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Characterisation and differentiation of oligotrophic waters by culturable particle-attached and free-living bacterial communities

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ABSTRACT

This study reports the development of a tool to characterise and differentiate northern Adriatic waters, particularly oligotrophic, high-salinity waters, based on the cellular fatty acids of culturable heterotrophic bacterioplankton. The growth abilities and population dominance were observed for particle-attached and free-living bacteria cultured in three types of media: Marine Broth, diluted Marine Broth (1:10) and R2 broth. Three groups of water layers were distinguished by Hierarchical clustering analysis: eutrophic, oligotrophic and oligotrophic nutrient-selected. Significant differences between the resulting groups were tested by two-way ANOVA (with replication). Eutrophic layers were characterised by readily culturable particle-attached and free-living fractions of the bacterial community in all three media, all dominated by fast-growing γ-Proteobacteria. In contrast, oligotrophic water layers with low productivity had a much weaker culturability and a different population dominance for the free-living community, as compared to their attached or growth-arrested counterparts, for all media. The free-living bacteria from strictly oligotrophic environments demonstrated minimum culturability in Marine Broth, while those from selective oligotrophic environments were culturable and were dominated either by Cytophaga-Flavobacter complex, α-Proteobacteria or γ-Proteobacteria. The conclusive evidence regarding the selective and refractory nature of organic compounds in these waters demonstrates the dominant culturability of the Cytophaga-Flavobacter complex and α-Proteobacteria in free-living communities in all growth-media. The response of fatty acid dominance ratios depends significantly on the trophic state and fraction ($p < 0.05$), although the effect of the trophic state is completely different in attached and free fractions. Both fractions were tested separately, demonstrating a significant influence of the trophic state ($p < 0.05$), while the effect of the media on the fatty acid response was not significant ($p > 0.05$). An interaction between media and trophic status was present in the attached fraction ($p < 0.05$), yet this was not observed in the free fraction ($p > 0.05$), indicating that any systematic difference between trophic states was the same for each media tested. Accordingly, the free-living fraction of bacterioplankton is a more informative attribute and can be used solely as an indicator of the water layer trophic condition.

Key words: culturable bacteria, fatty acids, northern Adriatic Sea, oligotrophic bacteria

1. Introduction

The population of heterotrophic bacterioplankton can be separated into two major groups based on the uptake of organic matter. Bacterial groups that successfully colonise organic aggregates or other nutrient rich micro-niches are considered to be copiotrophs. These are opportunistic species with low nutrient affinity and a rapid growth strategy, and they depend on high concentrations of organic substances (Pinhassi et al., 2004; Palijan and Fuks, 2006). In contrast, most typical free-living marine bacteria with unique physiological characteristics are able to survive and grow under conditions of an extremely low and discontinuous supply of nutrients and are defined as oligotrophs (Cavicchioli et al., 2003). Particle-attached bacterial communities are usually larger and more active than the free-living bacteria. Although there may be an interchange of members between both communities, marked taxonomical and physiological differences between them have been found (DeLong et al., 1993; Acinas et al., 1999). Since the physical and chemical properties of the microenvironments of these communities are extremely different, it was assumed that their enzymes have adapted to work efficiently under different conditions. Accordingly, the degree of similarity between those two fractions is variable and depends on environmental conditions (LaMontagne and Holden, 2003).

Functional differences between microbial communities have already been recognised as an indicator of local environmental conditions, and have potential applications in water quality assessment (Ahn et al., 2007; Marshall et al., 2008). In natural environments, as in different culture media, heterotrophic bacteria adapt their growth rate to both the nature and concentration of organic nutrients. Bacteria generally take on distinct properties when starved for each class of essential nutrients. The related depletions require completely different physiological and regulatory responses (Ferenci, 2001). Therefore, changes in microorganisms' taxonomic and physiological status caused by external factors can be successfully inferred from measurements of their cellular fatty acids (Blažina et al., 2005).

The aim of this study was to develop a tool based on the cellular fatty acids of the culturable fraction of heterotrophic bacterioplankton in order to better characterise and

differentiate northern Adriatic water masses, particularly oligotrophic high-salinity waters. This study employs the novel approach of comparing the responses to fatty acids of the particle-attached and free-living fractions of the natural bacterioplankton communities after cultivation in three different media.

2. Materials and Methods

Seawater was collected at seven stations in the northern Adriatic Sea during several cruises (June-September, 2006) aboard the Vila Velebita research vessel. The choice of sampling points was made according to salinity data obtained by CTD profile, and choices were made to correspond to water layers with different levels of salinity. Samples were transferred into sterile polycarbonate tubes and processed onboard the research vessel.

In total, 30 samples for bacteria enumeration by epifluorescence microscopy were fixed with 2 % formalin and stained with DAPI (Porter and Feig, 1980). In order to determine the number of colony-forming units (CFU) and the proportion of the total number of bacteria, as well as to isolate single bacterial colonies, samples were spread (100 µL) on three diverse solid media. Bacterial colonies were grown on agar for 10 days at 20°C.

Marine Agar (MA) and Marine Broth (MB) (Difco 2216) were used as representative of nutrient-rich media. Two different types of media were used to represent oligotrophic conditions, prepared by the following procedure. Marine Broth and Agar were diluted (dM) to 1:10 (Palijan and Fuks, 2006) with aged seawater (after 2 months in the dark) filtered through 0.22 µm GSM filters (Millipore, USA). The second media for growth of oligotrophic bacteria, R2A (Difco), was also prepared with aged seawater. R2 liquid media (R2) was prepared by addition of glucose, starch, sodium pyruvate and free casamino acids in the same quantities as used in original R2A solid media.

Additionally, 100 mL of each water sample was filtered on 0.7 µm GF/F filters (Whatman, USA) to retain particle-attached bacteria. Then, the filtrate was filtered on 0.22 µm filters (Millipore, USA) to retain free-living bacteria. Each filter was cut into 3 equal parts with a sterile blade, inoculated in 100 mL of three different liquid media, and

incubated for 48 hours at 20°C to obtain the culturable part of particle-attached and free-living communities, respectively, for Fatty Acid Methyl Ester (FAME) analyses. Before further processing, 0.8 mL aliquots of bacterial cultures were separated, added to 0.2 mL of 50% glycerol and preserved at -80°C for further molecular analysis. Liquid cultures of bacterial isolates and communities were centrifuged at 4000 x g for 20 minutes and washed twice with deionised water. The bacterial pellets (100-400 mg fresh weight), were saponified, methylated and analysed (Blažina et al., 2005). The samples were added to 1.2 M NaOH in 50% aqueous methanol solution. The tubes were placed in a boiling bath for 30 min. After cooling, the saponificate was acidified with 6 M HCl (pH<2). Next, 12% BF_3 in methanol was added, and the samples were heated for 10 min in a near boiling water bath. After cooling, the FAME were extracted in dichloromethane.

The FAME were analysed by gas-liquid chromatography on a 6890N Network GC System equipped with a 5973 Network Mass Selective Detector with a capillary column (30 m x 0.25 mm x 0.25 µm; cross linked 5% phenylmethylsiloxan), using ultra-high purity helium as the carrier gas. The GLC settings were as follows: the programmed column temperature was 145°C, increasing at 4°C/min up to 270°C, and a constant column pressure of 15 psi was used. The retention time and peak areas were recorded using Chemstation Software. Bacterial FAME were identified by mass spectral data, and by a family plot of equivalent chain length data obtained by GC standards (Blažina et al., 2005).

For each fraction (attached and free-living), 3 dominance ratios were calculated for each of three culture media (M, dM and R2): DRgamma = C16:1/(C18:1+BRtot), DRalpha = C18:1/(C16:1+BRtot) and DRCF = BRtot/(C18:1+ C16:1). A multivariate classification procedure (Hierarchical clustering method), performed over dominance ratios, was used to detect natural sample groupings. These ratios emphasise the dominance of bacterial populations in the culturable community (DRgamma of γ-Proteobacteria; DRalpha of α-Proteobacteria; DRCF of Cytophaga-Flavobacter complex), are the highest for the dominating population (>1), and do not overlap with ratios of the other populations (Blažina et al., 2005). Significant differences between groups obtained by Hierarchical clustering were tested by two-way ANOVA with replication with

statistical significance set at the conventional 5 % level ($p>0.05$). The statistical data analyses were performed on a PC Systat 10.

A fragment of bacterial 16S rRNA (positions 28 to 518 of the *Escherichia coli* numbering) was PCR amplified using primers: 5′-CTCGAGGTCGACGGTATCGGAG TTTGATCCTGGCTCAG-3′ and 5′-CACGCTCTAGA-ACTAGTGGAT(AT)ATTACC GCGGC(GT)GCTG-3′. The 5′- terminal twenty nucleotides of the two primers were added as annealing sites for sequencing primers (Mitsui *et al*., 1997). The reaction mixture contained 1 µM of each primer, 200 µM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase, 1x *Taq* buffer with KCl, and 1 µL of each bacterial culture grown on different types of media as templates. The mixture was layered with mineral oil, and the following protocol was used: a 2 min initial denaturation at 94ºC followed by 35 cycles of 94ºC for 1 min, 53ºC for 1 min and 72ºC for 1 min, with a final extension step at 72ºC for 5 min after the last cycle. The PCR products then underwent electrophoresis in a 1% agarose gel in 1xTAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3). Sequencing of PCR products was carried out by Macrogen (Seoul, Korea) and conducted under BigDyeTM terminator cycling conditions. The sequences determined in this study were compared and analysed for similarity to other known sequences in public-domain databases (NCBI GeneBank [http://www.ncbi.nlm.nih.gov/] and the Ribosomal Database Project [http://rdp.cme.msu.edu/]).

3. Results

The data set for each water layer consisted of 18 variables: 2 fractions x 3 growth media x 3 FAME dominance ratios. Multivariate hierarchical clustering procedures performed on more than 30 different water layers indicated 3 major groupings: GI, GII and GIII, each consisting of 10 water layers. The dendrogram is presented in Fig 1. The environmental conditions met in the water layers joined to groups GI-GIII are summarised in Table 1. Water layers in GI were mostly had higher chlorophyll a concentrations, a higher total number of bacteria, and lower salinity levels. CFUs on all three solid media were highly similar. In contrast, the water layers in groups GII and GIII had higher salinity, lower chlorophyll a concentrations and a lower number of total bacteria. CFUs on each of the

three media were similar between the groups, and 20 times lower than in GI. A different trophic status was assigned to each of the three groups: eutrophic (GI), oligotrophic (GII) and oligotrophic-nutrient selected (GIII).

Eutrophic layers (GI) were characterised by culturability and the dominance of γ-Proteobacteria in both fractions and all growth media. In the oligotrophic layers (GII), a decrease in the proportion of γ-Proteobacteria dominance was observed in attached fractions. The free fractions in all three media were poorly culturable and when culturable they were dominated by either α-Proteobacteria or Cytophaga-Flavobacter complex. In oligotrophic-nutrient selected water layers (GIII), particle-attached fractions were culturable in 87% of the samples, and these layers were mostly dominated by γ-Proteobacteria. Culturability in the free fraction was significantly higher than in GII, particularly in MB media. In the other two media, the relationships between dominating populations were similar, with proportionately higher culturability than in GII (Fig 2).

The significance of each of two experimental variables (trophic state and fraction) with respect to the DRgamma response was tested by two-way ANOVA with replication (Table 2a). The results show that DRgamma significantly varies with trophic state ($p=0.000$) and with fraction ($p=0.000$). Also, the trophic state and fraction interact in their effect on DRgamma ($p=0.000$), meaning that the effect of trophic state on DRgamma is completely different in attached and free fractions. In free fractions of the oligotrophic groups (GII and GIII), there was a pronounced contribution of bacterioplankton not belonging to γ-Proteobacteria (DRgamma < 1). To verify the influence of media on the DRgamma response within each fraction, an ANOVA was performed separately for the free and attached fractions (Table 2b). In both fractions, a significant influence of trophic state was present ($p = 0.000$), while the effect of media on the DRgamma response was insignificant ($p > 0.05$). However, a certain interaction between media and trophic status was observed in the attached fraction ($p = 0.031$). In the free fraction, the media and trophic status did not interact ($p = 0.156$), indicating that any systematic differences between trophic states were the same for each media tested.

The accession numbers and phylogenetic affiliation of the numerically prevailing isolates (≥10 %) belonging to Groups I - III are presented in Table 3. The tested strains were identified at the RDP II web site, all with a probability of at least 94 % and

sequence similarities to known bacterial species (deposited in GenBank) of at least 98.5 %. The diversity of the most abundant bacterioplankton cultured on solid plates was consistent with the dominance ratio responses in liquid cultures for waters with different trophic statuses.

4. Discussion

Since the growth abilities and population dominance of particle-attached and free-living bacterioplankton strongly vary with trophic state when cultured in different nutrient media, it is possible to discriminate between waters with different trophic characteristics by their fatty acid responses.

The eutrophic water layers (GI) were characterised in both particle-attached and free-living fractions by overall growth ability in all liquid media, and all were without exception dominated by fast-growing γ-Proteobacteria. These rather typical members of marine bacterioplankton indicate elevated concentrations of nutrients and easily assimilated organic carbon sources, such as low molecular weight dissolved organic matter (DOM) (Pinhassi and Berman, 2003). Additionally, the formation of the highest number of colonies on all three agar plates is in agreement with their presumed opportunistic nature, versatile nutritional and enzymatic characteristics, and ability to exploit favourable growth conditions (DeLong et al., 1993). All these features reflect the rather eutrophic character of the original bacterioplankton environment, with higher production and thus elevated concentrations of polysaccharides, proteins, lipids and even some LMW compounds. The phylogenetically determined representatives of γ-Proteobacteria (ADRIF 1, ADRIF 6 and ADRIF 7) and α-Proteobacteria (ADROW 4, ADROW 5; Table 3) were identified as Vibrio, Pseudomonas and Erythrobacter sp., respectively. All these genera are common members of marine bacterioplankton with markedly heterogeneous nutritional properties enabling their omnipresence, and they particularly thrive under conditions where elevated and diverse nutrients are available (Pinhassi and Berman, 2003).

However, a number of bacteria isolated from oligotrophic marine environments also belong to γ-Proteobacteria (Antunes et al., 2003; Cho and Giovannoni, 2004). In

agreement with these findings, the numerous ADROW7 isolates (GII and GIII) are very closely related to Salinisphera shabanense, an oligotroph isolated from the brine-seawater interface of the Red Sea (Antunes et al., 2003). Also found were ADRIFs 2 - 4, closely related to Halomonas and Pseudoalteromonas species. The latter are known as opportunistic strategists in culture as well as promoters of eukaryote surface fouling due to the degradation of complex polysaccharides in the cell wall (Skovhus et al., 2007). Overall, results suggest that the part of the γ-Proteobacteria population that thrives in low-productivity environments is primarily related to the attached fraction.

The loss of culturability of free-living bacterial communities in response to all types of enrichment, observed in water layers of elevated salinity and minimum phytoplankton growth (GII, Table 1), characterises these communities as oligotrophic, which are best adapted to low nutrient- and energy- flux environments. Moreover, the communities from such an environment experience reduced or inhibited growth even in R2 media enriched in easily available organic nutrients, formulated to enhance recovery of bacteria with low nutrient requirements. The typical free-living bacteria isolated from strictly oligotrophic environments lack the ability to form colonies on solid media, since their growth is negatively affected by enhanced substrate levels (Straskrabovâ, 1983). Thus, the growth arrest by nutritional enrichment indicates the severe nutrient scarcity of these water layers.

The different population dominance of the free-living community versus the attached community in the same media, and/or thee different population dominance in different media for the same fraction, indicates that a nutrient selective environment was the source of the related bacteria. The growth of free-living communities in Group III was regularly supported by diverse complex nutrients in excess (MB). This singularity also differentiated low productivity and high salinity waters in GII and GIII. Culturable bacteria structures, mostly dominated either by α-Proteobacteria or by Cytophaga-Flavobacter complex, suggests the presence of both easily available and complex DOM, since their main roles in pelagic ecosystems are enzymatic breakdown of macromolecular DOM and utilisation of small mass carbon units, respectively (Cotrell and Kirchman, 2000). The attached bacteria are responsible for rapid solubilisation of detrital particles via exoenzymatic activities, thereby releasing DOM and their own progeny as free-living

cells. The low molecular weight compounds are subsequently utilised by the free-living microbial community. All of these cells might turn to particles in order to grow (DeLong et al., 1993). The most numerous among phylogenetically determined representatives of α-Proteobacteria were identified as Sulfitobacter, Sphingomonas and Novosphingobium. These genera are common members of marine bacterioplankton, and some of them are known as obligate oligotrophs (Cavicchioli et al., 2003). Even though the CF-complex is highly diverse, all of its cultured representatives belonged to one single family, Flavobacteriaceae. A number of the sequenced strains (GII-GIII) were closely related to species recovered from the colder Antarctic, the north-western Pacific Ocean, and some Mediterranean oligotrophic environments, e.g., Leeuwenhoekiella (Pinhassi et al., 2006). Most abundant bacterioplankton species isolated from the northern Adriatic waters possess metabolic traits that link them to specific environmental conditions: mezotrophic, eutrophic, or oligotrophic.

These results show that the effects of the trophic state of the marine environment and the fraction of bacterioplankton are critical in the bacterial growth response to culturing on different nutrient sources. The DRgamma fatty acid ratio of culturable bacterial communities had significant differences, showing its potential value as a tool in characterisation and discrimination between waters of different productivities and nutritional states. Also, the fraction of the free-living bacterioplankton provides more informative attributes and can be used as the sole discrimination tool, particularly for oligotrophic waters. Consequently, the microbial fatty acids can be used as bioindicators for fast assessment of the marine ecosystems' condition and monitoring of its changes.

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Figure captions

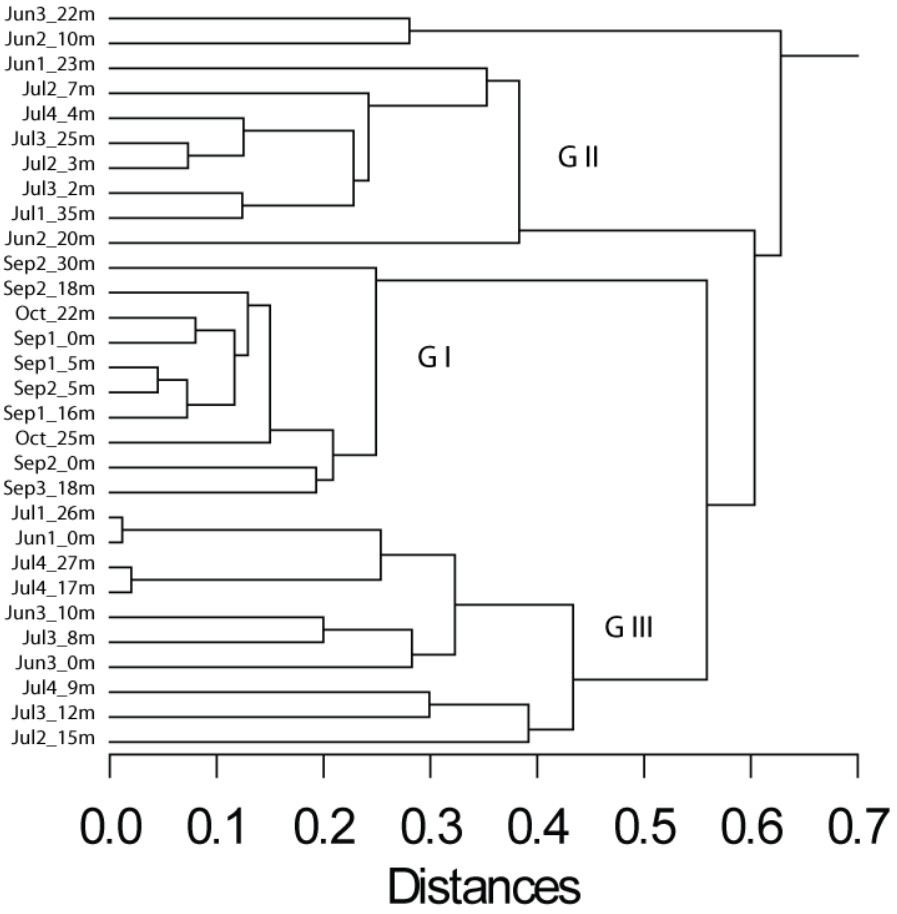

Fig. 1. Dendrogram obtained by numerical analysis (Hierarchical clustering) performed on fatty acid dominance ratios (DRgamma, DRalpha, DRCF) of culturable free-living and particle-attached bacterioplankton collected from the different water gradients from June to October 2006, cultured in 3 growth media: Marine broth (MB), R2 and 1:10 diluted Marine broth (dM). The designation 1-4 next to month stands for different sampling locations in the northern Adriatic.

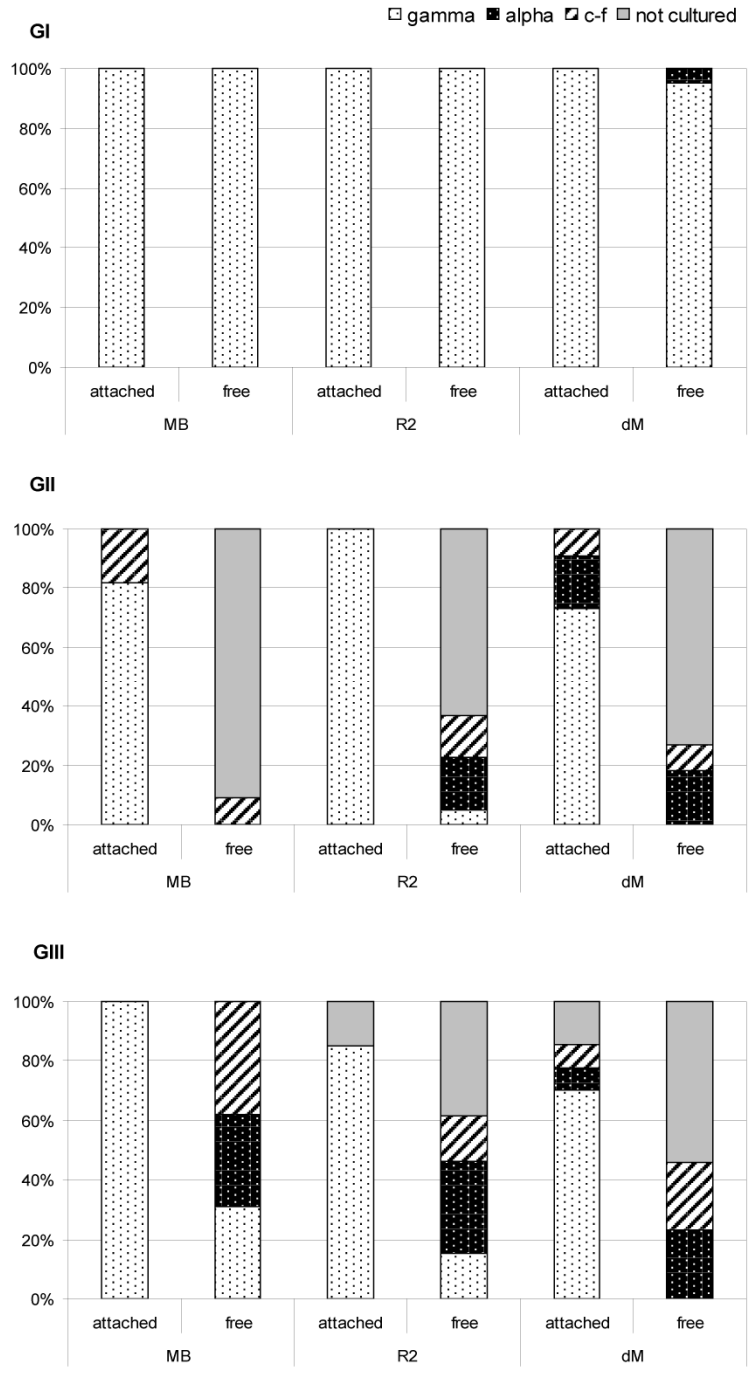

Fig. 2. Proportions of the dominant bacterial populations (alpha: α-Proteobacteria, gamma: γ-Proteobacteria, or c-f: Cytophaga-Flavobacter complex) within free-living and particle-attached bacterioplankton from the gradient waters cultured in 3 growth media: Marine broth (MB), R2 and 1:10 diluted Marine broth (dM), divided into 3 groups (GI-GIII) by the Hierarchical clustering method (Fig. 1).

Table 1. Means and standard deviations of salinity (S), total bacterial number (HB), chlorophyll a (Chl a) and colony-forming units (% of total bacterial number) on 3 solid media; Marine agar (CFU, MA), R2 agar (CFU, R2A) and 1:10 diluted Marine agar (CFU, dMA) assigned to 3 groups of water layers.

group	S (PSU) M ± SD	HB*10^8/L M ± SD	Chl *a* (mg/m^3) M ± SD	CFU (MA) M ± SD	CFU (R2A) M ± SD	CFU (dMA) M ± SD
G I	37.27 ± 0.67	6.3 ± 2.3	0.69 ± 0.27	2.3 ± 4.3	2.2 ± 4.8	2.0 ± 4.2
G II	37.93 ± 0.41	3.5 ± 1.0	0.19 ± 0.16	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
G III	37.83 ± 1.00	3.2 ± 1.2	0.18 ± 0.18	0.1 ± 0.1	< 0.1	0.1 ± 0.2

Table 2a. Two-way ANOVA with replication, carried out over DRgamma in two fractions (attached and free) and three groups (GI-GIII), each representing a different trophic status (significant differences at confidence level $p \leq 0.05$ are given in bold).

	ATTACHED	FREE
EUTROPHIC (GI)		
Count	30	30
Average *DRgamma*	3.87	3.70
Variance	0.71	6.93
OLIGOTROPHIC (GII)		
Count	30	30
Average *DRgamma*	3.00	0.13
Variance	1.36	0.11
NUTRIENT SELECTED (GIII)		
Count	30	30
Average *DRgamma*	2.62	0.83
Variance	1.86	1.11

Source of Variation	*F*	*P-value*	*F crit*
GROUPS	45.67	**0.000**	3.05
FRACTIONS	57.80	**0.000**	3.90
Interaction	13.79	**0.000**	3.05

Table 2b. Two-way ANOVA with replication, carried out over DRgamma, separately for each fraction, for three different liquid media (MB, R2, dM) and three groups (GI-GIII), each representing a different trophic status (significant differences at $p \leq 0.05$ are given in bold).

	ATTACHED			FREE		
EUTROPHIC (GI)	*MB*	*R2*	*dM*	*MB*	*R2*	*dM*
Count	10	10	10	10	10	10
Average *DRgamma*	3.92	3.88	3.80	3.21	4.57	3.33
Variance	0.72	1.36	0.21	0.87	18.15	2.05
OLIGOTROPHIC (GII)						
Count	10	10	10	10	10	10
Average *DRgamma*	3.68	3.28	2.03	0.00	0.27	0.13
Variance	0.96	0.79	1.00	0.00	0.29	0.04
NUTRIENT SELECTED (GIII)						
Count	10	10	10	10	10	10
Average *DRgamma*	2.68	2.25	2.93	1.68	0.57	0.23
Variance	0.45	0.82	4.45	1.41	0.65	0.25

Source of Variation	*F*	*P-value*	*F crit*	*F*	*P-value*	*F crit*
GROUPS	10.21	**0.000**	3.11	40.87	**0.000**	3.11
MEDIA	1.64	0.201	3.11	0.99	0.376	3.11
Interaction	2.81	**0.031**	2.48	1.71	0.156	2.48

Table 3. Bacterial isolates collected from northern Adriatic waters with different trophic statuses (GI-GIII). Related bacteria in GenBank are noted with accession number, 16S rDNA sequence similarity and phylotype.

group	Isolate; Accession no.	Closest relatives in Genbank; Accession no.	Similarity (%)	Phylotype
G I	ADRIF 1 EU649682	Vibrio campbellii ATCC 25920T, X74692.1	100	γ-Proteobacteria
	ADRIF 6 EU649686	Psychrobacter pacificensis strain NIBH P2K17, AR016058.1	98	
	ADRIF 7 EU649687	Pseudomonas stutzeri, strain 1908 clone sample 2, DQ888887	100	
	ADROW4 EU649688	Erythrobacter sp. AP38p, EU374890	100	α-Proteobacteria
	ADROW5 EU649689	Erythrobacter sp. JL893, DQ985055.1	100	
G II, GIII	ADRIF 8 EU798941	Sufflavibacter litoralis strain IMCC 1001, DQ 868538	100	Flavobacteria
	ADROW2 EU685827	Croceibacter atlanticus HTCC2559T, AY163576.1	100	
	ADROW3 EU649690	Leeuwenhoekiella aequorea, strain LMG 22550T, AY278780	100	
	ADRIF 2 EU649683	Halomonas meridiana DSM5425, AJ306891.1	99	γ-Proteobacteria
	ADROW7 EU649691	Salinisphaera sp. ARD M17, AB167073	99.1	
	ADRIF 3 EU649684	Pseudoalteromonas sp. strain 03/034 AJ874351.1	100	
	ADRIF 4 EU649685	Pseudoalteromonas citrea (NICMB 1889T) X82137.1	98	
	ADRIF 11 EU798938	Alcanivorax dieselolei, strain B-5 clone 1, AY683537	99	
	ADRIF 9 EU798942	Sulfitobacter sp. SPB-4, DQ 412074	99	α-Proteobacteria
	ADRIF 10 EU798939	Sphingomonas sp. KH3-2, AF282616	100	
	ADRIF 12 EU798940	Novosphingobium sp. JL832, EF512739	100	