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Substrates specialization in lipid compounds and hydrocarbons of *Marinobacter* genus

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Abstract: The impact of petroleum contamination and of burrowing macrofauna on abundances of *Marinobacter* and denitrifiers was tested in marine sediment mesocosms after 3 months incubation. Quantification of this genus by qPCR with a new primer set showed that the main factor favoring *Marinobacter* abundance was hydrocarbon amendment followed by macrofauna presence. In parallel, proportion of nosZ-harboring bacteria increased in the presence of macrofauna. Quantitative findings were explained by physiological data from a set of 34 strains and by genomic analysis of 16 genomes spanning 15 different *Marinobacter* validated species (*M. hydrocarbonoclasticus*, *M. daeopensis*, *M. santoriniensis*, *M. pelagius*, *M. flavimaris*, *M. adhaerens*, *M. xestospongiae*, *M. algicola*, *M. vinifirmus*; *M. maritimus*, *M. psychrophilus*, *M. lipoliticus*, *M. manganooxydans*, *M. excellens*, *M. nanhaiticus*) and 4 potential novel ones. Among the 105 organic electron donors tested in physiological analysis, *Marinobacter* pattern appeared narrow for almost all kinds of organic compounds except lipid ones. Strains of this set could oxidize a very large spectrum of lipids belonging to glycerolipids, branched-, fatty acyl-, and aromatic hydrocarbon classes. Physiological data were confirmed by genomic analysis and genes of alkane 1-monooxygenase, haloalkane dehalogenase and flavin-binding monooxygenase were detected in most genomes. Denitrification was assessed for several strains belonging to *M. hydrocarbonoclasticus*, *M. vinifirmus*, *M. maritimus*, *M. pelagius* species indicating the possibility to use nitrate as alternative electron acceptor. Higher occurrence of *Marinobacter* in presence of petroleum appeared to be the result of a broader physiological trait allowing this genus to use lipids including hydrocarbon as principal electron donors.

Introduction

The *Marinobacter* genus was first described in 1992 (Gauthier et al. 1992) and belongs to the γ -Proteobacteria class and the *Alteromonadaceae* family. They are Gram negative and mostly facultative aerobic heterotrophic bacteria. The genus includes 30 *Marinobacter* species currently validated. Some of them can grow anaerobically by nitrate reduction (20 /30) and some of them (6/30) have been further characterized as denitrifiers (able to perform reduction of nitrate to N₂ by respiratory process) (Handley and Lloyd 2013)

Presently, more than 3871 sequences of 16S rRNA genes affiliated to the genus *Marinobacter* are available in the RDP ribosomal database from either isolates or clone strains. Heighten genomes are deposited in different banks. Bacteria belonging to genus *Marinobacter* are commonly detected in diverse marine environments such as coastal marine sediments (Gauthier et al. 1992; Gorshkova et al. 2003), waters (Yoon et al. 2004), and sea sand environments (Kim et al. 2006), deep-water sediments (Cui et al. 2008; Edwards et al. 2003) hydrothermal vents (Shieh et al. 2003), mud volcanoes (Yakimov et al. 2002), hypersaline microbial mats (Abed et al. 2007; Bordenave et al. 2007), sediments of mangrove (Brito et al. 2006), alkaline seabed (Takai et al. 2005), sea ice of the Arctic (Deppe et al. 2005) or the Antarctica (Bowman et al. 1997; Mikucki and Prisco 2007), sediments of the deep Arctic waters (Martin et al. 2003). Representatives of this genus have also been identified associated with the bacterial flora of marine organisms. Indeed, they were isolated from photosynthetic unicellular organisms such as dinoflagellates secreting paralyzing toxins (*Gymnodinium catenatum* and *Alexandrium tamarense*) (Green et al. 2006) as well as multicellular organisms such as bryozoans (Romanenko et al. 2005) and sponges (Lee et al. 2012). Thus *Marinobacter* seems to colonize a wide variety of marine ecosystems around the world ranging from psychrophilic to thermophilic environments with a high tolerance to salinity and pH. Their ability to occupy diverse physicochemical regimes seems an indication of large phenotypic versatility.

Recently metagenomic analyses have highlighted the presence of *Marinobacter* sequences at relatively high percentage in oil polluted environments. Ribosomal or denitrifying genes affiliated to the genus *Marinobacter* have been identified in oil spill simulation mesocosms with marine coastal sediment (Stauffert et al. 2014), estuarine sediment (Gray et al. 2011) or mangrove sediment (dos Santos et al. 2011). Metatranscriptomic analysis revealed that strains belonging to *Marinobacter* genus are active in polluted environments. In a study on the Deep Horizon spill, *Marinobacter aquaolei* strain VT8 genome (now affiliated with *M hydrocrabonoclasticus*) was the closest match to metatranscriptomes (Lamendella et al. 2014). Moreover, in anoxic mesocosm, *Marinobacter* strain alkB cDNA, coding for a hydroxylase involved in degradation of short-chain alkanes, has been quantitatively detected by qPCR (Genovese et al. 2014). These results led some authors to propose the use of sequences of the genus *Marinobacter* as one of the proxies for oil pollution monitoring (dos Santos et al. 2011; Genovese et al. 2014; Gray et al. 2011). Molecular approaches alone are

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4 however, unable to establish a relationship between the presence of a genus and its real ecophysiological role
5 in the studied ecosystem. In order to tackle this drawback, it is necessary to complement molecular
6 information with physiological data on hydrocarbon degradation capacities of strains belonging to different
7 *Marinobacter* species. Although the properties towards hydrocarbon degradation of some type strains have
8 been investigated (for a review see (Duran 2010), it should extend to more strains and to several isolates of the
9 same species. In a broader approach, the determination of the ecological role of *Marinobacter* needs further
10 investigation on their electron donor pattern.

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13 With the exception of the most superficial layer, bulk of marine sediments are assumed to be anoxic (Canfield
14 et al. 1993) especially when contaminated with hydrocarbons due to the reduction of oxygen diffusion. The
15 ability to use alternative electron acceptors is then an ecological advantage for bacterial communities. The
16 capability to perform nitrate reduction in anaerobiosis has been frequently reported for *Marinobacter* and
17 denitrification for at least some strains. This feature need also to be further explored.

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22 This study intends to analyze the robustness of *Marinobacter* proxy as hydrocarbon pollution applying genus
23 dynamic in the face of oil contamination, physiological analysis toward hydrophobic and hydrocarbons
24 compounds and anaerobic respiration. Their dynamic upon contamination with petroleum has been
25 investigated by qPCR with new primer set, in marine sediment mesocosms. Due to the impact of burrowing
26 macrofauna on oxygen supply and on in situ nitrate production resulting of the activity of nitrifying
27 prokaryotes, this condition has been also tested. In parallel investigations on the physiological characteristics
28 of *Marinobacter* has been undertaken with 34 isolates pertaining to 7 validated species and 3 potential novel
29 ones in order to determine their spectrum of electron donors and their potentiality regarding denitrification.
30 The analysis of 16 genomes has permitted to enlarge these observations to 8 additional validated species and
31 to one potential novel one.

32 33 34 35 36 37 38 39 40 41 42 **Materials and Methods**

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45 ***Bacterial strains and isolation procedures:*** The bacterial strains studied belong to the genus *Marinobacter*.
46 The set of strain comprised collection strains (*M. hydrocarbonuclasticus*, *M. aquaolei*, *M. algicola*) and strains
47 isolated from previous studies from Mediterranean sediments (Bonin et al. 1987; 1992; Goregues et al. 2004;
48 Rontani et al. 1997) (Fig. 1).

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51 Other strains (23w2b, 23tet5b, cubw5a, cubw5b, cubtrip2b and cubtet1b) have been isolated in the frame of
52 this study from North Sea water (Fig. 1). Samples were collected on board the RV Pelagia during May 2008.
53 Strains 23w2b, 23tet5b were isolated from samples of surface water contaminated by an oil slick collected at
54 53° 45' 50.4"N 4° 11' 15.7914"E. Strains, cubw5a, cubw5b, cubtrip2b and cubtet1b were isolated from
55 samples retrieved from a 1 m³ on-board mesocosm filled with seawater collected at 55° 8' 23.208" N 3° 11'

1 9.672" E at 29 m deep and contaminated with crude oil. Mesocosm were incubated seven days at ambient
2 temperature on the deck before sampling for strain isolation. Seawater samples, either from sea or mesocosms
3 were used to inoculate enrichment cultures in ASW (Baumann and Baumann 1981) containing tetradecane,
4 paraffin or tripalmitate as sole carbon source. Colonies isolation was performed on agar ASW plates with
5 acetate lactate glucose and yeast extract as carbon sources.

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8 **Electron donors screening:** Electron donors were tested with Biolog GN microplates (Biolog TNC,
9 Hayward, CA,USA). Cells were grown in ASW containing ammonium (1g/L) and a mixture of different
10 carbon sources (lactate 1g/L, acetate 1g/L , glucose 1g/L , yeast extract 2g/L, peptone 2g/L). Cultures in
11 exponential phase of growth were pelleted, washed once in ASW without carbon source. Biolog GN
12 microplates were inoculated with the cell suspensions as described in the manufacturer instructions. Utilization
13 of carbon sources was detected as an increase in the respiration rate of cells in the well of a microplate, leading
14 to irreversible reduction of the tetrazolium dye INT [2-(p-iodopheny)-3-(p-nitropheny)-5-phenyl tetrazolium
15 chloride]. A positive utilization reaction was indicated when a purple color formed due to INT-formazan
16 formation in a well after one week incubation at 20°C. Biolog GN microplates contained the following carbon
17 sources that could be used as electron donor in aerobiosis: 30 carbohydrates (α -Cyclodextrin, Dextrin,
18 Glycogen, N-Acetyl-D-Galactosamine, N-Acetyl-D-Glucosamine, Adonitol, L-Arabinose, D-Arabitol, D-
19 Cellobiose, i-Erythritol, D-Fructose, D-Fucose, D-Galactose, Gentiobiose, α -D-Glucose, α -D-Lactose,
20 Lactulose, Maltose, D-Mannitol, D-Mannose, D-Melibiose, β -Methyl-D-Glucoside, D-Psicose, D-Raffinose,
21 L-Rhamnose, D-Sorbitol, Sucrose, D-Trehalose, Turanose, Xylitol), 19 amino-acids (D-Alanine, L-Alanine,
22 L-Alanyl-Glycine, L-Asparagine, L-Aspartic Acid, L-Glutamic Acid, Glycyl-L-Aspartic Acid, Glycyl-L-
23 Glutamic Acid, L-Histidine, Hydroxy-L-Proline, L-Leucine, L-Ornithine, L-Phenylalanine, L-Proline, D-
24 Serine, L-Serine, L-Threonine, D,L-Carnitine, γ -Aminobutyric Acid), 30 organic acids (Pyruvic Acid Methyl
25 Ester, Succinic Acid Mono-Methyl Ester, Acetic Acid, Cis-Aconitic Acid, Citric Acid, Formic Acid, D-
26 Galactonic Acid Lactone, D-Galacturonic Acid, D-Gluconic Acid, D-Glucosaminic Acid, D-Glucuronic Acid,
27 α -Hydroxybutyric Acid, β -Hydroxybutyric Acid, γ -Hydroxybutyric Acid, p-Hydroxy-phenylacetic Acid,
28 Itaconic Acid, α -Ketobutyric, α -Ketoglutaric Acid, α -Ketovaleric Acid, D,L-Lactic Acid, Malonic Acid,
29 Propionic Acid, Quinic Acid, D-Saccharic Acid, Sebacic Acid, Succinic Acid, Bromosuccinic Acid,
30 Succinamic Acid, L-Pyroglutamic Acid, Urocanic Acid), 3 alcohols (m-Inositol, 2,3-Butanediol, Glycerol) et 13
31 other substrates (tween 40, tween 80, Glucuronamide, L-Alaninamide, Inosine, Uridine, Thymidine,
32 Phenylethyl-amine, Putrescine, 2-Aminoethanol, D,L, α -Glycerol Phosphate, α -D-Glucose 1-Phosphate, D-
33 Glucose-6-Phosphate) .

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54 For insoluble substrates, strains were inoculated on ASW agar plates containing ammonium (1g/L) and one of
55 the following electron donors : Aromatic Hydrocarbon: fluoranthene (1 mg/mL), phenantrene (1 mg/ mL),
56 dodecylbenzene (1 μ L/mL); Fatty acyl hydrocarbon: tetradecane (1 μ L/mL), heptadecane (1 μ L/mL), wax
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1 ester (hexadecyl d'hexadecanoate ; 0.625 mg/ mL), paraffin (mixture of C19 to C31 alkanes with melting
2 point 52°C ; 2.5 mg/mL); Branched hydrocarbon: pristane (1 µL/mL), glycerolipids: triolein (5 µL/mL),
3 tripalmitin (7.5 mg/mL). Plates with a mixture of soluble carbon sources or no carbon source were also used as
4 positive and negative control respectively. The plates were incubated aerobically at 20°C for two weeks and
5 then overlaid with 10 mL molten soft agar containing INT (0.2g per liter ASW) and the same carbon source
6 than that used for the growth step. Oxygen respiring colonies in the presence of the selected substrates were
7 identified as those reducing colorless INT into red INT-formazan (Goregues et al. 2004). Results were
8 recorded after 30 minutes incubation.
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16 **Mesocosm set up:** Sediment was collected at Brittany coast (Celtic Sea, Atlantic Ocean, 48° 34' 28.2''N 4°
17 36'40.2'' E) in October 2013. After removing macrofauna, 72 L of sediments were distributed in 12
18 mesocosms (boxes 15x20x 30 cm). Four conditions were established in triplicate (1) No Nereis (*Hediste*.
19 *diversicolor*) addition and no hydrocarbon amendment (-HC-N), (2) Nereis addition at density of 1000
20 organism/m² and no hydrocarbon amendment (-HC+N) (3) no Nereis addition and hydrocarbon amendment at
21 7g/kg (+HC-N), (4) Nereis addition at density of 1000 organism /m² and hydrocarbon amendment at 7g/kg
22 (+HC+N). Hydrocarbon addition consist in Ural type petroleum topped at 100°C (removal of HC < 7C).
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28 **Nucleic acid extraction, quantification by qPCR in mesocosms and characterization of genes of** 29 **denitrification on strains set** 30

31 After 3 months of incubation at 20°C, 5 g of the 2 first centimeters of sediment in each mesocosm were
32 sampled and nucleic acids were extracted using the MoBio Power soil RNA/DNA extraction kit (MoBio
33 Laboratories, California) according to the manufacturer's protocol. DNAs from cultures of strains were
34 purified as previously reported (Goregues et al. 2005). Purified DNA was stored at 20°C until use.
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39 The determinations of 16S rRNA gene copy numbers were performed by real time PCR with SsoAdvanced
40 SYBR Green Supermix using a CFX96 Real Time System (C1000 Thermal Cycler, Bio-Rad Laboratories,
41 CA, USA). The primer sets GML5F-Uni516R (Michotey et al. 2012) and marinob300F (5'-
42 GACTGAGACACGGCCCGA-3') - marinob600R (5'-CCGGGGCTTTCACATCTCG-3') were used for
43 quantification of total bacterial 16S rRNA genes and *Marinobacter* 16S rRNA genes respectively. The real
44 time PCR cycles consisted of a denaturation step of 5 s, a hybridization step of 20 s at 56°C with an elongation
45 step of 40 s. Quantification of bacterial *nosZ* were performed using nosZ 1211F and nosZ 1897R primers
46 (Rosch et al. 2002) and SsoFast EVA Green Supermix (Bio-Rad Laboratories, CA, USA). The real time PCR
47 cycles consist of a denaturation step of 15 s, a hybridization step of 15 s at 64°C with an elongation step of 40 s
48 at 72°C. Gene abundance in microcosm DNA samples was determined in relation with a calibration standards
49 corresponding to dilution of plasmids harboring target genes.
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Denitrification activity of strains were determined as previously described (Bonin et al. 1987; 1992; Goregues et al. 2004; Rontani et al. 1997). The detection by PCR on genome of the strains of *narG*, *nirS* and *nosZ* were performed as previously reported (Goregues et al. 2005, Michotey et al. 2000, Rosch et al. 2002) respectively. All amplified DNA fragments were checked by sequencing. The nucleotide sequence data generated in this study have been deposited in the Genbank database under accession numbers KM816585 to KM816618.

Genomes analysis: Sixteen genomes are currently available, *M. adhaerens* DSM 23420 (CP001978), *M. algicola* DG893 (GCA_000170835.1), *M. hydrocarbonoclasticus* strain VT8 (GCA_000015365.1), *M. hydrocarbonoclasticus* strain 17 ATCC 49840 (FO203363), *M. excellens* (PRJNA195885), *M. daepoensis* DSM 16072 (GCA_000421165.1), *M. lipolyticus* strain BF04 (GCA_000372805.1), *M. lipolyticus* strain SM19 (ASAD01000001- ASAD01000006), *M. maganoxydans* (AGTR00000000), *M. nanhaiticus* (APLQ01000000), *M. santoriniensis* (APAT01000000), *M.* strain AK21 (ANIE01000001- ANIE01000006), *M.* strain BSs20148 (CP003735), *M.* strain ELB17 (GCA_000169375.1), *M.* strain MCTG268 (JQMK00000000), *M.* strain HL-58 (GCA_000686085.1). The metabolic properties harbored by the strain according to their genomes analyses was assessed with IMG-ER (DOE-JGI) platform (<https://img.jgi.doe.gov>)

Statistical analysis: An ANOVA analysis was used to determine the influence of hydrocarbon amendment and presence of Nereis on bacterial numeration in microcosms. The significance level for all tests was set at $p < 0.05$. All tests were performed with XLSTAT® (XLSTAT software® 2008, Addinsoft, Paris).

Results

In this study we propose new qPCR primers for the detection of *Marinobacter* ribosomal 16S rDNA (Table 2). Primer set Marinob300f-Marinob600R matches with 64.5% of ribosomal sequences affiliated to *Marinobacter* genus in RDP database and 90% of ribosomal sequence of *Marinobacter* type strains. Previous qPCR primers (Mab451f-Mab573r) and CARD-FISH probe presented a narrower spectrum of detection since they matched with 6.4 and 26.6 % of *Marinobacter* ribosomal sequences in RDP, respectively. These percentages dropped to 3 and 20% for type strains. (Tab.2). Both qPCR primer sets present lower percentage of match toward non-target sequences (1.2% and 2.8%) in comparison of CARD-FISH probes (20%). Optimization of hybridization temperature of the qPCR was determined ($T=56^{\circ}\text{C}$) in relation with positive (*M. hydrocarbonoclasticus*) and negative (*E. coli*) Gammaproteobacteria controls. The resulting conditions lead to a qPCR efficiency of 97.1% ($R^2=0.994$).

Marinobacter dynamic was monitored upon hydrocarbon pollution in sediment mesocosms. *Marinobacter* and bacterial 16S rRNA gene abundances were measured by qPCR in all mesocosms after 3 months incubations

(Fig. 1). The highest number of bacterial ribosomal genes (about 10^{11} /g sediment) was observed for the condition without macrofauna and hydrocarbon. For the other conditions, no significant difference in numeration of bacterial 16S rRNA genes was recorded in the mesocosms (about 10^{10} /g sediment) (Fig. 1). The analysis of abundance of *Marinobacter* ribosomal genes emphasized the predominant impact of hydrocarbon to presence of macrofauna. Indeed, all mesocosms containing hydrocarbon presented significant higher numbers of *Marinobacter* (+HC+N, +HC-N> -HC+N, -HC-N; $p < 0.05$). Their abundance reached 0.3-0.09% of the bacterial population in presence of HC in comparison of 0.02-0.004% in its absence. Whatever the presence of hydrocarbon, the mesocosms without macrofauna harbored lower percentage of *Marinobacter* than those in which *Nereis* has been introduced (0.3 vs 0.09 and 0.022 vs 0.004 for condition with and without hydrocarbons).

The denitrifying community was quantified using *nosZ* gene with primers previously used for competitive PCR (Rontani et al. 2010, Fernandes et al. 2012). In qPCR the efficiency of the reaction reached 91.7%. In mesocosms, the number of bacterial *nosZ* varied from $6.66 \cdot 10^6$ to $3.24 \cdot 10^7$ /g sediment (Fig.1). The ratio *nosZ*/16S rRNA bacterial genes ranged from $0.14 \cdot 10^{-3}$ to $0.07 \cdot 10^{-3}$ in mesocosms without hydrocarbon amendment to $1.47 \cdot 10^{-3}$ to $0.57 \cdot 10^{-3}$ in its presence. Within a condition (+ or – hydrocarbon) this ratio was higher in presence of macrofauna. In contrast, the ratio denitrifier /*Marinobacter* (*nosZ*/*Marinobacter* 16S rRNA gene) seemed relatively stable and range between 0.48 and 1.72 (Fig. 1).

The electron donor pattern was determined on a set of 34 isolates which according to phylogenetic tree belong to 7 validated species and 3 potential novel ones referred as phylotype # 2, #8, #9 (S1). Hereafter, validated species and potential novel ones were referred as taxa. Of the 34 tested strains, only 8 were able to use a few carbohydrates as electron donors (S2). All the strains were only able to use less than 4 different carbohydrates, excepted strain T33 (phylotype #8) oxidizing 16 of them. Glucose was the most commonly utilized (6 isolates) but only occasionally within a taxon (1/11 for *M. hydrocarbonoclasticus*, 2/8 for *M. vinifirmus*; 1/ 2 for *M. maritimus*, 2/4 for phylotype #8). The same trend was observed for dextrin since only 4 strains were able to use it (S2). Likewise, amino acid utilization range of *Marinobacter* strains of this study was restricted to few them; however in contrast to carbohydrates, most of the strains of a taxon shared the same trend toward a given Amino acid. L-proline was the most frequently used by the isolates (8/10 taxa) followed by L-glutamic acid (5/10 taxa) and then L-leucine, (4/10 taxa) (Fig.2). L alanine and D-alanine were oxidized by only few strains within 3 and 4 taxa respectively (S2). Eight out of 30 organic acids tested could be used as electron donors by a least one *Marinobacter* strain. The most widely used acid was β -hydroxybutyric acid that was electron donor for 8/10 taxa followed by pyruvic acid methyl ester or D,L lactic acid that were oxidized by 4/10 taxa (Fig.2). The utilization of these 3 organic acids was shared by most of the strains in a taxon (Fig.2

245 and S2). In contrast, propionic acid, acetic acid, citric acid, succinic acid and bromo-succinic acid were
1 oxidized at a lesser extent and only occasionally within a taxon.
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4 Among the investigated panel of electron donors, the most used were lipid compounds. All tested molecules
5 were used by several taxa of *Marinobacter* and all taxa, excepted *M. xestospongia* strain BP42, could utilized
6 more than half of them. *M. hydrocarbonoclasticus* and *M. vinifirmus*, represented by 11 and 8 isolates
7 respectively, were used to analyze the representativeness of trait within a taxon. Tween 40 (Polyoxyethylene
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250 (20) sorbitan monopalmitate) and Tween 80 (Polyoxyethylene (20) sorbitan monooleate) that are emulsifiers,
10 or triolein that is a glycerolipid were oxidized by all taxa. This feature is also observed for heptadecane, a
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12 C17- alkane belonging to fatty acyls. These latter patterns were shared by more than 75% of the isolates of a
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14 taxon in most all cases. The two other alkanes tested, tetradecane, a linear -C14 and pristane, a branched C19
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16 molecule, were oxidized by 9/10 and 6/10 taxa, respectively. Pristane utilization is sparse since only 9% of
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255 isolates of *M. hydrocarbonoclasticus* (9%) and 25% of *M. vinifirmus* (25%) were positive, suggesting that it
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19 cannot be considered as specific trait of a species. The other substrate molecules were characterized by their
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21 low lability due to the presence of one or more aromatic cycles or due to higher melting point. The 3 aromatic
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23 molecules, dodecylbenzene, phenanthrene or fluorethene were oxidized by 6/10, 5/10 and 6/10 taxa
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25 respectively. As for pristane, the utilization pattern of aromatic hydrocarbon was not identical for most of the
260 strain within a taxon. For example, only 18% and 64% of *M. hydrocarbonoclasticus* and 12% and 25% of
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28 *M. vinifirmus* could use fluorethene or dodecylbenzene respectively. This trend was also observed for very
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30 insoluble substrates pertaining to fatty acyls or glycerolipids such as tripalmitate paraffin and wax. Between 5
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32 and 7 taxa were positive for their utilization, with positive strains varying between 12 to 55% for *M.*
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345 *hydrocarbonoclasticus* and *M. vinifirmus*.
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37 The available genomes corresponded to 10 validated species (*M. adhaerens*, *M. algicola*, *M*
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39 *hydrocarbonoclasticus*, *M excellens*, *M. daepoensis*, *M. lipolyticus*, *M. maganoxydans*, *M. nanhaiticus*, *M.*
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41 *santoriniensis*, *M. psychrophilus*) and a potential novel one (*M. sp* AK21) (S1). From the analysis of available
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43 genomes, similar trends could be emitted on nature of electron donor of *Marinobacter*. The genomic analysis
270 was focused on proteins mediating the entry of substrates into the cell (translocation protein, transporter,
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45 permease, symport) or the first enzyme of a pathway for lipids (Tab. 3). To import many carbohydrates
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47 (glucose, mannose, fructose...), Bacteria have developed an unique mechanism the
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49 phosphoenolpyruvate:sugar phosphotransferase system (PTS). This system synchronizes the transport of the
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51 sugar through the cellular membrane and its phosphorylation. With some variations, the PTS comprises
5275 several proteins, some of them are common for all sugars such as EnZI and HPr and others that are specific of
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54 a carbohydrate or a group of carbohydrates such as EnZII composed of domain IIA, IIB, IIC. This latter one is
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56 located in the membrane and mediates specific sugar translocation. Whereas HPR has been identified in all
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58 genomes of *Marinobacter*, EnZII were scarce (Tab.3). From the panel of genomes, only EnZII A and B genes
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60 specific to fructose have been identified in 8 and 5 genomes, respectively; in contrast to EnZII C for which
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280 whatever the sugar, no gene could be identified. The absence of the protein responsible for of sugars
1 translocation led to the impossibility to use these compounds as electron donors. For some organic acids such
2 lactate or succinate, putative genes annotated as transporters were found in 5 and 16 genomes, respectively.
3 Proline appeared as an important amino acid since all genomes harbored a sodium/proline symporter. Except
4 the Carboxylate/Amino Acid/Amine transporter that was present on all genomes, the other specific amino acid
5 transporters were either absent or present in only one genome. In contrast, all the strains contained the fatty
6 oxidation complexes *FadAB* and a complete β -oxidation pathway (data not shown). Furthermore, the
7 occurrence of both alkane mono-oxygenase (*alkA*) and haloalkane dehalogenase genes in genomes was above
8 87%. The only genome in which *alkA* has not been detected is *M. lipoliticus* strain BF04. The genome of this
9 strain did not contain also the gene coding for haloalkane dehalogenase as that of *M psychrophylus* BSs20148.
10 Long chain alkanes (C_{32} and longer) are oxidized by the product of gene *almaA* that encodes a flavin-binding
11 monooxygenase. Blast search with *almaA* sequence of *M. lipolyticus* (FJ263110) from the study of Wang and
12 Shao (2012) and with an E-value cutoff at 10^{-5} , permit the identification of 13 homologous genes in
13 *Marinobacter* set of genome. The two strains of *M. hydrocarbonoclasticus*, all 3 strains of *M algicola*, and
14 strains of *M adhaerens*, *M. manganoxydans*, *M. daepoensis*, *M. santoriniensis*, *M. nanhaiticus*, *M. excellens*,
15 *M. lipolyticus* SP19 and *M* strain AK21 were positive.
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28 During denitrification, nitrate is reduced into N_2 through several steps. Nitrate can be reduced to nitrite by
29 NarG or NapA, nitrite to nitric oxide by NirS or nirK, nitric oxide to nitrous oxide by NorB, and nitrous oxide
30 to N_2 by NosZ. Three genes out of four were tested. Analyses of denitrifying genes revealed the preferential
31 presence of *narG*, *nirS* among our denitrifying *Marinobacters* set (Table 4). Most of the nitrate reducers
32 among our set of strains were able to perform total denitrification and harbored also *nosZ*. This tendency was
33 also found by genomic approach (Table 3). Denitrification genes the most frequently recovered were *narG* and
34 *nosZ* and 75% of the genomes possessed at least one gene of denitrification.
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42 Discussion

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45 505 Hydrocarbons occur as biosynthetic products of living organisms in the biosphere (Ladygina et al. 2006) or as
46 abiotic transformation products of biogenic organic matter in the geosphere (Seewald 2003). They are the
47 main constituents of petroleum and thus are extremely abundant in geological systems. They can be
48 transferred from geosphere to biosphere by different manners such as hydrothermal vent, mud volcano
49 (Whiticar and Suess, 1990; Timmis 2010) or drilling problems. In consequence their presence is probably
50 ubiquitous but in various proportions. Marine ecosystems are frequently exposed to oil pollution. After a spill,
51 the n-alkanes, short chains tend to evaporate or dissolve in seawater. In contrast to short and medium-chain
52 alkanes that are quickly degraded after the spill, the long-chain molecules with more than 18 carbon atoms, do
53 not dissolve nor degrade efficiently and stay for longer periods of time in the environment. Hydrocarbons of a
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spill or from biogenic origin enter with other compounds in the composition of marine organic matter resulting in a complex mixture of molecules having various compositions, physical structures and reactivity. This complexity is probably a major factor for maintaining a high degree of diversity among prokaryotes in oceans. Heterotrophic bacteria recycle dissolved and particulate organic matter and contribute to the productivity of the ecosystem. Isolation campaigns have demonstrated the presence of the heterotrophic Gammaproteobacteria *Marinobacter* in many marine environments exhibiting disparate characteristics. This finding would suggest that they are capable of oxidizing a wide range of carbon substrates. This physiological studies of 34 isolates and analyses of 16 genomes from diverse marine habitats such as sediment, water column or phycosphere and partitioning into 15 species and 4 potential novel ones within *Marinobacter* genus give rise to a different view. Except for some strains, very few molecules classified as labile organic matter are used. The spectra of carbohydrates utilization as electron donors determined by physiological as well as genomic approaches are extremely reduced. Comparison of physiological and genomic approaches focusing on transporters was evaluated by comparison of patterns obtained from *Marinobacter hydrocarbonoclasticus* strain 17. From its genome fully annotated (Grimaud et al. 2012), no EnzIIC of sugar could be detected in congruence with results of physiological study. The inability to use carbohydrates or to degrade the polysaccharides has been reported previously for some *Marinobacter* strains (Bowman and McMeekin 2005). However, our results including a wider list of species and with several strains within a species strengthen this observation that appears as a common trait of this genus.

The ability of *Marinobacter* to use external amino acids as electron donors was reported. The list of the preferential ones included L-alanine, D glutamate and L phenylalanine (Handley and Lloyd 2013). From our data, the most frequently used by our set of strains is proline and all analyzed genomes contained a specific transporter for this amino acid. This feature was described previously for *M. gudaonensis* (Gu et al. 2007), and *M. koreensi* (Kim et al. 2006). With the present study, this characteristic is enlarged to at least 16 species and 4 potential novel ones. Furthermore this amino acid appeared to have an important physiological role due to the very high frequency of its utilization. Proline, is uncommon in proteins but has the characteristic to be compatible solute, that is to say, a molecule involved in resistance to the osmotic pressure, thermal stress for many heterotrophs (Welsh 2000). This ability could allow *Marinobacter* strains to resist to a wide range of physical parameters.

The main feature which emerges from the analyses of the substrates pattern is the capacity of many strains of *Marinobacter* to oxidize a very large spectrum of lipid molecules of various type and molecular weight (including Tween). This physiological aspect is not frequently explored in species characterization (Handley and Lloyd 2013). Degradation capacity towards tween is often reported in the literature due to its presence in Biolog panel. For example *M. koreensis* (Kim et al. 2006), *M. lipolyticus* strain SM19, *M. litoralis* (Martin et al. 2003) and *M. bryozoorum* or *M. sediminum* (Romanenko et al. 2005) were found positive. For other lipid molecules, except few studies, the degradation pattern toward these compounds remains often untested. *M.*

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hydrocarbonoclasticus is may be one of the best characterized. It can utilize *n*-alkanes ranging from C₆-C₃₀ (Doumenq et al. 2001; Duran 2010; Grimaud 2010). This trait seemed largely shared since genomic approach has highlighted the presence of alkane mono-oxygenase, an enzyme responsible for the first step oxidation of fatty acyl hydrocarbon in 15 out of 16 genomes. According to literature, 9 *Marinobacter* species were identified as hydrocarbon degraders (Handley and Lloyd 2013); our results have identified 9 additional species. This feature was observed for strains isolated from environment enriched with petroleum such as oil producing well in the case of *M. hydrocarbonoclasticus* VT8 but was also observed for strains retrieved from potential petroleum-free samples such as dinoflagelates cultures for *M. algicola* (Green et al. 2006), or microbial mat for the two other genomes affiliated to *M. algicola* (HL-58 and MCTG268), marine particles for *M. adhaerens* (Gärdes et al 2010) or Arctic ocean sediment for *M. psychrophilus* BSs20148 (Song et al. 2013) for examples. Usually when hydrocarbon utilization is included in physiological characterization, only short chain hydrocarbon are tested. For screening the capacity to degrade long –chain alkanes with more than 18 carbon atoms, the use of *almA* gene has been recently suggested by Wang and Shao (Wang and Shao 2012). Strains belonging to *M. koreensis*, *M. alkaliphilus*, *M. lipolyticus*, *M. flavimaris* and *M. hydrocarbonoclasticus* were found positive by these authors (Wang and Shao 2012). This gene was recovered by specific BlastP on most genomes of our set and this trend was extended to species *M. adhaerens*, *M. manganoxydans*, *M. algicola*, *M. daepoensis*, *M. santoriniensis*, *M. nanhaiticus*, and *M. excellens*. In this study, the panel of electron donors include short and long chain hydrocarbons but also non-hydrocarbon lipids (glycerolipids). Most of the strains appeared positive. The results of this study highlighted also the need to screen several strains of a same species to find common physiological traits within a species. From previous studies (Timmis 2010 for a review), some *Marinobacter* type strains were described as short chain alkane degraders, this trend could now be expanded through a larger number of species including several strains within a species and to large spectrum of lipid molecules. These compounds appeared as preferred electron donors for *Marinobacter* species included in this set.

This capacity could account for the 10 fold increase of *Marinobacter*s abundance in mesocosm contaminated with petroleum of this study. This value is higher than the 2 fold increase observed in anaerobic mesocosm with a CARD-FISH probe exploring only 26% of *Marinobacter* sequence (Genovese et al. 2014) but are in agreement with augmentation of *Marinobacter* ribosomal sequences found by pyrosequencing before and after oil pollution in mangrove (dos Santos et al. 2011). In our study, *Marinobacter* reached about 0.3% of the bacterial community in mesocosms containing hydrocarbon which is lower than the value found with less specific probe in anaerobic mesocosm by Genovese et al. (2014). However comparison between different studies should be taken with caution since other factors could be involved such as sensitivity to the load of petroleum or the selection of some species not detected by qPCR or CAR-FISH or a kinetic effect. The highest abundance of *Marinobacter* was observed in presence of petroleum and burrowing macrofauna. The presence and activity of burrowing macrofauna such as *H. diversicolor*, in sediment can significantly affect the

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repartition of electron acceptors and nutrient availability (Banta and Andersen 2003). In marine sediment, oxygen rapidly decreases with depth but several alternative electron acceptors are available. In absence of oxygen, nitrate is the most favorable electron acceptor since its reduction to N_2 yields almost as much energy as aerobic respiration (Canfield et al. 2005). However nitrate concentrations in sediments are low probably due to low in situ production or rapid turnover. Bioturbation by macrofauna can enhance oxygen supply to deeper layers, favoring activity of nitrifying prokaryotes than can provide nitrate for nitrate respiring organisms (Laverock et al. 2011) such as denitrifiers. Some strains of *Marinobacter* have been shown as able to respire inorganic compounds such as arsenic (Handley et al 2013) or more frequently nitrate (i.e; denitrifier (Bonin et al. 1987; Gilewicz et al. 1997; Rontani et al. 2002; Goregues et al. 2004). The use of molecular technics has shown that *narG*, *nirS* and *nosZ* are present in most of the nitrate reducers included in this study. These genes are frequently identified in genomes also. The denitrification property of type strain of *M. pelagius*, *M. maritimus* and *M. vinifirmus* was either incomplete or negative in previous studies. Our results have shown that at least some member of these species can perform this process. The impact of oil on denitrifying community was also addressed in this study. This community was enumerated with *nosZ* as validated earlier (Rontani et al. 2010; Fernandes et al. 2012; Nogales et al. 2002). This gene was preferred to *nirS* since it presents the advantage to be the sole one coding for this enzymatic step. The percentage of *nosZ*-harboring cells increased under hydrocarbon amendment suggesting a probable role in petroleum degradation. Indeed some studies on isolated strains have reported the property of some *Marinobacter* to degrade hydrocarbon in anaerobiosis (Rontani et al. 2002).

From the results obtained in this study, primers amplifying a broader spectrum of *Marinobacter* ribosomal gene have confirmed the increasing prevalence of this genus when confronted with petroleum in mesocosms as previously observed with other technics. This phenomenon seems to be due to the pattern of electron donors of this genus, presenting a large range of lipid compounds and narrow ones with other organic molecules. This result has been confirmed by screening of several species and several strains within a species. *Marinobacter* development in sediment contaminated by petroleum was favored by burrowing macrofauna which facilitate diffusion of oxygen but also the supply nitrate to denitrifiers, some of whom belonging to *Marinobacter* genus. The high frequency of genes involved in alkane degradation of this genus would favor the utilization of this genus as proxy of petroleum monitoring. For example, their occurrence in surrounding area of hydrothermal vents (Wang et al 2012; Handley et al 2013) could be an indication of the diffusion of hydrocarbon effluents. However, the increase of *Marinobacter* in presence to oil pollution could be also the result of an opportunistic physiological trait toward lipid substrates.

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10 Figure captions

11 **Fig.1** Abundance of ribosomal 16S rRNA gene from bacteria or *Marinobacter*, and of nitrous oxide reductase
12 gene (*nosZ*) in mesocosm in presence of Hydrocarbon or of burrowing macrofauna (*H. diversicolor*)
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15 Neither Hydrocarbon amendment nor *H. diversicolor* addition (-HC-N), no Hydrocarbon amendment and *H.*
16 *diversicolor* addition (-HC+N), hydrocarbon amendment and no *H. diversicolor* addition (+HC-N),
17 hydrocarbon amendment and *H. diversicolor* addition (+HC+N); These values mean the average number of
18 genes detected in triplicated mesocosms. Several subsamples were collected from each mesocosm.
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230 **Fig.2** Main electron donors pattern of 10 taxa of *Marinobacter*, expressed as percentage of positive strains
24 among a taxon. Substrates tested: 30 carbohydrates, 19 AA, 30 organic acids, 12 lipid molecules and 17 other
25 compounds, only relevant results were indicated
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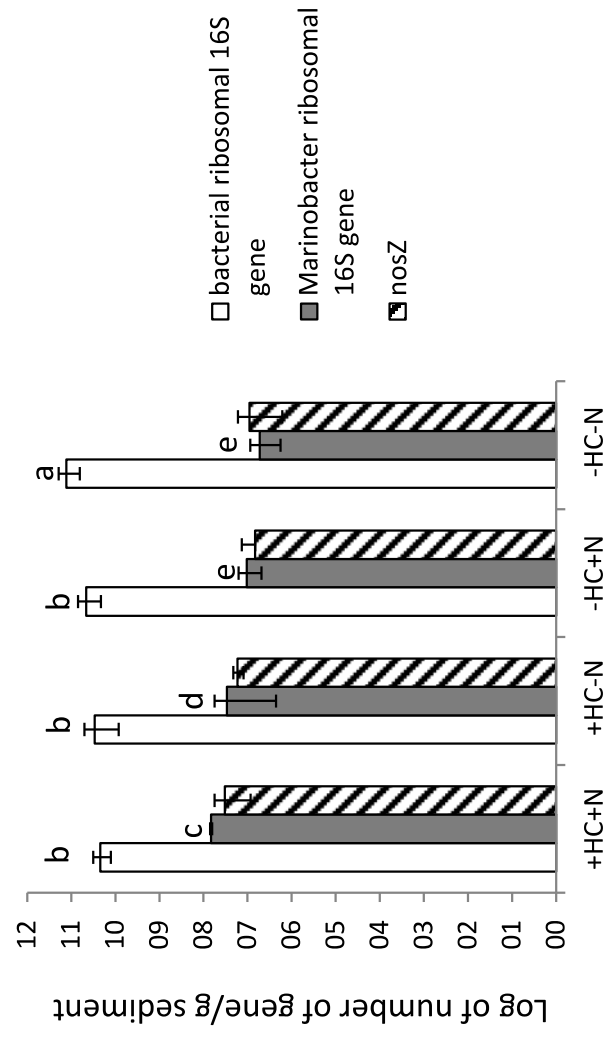


Fig.1 Abundance of ribosomal 16S rRNA gene from bacteria or *Marinobacter*, and of nitrous oxide reductase gene (*nosZ*) in mesocosm in presence of Hydrocarbon or of burrowing macrofauna (*H. diversicolor*) Neither Hydrocarbon amendment nor *H. diversicolor* addition (-HC-N), no Hydrocarbon amendment and *H. diversicolor* addition (-HC+N), hydrocarbon amendment and no *H. diversicolor* addition (+HC-N), hydrocarbon amendment and *H. diversicolor* addition (+HC+N); These values mean the average number of genes detected in triplicated mesocosms. Several subsamples were collected from each mesocosm.

Fig.2 Main electron donors pattern of 10 taxa of *Marinobacter*, expressed as percentage of positive strains among a taxon. Substrates tested: 30 carbohydrates, 19 AA, 30 organic acids, 12 lipid molecules and 17 other compounds, only relevant results were indicated

Table 1 : list of *Marinobacter* strains used in this study

species	strains	Location of isolation	Type of sample	reference	Physiological (P), genomic (G) analysis
<i>M. hydrocarbonoclasticus</i>	VT8, DSM 11845*	Vietnam	Oil producing well	(Huu et al. 1999)	P, G
	DN1*	Gulf of Fos, Med Sea	sediment	(Bonin et al. 1987)	P
	17 ATCC 49840	Gulf of Fos, Med Sea	sediment	(Gauthier et al. 1992)	P, G
	CAB	Gulf of Fos, Med Sea	sediment	(Gilewicz et al. 1997)	P
	BC36, BC38, R153	Berre lagoon, Med Sea	sediment	(Goregues et al. 2004)	P
	23W2B, CUBW5A, CUBW5B, 23tet5B	North Sea	Water column	This study	P
<i>M. vinifirmus</i>	BC45, NBC44, NBC35, NBC34, NBC33, NBC32, BC43, BC34	Berre lagoon, Med Sea	sediment	(Goregues et al. 2004)	P
<i>M. maritimus</i>	2Sq31, 1sq53	Gulf of Fos, Med Sea	sediment	(Rontani et al. 2002)	P
<i>M. flavimari</i> <i>M. adhaerens</i> <i>M. manganoxydans</i>	Cubtrip2B, Cubtet1B	North Sea	Water column	This study	P
<i>M. adhaerens</i>	HP15 DSM 23420	German Wadden Sea	Diatom interaction	(Gärdes et al 2010)	G
<i>M. manganoxydans</i>	MnI7-9	Indian Ocean	deep-sea hydrothermal vent sediment	(Wang et al 2012)	G
<i>M. pelagius</i>	R171, R42	Berre lagoon, Med Sea	sediment	(Goregues et al. 2004)	P
<i>M. algicola</i>	DG893	culture	dinoflagellates	(Green et al. 2006)	P, G
	HL-58	-	Microbial mat	unpublished	G
	MCTG268	-	Microbila mat	unpublished	G
<i>M. xestospongiae</i>	BP42	Berre lagoon, Med Sea	sediment	(Goregues et al. 2004)	P
<i>M. daepoensis</i>	SW-156 ^T (DSM 16072)	South Korea	Water column	(Yoon et al 2004)	G
<i>M. santoriniensis</i>	NKSG1	Santorini, Med Sea	Shallow marine hydrothermal sediment;	(Handley et al 2013)	G
<i>M. lipolyticus</i>	SM19	Spain	saline soil	(Papke et al 2013)	G

	BF04_CF-4	-	-	unpublished	G
<i>M. nanhaiticus</i>	D15-8W	South China Sea	Sediment	(Cui et al 2013)	G
<i>M. excellens</i>	HL-55	-	-	unpublished	G
<i>M. psychrophilus</i>	ELB17	Antarctica	waters of a permanently ice-covered lake	unpublished	G
	BSs20148	Arctic Ocean	sediment	(Song et al 2013)	G
<i>M. strain AK21</i>	AK21	India	Water column	unpublished	G
<i>M. sp. phylotype #2</i>	BP45, NBP42	Berre lagoon, Med Sea	sediment	(Goregues et al. 2004)	P
<i>M. sp. phylotype #8</i>	U32, S64, T33, U31	Berre lagoon, Med Sea	sediment	(Goregues et al. 2004)	P
<i>M. sp. phylotype #9</i>	R53	Berre lagoon Med Sea	sediment	(Goregues et al. 2004)	P

* *Pseudomonas nautica* (DN1) (Sproer et al. 1998) and *Marinobacter aquaeolei* (VT8) ((Marquez & Ventosa 2005) are synonymous with the type species *Marinobacter hydrocarbonoclasticus* (Gauthier et al. 1992)

Table 2 Comparison of different PCR primers or probes for detection of *Marinobacter* 16S rDNA using probe-match (RDP probe match tool)

Primer set	Marinob300F-Marinob600R Number of hits (% recovery)	Mab451f-Mab573r Number of hits (% recovery)	CARD-FISH probe Number of hits (% recovery)
reference	This study	(Gray et al. 2011)	(Karner & Fuhrman 1997)
Bacteria	2414/2879170	242/2879170	885/2879170
<i>Marinobacter</i> genus	2374/3639 (64.5%)	235/3639 (6.4%)	714/3639 (20.6%)
<i>Marinobacter</i> type strains	27/30 (90%)	1/30 (3%)	8/30 (26.6%)
Non-target matches	30/ 2414 (1.2%)	7/242(2.8%)	171/885(20%)

Table 3: Main features of *Marinobacter* capacity to use electron donors and acceptors from the analysis of 16 genomes with the focus on external transporters or crucial enzymatic steps.

Classification	name	Number of positive genomes out of 16
Carbohydrate, organic alcohol and acids transport		
<i>TIGR00786</i>	<i>TRAP transporter, DctM subunit (malate, fumarate, succinate)</i>	16
<i>TIGR00795</i>	<i>L-lactate transport</i>	5
<i>TIGR00792</i>	<i>sugar (Glycoside-Pentoside-Hexuronide) transporter</i>	0
<i>TIGR01003</i>	<i>Phosphotransferase System HPr (HPr) Family</i>	16
<i>TIGR00822</i>	<i>PTS system, mannose/fructose/sorbose family, IIC component</i>	0
<i>TIGR00410</i>	<i>PTS system, lactose/cellobiose family IIC component</i>	0
<i>TIGR00821</i>	<i>PTS system, glucitol/sorbitol-specific, IIC component</i>	0
<i>TIGR00852</i>	<i>PTS system, maltose and glucose-specific subfamily, IIC component</i>	0
AA transport		
<i>TIGR02121</i>	<i>sodium/proline symporter</i>	16
<i>TIGR00814</i>	<i>serine transporter</i>	0
<i>TIGR00837</i>	<i>aromatic amino acid transport protein</i>	0
<i>TIGR00905</i>	<i>transporter, basic amino acid/polyamine antiporter (APA) family</i>	1
<i>TIGR00950</i>	<i>Carboxylate/Amino Acid/Amine Transporter</i>	16
<i>TIGR00906</i>	<i>cationic amino acid transport permease</i>	0
Lipid degradation		
<i>TIGR02437</i>	<i>fatty oxidation complex, alpha subunit FadB</i>	16
<i>TIGR02445</i>	<i>fatty oxidation complex, beta subunit FadA</i>	16
<i>EC:1.14.15.3</i>	<i>Alkane 1-monooxygenase. AlkA</i>	15
<i>EC:1.14.13.8</i>	<i>flavin-binding monooxygenase AlmA</i>	13
<i>EC:3.8.1.5</i>	<i>Haloalkane dehalogenase.</i>	14
Nitrate respiration/denitrification		
<i>TIGR01706</i>	<i>periplasmic nitrate reductase, large subunit NapA</i>	2
<i>TIGR01580</i>	<i>respiratory nitrate reductase, alpha subunit NarG</i>	12
<i>TIGR02161</i>	<i>periplasmic nitrate (or nitrite) reductase c-type cytochrome, NapC/NirT family NirS</i>	4
<i>TIGR02376</i>	<i>nitrite reductase, copper-containing, nirK</i>	4
<i>TIGR04244</i>	<i>nitrous-oxide reductase, TAT-dependent NosZ</i>	10

Genome list: *M. adhaerens*, *M. algicola*, *M. hydrocarbonoclasticus* VT8, *M. hydrocarbonoclasticus* sp17, *M. excellens*, *M. daepoensis*, *M. lipolyticus* BF04, *M. lipolyticus* SM14, *M. maganoxydans*, *M. nanhaiticus*, *M. santoriniensis*, *M. sp* AK21, *M. sp.* BSs20148, *M. sp.* ELB17, *M. sp.* MCTG268, *M. sp.* HL-58

Table 4 denitrifying strains among *Marinobacter* set and presence of 3 out of the 4 steps of denitrification

	<i>M. Hydrocarbonoclasticus</i>	<i>M. vinifirmus</i>	<i>M. maritimus</i>	<i>M. pelagius</i>	<i>M</i> sp. phytotype #8	<i>M</i> sp. phytotype #2	<i>M</i> sp. phytotype #9	<i>M</i> sp. phytotype #20
Denitrification*	n=6 ¹	n=8	n=2	n=2	n=4	n=2	n=1	n=1
<i>narG</i>	6	7	1	2	3	1	1	1
<i>nirS</i>	6	5	2	2	4	2	1	1
<i>nosZ</i>	6	7	2	2	3	2	1	1
	6	7	2	2	4	1	1	1

Denitrification property were determined previously (Bonin et al. 1987, 1992, Goregues et al. 2004, Rontani et al. 1997)

Data concerning nitric oxide reductase (NorB) are not available

¹ DN1, Sp 17, CAB, BC36, BC38, R153

Supplementary Material

[Click here to download Supplementary Material: Bonin et al S1 Marinobacter.pptx](#)

Supplementary Material

[Click here to download Supplementary Material: Bonin et al S2 Marinobacter.docx](#)

Substrate utilization profile of *Marinobacter* genus indicates a specialization in lipid compounds including hydrocarbons

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Abstract: The impact of petroleum contamination and of burrowing macrofauna on abundances of *Marinobacter* and denitrifiers was tested in marine sediment mesocosms after 3 months incubation. Quantification of this genus by qPCR with a new primer set showed that the main factor favoring *Marinobacter* abundance was hydrocarbon amendment followed by macrofauna presence. In parallel, proportion of nosZ-harboring bacteria increased in the presence of macrofauna. Quantitative findings were explained by physiological data from a set of 34 strains and by genomic analysis of 16 genomes spanning 15 different *Marinobacter* validated species (*M. hydrocarbonoclasticus*, *M. daeopensis*, *M. santoriniensis*, *M. pelagius*, *M. flavimaris*, *M. adhaerens*, *M. xestospongiae*, *M. algicola*, *M. vinifirmus*; *M. maritimus*, *M. psychrophilus*, *M. lipoliticus*, *M. manganooxydans*, *M. excellens*, *M. nanhaiticus*) and 4 potential novel ones. Among the 105 organic electron donors tested in physiological analysis, *Marinobacter* pattern appeared narrow for almost all kinds of organic compounds except lipid ones. Strains of this set could oxidize a very large spectrum of lipids belonging to glycerolipids, branched-, fatty acyl-, and aromatic hydrocarbon classes. Physiological data were confirmed by genomic analysis and genes of alkane 1-monooxygenase, haloalkane dehalogenase and flavin-binding monooxygenase were detected in most genomes. Denitrification was assessed for several strains belonging to *M. hydrocarbonoclasticus*, *M. vinifirmus*, *M. maritimus*, *M. pelagius* species indicating the possibility to use nitrate as alternative electron acceptor. Higher occurrence of *Marinobacter* in presence of petroleum appeared to be the result of a broader physiological trait allowing this genus to use lipids including hydrocarbon as principal electron donors.

145 Introduction

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4 The *Marinobacter* genus was first described in 1992 (Gauthier et al. 1992) and belongs to the γ -
5 Proteobacteria class and the *Alteromonadaceae* family. They are Gram negative and mostly facultative aerobic
6 heterotrophic bacteria. The genus includes 30 *Marinobacter* species currently validated. Some of them can
7 grow anaerobically by nitrate reduction (20 /30) and some of them (6/30) have been further characterized as
8 denitrifiers (able to perform reduction of nitrate to N₂ by respiratory process) (Handley and Lloyd 2013)
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1150 Presently, more than 3871 sequences of 16S rRNA genes affiliated to the genus *Marinobacter* are available in
12 the RDP ribosomal database from either isolates or clone strains. Heighten genomes are deposited in different
13 banks. Bacteria belonging to genus *Marinobacter* are commonly detected in diverse marine environments such
14 as coastal marine sediments (Gauthier et al. 1992; Gorshkova et al. 2003), waters (Yoon et al. 2004), and sea
15 sand environments (Kim et al. 2006), deep-water sediments (Cui et al. 2008; Edwards et al. 2003)
16 hydrothermal vents (Shieh et al. 2003), mud volcanoes (Yakimov et al. 2002), hypersaline microbial mats
17 (Abed et al. 2007; Bordenave et al. 2007), sediments of mangrove (Brito et al. 2006), alkaline seabed (Takai et
18 al. 2005), sea ice of the Arctic (Deppe et al. 2005) or the Antarctica (Bowman et al. 1997; Mikucki and Prisco
19 2007), sediments of the deep Arctic waters (Martin et al. 2003). Representatives of this genus have also been
20 identified associated with the bacterial flora of marine organisms. Indeed, they were isolated from
21 photosynthetic unicellular organisms such as dinoflagellates secreting paralyzing toxins (*Gymnodinium*
22 *catenatum* and *Alexandrium tamarense*) (Green et al. 2006) as well as multicellular organisms such as
23 bryozoans (Romanenko et al. 2005) and sponges (Lee et al. 2012). Thus *Marinobacter* seems to colonize a
24 wide variety of marine ecosystems around the world ranging from psychrophilic to thermophilic environments
25 with a high tolerance to salinity and pH. Their ability to occupy diverse physicochemical regimes seems an
26 indication of large phenotypic versatility.
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41 Recently metagenomic analyses have highlighted the presence of *Marinobacter* sequences at relatively high
42 percentage in oil polluted environments. Ribosomal or denitrifying genes affiliated to the genus *Marinobacter*
43 have been identified in oil spill simulation mesocosms with marine coastal sediment (Stauffert et al. 2014),
44 estuarine sediment (Gray et al. 2011) or mangrove sediment (dos Santos et al. 2011). Metatranscriptomic
45 analysis revealed that strains belonging to *Marinobacter* genus are active in polluted environments. In a study
46 on the Deep Horizon spill, *Marinobacter aquaolei* strain VT8 genome (now affiliated with *M*
47 *hydrocrabonoclasticus*) was the closest match to metatranscriptomes (Lamendella et al. 2014). Moreover, in
48 anoxic mesocosm, *Marinobacter* strain alkB cDNA, coding for a hydroxylase involved in degradation of
49 short-chain alkanes, has been quantitatively detected by qPCR (Genovese et al. 2014). These results led some
50 authors to propose the use of sequences of the genus *Marinobacter* as one of the proxies for oil pollution
51 monitoring (dos Santos et al. 2011; Genovese et al. 2014; Gray et al. 2011). Molecular approaches alone are
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4 however, unable to establish a relationship between the presence of a genus and its real ecophysiological role
5 in the studied ecosystem. In order to tackle this drawback, it is necessary to complement molecular
6 information with physiological data on hydrocarbon degradation capacities of strains belonging to different
7 *Marinobacter* species. Although the properties towards hydrocarbon degradation of some type strains have
8 been investigated (for a review see (Duran 2010), it should extend to more strains and to several isolates of the
9 same species. In a broader approach, the determination of the ecological role of *Marinobacter* needs further
10 investigation on their electron donor pattern.

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13 With the exception of the most superficial layer, bulk of marine sediments are assumed to be anoxic (Canfield
14 et al. 1993) especially when contaminated with hydrocarbons due to the reduction of oxygen diffusion. The
15 ability to use alternative electron acceptors is then an ecological advantage for bacterial communities. The
16 capability to perform nitrate reduction in anaerobiosis has been frequently reported for *Marinobacter* and
17 denitrification for at least some strains. This feature need also to be further explored.

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22 This study intends to analyze the robustness of *Marinobacter* proxy as hydrocarbon pollution applying genus
23 dynamic in the face of oil contamination, physiological analysis toward hydrophobic and hydrocarbons
24 compounds and anaerobic respiration. Their dynamic upon contamination with petroleum has been
25 investigated by qPCR with new primer set, in marine sediment mesocosms. Due to the impact of burrowing
26 macrofauna on oxygen supply and on in situ nitrate production resulting of the activity of nitrifying
27 prokaryotes, this condition has been also tested. In parallel investigations on the physiological characteristics
28 of *Marinobacter* has been undertaken with 34 isolates pertaining to 7 validated species and 3 potential novel
29 ones in order to determine their spectrum of electron donors and their potentiality regarding denitrification.
30 The analysis of 16 genomes has permitted to enlarge these observations to 8 additional validated species and
31 to one potential novel one.

32 33 34 35 36 37 38 39 40 41 42 **Materials and Methods**

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45 **Bacterial strains and isolation procedures:** The bacterial strains studied belong to the genus *Marinobacter*.
46 The set of strain comprised collection strains (*M. hydrocarbonuclasticus*, *M. aquaolei*, *M. algicola*) and strains
47 isolated from previous studies from Mediterranean sediments (Bonin et al. 1987; 1992; Goregues et al. 2004;
48 Rontani et al. 1997) (Fig. 1).

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51 Other strains (23w2b, 23tet5b, cubw5a, cubw5b, cubtrip2b and cubtet1b) have been isolated in the frame of
52 this study from North Sea water (Fig. 1). Samples were collected on board the RV Pelagia during May 2008.
53 Strains 23w2b, 23tet5b were isolated from samples of surface water contaminated by an oil slick collected at
54 53° 45' 50.4"N 4° 11' 15.7914"E. Strains, cubw5a, cubw5b, cubtrip2b and cubtet1b were isolated from
55 samples retrieved from a 1 m³ on-board mesocosm filled with seawater collected at 55° 8' 23.208" N 3° 11'

1 9.672" E at 29 m deep and contaminated with crude oil. Mesocosm were incubated seven days at ambient
2 temperature on the deck before sampling for strain isolation. Seawater samples, either from sea or mesocosms
3 were used to inoculate enrichment cultures in ASW (Baumann and Baumann 1981) containing tetradecane,
4 paraffin or tripalmitate as sole carbon source. Colonies isolation was performed on agar ASW plates with
5 acetate lactate glucose and yeast extract as carbon sources.

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8 **Electron donors screening:** Electron donors were tested with Biolog GN microplates (Biolog TNC,
9 Hayward, CA,USA). Cells were grown in ASW containing ammonium (1g/L) and a mixture of different
10 carbon sources (lactate 1g/L, acetate 1g/L, glucose 1g/L, yeast extract 2g/L, peptone 2g/L). Cultures in
11 exponential phase of growth were pelleted, washed once in ASW without carbon source. Biolog GN
12 microplates were inoculated with the cell suspensions as described in the manufacturer instructions. Utilization
13 of carbon sources was detected as an increase in the respiration rate of cells in the well of a microplate, leading
14 to irreversible reduction of the tetrazolium dye INT [2-(p-iodopheny)-3-(p-nitropheny)-5-phenyl tetrazolium
15 chloride]. A positive utilization reaction was indicated when a purple color formed due to INT-formazan
16 formation in a well after one week incubation at 20°C. Biolog GN microplates contained the following carbon
17 sources that could be used as electron donor in aerobiosis: 30 carbohydrates (α -Cyclodextrin, Dextrin,
18 Glycogen, N-Acetyl-D-Galactosamine, N-Acetyl-D-Glucosamine, Adonitol, L-Arabinose, D-Arabitol, D-
19 Cellobiose, i-Erythritol, D-Fructose, D-Fucose, D-Galactose, Gentiobiose, α -D-Glucose, α -D-Lactose,
20 Lactulose, Maltose, D-Mannitol, D-Mannose, D-Melibiose, β -Methyl-D-Glucoside, D-Psicose, D-Raffinose,
21 L-Rhamnose, D-Sorbitol, Sucrose, D-Trehalose, Turanose, Xylitol), 19 amino-acids (D-Alanine, L-Alanine,
22 L-Alanyl-Glycine, L-Asparagine, L-Aspartic Acid, L-Glutamic Acid, Glycyl-L-Aspartic Acid, Glycyl-L-
23 Glutamic Acid, L-Histidine, Hydroxy-L-Proline, L-Leucine, L-Ornithine, L-Phenylalanine, L-Proline, D-
24 Serine, L-Serine, L-Threonine, D,L-Carnitine, γ -Aminobutyric Acid), 30 organic acids (Pyruvic Acid Methyl
25 Ester, Succinic Acid Mono-Methyl Ester, Acetic Acid, Cis-Aconitic Acid, Citric Acid, Formic Acid, D-
26 Galactonic Acid Lactone, D-Galacturonic Acid, D-Gluconic Acid, D-Glucosaminic Acid, D-Glucuronic Acid,
27 α -Hydroxybutyric Acid, β -Hydroxybutyric Acid, γ -Hydroxybutyric Acid, p-Hydroxy-phenylacetic Acid,
28 Itaconic Acid, α -Ketobutyric, α -Ketoglutaric Acid, α -Ketovaleric Acid, D,L-Lactic Acid, Malonic Acid,
29 Propionic Acid, Quinic Acid, D-Saccharic Acid, Sebacic Acid, Succinic Acid, Bromosuccinic Acid,
30 Succinamic Acid, L-Pyroglutamic Acid, Urocanic Acid), 3 alcohols (m-Inositol, 2,3-Butanediol, Glycerol) et 13
31 other substrates (tween 40, tween 80, Glucuronamide, L-Alaninamide, Inosine, Uridine, Thymidine,
32 Phenylethyl-amine, Putrescine, 2-Aminoethanol, D,L, α -Glycerol Phosphate, α -D-Glucose 1-Phosphate, D-
33 Glucose-6-Phosphate).

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54 For insoluble substrates, strains were inoculated on ASW agar plates containing ammonium (1g/L) and one of
55 the following electron donors : Aromatic Hydrocarbon: fluoranthene (1 mg/mL), phenantrene (1 mg/ mL),
56 dodecylbenzene (1 μ L/mL); Fatty acyl hydrocarbon: tetradecane (1 μ L/mL), heptadecane (1 μ L/mL), wax
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1 ester (hexadecyl d'hexadecanoate ; 0.625 mg/ mL), paraffin (mixture of C19 to C31 alkanes with melting
2 point 52°C ; 2.5 mg/mL); Branched hydrocarbon: pristane (1 µL/mL), glycerolipids: triolein (5 µL/mL),
3 tripalmitin (7.5 mg/mL). Plates with a mixture of soluble carbon sources or no carbon source were also used as
4 positive and negative control respectively. The plates were incubated aerobically at 20°C for two weeks and
5 then overlaid with 10 mL molten soft agar containing INT (0.2g per liter ASW) and the same carbon source
6 than that used for the growth step. Oxygen respiring colonies in the presence of the selected substrates were
7 identified as those reducing colorless INT into red INT-formazan (Goregues et al. 2004). Results were
8 recorded after 30 minutes incubation.
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16 **Mesocosm set up:** Sediment was collected at Brittany coast (Celtic Sea, Atlantic Ocean, 48° 34' 28.2''N 4°
17 36'40.2'' E) in October 2013. After removing macrofauna, 72 L of sediments were distributed in 12
18 mesocosms (boxes 15x20x 30 cm). Four conditions were established in triplicate (1) No Nereis (*Hediste*.
19 *diversicolor*) addition and no hydrocarbon amendment (-HC-N), (2) Nereis addition at density of 1000
20 organism/m² and no hydrocarbon amendment (-HC+N) (3) no Nereis addition and hydrocarbon amendment at
21 7g/kg (+HC-N), (4) Nereis addition at density of 1000 organism /m² and hydrocarbon amendment at 7g/kg
22 (+HC+N),. Hydrocarbon addition consist in Ural type petroleum topped at 100°C (removal of HC < 7C).
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28 **Nucleic acid extraction, quantification by qPCR in mesocosms and characterization of genes of** 29 **denitrification on strains set**

30 After 3 months of incubation at 20°C, 5 g of the 2 first centimeters of sediment in each mesocosm were
31 sampled and nucleic acids were extracted using the MoBio Power soil RNA/DNA extraction kit (MoBio
32 Laboratories, California) according to the manufacturer's protocol. DNAs from cultures of strains were
33 purified as previously reported (Goregues et al. 2005). Purified DNA was stored at 20°C until use.
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39 The determinations of 16S rRNA gene copy numbers were performed by real time PCR with SsoAdvanced
40 SYBR Green Supermix using a CFX96 Real Time System (C1000 Thermal Cycler, Bio-Rad Laboratories,
41 CA, USA). The primer sets GML5F-Uni516R (Michotey et al. 2012) and marinob300F (5'-
42 GACTGAGACACGGCCCGA-3') - marinob600R (5'-CCGGGGCTTTCACATCTCG-3') were used for
43 quantification of total bacterial 16S rRNA genes and *Marinobacter* 16S rRNA genes respectively. The real
44 time PCR cycles consisted of a denaturation step of 5 s, a hybridization step of 20 s at 56°C with an elongation
45 step of 40 s. Quantification of bacterial *nosZ* were performed using nosZ 1211F and nosZ 1897R primers
46 (Rosch et al. 2002) and SsoFast EVA Green Supermix (Bio-Rad Laboratories, CA, USA). The real time PCR
47 cycles consist of a denaturation step of 15 s, a hybridization step of 15 s at 64°C with an elongation step of 40 s
48 at 72°C. Gene abundance in microcosm DNA samples was determined in relation with a calibration standards
49 corresponding to dilution of plasmids harboring target genes.
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Denitrification activity of strains were determined as previously described (Bonin et al. 1987; 1992; Goregues et al. 2004; Rontani et al. 1997). The detection by PCR on genome of the strains of *narG*, *nirS* and *nosZ* were performed as previously reported (Goregues et al. 2005, Michotey et al. 2000, Rosch et al. 2002) respectively. All amplified DNA fragments were checked by sequencing. The nucleotide sequence data generated in this study have been deposited in the Genbank database under accession numbers KM816585 to KM816618.

Genomes analysis: Sixteen genomes are currently available, *M. adhaerens* DSM 23420 (CP001978), *M. algicola* DG893 (GCA_000170835.1), *M. hydrocarbonoclasticus* strain VT8 (GCA_000015365.1), *M. hydrocarbonoclasticus* strain 17 ATCC 49840 (FO203363), *M. excellens* (PRJNA195885), *M. daepoensis* DSM 16072 (GCA_000421165.1), *M. lipolyticus* strain BF04 (GCA_000372805.1), *M. lipolyticus* strain SM19 (ASAD01000001-ASAD01000006), *M. maganoxydans* (AGTR00000000), *M. nanhaiticus* (APLQ01000000), *M. santoriniensis* (APAT01000000), *M.* strain AK21 (ANIE01000001-ANIE01000006), *M.* strain BSs20148 (CP003735), *M.* strain ELB17 (GCA_000169375.1), *M.* strain MCTG268 (JQMK00000000), *M.* strain HL-58 (GCA_000686085.1). The metabolic properties harbored by the strain according to their genomes analyses was assessed with IMG-ER (DOE-JGI) platform (<https://img.jgi.doe.gov>)

Statistical analysis: An ANOVA analysis was used to determine the influence of hydrocarbon amendment and presence of Nereis on bacterial numeration in microcosms. The significance level for all tests was set at $p < 0.05$. All tests were performed with XLSTAT® (XLSTAT software® 2008, Addinsoft, Paris).

Results

In this study we propose new qPCR primers for the detection of *Marinobacter* ribosomal 16S rDNA (Table 2). Primer set Marinob300f-Marinob600R matches with 64.5% of ribosomal sequences affiliated to *Marinobacter* genus in RDP database and 90% of ribosomal sequence of *Marinobacter* type strains. Previous qPCR primers (Mab451f-Mab573r) and CARD-FISH probe presented a narrower spectrum of detection since they matched with 6.4 and 26.6 % of *Marinobacter* ribosomal sequences in RDP, respectively. These percentages dropped to 3 and 20% for type strains. (Tab.2). Both qPCR primer sets present lower percentage of match toward non-target sequences (1.2% and 2.8%) in comparison of CARD-FISH probes (20%). Optimization of hybridization temperature of the qPCR was determined ($T=56^{\circ}\text{C}$) in relation with positive (*M. hydrocarbonoclasticus*) and negative (*E. coli*) Gammaproteobacteria controls. The resulting conditions lead to a qPCR efficiency of 97.1% ($R^2=0.994$).

Marinobacter dynamic was monitored upon hydrocarbon pollution in sediment mesocosms. *Marinobacter* and bacterial 16S rRNA gene abundances were measured by qPCR in all mesocosms after 3 months incubations

(Fig. 1). The highest number of bacterial ribosomal genes (about 10^{11} /g sediment) was observed for the condition without macrofauna and hydrocarbon. For the other conditions, no significant difference in numeration of bacterial 16S rRNA genes was recorded in the mesocosms (about 10^{10} /g sediment) (Fig. 1). The analysis of abundance of *Marinobacter* ribosomal genes emphasized the predominant impact of hydrocarbon to presence of macrofauna. Indeed, all mesocosms containing hydrocarbon presented significant higher numbers of *Marinobacter* (+HC+N, +HC-N> -HC+N, -HC-N; $p < 0.05$). Their abundance reached 0.3-0.09% of the bacterial population in presence of HC in comparison of 0.02-0.004% in its absence. Whatever the presence of hydrocarbon, the mesocosms without macrofauna harbored lower percentage of *Marinobacter* than those in which *Nereis* has been introduced (0.3 vs 0.09 and 0.022 vs 0.004 for condition with and without hydrocarbons).

The denitrifying community was quantified using *nosZ* gene with primers previously used for competitive PCR (Rontani et al. 2010, Fernandes et al. 2012). In qPCR the efficiency of the reaction reached 91.7%. In mesocosms, the number of bacterial *nosZ* varied from $6.66 \cdot 10^6$ to $3.24 \cdot 10^7$ /g sediment (Fig.1). The ratio *nosZ*/16S rRNA bacterial genes ranged from $0.14 \cdot 10^{-3}$ to $0.07 \cdot 10^{-3}$ in mesocosms without hydrocarbon amendment to $1.47 \cdot 10^{-3}$ to $0.57 \cdot 10^{-3}$ in its presence. Within a condition (+ or – hydrocarbon) this ratio was higher in presence of macrofauna. In contrast, the ratio denitrifier /*Marinobacter* (*nosZ*/*Marinobacter* 16S rRNA gene) seemed relatively stable and range between 0.48 and 1.72 (Fig. 1).

The electron donor pattern was determined on a set of 34 isolates which according to phylogenetic tree belong to 7 validated species and 3 potential novel ones referred as phylotype # 2, #8, #9 (S1). Hereafter, validated species and potential novel ones were referred as taxa. Of the 34 tested strains, only 8 were able to use a few carbohydrates as electron donors (S2). All the strains were only able to use less than 4 different carbohydrates, excepted strain T33 (phylotype #8) oxidizing 16 of them. Glucose was the most commonly utilized (6 isolates) but only occasionally within a taxon (1/11 for *M. hydrocarbonoclasticus*, 2/8 for *M. vinifirmus*; 1/ 2 for *M. maritimus*, 2/4 for phylotype #8). The same trend was observed for dextrin since only 4 strains were able to use it (S2). Likewise, amino acid utilization range of *Marinobacter* strains of this study was restricted to few them; however in contrast to carbohydrates, most of the strains of a taxon shared the same trend toward a given Amino acid. L-proline was the most frequently used by the isolates (8/10 taxa) followed by L-glutamic acid (5/10 taxa) and then L-leucine, (4/10 taxa) (Fig.2). L alanine and D-alanine were oxidized by only few strains within 3 and 4 taxa respectively (S2). Eight out of 30 organic acids tested could be used as electron donors by a least one *Marinobacter* strain. The most widely used acid was β -hydroxybutyric acid that was electron donor for 8/10 taxa followed by pyruvic acid methyl ester or D,L lactic acid that were oxidized by 4/10 taxa (Fig.2). The utilization of these 3 organic acids was shared by most of the strains in a taxon (Fig.2).

245 and S2). In contrast, propionic acid, acetic acid, citric acid, succinic acid and bromo-succinic acid were
1 oxidized at a lesser extent and only occasionally within a taxon.
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4 Among the investigated panel of electron donors, the most used were lipid compounds. All tested molecules
5 were used by several taxa of *Marinobacter* and all taxa, excepted *M. xestospongia* strain BP42, could utilized
6 more than half of them. *M. hydrocarbonoclasticus* and *M. vinifirmus*, represented by 11 and 8 isolates
7 respectively, were used to analyze the representativeness of trait within a taxon. Tween 40 (Polyoxyethylene
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250 (20) sorbitan monopalmitate) and Tween 80 (Polyoxyethylene (20) sorbitan monooleate) that are emulsifiers,
10 or triolein that is a glycerolipid were oxidized by all taxa. This feature is also observed for heptadecane, a
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12 C17- alkane belonging to fatty acyls. These latter patterns were shared by more than 75% of the isolates of a
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14 taxon in most all cases. The two other alkanes tested, tetradecane, a linear -C14 and pristane, a branched C19
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16 molecule, were oxidized by 9/10 and 6/10 taxa, respectively. Pristane utilization is sparse since only 9% of
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255 isolates of *M. hydrocarbonoclasticus* (9%) and 25% of *M. vinifirmus* (25%) were positive, suggesting that it
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19 cannot be considered as specific trait of a species. The other substrate molecules were characterized by their
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21 low lability due to the presence of one or more aromatic cycles or due to higher melting point. The 3 aromatic
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23 molecules, dodecylbenzene, phenanthrene or fluorethene were oxidized by 6/10, 5/10 and 6/10 taxa
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25 respectively. As for pristane, the utilization pattern of aromatic hydrocarbon was not identical for most of the
260 strain within a taxon. For example, only 18% and 64% of *M. hydrocarbonoclasticus* and 12% and 25% of *M.*
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28 *vinifirmus* could use fluorethene or dodecylbenzene respectively. This trend was also observed for very
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30 insoluble substrates pertaining to fatty acyls or glycerolipids such as tripalmitate paraffin and wax. Between 5
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32 and 7 taxa were positive for their utilization, with positive strains varying between 12 to 55% for *M.*
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345 *hydrocarbonoclasticus* and *M. vinifirmus*.
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36 The available genomes corresponded to 10 validated species (*M. adhaerens*, *M. algicola*, *M*
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38 *hydrocarbonoclasticus*, *M excellens*, *M. daepoensis*, *M. lipolyticus*, *M. maganoxydans*, *M. nanhaiticus*, *M.*
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40 *santoriniensis*, *M. psychrophilus*) and a potential novel one (*M. sp* AK21) (S1). From the analysis of available
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42 genomes, similar trends could be emitted on nature of electron donor of *Marinobacter*. The genomic analysis
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270 was focused on proteins mediating the entry of substrates into the cell (translocation protein, transporter,
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45 permease, symport) or the first enzyme of a pathway for lipids (Tab. 3). To import many carbohydrates
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47 (glucose, mannose, fructose...), Bacteria have developed an unique mechanism the
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49 phosphoenolpyruvate:sugar phosphotransferase system (PTS). This system synchronizes the transport of the
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51 sugar through the cellular membrane and its phosphorylation. With some variations, the PTS comprises
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275 several proteins, some of them are common for all sugars such as EnZI and HPr and others that are specific of
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54 a carbohydrate or a group of carbohydrates such as EnzII composed of domain IIA, IIB, IIC. This latter one is
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56 located in the membrane and mediates specific sugar translocation. Whereas HPR has been identified in all
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58 genomes of *Marinobacter*, EnZII were scarce (Tab.3). From the panel of genomes, only EnZII A and B genes
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60 specific to fructose have been identified in 8 and 5 genomes, respectively; in contrast to EnZII C for which
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280 whatever the sugar, no gene could be identified. The absence of the protein responsible for of sugars
1 translocation led to the impossibility to use these compounds as electron donors. For some organic acids such
2 lactate or succinate, putative genes annotated as transporters were found in 5 and 16 genomes, respectively.
3 Proline appeared as an important amino acid since all genomes harbored a sodium/proline symporter. Except
4 the Carboxylate/Amino Acid/Amine transporter that was present on all genomes, the other specific amino acid
5 transporters were either absent or present in only one genome. In contrast, all the strains contained the fatty
6 oxidation complexes *FadAB* and a complete β -oxidation pathway (data not shown). Furthermore, the
7 occurrence of both alkane mono-oxygenase (*alkA*) and haloalkane dehalogenase genes in genomes was above
885 87%. The only genome in which *alkA* has not been detected is *M. lipoliticus* strain BF04. The genome of this
9 strain did not contain also the gene coding for haloalkane dehalogenase as that of *M psychrophylus* BSs20148.
10 Long chain alkanes (C_{32} and longer) are oxidized by the product of gene *almaA* that encodes a flavin-binding
11 monooxygenase. Blast search with *almaA* sequence of *M. lipolyticus* (FJ263110) from the study of Wang and
12 Shao (2012) and with an E-value cutoff at 10^{-5} , permit the identification of 13 homologous genes in
13 *Marinobacter* set of genome. The two strains of *M. hydrocarbonoclasticus*, all 3 strains of *M algicola*, and
14 strains of *M adhaerens*, *M. manganoxydans*, *M. daepoensis*, *M. santoriniensis*, *M. nanhaiticus*, *M. excellens*,
15 *M.lipolyticus* SP19 and *M* strain AK21 were positive.
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28 During denitrification, nitrate is reduced into N_2 through several steps. Nitrate can be reduced to nitrite by
29 NarG or NapA, nitrite to nitric oxide by NirS or nirK, nitric oxide to nitrous oxide by NorB, and nitrous oxide
30 to N_2 by NosZ. Three genes out of four were tested. Analyses of denitrifying genes revealed the preferential
31 presence of *narG*, *nirS* among our denitrifying *Marinobacters* set (Table 4). Most of the nitrate reducers
32 among our set of strains were able to perform total denitrification and harbored also *nosZ*. This tendency was
33 also found by genomic approach (Table 3). Denitrification genes the most frequently recovered were *narG* and
340 *nosZ* and 75% of the genomes possessed at least one gene of denitrification.
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42 Discussion

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4505 Hydrocarbons occur as biosynthetic products of living organisms in the biosphere (Ladygina et al. 2006) or as
46 abiotic transformation products of biogenic organic matter in the geosphere (Seewald 2003). They are the
47 main constituents of petroleum and thus are extremely abundant in geological systems. They can be
48 transferred from geosphere to biosphere by different manners such as hydrothermal vent, mud volcano
49 (Whiticar and Suess, 1990; Timmis 2010) or drilling problems. In consequence their presence is probably
50 ubiquitous but in various proportions. Marine ecosystems are frequently exposed to oil pollution. After a spill,
51 the n-alkanes, short chains tend to evaporate or dissolve in seawater. In contrast to short and medium-chain
52 alkanes that are quickly degraded after the spill, the long-chain molecules with more than 18 carbon atoms, do
53 not dissolve nor degrade efficiently and stay for longer periods of time in the environment. Hydrocarbons of a
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1 spill or from biogenic origin enter with other compounds in the composition of marine organic matter resulting
2 in a complex mixture of molecules having various compositions, physical structures and reactivity. This
3 complexity is probably a major factor for maintaining a high degree of diversity among prokaryotes in oceans.
4 Heterotrophic bacteria recycle dissolved and particulate organic matter and contribute to the productivity of
5 the ecosystem. Isolation campaigns have demonstrated the presence of the heterotrophic Gammaproteobacteria
6 *Marinobacter* in many marine environments exhibiting disparate characteristics. This finding would suggest
7 that they are capable of oxidizing a wide range of carbon substrates. This physiological studies of 34 isolates
8 and analyses of 16 genomes from diverse marine habitats such as sediment, water column or phycosphere and
9 partitioning into 15 species and 4 potential novel ones within *Marinobacter* genus give rise to a different view.
10 Except for some strains, very few molecules classified as labile organic matter are used. The spectra of
11 carbohydrates utilization as electron donors determined by physiological as well as genomic approaches are
12 extremely reduced. Comparison of physiological and genomic approaches focusing on transporters was
13 evaluated by comparison of patterns obtained from *Marinobacter hydrocarbonoclasticus* strain 17. From its
14 genome fully annotated (Grimaud et al. 2012), no EnzIIC of sugar could be detected in congruence with
15 results of physiological study. The inability to use carbohydrates or to degrade the polysaccharides has been
16 reported previously for some *Marinobacter* strains (Bowman and McMeekin 2005). However, our results
17 including a wider list of species and with several strains within a species strengthen this observation that
18 appears as a common trait of this genus.

19 The ability of *Marinobacter* to use external amino acids as electron donors was reported. The list of the
20 preferential ones included L-alanine, D glutamate and L phenylalanine (Handley and Lloyd 2013). From our
21 data, the most frequently used by our set of strains is proline and all analyzed genomes contained a specific
22 transporter for this amino acid. This feature was described previously for *M. gudaonensis* (Gu et al. 2007),
23 and *M. koreensi* (Kim et al. 2006). With the present study, this characteristic is enlarged to at least 16 species
24 and 4 potential novel ones. Furthermore this amino acid appeared to have an important physiological role due
25 to the very high frequency of its utilization. Proline, is uncommon in proteins but has the characteristic to be
26 compatible solute, that is to say, a molecule involved in resistance to the osmotic pressure, thermal stress for
27 many heterotrophs (Welsh 2000). This ability could allow *Marinobacter* strains to resist to a wide range of
28 physical parameters.

29 The main feature which emerges from the analyses of the substrates pattern is the capacity of many strains of
30 *Marinobacter* to oxidize a very large spectrum of lipid molecules of various type and molecular weight
31 (including Tween). This physiological aspect is not frequently explored in species characterization (Handley
32 and Lloyd 2013). Degradation capacity towards tween is often reported in the literature due to its presence in
33 Biolog panel. For example *M. koreensis* (Kim et al. 2006), *M. lipolyticus* strain SM19, *M. litoralis* (Martin et
34 al. 2003) and *M. bryozoorum* or *M. sediminum* (Romanenko et al. 2005) were found positive. For other lipid
35 molecules, except few studies, the degradation pattern toward these compounds remains often untested. *M.*

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hydrocarbonoclasticus is may be one of the best characterized. It can utilize *n*-alkanes ranging from C₆-C₃₀ (Doumenq et al. 2001; Duran 2010; Grimaud 2010). This trait seemed largely shared since genomic approach has highlighted the presence of alkane mono-oxygenase, an enzyme responsible for the first step oxidation of fatty acyl hydrocarbon in 15 out of 16 genomes. According to literature, 9 *Marinobacter* species were identified as hydrocarbon degraders (Handley and Lloyd 2013); our results have identified 9 additional species. This feature was observed for strains isolated from environment enriched with petroleum such as oil producing well in the case of *M. hydrocarbonoclasticus* VT8 but was also observed for strains retrieved from potential petroleum-free samples such as dinoflagelates cultures for *M. algicola* (Green et al. 2006), or microbial mat for the two other genomes affiliated to *M. algicola* (HL-58 and MCTG268), marine particles for *M. adhaerens* (Gärdes et al 2010) or Artic ocean sediment for *M. psychrophilus* BSs20148 (Song et al. 2013) for examples. Usually when hydrocarbon utilization is included in physiological characterization, only short chain hydrocarbon are tested. For screening the capacity to degrade long –chain alkanes with more than 18 carbon atoms, the use of *almA* gene has been recently suggested by Wang and Shao (Wang and Shao 2012). Strains belonging to *M. koreensis*, *M. alkaliphilus*, *M. lipolyticus*, *M. flavimaris* and *M. hydrocarbonoclasticus* were found positive by these authors (Wang and Shao 2012). This gene was recovered by specific BlastP on most genomes of our set and this trend was extended to species *M. adhaerens*, *M. manganoxydans*, *M. algicola*, *M. daepoensis*, *M. santoriniensis*, *M. nanhaiticus*, and *M. excellens*. In this study, the panel of electron donors include short and long chain hydrocarbons but also non-hydrocarbon lipids (glycerolipids). Most of the strains appeared positive. The results of this study highlighted also the need to screen several strains of a same species to find common physiological traits within a species. From previous studies (Timmis 2010 for a review), some *Marinobacter* type strains were described as short chain alkane degraders, this trend could now be expanded through a larger number of species including several strains within a species and to large spectrum of lipid molecules. These compounds appeared as preferred electron donors for *Marinobacter* species included in this set.

This capacity could account for the 10 fold increase of *Marinobacters* abundance in mesocosm contaminated with petroleum of this study. This value is higher than the 2 fold increase observed in anaerobic mesocosm with a CARD-FISH probe exploring only 26% of *Marinobacter* sequence (Genovese et al. 2014) but are in agreement with augmentation of *Marinobacter* ribosomal sequences found by pyrosequencing before and after oil pollution in mangrove (dos Santos et al. 2011). In our study, *Marinobacter* reached about 0.3% of the bacterial community in mesocosms containing hydrocarbon which is lower than the value found with less specific probe in anaerobic mesocosm by Genovese et al. (2014). However comparison between different studies should be taken with caution since other factors could be involved such as sensitivity to the load of petroleum or the selection of some species not detected by qPCR or CAR-FISH or a kinetic effect. The highest abundance of *Marinobacter* was observed in presence of petroleum and burrowing macrofauna. The presence and activity of burrowing macrofauna such as *H. diversicolor*, in sediment can significantly affect the

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repartition of electron acceptors and nutrient availability (Banta and Andersen 2003). In marine sediment, oxygen rapidly decreases with depth but several alternative electron acceptors are available. In absence of oxygen, nitrate is the most favorable electron acceptor since its reduction to N_2 yields almost as much energy as aerobic respiration (Canfield et al. 2005). However nitrate concentrations in sediments are low probably due to low in situ production or rapid turnover. Bioturbation by macrofauna can enhance oxygen supply to deeper layers, favoring activity of nitrifying prokaryotes than can provide nitrate for nitrate respiring organisms (Laverock et al. 2011) such as denitrifiers. Some strains of *Marinobacter* have been shown as able to respire inorganic compounds such as arsenic (Handley et al 2013) or more frequently nitrate (i.e; denitrifier (Bonin et al. 1987; Gilewicz et al. 1997; Rontani et al. 2002; Goregues et al. 2004). The use of molecular technics has shown that *narG*, *nirS* and *nosZ* are present in most of the nitrate reducers included in this study. These genes are frequently identified in genomes also. The denitrification property of type strain of *M. pelagius*, *M. maritimus* and *M. vinifirmus* was either incomplete or negative in previous studies. Our results have shown that at least some member of these species can perform this process. The impact of oil on denitrifying community was also addressed in this study. This community was enumerated with *nosZ* as validated earlier (Rontani et al. 2010; Fernandes et al. 2012; Nogales et al. 2002). This gene was preferred to *nirS* since it presents the advantage to be the sole one coding for this enzymatic step. The percentage of *nosZ*-harboring cells increased under hydrocarbon amendment suggesting a probable role in petroleum degradation. Indeed some studies on isolated strains have reported the property of some *Marinobacter* to degrade hydrocarbon in anaerobiosis (Rontani et al. 2002).

From the results obtained in this study, primers amplifying a broader spectrum of *Marinobacter* ribosomal gene have confirmed the increasing prevalence of this genus when confronted with petroleum in mesocosms as previously observed with other technics. This phenomenon seems to be due to the pattern of electron donors of this genus, presenting a large range of lipid compounds and narrow ones with other organic molecules. This result has been confirmed by screening of several species and several strains within a species. *Marinobacter* development in sediment contaminated by petroleum was favored by burrowing macrofauna which facilitate diffusion of oxygen but also the supply nitrate to denitrifiers, some of whom belonging to *Marinobacter* genus. The high frequency of genes involved in alkane degradation of this genus would favor the utilization of this genus as proxy of petroleum monitoring. For example, their occurrence in surrounding area of hydrothermal vents (Wang et al 2012; Handley et al 2013) could be an indication of the diffusion of hydrocarbon effluents. However, the increase of *Marinobacter* in presence to oil pollution could be also the result of an opportunistic physiological trait toward lipid substrates.

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2 the Royal Netherlands Institute for Sea Research (NIOZ) for the support by the NIOZ-Marine Research
420 Facilities (MRF) on-shore and on board.
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10 Figure captions

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12 **Fig.1** Abundance of ribosomal 16S rRNA gene from bacteria or *Marinobacter*, and of nitrous oxide reductase
13 gene (*nosZ*) in mesocosm in presence of Hydrocarbon or of burrowing macrofauna (*H. diversicolor*)
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16 Neither Hydrocarbon amendment nor *H. diversicolor* addition (-HC-N), no Hydrocarbon amendment and *H.*
17 *diversicolor* addition (-HC+N), hydrocarbon amendment and no *H. diversicolor* addition (+HC-N),
18 hydrocarbon amendment and *H. diversicolor* addition (+HC+N); These values mean the average number of
19 genes detected in triplicated mesocosms. Several subsamples were collected from each mesocosm.
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24 **Fig.2** Main electron donors pattern of 10 taxa of *Marinobacter*, expressed as percentage of positive strains
25 among a taxon. Substrates tested: 30 carbohydrates, 19 AA, 30 organic acids, 12 lipid molecules and 17 other
26 compounds, only relevant results were indicated
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31 references

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Table 1

List of *Marinobacter* strains used in this study

Species	Strains	Location of isolation	Type of sample	Reference	Physiological (P), genomic (G) analysis
<i>M. hydrocarbonoclasticus</i>	VT8, DSM 11845 ^a	Vietnam	Oil producing well	(Huu et al. 1999)	P, G
	DN1 ^a	Gulf of Fos, Med Sea	sediment	(Bonin et al. 1987)	P
	17 ATCC 49840	Gulf of Fos, Med Sea	sediment	(Gauthier et al. 1992)	P, G
	CAB	Gulf of Fos, Med Sea	sediment	(Gilewicz et al. 1997)	P
	BC36, BC38, R153	Berre lagoon, Med Sea	sediment	(Goregues et al. 2004)	P
	23W2B, CUBW5A, CUBW5B, 23tet5B	North Sea	Water column	This study	P
<i>M. vinifirmus</i>	BC45, NBC44, NBC35, NBC34, NBC33, NBC32, BC43, BC34	Berre lagoon, Med Sea	sediment	(Goregues et al. 2004)	P
<i>M. maritimus</i>	2Sq31, 1sq53	Gulf of Fos, Med Sea	sediment	(Rontani et al. 2002)	P
<i>M. flavimari</i> <i>M. adhaerens</i> <i>M. manganoxydans</i>	Cubtrip2B, Cubtet1B	North Sea	Water column	This study	P
<i>M. adhaerens</i>	HP15 DSM 23420	German Wadden Sea	Diatom interaction	(Gärdes et al. 2010)	G
<i>M. manganoxydans</i>	MnI7-9	Indian Ocean	Deep-sea hydrothermal vent sediment	(Wang et al. 2012)	G
<i>M. pelagius</i>	R171, R42	Berre	Sediment	(Goregues	P

Species	Strains	Location of isolation	Type of sample	Reference	Physiological (P), genomic (G) analysis
		lagoon, Med Sea		et al. 2004)	
<i>M. algicola</i>	DG893	Culture	Dinoflagellates	(Green et al. 2006)	P, G
	<i>HL-58</i>	–	Microbial mat	unpublished	G
	MCTG268	–	Microbila mat	unpublished	G
<i>M. xestospongiae</i>	BP42	Berre lagoon, Med Sea	sediment	(Goregues et al. 2004)	P
<i>M. daepoensis</i>	<i>SW-156^T</i> (DSM 16072)	South Korea	Water column	(Yoon et al. 2004)	G
<i>M. santoriniensis</i>	<i>NKSG1</i>	Santorini, Med Sea	Shallow marine hydrothermal sediment;	(Handley et al. 2013)	G
<i>M. lipolyticus</i>	<i>SM19</i>	Spain	saline soil	(Papke et al. 2013)	G
	BF04_CF-4	–	–	unpublished	G
<i>M. nanhaiticus</i>	D15-8 W	South China Sea	Sediment	(Cui et al. 2013)	G
<i>M. excellens</i>	HL-55	–	–	unpublished	G
<i>M. psychrophilus</i>	ELB17	Antarctica	Waters of a permanently ice-covered lake	unpublished	G
	BSs20148	Arctic Ocean	Sediment	(Song et al. 2013)	G
<i>M. strain AK21</i>	AK21	India	Water column	unpublished	G
<i>M. sp. phylotype no. 2</i>	BP45, NBP42	Berre lagoon, Med Sea	Sediment	(Goregues et al. 2004)	P
<i>M. sp. phylotype no. 8</i>	U32, S64, T33, U31	Berre lagoon, Med Sea	Sediment	(Goregues et al. 2004)	P
<i>M. sp. phylotype no. 9</i>	R53	Berre lagoon Med Sea	Sediment	(Goregues et al. 2004)	P

Table 2

Comparison of different PCR primers or probes for detection of *Marinobacter* 16S rDNA using probe-match (RDP probe match tool)

Primer set	Marinob300F- Marinob600R	Mab451f-Mab573r	CARD-FISH probe
	Number of hits (% recovery)	Number of hits (% recovery)	Number of hits (% recovery)
Reference	This study	(Gray et al. 2011)	(Karner and Fuhrman 1997)
Bacteria	2414/2879170	242/2879170	885/2879170
<i>Marinobacter</i> genus	2374/3639 (64.5 %)	235/3639 (6.4 %)	714/3639 (20.6 %)
<i>Marinobacter</i> type strains	27/30 (90 %)	1/30 (3 %)	8/30 (26.6 %)
Non-target matches	30/ 2414 (1.2 %)	7/242 (2.8 %)	171/885 (20 %)

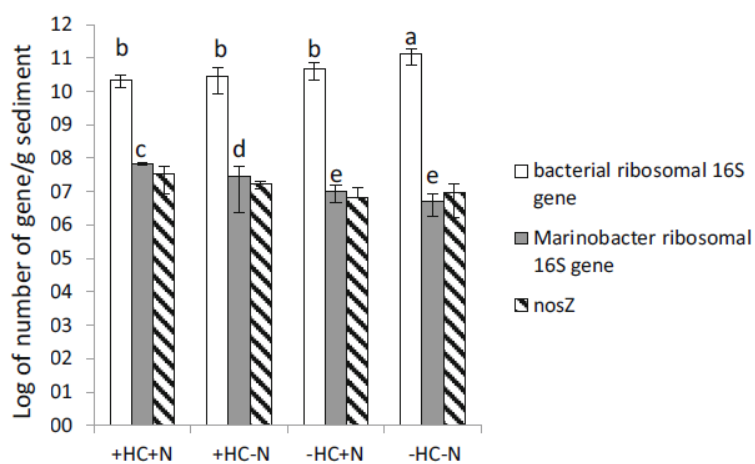


Fig. 1 Abundance of ribosomal 16S rRNA gene from bacteria or *Marinobacter* and of nitrous oxide reductase gene (*nosZ*) in mesocosm in the presence of hydrocarbon or of burrowing macrofauna (*H. diversicolor*). Neither hydrocarbon amendment nor *H. diversicolor* addition (-HC-N), no Hydrocarbon amendment and *H. diversicolor*

addition (-HC+N), hydrocarbon amendment and no *H. diversicolor* addition (+HC-N), hydrocarbon amendment and *H. diversicolor* addition (+HC+N). These values mean the average number of genes detected in triplicated mesocosms. Several subsamples were collected from each mesocosm

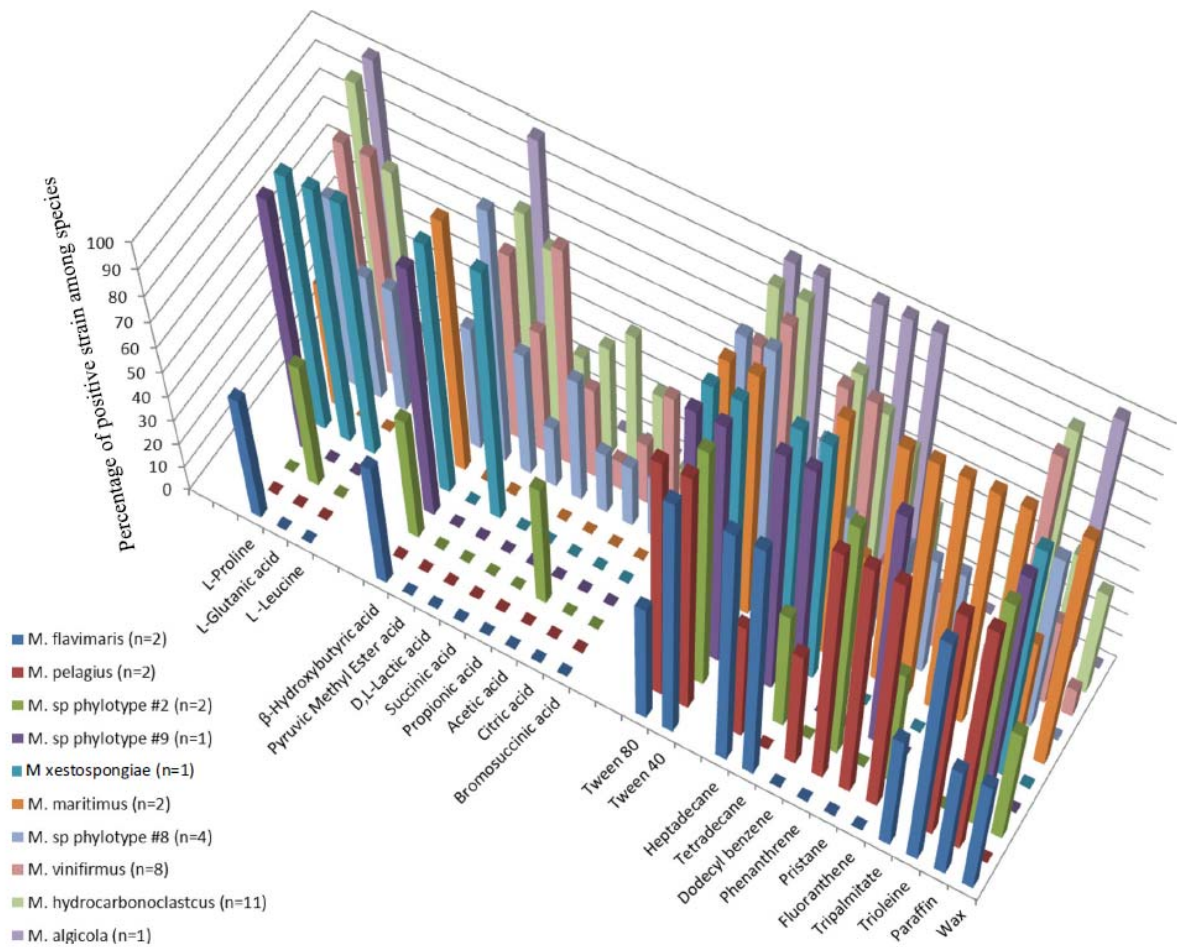


Fig. 2 Main electron donors pattern of 10 taxa of *Marinobacter*, expressed as percentage of positive strains among a taxon. Substrates

tested: 30 carbohydrates, 19 AA, 30 organic acids, 12 lipid molecules, and 17 other compounds; only relevant results were indicated