Species diversity, spatial distribution, and virulence associated genes of culturable vibrios in a brackish coastal Mediterranean environment

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Abstract

The *Vibrio* genus is widespread in marine and brackish environments, and several species are human and animal pathogens of global importance. Vibrios adapt rapidly to many environmental stresses, so that brackish environments can be both a suitable niche and a possible reservoir for them. To test the occurrence of culturable vibrios and their possible correlation with environmental factors in a temperate brackish environment, a 1-year sampling study was performed in three brackish ponds located along the Central Thyrrenian coast in the Macchiatonda Nature Reserve (Santa Marinella, district of Rome, Italy). Molecular methods were used to detect *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* pathogenicity-associated genes among the *Vibrio*

isolates. Out of 130 *Vibrio* isolates identified by sequencing a *recA* fragment, 70 harbored virulence-associated genes including *ctx*, *ace*, *tcpA*, *tdh*, *trh*, *vvhA*, *vllY*, and *toxRS*, so confirming the spread of virulence determinants across the environmental isolates. Ecological analysis showed that, although the water temperature is known to be a strong predictor of abundance and distribution of vibrios, its influence accounts for 27 % of the observed variance in the Macchiatonda samples, increasing to 40 % when combined with salinity.

Keywords

Vibrios
Brackish environment
Virulence-associated genes

Introduction

The family *Vibrionaceae* is a large group within the Gamma-proteobacteria, encompassing species, which are common natural members of marine and estuarine bacterial communities. The genus *Vibrio* includes 99 recognized species (Association of *Vibrio* Biologists, http://www2.ioc.fiocruz.br/vibrio/AVib/Vibrio.html, last consultation September 2014) widely distributed in most aquatic environments (Ki et al. 2009), either free living or associated with corals, fish, mollusks, crustaceans, algae, and zooplankton (Thompson et al. 2004b).

Some species, including *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, are a recognized cause of severe human infections. Many other species found in the aquatic environment and defined as halophilic 'non-cholera vibrios' (NCVs), such as *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio harveyi*, *Vibrio fluvialis*, *Vibrio furnissii*, *Vibrio metschnikovii*, and *Vibrio mimicus*, are known as mainly marine animal pathogens. They have been isolated only occasionally in association with infections in humans (Austin 2010), mostly via ingestion or direct skin penetration (Ottaviani et al. 2001). Among these, *Vibrio anguillarum* behaves as a major pathogen for a variety of aquatic organisms (fishes, eels, crustaceans, marine mammals, and corals). Although the environmental isolates are usually lacking the virulence-associated genes found in the clinical ones, some recent studies showed that they can also carry such genes, or homologues

thereof, acquired through horizontal transfer events (Kirkup et al. 2010; Gennari et al. 2012). Both the persistence and abundance of vibrios are related to several parameters, whose influence can differ according to the geographic region (Caburlotto et al. 2012). Moreover, the available dissolved organic matter and the plankton, temperature and salinity seem to be related to the abundance, distribution, and persistence of *Vibrio* populations in aquatic environments (Eiler et al. 2006; Hughes et al. 2013), although the different species vary in both minimal Na^+ concentrations and temperature requirements (Farmer et al. 2005; Urakawa and Rivera 2006). The ability to enter a viable but not culturable state (Rollins and Colwell 1986) prolongs their survival in the environment, especially during winter (Maugeri et al. 2004). Observations collected over a range of environmental parameters affecting *Vibrio* spp. occurrence across different regions, allowed to develop predictive ecological models to estimate the role of climate and ecological variables on *Vibrionaceae* populations (Lobitz et al. 2000; Codeço and Coelho 2006). Investigations on vibrios communities in particular environments, such as that investigated in the present study, are useful to improve the predictive models about their dynamics. Vibrios are common in the Mediterranean Sea, but most studies have focused on specific pathogenic members of the group and, so far, there are limited data on the free-living *Vibrio* populations along the Italian Tyrrhenian coasts, and about their correlation with environmental factors. The Macchiatonda coastal ponds are brackish lakes located in a temperate area and subjected to seasonal changes in temperature and to wide fluctuations of salinity. They were, therefore, considered ideal for evaluating the effects of natural fluctuations of temperature and salinity on *Vibrio* populations. The present study is aimed at investigating for the first time the occurrence, diversity, and distribution of culturable *Vibrio* species in this peculiar habitat and their correlation with both temperature and salinity variations. The occurrence of virulence-associated genes in isolates belonging to species defined as non-pathogenic was also evaluated.

Materials and methods

Study area

Macchiatonda Natural Reserve includes an area of 0.7 km^2 of coastal ponds made up of nearly static brackish water on the Tyrrhenian coast, located 50 km North of Rome, within the Santa Marinella municipality

(UTM-ED50 zone 32 T). *Alberobello* is a system of channels that carry water to a major water hole and connect to the sea. *Piscinula* is a continuous wetland with little islands inside. It has a less deep portion (max 60 cm depth, undergoing seasonal dry periods) and a deeper area, converted into a pond thanks to a constant seawater supply; both of them are close to the waterline and may undergo marine ingressions. *Didattico* is a small circular artificial basin, located more inland, where seawater input does not occur and freshwater is supplied. Surface water samples were collected in the three coastal ponds. At each pond, 1, 2, or 3 sites were chosen, making a total of six sampling sites. A seventh control site was located 5 m offshore from the Santa Marinella coast, in the Central Tyrrhenian Sea.

Sampling strategy

Samplings were carried out every 45 days, from September 2010 to July 2011. In each sampling site, a 50 ml fraction of water was collected in sterile vials, at a depth of about 1 m. All the samples were placed in coolers, transferred to the laboratory, and processed within 3 h after collection.

Temperature and salinity measurement

Water temperature (T C°) and salinity (SAL) values were recorded simultaneously *in situ* at each sampling station using the multiparametric probe Multi 340i (WTW, Udine, Italy) in the upper 1 m of the surface water.

Enumeration and typing of *Vibrio spp*

A 100 μl volume of each sample was plated on thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Difco) both directly and after a tenfold concentration by centrifugation at 15,000 × g for 15 min. CFU were counted after a 48 h incubation at 20–22 °C in order to retrieve all the culturable heterotrophic bacteria able to grow on TCBS. From each plate, isolated colonies of each size and morphology were picked for purity onto Tryptic Soy Agar with 2 % NaCl added (sTSA) and incubated for 24–48 h at 25 °C, to be submitted to a preliminary biochemical screening and molecular identification as detailed below.

Presumptive *Vibrio* strains were identified by colony shape and

pigmentation on TCBS, Gram staining, cytochrome-oxidase activity, and glucose fermentation. The oxidase-positive, gram-negative, glucose acidifying isolates were selected for molecular analysis. Molecular identification at the species level was obtained by amplifying and sequencing a 739 bp *recA* gene fragment according to Thompson et al. (2004a). The amplification products were sequenced by Macrogen Europe (The Netherlands) with an automated capillary sequencing. The isolates identified as *V. cholerae* were also tested with the *rfb* primers, specific to *V. cholerae* O1 and O139, according to Hoshino et al. (1998).

Detection of pathogenicity-associated genes in *Vibrio* isolates

For an evaluation of the health risk associated with the occurrence of potentially pathogenic vibrios, the possible presence of virulence genes was analysed by PCR.The targeted genes were: the *V. cholerae ctx*, *zot*, *ace*, *tcpA*, *toxR*, and *toxS* genes, the *V. parahaemolyticus tdh* and *trh* genes, and the *V. vulnificus vvhA* and *vllY* genes. Primers were purchased from Sigma-Aldrich Company Ltd. Primer sequences, reaction conditions, and cycling were as described by Baffone et al. (2006), other than for the primer pair vvhA-1 F and vvhA-1R that were according to Senoh et al. (2005).

Statistical analyses

Vibrios relations with surface water salinity and temperature have been investigated for each pond and sampling session. For seasonal analysis, autumn was considered to be the November and December sampling sessions, winter was February and March, spring was May and June, and summer was July and September.

Concentrations of culturable *Vibrio* as CFU ml^{-1} were $\log_{10}$ transformed to fit a normal distribution. Differences in the mean ranks of CFU counts, temperature, and salinity values among sampling sites, were tested for significance using Kruskal-Wallis test (to test whether samples originated from the same distribution). Differences were considered significant at $p \leq$ 0.05.

A Spearman's rank correlation test was run to determine the relationship between the mean $\log_{10}$ CFU abundance and individual environmental

variables. The closer the Spearman correlation coefficient, r_s, is to zero, the weaker the association between the ranks of the variables. The significance level for considering variables to be associated was set at $p \leq 0.05$.

Moreover, environmental parameters were tested together for their impact on the abundances of vibrios with Multiple Linear Regression analyses, in the attempt to reveal the main abiotic factor(s) controlling the occurrence of vibrios in Macchiatonda ponds.

All statistical analyses were performed using PAST (PAleontological Statistics, version 3.0).

Results and discussion

Temperature and salinity ranges in the sampled ponds

The Macchiatonda Nature Reserve wetland (Fig. 1) is situated along the Central Tyrrhenian coast in Santa Marinella (60 km North of Rome, Italy). The reserve includes a brackish area of 0.7 km^2 that offers the unique feature of the coexistence of three ponds with different degrees of salinity but substantially similar temperature at each sampling time. This makes it possible to compare the effect of salinity in addition to the seasonal effect of temperature shifts, and where the environmental factors can sometimes fluctuate suddenly within a few days, due to tides, rain, and evaporation. The artificial pond *Didattico* (station 1) has a constant depth of about 1.5 mt ensured also by means of periodical input of fresh water. *Alberobello* is a net of ditches about 60 cm deep (stations 2 and 3), with a major water hole (maximum depth about 90 cm, station 4). *Piscinula* has a shallower (maximum 60 cm, station 5) and a deeper area (maximum 1 mt, station 6). A seventh station was placed in the coastal seawater in front of the ponds, as a control site with seasonal temperature shift and the constant salinity typical of the Mediterranean Sea. In the ponds, the water temperature ranged from a minimum of 2.8 °C to a maximum of 29.3 °C with a clear cut seasonal trend, and no significant differences among ponds (Kruskal-Wallis, $p = 0.99$). Seawater was markedly less cold than the ponds in December and February, and slightly cooler in summer. The salinity mean value was significantly different among ponds (Kruskal-Wallis, $p = 0.02$), as each pond was characterized by a peculiar salinity rang, that was rather constant in the period November to July in both *Didattico* and *Alberobello*. In the same period, *Piscinula*, which is more exposed to dilution in rainy

months and evaporation in summer, particularly in the shallow station 5, underwent wider and abrupt fluctuations. In September, salinity dramatically increased due to the very dry summer season, usual in this area, and to a strong evaporation. The values went from 22 ppt in *Didattico* up to 54.5 ppt in *Piscinula* where only station 6 was sampled, as station 5 was completely dry. In each pond, however, both temperature and salinity were substantially the same at the different sampling times so that the data obtained from the same pond have been pooled whenever possible.

Fig. 1

Sampling sites: 1, *Didattico* artificial pond; 2, 3, 4, *Alberobello* pond; 5, 6, *Piscinula* pond; 7, coastal seawater in front of the Macchiatonda wetland

AQ1
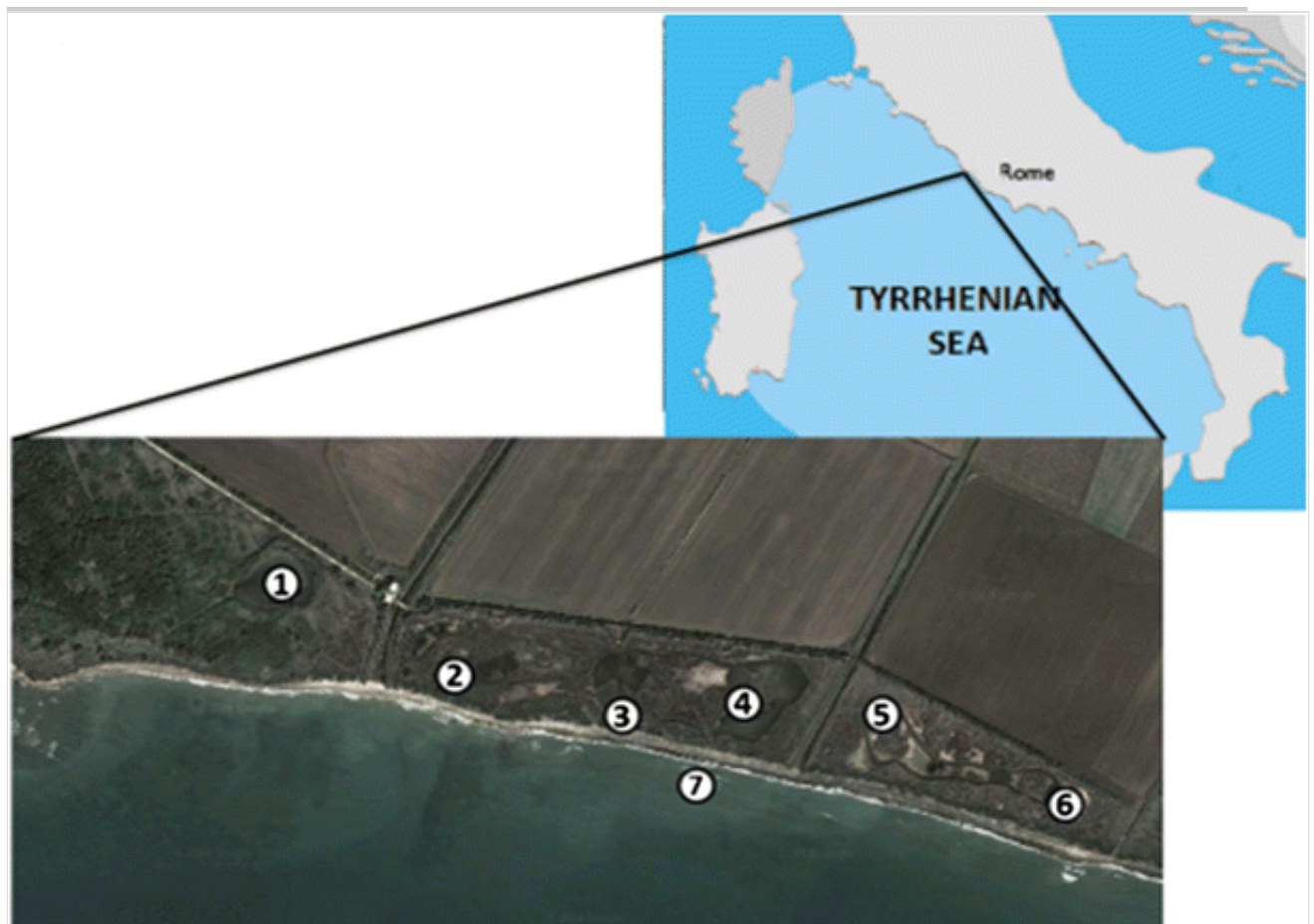
Vibrio counts and correlations with temperature and salinity

In the Macchiatonda wetland, the CFU counts on TCBS ranged from 20 CFU ml^{-1} in *Didattico*-March and *Piscinula*-May, up to $5.76*10^3$ CFU ml^{-1} in *Alberobello*-September, following a general seasonal trend consistent with the ecology of culturable vibrios (Fig. 2).

Fig. 2

Monthly variation, over the investigated period, of temperature, salinity, and *Vibrio* spp. abundance in Macchiatonda wetland. **a** Mean seasonal values of temperature and salinity in the Macchiatonda wetland; **b** Mean CFU/mL abundance with confidence intervals 95 %. *Alb*: Alberobello pond; *Did*: Didattico pond; *Pis*: Piscinula pond; *Tyr*: Tyrrhenian Sea
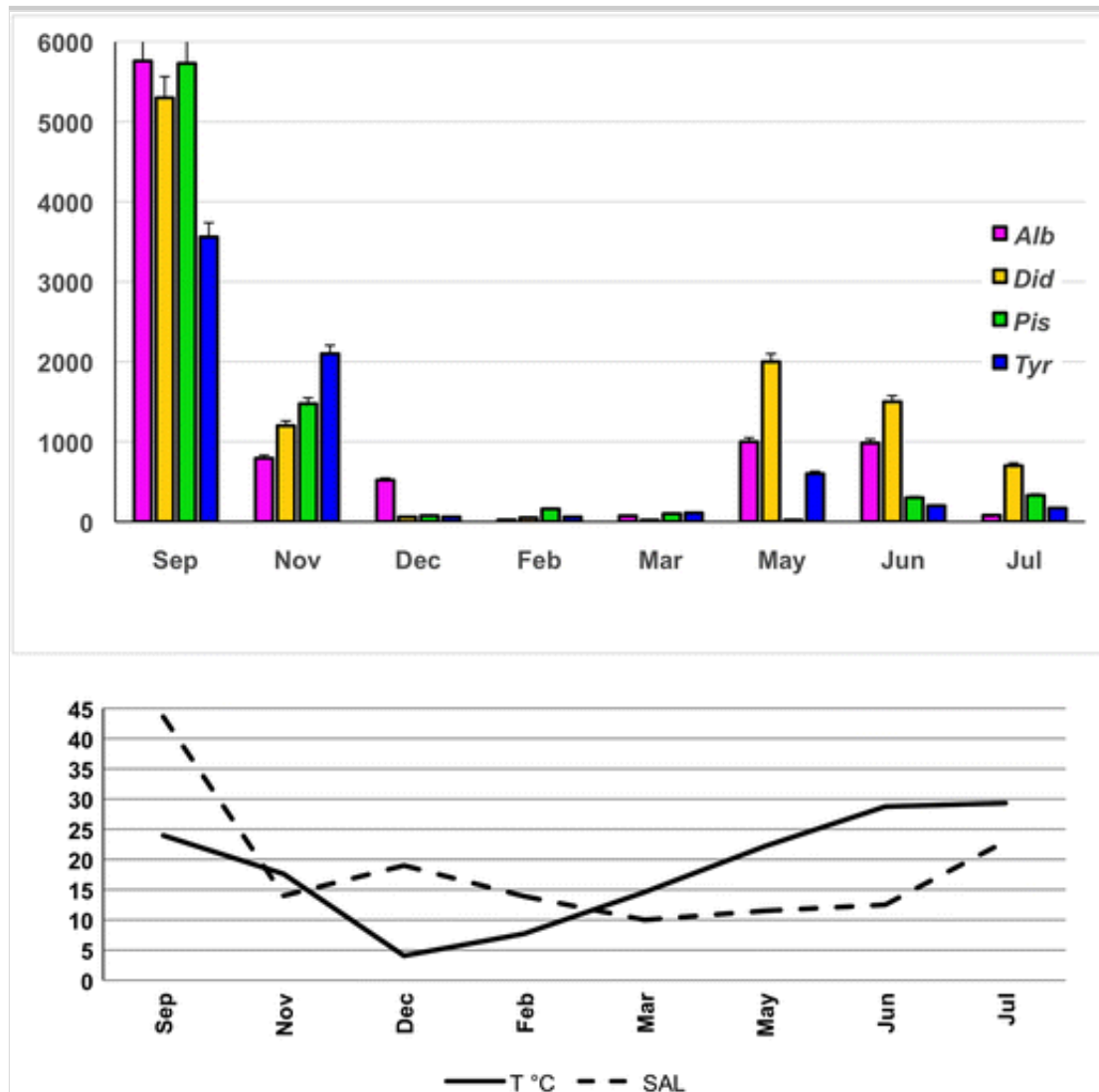
These values exclude unculturable and injured *Vibrio*, and are comprehensive for species, other than vibrios, able to grow on TCBS, commonly *Aeromonas* and *Photobacterium* but, in our samples, mostly *Lucibacterium* and *Shewanella*. In the sea station the counts ranged from a minimum of 60 CFU ml^{-1} in December and February, to a maximum of 3.56 *10^3 CFU ml^{-1} in September. The overall CFU abundance in the whole Macchiatonda area was high in September, sloping down in November to reach the minimum during the winter months, and increased again in May, other than in *Piscinula*, where the increase was delayed to

June. During the sampling period, neither the differences in CFU number among the ponds (Kruskal-Wallis $p = 0.92$) nor those between the brackish ponds and seawater were significant (Kruskall-Wallis, $p = 0.25$). A significant positive correlation was found between CFU counts and water temperature (Spearman's rs $= 0.67$; p (uncorr) $= 0.002$) for samples collected up to 25.6 °C in agreement with several data in the literature on Mediterranean coastal environments and brackish waters (*e.g.*, Maugeri et al. 2000; Baffone et al. 2006; Covazzi-Harriague et al. 2008; Vezzulli et al. 2009; Caburlotto et al. 2012). At higher temperatures, however, the correlation is negative (Spearman's rs $= -0.63$; p (uncorr) $= 0.07$). Within the range observed during the sampling campaign, the CFU counts were not significantly correlated with the salinity values (Spearman's rs $= 0.27$; p (uncorr) $= 0.19$), as could be expected as the variations were low within each pond, other than for the September samples. Moreover, the influence of salinity varies across the *Vibrio* genus (Thompson et al. 2004b) and often also depends on the range of temperature in the system (Randa et al. 2004). Both temperature and salinity, however, influenced the CFU number variations. By incorporating CFU number, temperature, and salinity values, the Multiple Linear Regression model estimated temperature and salinity, together, to explain 40 % of the CFU number variation, whereas temperature accounted for the 27 % of the model variation (MRL $p = 0.01$; adjusted $R2 = 0.27$; Table 1). According to these data, the CFU abundance on TCBS in the brackish ponds depends by both temperature and salinity, with temperature accounting for variance more than salinity (27 % and 20 %, respectively). On the other hand, as temperature and salinity together explain only 40 % of total variance of the CFU number, other environmental and biologic factors have to play a role in driving *Vibrio* abundance in brackish systems.

Table 1

Multiple Linear Regression model values

	Coeff.	Std. err.	t	p	R^2
T (°C)	0.039	0.015	2.66	0.01	0.27
SAL (ppt)	0.025	0.011	2.22	0.04	0.20
Multiple regression				0.004	0.40

Diversity and distribution of *Vibrio* species

In total, 130 Gram-negative, cytochrome-oxidase positive and glucose-fermenting isolates were selected from the 56 samples examined: 109 were from the brackish ponds and 21 from seawater. Sequencing of the amplicons obtained with genus specific primers within the *recA* gene, allowed to identify them as belonging to 20 named species and four unnamed groups within the genus *Vibrio* (Table 2).

Table 2

Vibrio spp. isolated from Macchiatonda brackish ponds (BP) and Tyrrhenian sea control site (SW)

Species	N. of isolates	Source(s)	Species	N. of isolates	Source(s)
V. agarivorans	3	SW	*V. mediterranei*	1	SW
V. alginolyticus	6	BP, SW	*V. navarrensis*	1	BP
V. anguillarum	44	BP, SW	*V. ordalii*	8	BP
V. atlanticus	1	SW	*V. pacinii*	3	BP
V. cholerae[a]	7	BP	*V. parahaemolyticus*	13	BP, SW
V. cyclotrophicus	1	BP	*V. pelagius*	1	BP
V. diabolicus	2	BP	*V. splendidus*	4	BP, SW
V. diazotrophicus	1	BP	*V. vulnificus*	3	BP
V. harveyi	6	BP	*Vibrio* sp. CAIM1833	1	SW
V. kanaloae	14	BP, SW	*Vibrio* sp. FAL1533	1	BP
V. lentus	1	BP	*Vibrio* sp. FALF273	4	BP
V. litoralis	2	BP	*Vibrio* sp. MWB21	2	SW
[a] Non O1/O139					

The unnamed groups have been distinguished by the name of the strain with the most similar sequence that were: CAIM 1833, belonging to a clade for which the name *Vibrio alfacsensis* has been proposed (Gomez-Gil et al. 2012) and similar to *Vibrio ponticus*; MWB21 originally isolated by Beijerinck in 1924, from surface coastal seawater at Scheveningen (Netherlands), labelled at that time as *Photobacterium phosphoreum* and belonging to a separate clade within the genus *Vibrio* (Figge et al. 2011); FALF 273 and FAL 1533, two unclassified strains from estuarine waters, loosely clustering with *Vibrio lentus* and *Vibrio pacinii*, respectively (Preheim et al. 2011). Out of the identified species, only *V. alginolyticus*, *V. anguillarum*, *Vibrio kanaloae*, *Vibrio parahaemolyticus*, and *Vibrio splendidus* were found in both brackish and seawater. Twelve named species and two groups were found only in the brackish ponds, and three species and two groups only in the coastal sea station.

Vibrio species distribution ~~vs.~~ *vs* temperature-salinity combinations

~~A~~ Vibrios presence in sea waters is often reported as temperature-(upon/below 15 or 17 °C) and salinity-related. In some brackish environments as the Macchiatonda one, both temperature and salinity are highly variable, over both season and sites. Therefore, many different conditions can be observed at the same time, within this system, and a more finely tuned approach is advisable. We have, therefore, furtherly categorized the temperature and salinity observed values in five narrow intervals each. Two temperature intervals were set under 15 °C, two in the range 15–26, and the last one starting at 27 °C, that is where temperature and CFU counts on TCBS start to have a negative correlation. The temperature intervals, therefore, were: A) <10 °C – low, in the ponds; B) 11–14 °C – low, at the sea site C) 15–20 °C, and D) 21–26 °C, the cooler and the warmer intervals within the permissive temperature range, respectively, and E) 27–30 °C.
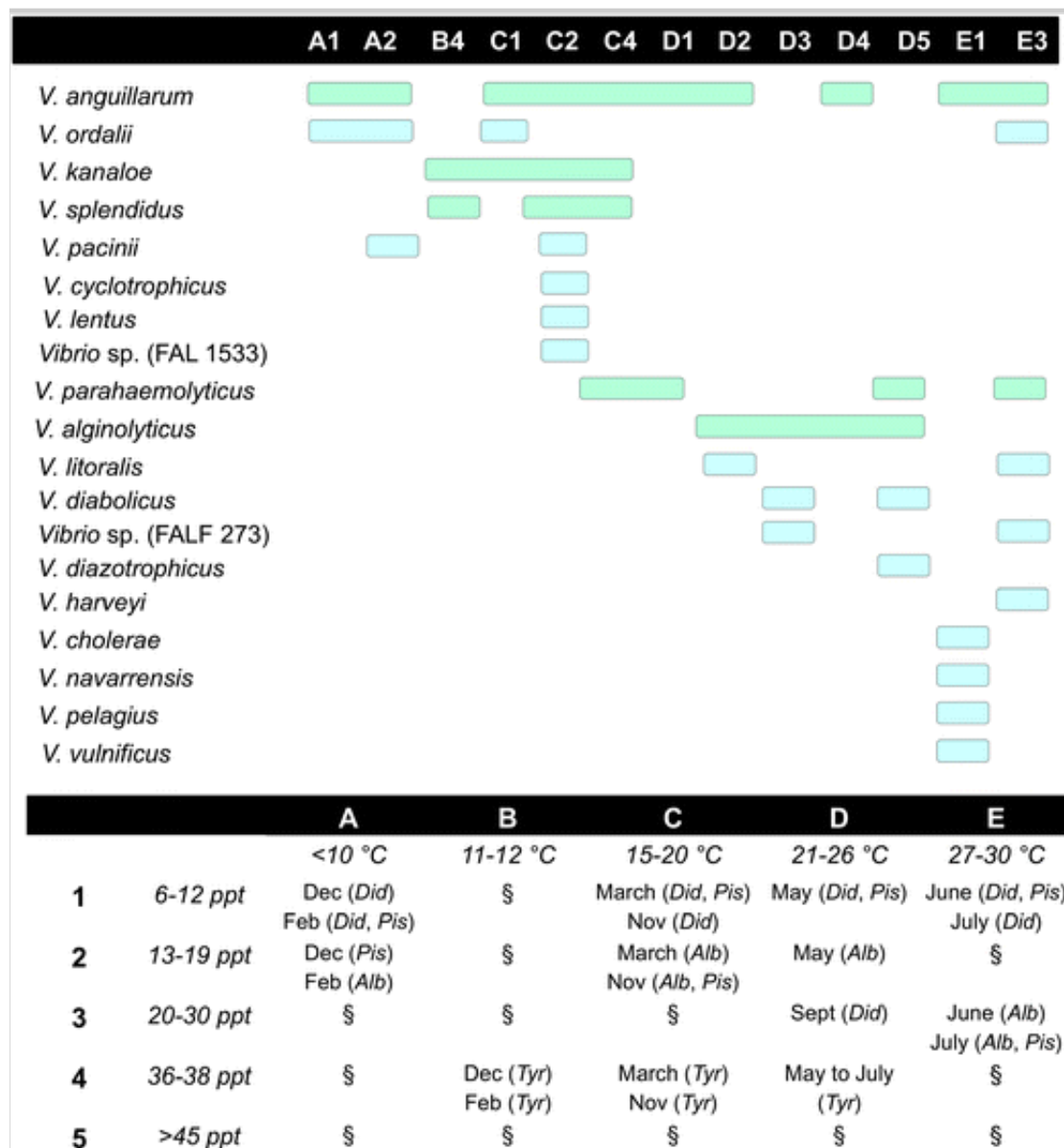
As to salinity, we have divided the wide range 0.5 to 30 ppt, usually referred to as brackish, in three sections: 1) 0.5–10 ppt -"Baltic-like"; 2) 11–20 ppt "Black-sea-like," and 3) 20–30 ppt, the higher salinity still to be regarded as truly brackish. The further intervals were 4) 31–40 typical of euhaline to metahaline seas, and 5) >40 ppt, that is already brine. All of these intervals were combined to identify different Temperature/Salinity (T/S) conditions, so to assemble together the similar ones, even if

registered in different ponds and/or months (in Fig. 3b are reported for each class the months and the stations in which they occurred). The distribution of *Vibrio* species, along the T/S actually occurring conditions and yielding vibrio isolates, is shown in Fig. 3a. *Vibrio* species are differently distributed among T/S classes. *Vibrio anguillarum*, steadily found from December to March, spanned several temperature (A to E) and salinity conditions (1 to 4); and a similar trend was observed for the related species *Vibrio ordalii*. Such a wide tolerance surely has advantages for these species in causing diseases to various fishes, bivalves, and crustaceans in marine, brackish and fresh waters and makes the brackish environment an optimal reservoir for them. The salt dependent *V. kanaloae* (Thompson et al. 2003) was mainly found in cool waters (B to C) and in a salinity range spanning from the Baltic to Mediterranean-like ones. A similar pattern was observed for *V. splendidus* that was, however never isolated at the lowest salinity interval, and spanned from the Black Sea to Mediterranean salinity values. The group formed by *V. pacinii*, *Vibrio cyclotrophicus*, *V. lentus*, and the unnamed isolate similar to the FAL1533 strain was found at cool temperatures, too, but in a narrow salinity range (mostly C2). The human pathogen *V. parahaemolyticus*, usually found in estuarine warm waters (Nigro et al. 2011; Thongchankaew et al. 2011) but also able to stand cooler and saltier environments (Martinez-Urtaza et al. 2012) was found in very different salinity conditions (1 to 5) but mostly above 20 °C. All of the other species were found above 20 °C (D and E intervals) and formed two separate groups: *V. alginolyticus Vibrio diabolicus*, *Vibrio litoralis* and unnamed isolate homologous to FALF273 were scattered along different salinity ranges, whilst *V. harvey*, *Vibrio diazotrophicus*, *V. cholerae*, *V. vulnificus*, *Vibrio navarrensis*, and *V. pelagius* were all observed within only one T/S class. *Vibrio harveyi* is often present in tropical environments (Oakey et al. 2003) and, in this campaign, it was only isolated in June from all the three stations of *Alberobello*, at 27–28 °C and 20–21 ppt, corresponding to the E3 class. The high salinity conditions of D5 class, restricted the species diversity to *V. diazotrophicus*, reported to be able to grow up to 40 °C and 100 ppt (Guerinot et al. 1982); *V. parahaemolyticus* and *V. diabolicus*. *Vibrio diabolicus* was first isolated from a deep sea hydrothermal vent, and its optimal temperature and ionic strength are reported in the range 30–45 °C and 20–50 ppt, respectively (Raguénès et al. 1997). It is, therefore, not surprising to find it also in very salty ponds at temperature values unlikely to be reached in the sea water.

Fig. 3

Vibrio species diversity along the T/S classes in the Macchiatonda wetland. §: Combination of temperature and salinity not occurring in the field. *Alb*: Alberobello pond; *Did*: Didattico pond; *Pis*: Piscinula pond; *Tyr*: Tyrrenian Sea.

▢ Only ponds

🟩 Ponds and sea

		A	B	C	D	E
		<10 °C	11-12 °C	15-20 °C	21-26 °C	27-30 °C
1	6-12 ppt	Dec (*Did*) Feb (*Did, Pis*)	§	March (*Did, Pis*) Nov (*Did*)	May (*Did, Pis*)	June (*Did, Pis*) July (*Did*)
2	13-19 ppt	Dec (*Pis*) Feb (*Alb*)	§	March (*Alb*) Nov (*Alb, Pis*)	May (*Alb*)	§
3	20-30 ppt	§	§	§	Sept (*Did*)	June (*Alb*) July (*Alb, Pis*)
4	36-38 ppt	§	Dec (*Tyr*) Feb (*Tyr*)	March (*Tyr*) Nov (*Tyr*)	May to July (*Tyr*)	§
5	>45 ppt	§	§	§	§	§

Within the last cluster, the human pathogen *V. cholerae* is well known to prefer low salt and warm conditions (Colwell et al. 1977; Takemura et al. 2014) and was indeed isolated only from warm, poorly salted waters (E1 class) in *Didattico* and in *Piscinula*, together with *V. vulnificus* that was also isolated from the same ponds in the same conditions, although its

presence, according to Randa et al. (2004), is unrelated to the temperature when salinity is low, but linked to warm waters at higher saltiness. Both *V. navarrensis*, first isolated from sewage in Spain (Urdaci et al. 1991) and *V. pelagius* were found only once, in *Didattico* (July) and in the deeper *Piscinula* station (station 6 in June), respectively.

Apart from a common core of the ubiquitous species *V. anguillarum*, *V. ordalii*, and *V. kanaloae*, the species profile varied among the three ponds according to their peculiar features. This observation stresses the influence of salinity for the single species. While the ponds do not differ substantially for the temperature trend, indeed, salinity was steadily low in *Didattico*, always higher than the other ponds in *Alberobello*, and widely fluctuating in *Piscinula* where the T/S conditions went alternating, being in the same class as *Didattico* in February, March, May, and June and as *Alberobello* in November, July, and September. In December, the T/S values, in *Piscinula*, were similar to those observed in *Alberobello* in February. According to the season, indeed, both the low salinity preferring species *V. cholerae* and *V. vulnificus*, were never encountered in *Alberobello*, and the salt-dependent *V. parahaemolyticus* and *V. alginolyticus*, were found in *Piscinula*.

The species exclusively found in sea-waters were all retrieved only once. Among them, only *Vibrio atlanticus* (Diéguez et al. 2011) was found below 15 °C while *Vibrio agarivorans* and *Vibrio mediterranei* so as the two unnamed isolates homologous to the Caim1833 and MWB21 strains were found above 20 °C, (D4) in June or May.

The distribution of the temperature and salinity values in narrow and discrete T/S classes helps also to understand how important other environmental factors can be: *e.g.*, the species diversity coincident to the C2 was mostly contributed by the November sampling in *Alberobello*, demonstrating that different parameters, such as the detrital particulate organic matter (Turner et al. 2009), rainfall (Yamazaki and Esiobu 2012), and/or both the amount and kind of plankton (Eiler et al. 2007; Turner et al. 2014) had to have exerted their influence within the ponds and/or seasons.

Occurrence of virulence-associated genes in the *Vibrio* isolates

The 130 *Vibrio* isolates from brackish and marine waters were screened for a battery of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* virulence-associated genes. Out of the genes included in the battery, four are involved in *V. cholerae* virulence: *ctxA*, *zot*, and *ace*, encoding the cholera toxin, the zonula occludens toxin, and the accessory enterotoxin, respectively, and all located in the CTX prophage, and *tcpA*, located in the VPI pathogenicity island, and encoding the major subunit of the toxin co-regulated pilus colonization factor. From *V. parahaemolyticus* the genes encoding the thermostable direct haemolysin (TDH) and the TDH-related haemolysin (*tdh* and *trh*) were considered. The *V. vulnificus* virulence associated genes were *vvhA* encoding a secreted cytolytic/haemolysin pore-forming toxin (Yamamoto et al. 1990); and *vllY* encoding another haemolysin, similar to the *Legionella pneumophila* legiolysin (Chang et al. 1997). A primer pair designed to detect the coding sequences of the ToxR and ToxS regulatory proteins were also used.

In total, 73 isolates (56 %) yielded positive PCR reactions with the selected primers. The very high frequency of positivity for *toxR* (61/130) and *toxS* (26/130) most probably relies on a greater-than-supposed variability in these ORFs, particularly in *toxR*. As a consequence, the primers (Sechi et al. 2000) expected to recognize *V. cholerae*, *V. parahaemolyticus*, and *V. alginolyticus*, according to the available sequences in the databases, actually succeed in amplifying the native genes of other species. For example, all of the available *Vibrio anguillarum* sequences share the same mismatches in the *toxR* primer pair, one in the forward and three in the reverse one, even at the very 3' end, but a large group of environmental isolates (in our sampling 26 out of 44) has probably a closer homology to *V. cholerae*, *V. parahaemolyticus* and *V. alginolyticus* in the primer regions. ToxRS, indeed are widely distributed among vibrios, mediating environmentally induced regulations (Reich and Schoolnik 1994; Di Rita and Mekalanos 1991; Wang et al. 2002) including some virulence gene expression such as *ctxAB*, *tcpA tdh*, and *vvhA* (Lin et al. 1993; Lee et al. 2000; Yu and Di Rita 2002). As demonstrated by Lee et al. (2000) for *V. vulnificus*, ToxRS are able to warrant both the expression of *vvhA* and *ctx* in an *Escherichia coli* background. Their presence, therefore, greatly enhances the probability of an actual expression of the horizontally transferred genes within the genus.

By removing the *toxRS* background (Table 3), the frequency of the

virulence associated determinants decreases to 33 isolates (25.4 %). The *V. cholerae* associated determinants were *tcpA* (9 isolates, mostly *V. anguillarum*) including a *tcpA* and *ace* positive *V. parahaemolyticus*, and two *ctx* positive isolates (*V. alginolyticus* and *V. parahaemolyticus*) that belong to one rare and one recognized pathogenic species for humans, respectively.

Table 3

~~Associations of virulence~~Virulence-associated determinants in the brackish *Vibrio* assemblage

Species	Ace/ tcpA	Ctx	TcpA	vvhA	vlly	tdh	trh
V. agarivorans							2
V. alginolyticus		1		1			
V. anguillarum			5	2			3
V. diabolicus					1		
V. harveyi			1			1	1
V. kanaloe						2	6
V. ordalii			1				
V. parahaemolyticus	1	1	1			1	1
V. sp. MWB21				1			
V. splendidus						1	

All of these genes are encoded on mobile genetic elements and can be horizontally transferred among *V. cholerae* strains, but neither Ctxϕ nor VPI prophage have been demonstrated to infect other species other than *V. cholerae* or the related one *V. mimicus* (Boyd et al. 2000). So, their presence in environmental species could be due to i) transducing phages (O'Shea and Boyd 2002) or ii) transformation, through the presence of a natural competence mechanism, similar to the one described in *V. cholerae* (Meibom et al. 2005).

Some positive amplifications were observed for both *tdh* and *trh*, involved in the virulence of *V. parahaemolyticus*. Terai et al. (1991) hypothesize that the *tdh* ancestor gene would probably originate in *V. hollisae* and spread to some strain of other environmental *Vibrionaceae*, as *V.*

parahaemolyticus, *V. cholerae* non-O1, and *V. mimicus*, probably via an insertion sequence. Moreover, according to Theethakaew et al. (2013), the presence of different haemolysin gene profiles within *V. parahaemolyticus*, accounts for horizontal gene transfers events involving both the *tdh* and *trh* encoding genes. The presence of *tdh* and *trh* negative isolates within *V. parahaemolyticus* is actually quite common in the environment (Robert-Pillot et al. 2004; Ceccarelli et al. 2013) and have been also found in clinical samples (Haley et al. 2014). In our sampling, indeed, we have found only one *tdh* and one *trh* positive *V. parahaemolyticus* isolate out of 13. Among the environmental species, the highest frequency was in *V. kanaloae* (2 *tdh* + and 6 *trh* + isolates) and, to our knowledge, this is the first report of the presence of these haemolysins in *V. kanaloae*. As *V. kanaloae* is often associated with molluscs (Romalde et al. 2014) and is endowed of a pathogenic potential for fish and crustaceans (Austin et al. 2005), the possible presence of such haemolysins in this species should be kept in mind whenever considering the risk of sea-food borne diseases.

The search for *vvha*, and *vllY* did not yield positive results within the *V. vulnificus* Macchiatonda isolates. The *vvha* gene belongs to the core genome of *V. vulnificus* (Morrison et al. 2012) but its sequence differs in clinical and environmental strains (Senoh et al. 2005). The failure to amplify the haemolysin-encoding genes with primers specific for the first group, assigns the Macchiatonda isolates to the non-pathogenic group. The negativity in *vllY* amplification was rather surprising, as both Wong et al. (2005) and Bier et al. (2013) regarded *vllY* as they are too frequently found to be useful for a discrimination within *V. vulnificus*. These authors, however, used a different primer pair. In this work the primers originally proposed by Chang et al. (1997) were used; these primers allowed amplification of *vllY* from about 40 strains in that study and yielded positive results in subsequent ones (*e.g.*, Baffone et al. 2006). A search in the five database available sequences, however, revealed three mismatches in the sense primer and two gaps in the antisense one (data not shown), so that a sequence heterogeneity can be speculated in the outer regions of this operon. The *V. vulnificus* haemolysins-encoding genes *vvhA* and *vllYA* were rarely and randomly found, the first in *V. anguillarum*, *V. alginolyticus*, and in a MWB21-like isolate; the second just once, in *V. diabolicus*.

Our data on the occurrence of culturable *Vibrio* species in the

Macchiatonda wetland are in general agreement with other reports concerning seawater in the Mediterranean Basin (*e.g.*, Macián et al. 2010; Narracci et al. 2014), other than for *V. anguillarum* being the most frequently isolated species. *Vibrio anguillarum* is the most important causative agent of haemorrhagic septicaemia in a great variety of farmed and wild fish species, crustaceans, and bivalves (Pedersen et al. 1994; Cavallo et al. 2012); the role of brackish coastal basins such as Macchiatonda as a possible reservoir for this species must, therefore, be kept in mind, a fortiori for the actual and forecasted presence of aquaculture settings in the surrounding areas. A concern for human health arises from the presence of acquired virulence determinants in environmental species that can keep them circulating even through environmental conditions unfavourable to the pathogens.

The different features of the ponds in Macchiatonda, indeed, allow *Vibrio* species with different environmental requirements to coexist in the area, so facilitating the exchange of virulence genes that, as suggested by Klein et al. (2014), could also increase the fitness and/or the scavenging of nutrients and lead to the emergence of additional virulent *Vibrio* species, perhaps with different environmental preferences and host ranges. Therefore, as stressed by our data, it is advisable to survey the global structure of the *Vibrio* communities rather than the mere presence of the human or animal pathogenic species. To this purpose, the approach suggested by Gennari et al. (2012) that analyses the transfer of fitness related traits could be the very expedient needed for future work.

Finally, studies conducted on brackish systems with a large variety of temperature and salinity combinations, as Macchiatonda, could be expedient to set predictive models on the effect of temperature shifts on the dynamics of vibrios, and other bacterial species, in different low salted marine systems. This is a current issue, as the increase of seawater-related infections, mainly wounds, experienced in Europe, is demonstrated even in the Northern regions since the mid-1990s (Baker-Austin et al. 2012; Böer et al. 2013).

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