in the other test. This incongruence can be related to the different type and
297 concentration of biosurfactant produced and / or to the different experimental conditions
298 (Youssef et al., 2004; Rodrigues et al 2006).

299

300 *3.4. GC×GC-Q MS/FID Analyses*

301 As shown in **Figure 1**, the Arabian Light Crude Oil represents a very complex sample
302 containing different chemical classes. To achieve a reliable identification and quantification of
303 the different chemical classes the separation power of a mono-dimensional GC system is not
304 enough. For such a reason, in the present research, a GC × GC was employed due to its higher
305 peak capacity and the capability to couple columns with different selectivity. Moreover, in such
306 a kind of samples, the use of an MS detector is mandatory for reliable identification. However,
307 the drawback of this detector is the different response according to the analyte, that does not
308 allow quantification in case of lack of standard materials. For this purpose, FID is the only
309 detector providing virtually the same response according to the chemical class (Zoccali et al.,
310 2016). Thus, in the present paper, we developed a GC × GC with a dual detection system able
311 to perform both identification and quantification of the different chemical classes in a single
312 run.

313 The goal of the GC × GC method optimization is to occupy as much as possible of the
314 two-dimensional space (without occurring in the wrap around effect) by achieving the best

315 separation possible and an ordered chromatogram. A medium polar column (35%) was used in
316 the first dimension and a non polar column (5%) was used in the second dimension, with such
317 a kind of configuration the non-polar components will occupy the upper part of the plot, while
318 the more polar ones the lower region. Compounds identification were carried out according to
319 MS spectral similarity, linear retention indices, and 2D position. In **Figure 1**, reporting the
320 Arabian Light Crude Oil analysis, the upper part of the 2D space was occupied by linear and
321 branched alkanes forming a fairly horizontal row of spots, and cyclic alkanes are positioned
322 below the row of the *n*-alkanes. As can be seen in **Figure 1** the retention in the second dimension
323 decreases according to the increase of the aromatic ring number, while increase in the first
324 dimension also considering the length of the alkyl chain. The use of the developed GC×GC-Q
325 MS/FID method, has allowed the quantification of the residual HC after incubation of the
326 different bacterial *Thalassospira* isolates. Totally, 6 different chemical classes were evaluated
327 namely: *i*) linear and branched alkanes, *ii*) cyclic alkanes, *iii*) C6-aromatics, *iv*) di-aromatics, *v)*
328 tri-aromatics + sulphur compounds and *vi*) tetra-aromatics + sulphur compounds. All the
329 analysis were carried out by using heptamethylnonane as internal standard and the values were
330 calculated considering the GC×GC-FID response. The obtained results were reported in the
331 histogram in **Figure 2**, expressed as percentage of degradation. Considering the total
332 contamination, interesting results were obtained for all the studied bacterial strains, in fact the
333 degradation of Arabian Light Crude Oil ranged between 77 to 91%. An important result was
334 obtained in the microcosm inoculated with the strain affiliated to *T. xianhensis* that gives the
335 best results with a degradation ranging between 87% for the Cyclic alkanes and 94% for Tetra-
336 aromatics + Sulphur compounds. These compounds, in particular, Tetra-aromatics, are
337 recalcitrant, toxic, persistent and this result could be used for future study.
338
339 **3. Discussion**

Increasing environmental pollution has involved scientific community to extend the research in this sector and in particular in bioremediation and biorecovery strategies. This relatively new technology exploits microorganisms' catabolism; indeed these last use the pollutant compound as carbon and energy source. Various studies demonstrated that this microbial processes convert pollutant compounds in non-toxic or less toxic products, and demonstrated that the bioremediation technologies are useful strategies to obtain the recovery polluted environment. Low cost and high efficiency of bioremediation identify this strategy as "*green technology*" and "eco-friendly" (Nicolopoulou et al, 2013, Crisafi et al., 2016). Several data about different kind of microorganisms with hydrocarbon degrading capability are present and described in the literature (Yakimov 2004; Rooling et al., 2004; Cappello et al., 2007; Catania et al. 2015; Bargiela et al. 2015, Bayat et al., 2015, Bayat et al., 2016). In this study we characterize and evaluate the biodegradation ability of three selected bacterial strains affiliated to *Thalassospira* genus, precisely *T. xianhensis* , *T. lucentensis* and *T. profundimaris* species, isolated from Priolo Bay, a chronically polluted marine coastal area in Mediterranean sea. Previous studies investigated the structure and diversity of indigenous microbial population in seawater and sediment samples of Priolo Bay, by cultural methods and culture independent approaches (Catania et al., 2015, Catania et al., 2018). The results have shown rich and diverse HC degrading microbial communities that contribute to natural attenuation of the area. The alkane catabolism was detected in both seawater and sediment samples, moreover in sub-surface sediments (5-10 cm) a degradation of HC in microaerophilic condition for co-metabolism was proposed (Catania et al., 2018). The highest diversity and abundance of culturable degrading bacteria was observed in the highest contaminated sediments (Catania et al., 2015). All sediment and seawater samples of Priolo Bay hosted high abundance and diversity of alkane and PAH degrading bacteria, assigned mainly to Alpha or Gammaproteobacteria, belonging to *Alcanivorax*, *Marinobacter*, *Alteromonas*, *Oleibacter*,

365 *Idiomarina*, *Pseudomonas*, *Halomonas* and *Thalassopira* genera, that are dominant in
366 contaminated environments and are known to play a key role in HC degradation.

367 The focus of this work has been the evaluation to features of three *Thalassospira* species
368 isolated from Priolo Bay and correlated with hydrocarbon degradation capability, in particular
369 tests of biosurfactant production, presence of specific catabolic genes involved in
370 biodegradation processes were carried out (*alkB, CYP450* and *bphA* genes); moreover
371 biodegradation tests in microcosms and analysis of the hydrocarbons fractions degraded were
372 investigated. Several test of biosurfactant production were performed, because these molecules
373 have different properties, besides low concentrations of these molecules could give incorrect
374 values (Mahjoubi et al 2013). Results of drop collapse and oil spreading test showed that all
375 isolates in study are biosurfactant producers; however, divergent data are obtained, in particular
376 data of superficial tension put in evidence that the isolate AU5AG4 *T. xiahnensis* is the best
377 producer of biosurfactant, while emulsification activity (E24%) was higher for AU6SG1 *T.*
378 *profundimaris*. These results could be dependent by the nature of biosurfactant, by the
379 concentration of this molecule and to the test applied. In the study of Youssef et al. (2004) the
380 assessment of surface tension is time- depending and showed a correlation coefficient stronger
381 than drop collapse and oil spreading test, which are indicated as more reliable detector of low
382 concentration of biosurfactants.

383 Results about the analysis of catabolic genes (alkano-monoxygenase and citocrome P450)
384 confirmed that *Thalassospira xianhensis* AU5AG4, *T. lucentensis* AU3AA1 and *T.*
385 *profundimaris* AU6SG1 present in their genome the ability to degrade hydrocarbons. Analysis
386 of oil degradation evidence as the strains in study were capable to degrade linear and branched
387 alkanes, cyclic alkanes, C6-aromatics, di-aromatics, tri-aromatics + sulphur compounds and
388 tetra-aromatics + sulphur compounds, with efficiency of biodegradation ranged between 61%

389 to 91% after 10 days of incubation in microcosm. However, the rates of biodegradation of
390 hydrocarbons substrate in the same isolates was different, in particular *T. xianhensis* was the
391 more performing among the *Thalassospira* strains, with more than 85% of HC degraded.
392 Previous studies showed the ability of *Thalassospira xianhensis* strain P-4T to degrade
393 naphthalene, phenanthrene and pyrene, but not fluoranthene or benzo[a]pyrene (Zhao, et al.,
394 2010). Several PAH-degrading strains belonging to *T. lucentensis* have been isolated from
395 sediments, and were able to degrade a more restrict group of PAHs respect to *T. lucentensis*
396 AU5AG4, including naphthalene, phenanthrene and pyrene, in 8 days (Zhao, et al., 2010). Other
397 strains of *T. lucentensis* degrade more than 90% of *n*-alkanes and more than 70% of (alkyl)
398 naphthalene in long times (30 days) and with addition of fertilizers (Sutiknowati, 2018), while
399 AU5AG4 was able to degrade HC in short time (10 days). *Thalassospira profundimaris*
400 WP0211T, isolated from deep-sea sediment of the west Pacific Ocean, showed the ability to
401 grow on hydrocarbon compounds with low and high concentration (Hütz et al., 2011). In
402 comparison to other reports, *T. xianhensis*, *T. lucentensis*, *T. profundimaris* isolated by Priolo
403 Bay were more able to grow and degrade hydrocarbon compounds with highest efficiency in
404 less time.

405 Although the degradation ability of *T. lucentensis, T. xianhensis* and *T. profundimaris*
406 have been studied, little is known about catabolic genes and production of biosurfactants. In
407 this study, in addition to evaluation of the potential and degradative abilities, we also study the
408 catabolic pathway, the production of biosurfactants and emulsification activity of *T.*
409 *lucentensis, T. xianhensis and T. profundimaris* isolated in Priolo Bay. This study contribute
410 to understand the role of *Thalassospira* in degradation processes, and provide new information
411 on species of *T. lucentensis, T. xianhensis and T. profundimaris*, until now not much
412 investigate, confirming that *Thalassospira* play a kay role in the restoration and recovery of
413 marine environments.

Recovery of oil polluted areas can be achieved by physic-chemical or biological methods. Due to negative aspect of the physic-chemical approach (Chibueze Azubuike et al., 2016), more attention is being given to the exploitation of biological alternatives (Okoh, 2006). Biological treatments are becoming more important, mainly due of the low environmental impact, costs (in general cheaper than other cleanup technologies), the capability to destroy/degrade organic contaminants and the possibility of advantageous use of treated sediments (Rulkens and Bruning, 2005). Several studies have shown better results using bioremediation strategies (Beolchini et al., 2010; Rocchetti et al., 2011, 2012; Cappello et al., 2015). In general, bioremediation is often based on *in situ* stimulation of the microbial community (biostimulation) or amending the microbial community with an inoculum of hydrocarbon-degrading bacteria (bioaugmentation). In both cases, the successful result of bioremediation depends on appropriate hydrocarbon-degrading consortia and environmental conditions. In this study, interesting results suggest that the isolates of *Thalassospira* species are important and relevant not only in the next studies of biotechnological processes, as the production and the extraction of significative biomolecules used in several industries including organic chemicals, petroleum, petrochemicals, mining, metallurgy (mainly bioleaching), agrochemicals, fertilizers, foods, beverages, cosmetics, pharmaceuticals and many others (Vijayakumar S. and Saravanan V. 2015), but also in the understanding of the bioremediation processes and in the application of the same microorganisms in bioremediation techniques (Scaffaro, et al., 2017, Catania et al., 2020).

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Conflict of Interest

No conflict of interest is declared by the authors and none of the authors of this paper has a direct financial relationship with the commercial identities mentioned in this article.

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671

672 **Tables**

673

674 **Tab. 1.** Closest relatives of the 16S rRNA gene sequences of bacteria isolated in this study and
675 results of the main classical biochemical.
676

677

Isolate code	Closest type strain	Identity (%)	Oxidase test	Catalase test	Mobility	Shape
AU5AG4	*T. lucentensis*	98	Positive	Positive	Yes	Curved-rod
AU3AA1	*T. profundimaris*	99	Positive	Positive	Yes	Curved-rods to spirilla
AU6SG1	*T. xianhensis*	98	Positive	Positive	no	Curved-rod

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682 **Tab. 2.** Superficial Tension, Drop Collapse test, Oil spreading test and Emulsification Activity
683 of strains in study. Positive data of Drop Collapse and Oil spreading test are indicated by the
684 symbol "+", in contrast negative data are indicated with the symbol "-".
685
686

Isolate code	Closest type strain	Superficial tension (mN m-1)	Drop collapse test	Oil spreading test	E24 test (%)
AU5AG4	*T. lucentensis*	49.7 ±0.5	+	+	22.2 ±0.5
AU3AA1	*T. profundimaris*	53.8 ±0.5	+	+	21.9 ±0.5
AU6SG1	*T. xianhensis*	53.2 ±0.5	+	+	11.1 ±0.5

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688

689 **Figure Captures**

690

691 **Fig. 1**. Phylogenetic tree based on 16S rRNA gene sequence analysis of the isolates in study
692 (AU3AA1, AU5SG4 and AU6SG1) and relationship with different species of the genus
693 *Thalassospira*, some genera related to the class of Alpha-proteobacteria (*Terasakiella and*
694 *Aquaspirillum*), and other hydrocarbon degrading bacteria affiliated to the class of Gamma-
695 proteobacteria. As Outgroup it was used the 16S rRNA gene of *Methanococcus jannaschii*
696 (M59126).

697

698 **Fig. 2.** GC×GC-Q MS analysis of Arabian Light Crude Oil. Abbreviations: B, benzene; N,
699 naphtalene; F, fluorene; DBT, dibenzothiophene; P, phenanthrene; Py, pyrene; BNT,
700 benzonapthothiophene; Cry, chrysene; Per, perylene. The abbreviations C1, C2, etc., refer to
701 the number of carbons in the alkyl substituents. The intensity of the peaks is related to color
702 (white minimum, red maximum).

703

704

705 **Fig. 3.** Rate (%) of biodegradation (calculated by using the GC×GC-FID response) of the
706 different bacterial strains studied in the present work: isolate AU6SG1 (T. *profundimaris*; white
707 bars), isolate AU3AA1 (T. *lucentensis*; light gray bars) and isolate AU5AG4 (T. *xianhensis*;
708 dark gray bars).

709