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Margherita Licciano, Antonio Terlizzi, Adriana Giangrande, Rosa Anna Cavallo, Loredana Stabili. Filter-feeder macroinvertebrates as key players in culturable bacteria biodiversity control: a case of study with (Polychaeta: Sabellidae). *Marine Environmental Research*, 2007, 64 (4), pp.504. 10.1016/j.marenvres.2007.04.004 . hal-00501917

HAL Id: hal-00501917

<https://hal.science/hal-00501917>

Submitted on 13 Jul 2010

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Accepted Manuscript

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PII: S0141-1136(07)00066-9
DOI: [10.1016/j.marenvres.2007.04.004](https://doi.org/10.1016/j.marenvres.2007.04.004)
Reference: MERE 3119

To appear in: *Marine Environmental Research*

Received Date: 8 January 2007
Revised Date: 13 April 2007
Accepted Date: 13 April 2007

Please cite this article as: Licciano, M., Terlizzi, A., Giangrande, A., Cavallo, R.A., Stabili, L., Filter-feeder macroinvertebrates as key players in culturable bacteria biodiversity control: a case of study with *Sabella spallanzanii* (Polychaeta: Sabellidae), *Marine Environmental Research* (2007), doi: [10.1016/j.marenvres.2007.04.004](https://doi.org/10.1016/j.marenvres.2007.04.004)

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Filter-feeder macroinvertebrates as key players in culturable bacteria biodiversity control: a case of study with *Sabella spallanzanii* (Polychaeta: Sabellidae)

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Keywords: Filter feeders, Polychaeta, culturable heterotrophic bacteria, diversity, *Sabella spallanzanii*, Mediterranean Sea

Abstract

The present study investigates the effect of the filtering activity of *Sabella spallanzanii* on the culturable heterotrophic bacterial community through the comparison of the bacterial diversity in transplanted polychaetes and the surrounding seawater. For isolation of culturable heterotrophic bacteria, seawater samples as well as polychaete homogenates were plated in triplicates onto Bacto Marine Agar 2216 (Difco). All the colonies grown were isolated, subcultured and identified by several morphological, biochemical and cultural methods. Some bacterial genera showed higher average abundances in polychaetes than in seawater (i.e. *Lucibacterium* and *Photobacterium*). *Aeromonas* represented a conspicuous component of the bacterial community both in *S. spallanzanii* and seawater. The presence of *Cytophaga* and *Pseudomonas* was also relevant in the examined seawater samples. The selective concentrations of some bacterial genera inside *S. spallanzanii* either by grazing on bacteria or their capability as bacterial reservoirs, provides evidence for the role of macrobenthic invertebrates as key determinants for microbial diversity.

1. Introduction

Research over the past three decades has dramatically changed views of food web structure in the aquatic ecosystems, establishing the central role of bacteria in the functioning of the microbial loop (Pomeroy, 1974, 1980). Less clear are, however, the mechanisms which regulate the structure of the pelagic microbial food web. Grazing by heterotrophic nanoplankton has been shown to exert a significant pressure on bacterioplankton dynamics in terms of biomass and structure (del Giorgio et al., 1996; Gasol et al., 2002a, 2002b). By contrast, the role of macrobenthic invertebrates feeding on bacteria, coupling the benthic-pelagic system, is only a recently explored issue (Cloern, 1996; Doering & Oviatt, 1986; Loo & Rosenberg, 1989; Petersen & Riisgård, 1992; Riisgård & Larsen, 2001a, 2001b).

The impact of macroinvertebrates on bacterial community has been evaluated mainly for molluscan populations. The removal from waters of several species of bacteria by the common mussel *Mytilus edulis* (L.) and the subsequent fate of some polymers of the bacteria have been investigated by Birkbeck & McHenry (1982). Moreover, oysters, clams and mussels have been described as reservoirs of vibrios (Cavallo & Stabili, 2002; Kaspar & Tamplin, 1993; Kaysner et al., 1989).

The trophic role of bacterioplankton has also been highlighted for other groups of suspension feeders, including polychaetes (Gili & Coma, 1998; Orejas et al., 2000; Prieur et al., 1990).

Recent field studies conducted on the filter-feeder polychaete *Sabella spallanzanii*, showed its ability to accumulate and concentrate bacteria from the surrounding environment with a higher efficiency for autochthonous bacteria (Stabili et al., 2006b). Further laboratory experiments carried out on this species demonstrated that bacteria, employed as only food source, were efficiently filtered and retained (Licciano et al., 2005).

Most of the studies on the trophic role of macroinvertebrates focused exclusively on the impact on bacterial abundance. Studies concerning the effects of filter-feeders on bacterial biodiversity are therefore needed, also in consideration of the fact that microbial diversity affects the involvement of bacteria in biogeochemical cycles (Fuhrman et al., 1994; Gonzàles & Moran, 1997; Rehnstam et al.,

1993). In this framework, both quantitative and qualitative removal throughout the filter-feeding activity of macroinvertebrates may have significant ecological implications.

Taking into account that the qualitative removal throughout the filter-feeding activity of *S. spallanzanii* is completely unexplored, the present study investigates the effects of filtration activity of this species on the culturable heterotrophic bacterial diversity through the comparison of the bacterial community in transplanted polychaetes and the surrounding seawater. It is well known that viable culturable bacteria account only for a small fraction of the total bacterial community and therefore it is not representative of the whole bacteria present in the environment, thus the interpretation of the data inferred from the count of the viable culturable bacteria is obviously constrained (Zweiffel & Hagström, 1995). However, cultural methods can still provide some information on potential metabolic activity and on the role of heterotrophic bacteria in the biogeochemical cycles (Zaccone et al., 2002). Moreover, several recent studies, using various hybridization protocols, have re-evaluated the role of bacteria able to form colonies on solid media (Fuhrman et al., 1994; Hagström et al., 2000; Pinhassi et al., 1999; Stabili et al., 2006a).

2. Materials and Methods

2.1 Study area and sampling methods

Sampling was undertaken in March (T_{i1}) and July 2002 (T_{i2}) at three oligotrophic sites (Lido Silvana (Si_1), S. Vito (Si_2), and Lido Gandoli (Si_3), Northern Ionian Sea, Mediterranean Sea) characterized by similar hydrological features (Fig. 1). More particularly, as measured by a multiparametric sounding-line “Ocean Seven 401” (Jolzonant, Italy), the mean temperature was $19.6 \pm 0.12^\circ\text{C}$ at S. Vito, $19.4 \pm 0.13^\circ\text{C}$ at Lido Silvana and 19.4 ± 0.12 at Lido Gandoli. The mean salinity was about 38‰ in the three sites. The oxygen mean percentage saturation was 110.7 ± 0.48 at S. Vito, 111.8 ± 0.43 at Lido Silvana and 112.6 ± 0.45 at Lido Gandoli. For each site, water samples were aseptically collected in three replicates using 5 L Niskin bottles at a depth of 0.5 m.

Samples were then transferred on ice to the laboratory for isolation of culturable heterotrophic bacteria within 4 h of sampling.

2.2 Collection of polychaetes

Adult specimens of *Sabella spallanzanii* were collected in the Gulf of Taranto (Italy) at a depth of 5-15 m by SCUBA divers. In the laboratory, after removal of tube epibionts, worms were placed in a 60 L tank at a salinity of 37‰ in a temperature-controlled room ($T = 22^{\circ}\text{C}$). The tank water was aerated and filtered through 0.22 μm pore size membranes (Millipore). Specimens were left unfed and acclimatised to these conditions for 2 days before the field studies. At T_{i1} (March) as well as T_{i2} (July), starved specimens were randomly assigned to 9 groups, each consisting of 10 individuals and suspended in mesh cages (mesh size 10x10 mm) at the three sampling sites (three cages for each site for a total number of 30 worms). After two weeks, specimens were collected together with the water samples.

2.3 Bacteriological methods

Bacteriological analyses were performed on seawater from the three sampling sites and on *S. spallanzanii* homogenates (for each cage the whole group of 10 individuals was pooled into one sample) prepared either immediately after the laboratory starvation period or after the field deployment (T_{i1} and T_{i2}). In both cases the worms were collected, extracted from the tubes in the laboratory, washed several times with sterile seawater to assure the elimination of any bacteria trapped within the external mucus, and homogenized for 90 s in a sterile Waring blender. The homogenates (ca. 100g) were filtered through sterile gauze, diluted with filtered (0.22 μm) seawater to obtain a 1:10 (w/v) dilution and processed in a similar manner to the seawater samples.

For isolation of culturable heterotrophic bacteria, undiluted seawater and serial dilutions of each water sample as well as polychaete homogenate were plated in triplicates onto Bacto Marine Agar 2216 (Difco) (seeding with 0.1 mL). The plates were incubated at 22°C for 7 days. At the end of the

incubation period all the colonies (276 from seawater and 161 from polychaetes) were isolated in axenic culture, subcultured and identified by several morphological, biochemical and cultural methods (Holt et al., 1994; Oliver, 1982). The miniaturized identification tests API 20 E and API 20 NE (Biomérieux Sa, France) were used to study the following biochemical characteristics of the isolated strains: hydrolysis of gelatin, nitrate reductase, presence of β -galactosidase, carbohydrate utilization: saccharose, arabinose, mannitol, fructose, glucose, maltose, starch, rhamnose, galactose, mannose, sorbitol, glycerol, urease, indole, H_2S and acetoin production, and citrate utilization. To adjust the salinity of the medium to a value more representative of seawater, and work in experimental conditions more adequate to the development of marine bacteria, a 3% NaCl solution was added to the suspension medium (Zdanowski & Figueiras, 1999). The galleries were incubated for 2 days at 22°C and the API profiles were compared with the Analytical Profile Index database (Apilab Plus, version 3.3.3; Biomérieux). Because of previously reported discrepancies between conventional and commercial assays (Biosca et al., 1993) several tests were re-examined. When necessary, additionally, other biochemical tests were performed. Overall, a total of 19 bacterial groups were identified.

2.4 Statistical analysis

The experimental design consisted of 3 factors: Time (Ti, 2 levels, random), Site (Si, 3 levels, namely Lido Gandoli, Lido Silvana and San Vito, random and crossed to Ti) and Polychaetes (Po, 2 levels, i.e. the 1 degree - of - freedom contrast of bacterial contents in polychaetes vs seawater, fixed and crossed to Ti and Si), with n with = 5 replicates.

The 19 identified bacterial groups were considered as variables in the multivariate dataset, which therefore consisted of 36 samples and 19 variables.

Non-parametric multivariate procedures have been developed in recent years, providing useful statistical tools that have been widely adopted in ecological studies (Clarke 1993). An important feature of these procedures is that they do not rely on the assumption of multivariate normality. This

is a requirement of traditional multivariate methods such as multivariate analysis of variance (MANOVA), but such an assumption is very unlikely to be met in the vast majority of ecological data sets (Legendre & Legendre 1998).

Here, tests for the significance of terms involved in the full model were made by using a distance-based permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) based on Bray-Curtis dissimilarities on untransformed data. Each term in the analysis was tested using 4999 random permutations of the appropriate units (Anderson & ter Braak, 2003). Where the number of possible permutable units was not enough to get a reasonable test by permutation, a *P*-value was obtained using a Monte Carlo random sample from the asymptotic permutation distribution (Anderson & Robinson, 2003).

If appropriate, terms found to be significant in the full model were examined individually using pair-wise tests using the square root of the PERMANOVA test statistic (a multivariate pseudo *t*-test) and 4999 random permutations. Multivariate patterns were portrayed by non-metric multidimensional scaling (nMDS) plots.

Similarity percentages (SIMPER) (Clarke, 1993) was used to identify the bacterial taxa most contributing to determine differences between Po and SW bacterial assemblages.

The analyses were done using the PERMANOVA.exe (Anderson, 2005) and PRIMER computer programs (Clarke & Gorley 2001).

3. Results

PERMANOVA highlighted the significance of the interaction term $T_i \times S_i \times P_o$, suggesting that the differences between polychaetes *versus* seawater (P_o vs SW) bacterial assemblages varied across sites and times of sampling (Table 1). Pairwise comparisons of these differences within each level of the factors T_i and S_i revealed that in T_{i1} (March) significant differences between P_o vs SW were detected only at S_{i2} (S. Vito). In T_{i2} (July), however, differences between P_o vs SW bacterial assemblages were significant at all sites (Table 1).

nMDS plots (Fig. 2a,b) reflected PERMANOVA outputs: points referring to the bacterial assemblages in polychaetes were separated from those referring to seawater. From the nMDS plots it was also clear that water samples clustered more closely than points referring to polychaetes which appeared more widespread. Global patterns of differences, therefore, were clearer in Ti_2 (Fig. 2b) whereas in Ti_1 (Fig. 2a) the variable pattern of differences across sites prevented a clear representation of clusters within bacterial differences Po and SW assemblages.

3.1 Heterotrophic bacterial diversity

Among the Gram-negative bacteria, with the exception of *Vibrio*, *Alteromonas* and *Flexibacter*, all the isolated genera were present at Ti_1 both in SW and Po (Table 2). *Vibrio* and *Alteromonas* were detected only in polychaetes; by contrast *Flexibacter* was isolated only from seawater.

Moreover, *Cytophaga* and *Pseudomonas* showed higher average abundances in seawater greatly accounting for the differentiation of Po vs SW bacterial assemblages (Table 2). A strong percentage contribution to the dissimilarity between Po vs SW was also provided by *Lucibacterium* that was more abundant in polychaetes. Gram-positive bacteria (cocci and bacilli) were found to occur with higher average abundances in seawater, contributing to differentiate Po vs SW assemblages (Table 2). At Ti_2 , among Gram-negative bacteria *Moraxella*, *Aeromonas*, *Cytophaga*, *Photobacterium*, *Pseudomonas* and the family of *Enterobacteriaceae* contributed mostly to the average dissimilarity between Po and SW (Table 2). *Aeromonas* represented a conspicuous component of the bacterial community both in *S. spallanzanii* and seawater. The bacterial genera isolated exclusively in polychaete samples were *Lucibacterium* and *Acinetobacter*. *Photobacterium* was more abundant in the polychaete samples. *Moraxella* and *Alcaligenes* were recorded only in seawater. Among Gram-positive bacteria, bacilli prevailed in seawater samples whilst cocci in polychaetes. SIMPER identified both categories as strongly contributing to the average dissimilarity between Po vs SW.

4. Discussion

Previous quantitative laboratory and field studies have demonstrated the capability of *Sabella spallanzanii* to utilize bacteria as food source as well as its ecological role as reservoir for survival and growth of some bacteria (Licciano et al., 2005; Stabili et al., 2006b).

In the present study we focused on the culturable heterotrophic bacteria diversity in transplanted *S. spallanzanii* and the surrounding seawater. The main separation between seawater vs polychaetes resulted to be significant in both times of sampling even though in the nMDS plots the main effect of differences between seawater vs polychaetes in T₁ is not immediately perceived due to the variability among sites. In the nMDS plots the clustering of points referred to polychaete homogenates across the 3 sites appeared more widespread than those referring to seawater. This feature suggests that the sampling site is unlikely to influence the polychaete microbial community which, by contrast, is quite heterogeneous. The filtration process predictably includes periods of inactivity or transition between feeding and inactivity by worms due to the intrusion of the branchial crown within the tube (Licciano et al., 2005). On this basis, we can suggest that the observed heterogeneous microbial community among the individuals of *S. spallanzanii* could depend either on the ability of this species in regulate its pumping rates and even stop filtering, according to physiological and behavioural needs, or the selective grazing on bacteria. Indeed, several studies estimating clearance rates based on the uptake of different trophic sources have reported a different retention efficiency indicating that macroinvertebrate filter feeders actively select particles (Frost, 1980; Navarro & Widdows, 1997; Riisgård & Larsen, 2001b; Shumway et al., 1988; Stabili et al., 2006c).

Two interesting features can be inferred from our data on the culturable bacteria biodiversity:

i)- bacterial genera detected in both polychaetes and seawater.

The analyses of culturable heterotrophic bacterial biodiversity showed that *Aeromonas* represented a conspicuous component of the bacterial community both in *S. spallanzanii* and seawater. Aeromonads are inhabitants of aquatic environments and also belong to the flora of fish, amphibia

and other marine organisms (Kueh & Chan, 1985; Stabili & Cavallo, 2004). The presence of *Cytophaga* and *Pseudomonas* was also relevant in the examined seawater samples. The enzymatic versatility of these genera is well known and has been suggested as an explanation of their importance in particle turnover (De Long et al., 1993). The isolation of *Cytophaga* during this study is in good agreement with other studies reporting frequent isolation of the *Cytophaga-Flavobacterium* group in marine water (Glöckner et al., 1999). *Pseudomonas* is often isolated from seawater, sediments, phytoplankton and zooplankton (Nair & Simidu, 1987).

ii)- selective grazing of bacteria by polychaetes.

Some bacterial genera such as *Lucibacterium* and *Photobacterium*, showed higher average abundances in polychaetes than in seawater in both Ti_1 and Ti_2 , whilst *Vibrio* and *Alteromonas* only at Ti_1 and *Acinetobacter* at Ti_2 . Microscope observations are therefore required to assess whether the captured microorganisms were phagocytosed and not yet digested, accounting for the higher average abundances, or whether *S. spallanzanii* may function as habitat “islands”, providing a unique set of environmental conditions for microbial colonization. In this framework, it is worth noting the presence in *S. spallanzanii* of the luminous bacteria ascribable to *Photobacterium* and *Lucibacterium*. Luminous bacteria are common in the marine environment and are isolated as “free-living bacteria” as well as mutualistic symbioses in the light organs of certain fishes (Baumann & Baumann, 1984; Neilson et al., 1984; Ruby & Morin, 1979). These bacteria also occur on the surfaces (saprophytic), in the vital fluids (parasitic) and within the gastrointestinal contents of marine organisms. The ecological role of the relationship between these associated bacterial populations and *S. spallanzanii* is not known; we could hypothesize only, as already suggested by Nair & Simidu (1987) for other marine organisms, that this sabellid may contribute to the survival and distribution of the luminous bacteria in the Northern Ionian Sea, without any inference on the type of association (symbiotic or saprophytic).

Our results agree with other studies carried out on other filter feeders macroinvertebrates such as mussels and sponges (Stabili et al. 2006c). Laboratory experiments have shown, in fact, that the mussels' filter-feeding activity may exert a control on microbial community in terms of abundance and biodiversity (Kiorboe et al., 1981). Among the few available studies on bivalves, Kueh & Chan (1985) observed that the most frequently detected microorganisms from *Crassostrea gigas* were mainly *Pseudomonas*, *Vibrio*, *Acinetobacter* and *Aeromonas*. Prieur et al. (1990) reported that *Photobacterium* and *Vibrio* were more abundant in *C. gigas* than in the surrounding seawater. Some of the bacterial genera found in our study, such as *Aeromonas*, *Pseudoalteromonas*, *Pseudomonas*, *Vibrio*, *Photobacterium* and the family *Enterobacteriaceae*, were also recently detected in the mussel *Mytilus trossulus*, (Beleneva et al., 2003). Taylor et al., (2004, 2005) studying the marine sponge-associated bacteria and the potential implications for marine microbial diversity showed that the microbial community in *Cymbastela concentrica* differed significantly from those found in other sponges, which were more similar to seawater. Kefalas et al., (2003) assessed that the bacterial load in *Spongia officinalis* extract was greater than that of the proximal seawater providing evidence for the sponge ability to concentrate bacteria in its body. Bacteria identified in the sponge extract belonged to *Escherichia*, *Morganella*, *Proteus*, *Pasteurella*, *Aeromonas*, *Pseudomonas* and *Acinetobacter* genera.

From present data we can assume that the filtering activity of *S. spallanzanii* results in higher average abundances of some bacteria genera inside the worms showing the role of this polychaete in determining microbial diversity. Estimates of total marine microbial diversity are usually mostly based on planktonic or sediment communities (Curtis et al., 2002; Hagström et al., 2002; Torsvik et al., 2002) with less emphasis on potential associations with macroinvertebrates. By contrast, these organisms can represent very different habitats from those in the plankton or sediments and they may retain the ingested bacteria thus harbouring a greater diversity of microbes. Moreover, dense populations of benthic filter-feeders could be very effective against some types of microorganisms or completely ineffective against others. This, in turn can play a significant role in separating

bacterial niches and reducing competition among bacteria in the environment (Kefalas et al., 2003; Prins et al., 1998; Taylor et al., 2005; Thoms et al., 2003).

Deriving global diversity estimates from measures of *S. spallanzanii* impact on microbial diversity is clearly speculative. However, taking into account that there are more than 200,000 species of marine eukaryotes (Ormond et al., 1997), if even a small percentage reflects the pattern observed for the investigated polychaete, we can assume that macrobenthic invertebrates either grazing on bacteria or functioning as bacterial reservoirs may have potential ecological implications, playing a role as key determinant for microbial diversity. As a consequence, macroinvertebrates control an extremely active process of oxidation of organic matter in the ecosystem (Cloern, 1996; Doering & Oviatt, 1986; Widdows et al., 1998) taking into account that culturable bacteria are the most active actors in performing the oxidation of organic matter in aquatic systems (Ostroumov, 2005) and that different bacteria, exhibiting different ectohydrolytic enzyme profiles, are able to degrade different organic compounds.

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Figure captions

Figure 1. Map of the Northern Ionian Sea indicating the three sampling sites.

Figure 2. Non-metric multidimensional scaling (nMDS) plots on the basis of the Bray-Curtis dissimilarities between bacterial assemblages in seawater (SW) and *S. spallanzanii* (Po) at each of the 2 sampling times.

Si= site; LS= Lido Silvana; SV= San Vito; LG= Lido Gandoli;

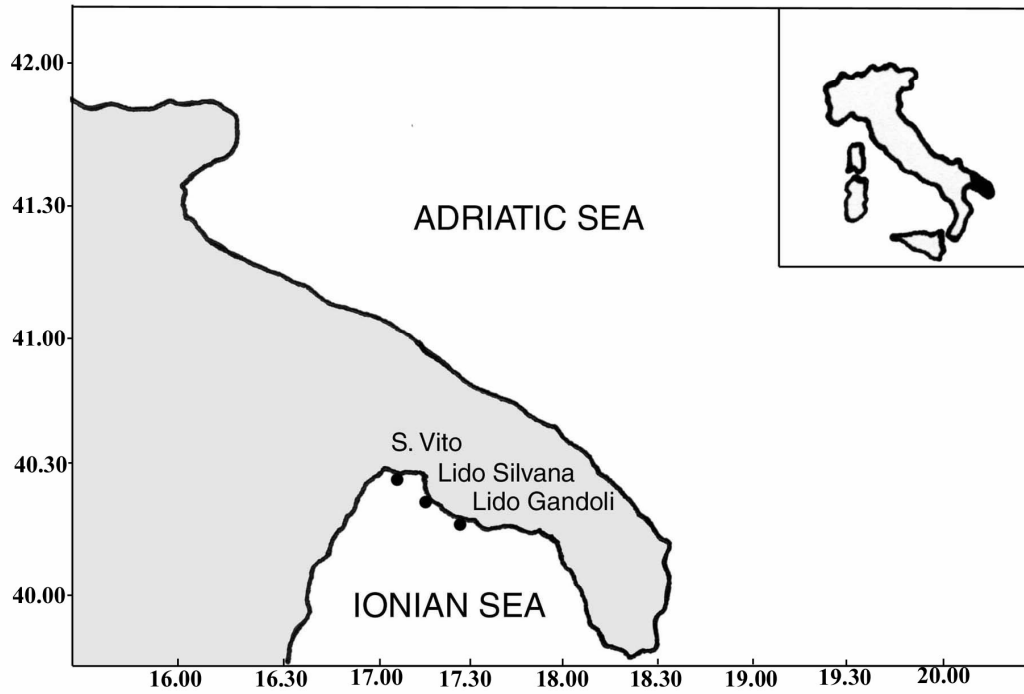


Fig. 1

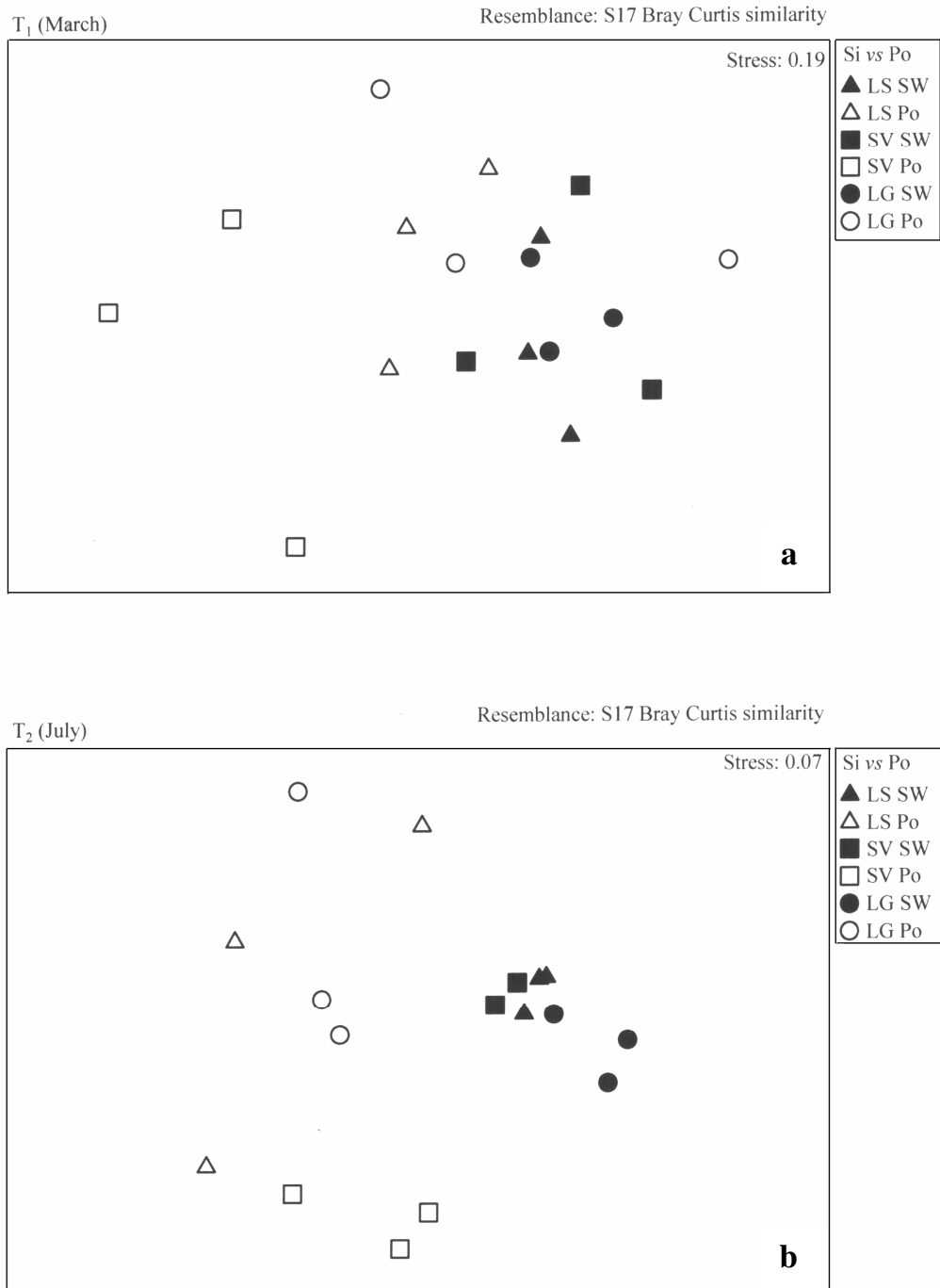


Fig. 2

Table 1 Results of PERMANOVA testing for differences in bacterial assemblages in polychaetes (Po) vs seawater (SW) across the three sites in time. Given the significance of the interaction term $T_i \times S_i \times P_o$ the analysis is followed by pairwise comparisons of bacterial assemblages in Po vs SW at each level of the T_i and S_i terms (see text for further details)

Source	Sum of Squares	Degrees of freedom	Mean Squares	F ratio	P(perm)
Time = T_i	7418.2078	1	7418.2078		
Site = S_i	5151.8617	2	2575.9309		
Polychaetes = P_o	12674.1527	1	12674.1527		
$T_i \times S_i$	3753.7746	2	1876.8873		
$T_i \times P_o$	3037.3550	1	3037.3550		
$S_i \times P_o$	5876.5201	2	2938.2600		
$T_i \times S_i \times P_o$	4650.5582	2	2325.2791	2.1941	0.0110
Residual	25434.5457	24	1059.7727		
Total	67996.9758	35			

	LS		SV		LG	
T_1 (March)	$t= 1.5270$	$P= 0.1088$	$t= 1.9492$	$P= 0.0350$	$t= 1.1334$	$P= 0.3180$
T_2 (July)	$t= 2.0253$	$P= 0.0448$	$t= 4.0303$	$P= 0.0060$	$t= 3.6258$	$P= 0.0044$

LS= Lido Silvana; SV= San Vito; LG= Lido Gandoli

Table 2 Output of SIMPER analysis: average abundance (AvAb) of bacterial genera contributing the most to the Bray-Curtis dissimilarity (data untransformed) values between seawater (SW) vs polychaetes (Po) groups in each of the two sampling times. For each of SW and Po groups data have been pooled across the three sites. The percentage contribute that each variable made to the observed value of dissimilarity is reported in the Contrib % column

Bacterial genera	Ti₁ (March)			Ti₂ (July)		
	AvAb SW	AvAb Po	Contrib %	AvAb SW	AvAb Po	Contrib %
<i>Moraxella</i>	0.89	0.89	6.94	2.00	0.00	13.05
<i>Aeromonas</i>	2.00	1.89	10.16	4.00	2.78	12.97
<i>Cytophaga</i>	3.44	0.11	21.85	2.22	0.22	12.96
<i>Photobacterium</i>	0.11	0.22	1.79	1.22	2.22	10.69
<i>Enterobacteriaceae</i>	0.78	0.67	5.21	2.00	0.44	10.00
<i>Pseudomonas</i>	2.00	0.67	11.75	1.67	0.22	9.92
<i>Flavobacterium</i>	0.44	0.67	4.59	0.44	0.22	3.01
<i>Lucibacterium</i>	0.11	1.00	6.47	0.00	0.33	2.17
<i>Flexibacter</i>	0.11	0.00	0.67	0.22	0.11	2.00
<i>Acinetobacter</i>	0.67	0.56	4.67	0.00	0.22	1.47
<i>Alcaligenes</i>	0.22	0.22	2.55	0.22	0.00	1.25
<i>Vibrio</i>	0.00	0.11	0.76	0.11	0.11	0.70
<i>Alteromonas</i>	0.00	0.11	0.94	-	-	-
<i>Agrobacterium</i>	0.11	0.11	1.15	-	-	-
<i>Chromobacterium</i>	0.67	0.67	4.61	-	-	-
Gram-positive (bacilli)	1.44	0.78	9.06	2.11	0.33	12.03
Gram-positive (cocci)	1.00	0.56	6.82	0.56	1.44	7.75

Ti₁ (March): Average Bray-Curtis dissimilarity between Po vs SW= 67.41

Ti₂ (July): Average Bray-Curtis dissimilarity between Po vs SW = 60.41