UNIVERSITA' DEGLI STUDI DI ROMA "TOR VERGATA"

FACOLTA' DI SCIENZE MATEMATICHE, FISICHE E NATURALI

DOTTORATO DI RICERCA IN
BIOLOGIA EVOLUZIONISTICA ED ECOLOGIA

CICLO XXVII

DEVELOPMENT OF A NEW MOLECULAR ASSAY FOR AN EASIER DETECTION AND IDENTIFICATION OF VIBRIONACEAE

Ph.D. Thesis

Giorgia Matteucci

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This work is dedicated to who has been waiting for me.

ABSTRACT

Vibrionaceae are widespread in marine and brackish environments, and several species are human and animal pathogens of global importance.

Preliminary studies suggest the role of coastal basins as a possible reservoir for vibrios and raise a concern for human health related to the presence of acquired virulence traits in environmental species that can keep them circulating even when environmental conditions are unfavorable to the well-known pathogens. Such events could facilitate the exchange of virulence genes that may lead to the emergence of additional virulent species besides the well-studied ones, perhaps with different environmental preferences and host ranges.

Accordingly, our data stressed the need of surveying the global structure of vibrio communities, rather than the mere presence of human or animal pathogenic species. An accurate identification of vibrios, indeed, is the basis for a proper surveillance. The difficult phenotypic and genotypic identification of environmental vibrios, however, greatly limits our understanding of Vibrionaceae ecology, the estimation of vibrio infection incidence, and the evaluation of the associated risk.

The question addressed in this Thesis rose to overcome this limitation, in order to develop a suitable and rapid molecular assay to properly detect these microorganisms to help tracking vibrios in the environment, as a very important challenge for both animals and human health.

A novel multiplex PCR-based protocol has been designed for the simultaneous differentiation and identification of isolates belonging to Vibrionaceae main genera *Aliivibrio*, *Photobacterium* and *Vibrio*, and *Vibrio* species through one genus-specific (*rpoD*) and two species-specific primer sets (*recG* and *rplB*), known as housekeeping genes for identification and phylogeny of Vibrionaceae.

A total of 84 vibrio isolates were used to validate the assay, representing 27 named species and including *Vibrio*, *Aliivibrio* and *Photobacterium* genera from various origins. Designed *rpoD* primers correctly identified all the genera. Although non-specific reactions by *recG* primers occurred in 4 test strains (4.8%), it did not interfere with the specificity of the PCR. Our results also show that *rplB* is unable to resolve *Vibrio* spp., therefore it is not recommended for differentiate them within the identification key.

To test the usefulness of the method, the identification key was eventually applied to evaluate the occurrence of vibrios in environmental samples collected from Oban (Scotland) and Eilat (Israel) coastal waters. The assay was able to correctly identify 88.1 % of the isolates as *Aliivibrio*, *Photobacterium* or *Vibrio*.

As a result, the work presents a validated multiplex PCR assay capable of simultaneous detection and differentiation of genus *Aliivibrio*, *Vibrio* and *Photobacterium*, and of *V. alginolyticus*, *V. diabolicus*, a group of species comprising *V. agarivorans*, *V. cholerae* and *V. harveyi*, and a group comprising *V. parahaemolyticus* and *V. splendidus*, thanks to the concatenated multiplex amplification, even in complex samples.

It represents a significant advance by providing an easy, fast and low-cost method for identification of closely related vibrios that are difficult to discriminate by existing routine approaches.

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1. INTRODUCTION

1.1. Historical perspectives and importance of the family Vibrionaceae

One hundred forty species are now described in the family Vibrionaceae (Table 1.1), which consists of six genera: *Vibrio* (Pacini, 1854), *Photobacterium* (Beijerink, 1889), *Salinivibrio* (Mellado *et al*., 1996), *Enterovibrio* (Thompson *et al*., 2002), *Grimontia* (Thompson *et al*., 2003) and *Aliivibrio* (Urbanczyk *et al*., 2007).

The first described genus was *Vibrio*, originally recognized by Pacini in 1854 with *Vibrio cholerae*, the agent of human cholera, as its type species, later isolated in pure culture from cholera patients by Robert Koch in 1883. According to the List of Bacterial Names with Standing in Nomenclature (http://www.bacterio.cict.fr/index.html, last consultation January 2015), *Vibrio* is the most representative genus among Vibrionaceae, with 99 recognized species, followed by genus *Photobacterium* (22 species), first described by Beijerinck in 1889 with *Photobacterium phosphoreum*, a bioluminescent species, as its type species.

Vibrionaceae belong to the Gammaproteobacteria and are defined as a group of gram-negative strains, rod shaped, with polar flagella. Primarily aquatic, most species require Na^+ for growth, although this requirement is minimal for *V. cholerae*, *V. mimicus*, *V. fluvialis* and *V. metschnikovii*. Most vibrios are mesophiles and, therefore, grow better in warm waters, whit an optimal growth temperature ranging from 20 to 25 °C.

A distinctive Vibrionaceae feature is that, unlike most bacteria, they possess two circular chromosomes (one large and one small). The large chromosome (Chromosome 1, Ch1) tends to contain housekeeping genes (DNA replication, transcription, translation, flagellar synthesis, metabolic pathways), while Chromosome 2 (Ch2) tends to contain accessory genes (pathogenicity, antibiotic resistance, host defense avoidance, survival in adverse environments).

Vibrios are facultative anaerobic organisms and the majority of the strains are oxidase positive, capable of fermenting D-glucose, reduce nitrate to nitrite and ferment D-fructose, maltose and glycerol. It has been shown that multiple species of *Aliivibrio* and *Vibrio* are bioluminescent, despite of the majority of members of *Photobacterium*. Vibrionaceae are metabolically versatile: some species show gas production, nitrogen fixation, and phototrophy.

Despite most vibrios are capable of motility thanks to a polar flagellum, there are species, notably *Vibrio halioticoli* and its closest relatives, that do not possess flagella and are non-motile (e.g. Sawabe *et al*., 2003), while *Aliivibrio fischeri* loses its flagella when in symbiosis with squid hosts, then regain them in the a free-living state (Ruby, 1996). Prior to the development of molecular biology methods, vibrios were largely identified and classified by these diverse and extensive traits with phenotypic tests.

Vibrios have mutualistic or commensal relationships with wide variety of hosts, including plants, providing the benefit of nitrogen fixation or antimicrobial activity. As an example, there are the species (*A. fischeri*, *A. logei*, *P. leiognathi*) that provide bioluminescence to the light organs of fishes and squids (Dunlap and McFall-Ngai, 1987; Ruby, 1996). The probiotic Vibrionaceae in the guts of abalone (Gastropoda: *Haliotidae*) are notable for their non-pathogenicity (e.g. *V. halioticoli*; Sawabe, 2006),

Even pathogenicity is a trait that often varies among strains of the same species, which may or may not be pathogenic (Gennari *et al.*, 2012). In addition to metabolic diversity, indeed, the pathogenic Vibrionaceae possess a variety of exotoxins, proteases, transport proteins, adhesion mechanisms and lipases that act as virulence factors. Since *Vibrio*s are gram-negative, they also possess endotoxin which differs in toxicity from strain to strain.

In general, vibrios may be opportunistic pathogens for both humans and marine animals and can cause systemic infections, skin lesions and gastroenteritis.

1.2. Taxonomy and phylogeny

Until the middle of the 1900s, bacterial taxonomy was dominated by morphological studies that tried to group strains on the basis of phenotypic features, e.g., flagellation, morphology, curvature of the cells, and cultural aspects. Obviously, classification and identification based on these features was quite artificial and it was concluded that at least three genera existed among the species examined (Davis and Park, 1962). In the 7th edition of Bergey's Manual of Determinative Bacteriology (Breed *et al.,* 1957), the genus *Vibrio* belonged to the family *Spirillaceae* and consisted of 34 species which, with the exception of *V. cholerae* and *V. metschnikovii*, were later reclassified into other genera, e.g., *Campylobacter*, *Commamonas* and *Pseudomonas* or no longer accepted as validly described species according to the Approved List of Bacterial Names (Skerman *et al.,* 1980). The genus *Photobacterium* harbored one species, i.e. *P. phosphoreum* and was allocated into the genus Bacterium of the family *Bacteriaceae* (Breed *et al.*, 1957).

Modern *Vibrio* taxonomy is laid by studies on numerical (phenetic) and/or polyphasic taxonomic made in the 1970s (Colwell, 1970). Such studies grouped strains on the basis of their ability to utilize different compounds (ca. 50–150) as sources of carbon and/or energy, enzyme activity (e.g., gelatinase, chitinase, DNase), salt tolerance, luminescence, growth at different temperatures, antibiograms, DNA base composition, morphological features and other biochemical tests (e.g., oxidase, catalase, Voges–Proskauer, indole, nitrate reduction, arginine dihydrolase, lysine and ornithine decarboxylases).

In the 8th edition of the Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974), the family Vibrionaceae, which was proposed by Veron in 1965, comprised *Vibrio* and *Photobacterium* along with *Beneckea*, *Aeromonas*, *Plesiomonas* and *Lucibacterium*.

According to the Bergey's Manual of Systematic Bacteriology (2002), the family Vibrionaceae comprises six genera: *Allomonas* (1 sp.), *Catenococcus* (1 sp.),

Enterovibrio (1 sp.), *Listonella* (2 spp.), *Photobacterium* (6 spp.), *Salinivibrio* (1 sp.) and *Vibrio* (59 spp.).

Based on 16S rRNA, *rpoA*, *recA*, and *pyrH* gene sequences and phenotypic features, Thompson and coworkers (2004) suggested that the family Vibrionaceae should be split into four families, *Entero*Vibrionaceae, *Photobacteriaceae*, *Salini*Vibrionaceae and Vibrionaceae *sensu stricto*. The new family Vibrionaceae would then comprise only the genus *Vibrio*.

The most recent edition of the Bergey's Manual of Systematic Bacteriology (2005) proposed a single family Vibrionaceae comprising three genera, *Vibrio* (44 spp.), *Photobacterium* (6 sp.), and *Salinivibrio* (1 sp.), based on 16S rRNA phylogeny (Farmer and Janda, 2005).

At current, the numbers of Vibrionaceae species has increased nearly 2.5-folds than those described in the 2005 Bergey's edition; for up-to-date status on the taxonomy and number of the approved species of vibrios, we based this work on the List of Bacterial Names with Standing in Nomenclature (http://www.bacterio.cict.fr/index.html) (Table 1.1).

It is worth noting that genus *Listonella* is proposed as a later heterotrophic synonym based on the 16S rRNA gene phylogeny and genome features (Thompson *et al.*, 2011) and now *V. anguillarum* should be used in place of *L. anguillarum*.

At present there are 3 *Vibrio* e 1 *Photobacterium* species name without Standing in Nomenclature: *Vibrio inhibens*, *Vibrio parilis*, *Vibrio zhanjiangensis* and *Photobacterium atrarenae* (Gomez-Gil *et al*., 2014).

Genus I. *Vibrio* Pacini 1854

- *Vibrio cholerae* (type species)
- *Vibrio aerogenes*
- *Vibrio aestuarianus*
- *Vibrio aestivus*
- *Vibrio agarivorans*
- *Vibrio alfacsensis*
- *Vibrio alginolyticus*
  Homotypic synonym: *Beneckea alginolytica.*
- *Vibrio anguillarum*
  Junior synonym: *Listonella anguillarum.*
- *Vibrio areninigrae*
- *Vibrio artaprorum*
- *Vibrio atlanticus*
- *Vibrio atypicus*
- *Vibrio azureus*
- *Vibrio brasiliensis*
- *Vibrio breoganii*
- *Vibrio campbellii*
  Basonym: *Beneckea campbellii.*
- *Vibrio carribeanicus*
- *Vibrio casei*
- *Vibrio chagasii*
- *Vibrio celticus*
- *Vibrio cincinnatiensis*
- *Vibrio comitans*
- *Vibrio communis*
- *Vibrio coralliilyticus*
- *Vibrio cortegadensis*
- *Vibrio crassostreae*
- *Vibrio cyclitrophicus*
- *Vibrio diabolicus*
- *Vibrio diazotrophicus*
- *Vibrio ezurae*
- *Vibrio fluvialis*
- *Vibrio fortis*
- *Vibrio furnissii*
- *Vibrio gallaecicus*
- *Vibrio gallicus*
- *Vibrio gazogenes*
  Previously classified as *Beneckea gazogenes.*
- *Vibrio gigantis*
- *Vibrio halioticoli*
- *Vibrio hangzhouensis*
- *Vibrio harveyi*
  Basonym: *Lucibacterium harveyi* Junior heterotypic synonyms: *Vibrio carchariæ, Vibrio trachuri*
- *Vibrio hepatarius*
- *Vibrio hemicentroti*
- *Vibrio hippocampi*
- *Vibrio hispanicus*
- *Vibrio ichthyoenteri*
- *Vibrio inusitatus*
- *Vibrio jasicida*
- *Vibrio kanaloae*
- *Vibrio lentus*
- *Vibrio litoralis*
- *Vibrio mangrovi*
- *Vibrio marisflavi*
- *Vibrio maritimus*
- *Vibrio mediterranei*
- *Vibrio metecus*
- *Vibrio metschnikovii*
- *Vibrio mimicus*
- *Vibrio mytili*
- *Vibrio natriegens*
  Basonym: *Beneckea natriegens.*
- *Vibrio navarrensis*
- *Vibrio neonatus*
- *Vibrio neptunius*
- *Vibrio nereis*
  Basonym: *Beneckea nereis.*
- *Vibrio nigripulchritudo*
  Basonym: *Beneckea nigripulchritudo*
- *Vibrio ordalii*
- *Vibrio orientalis*
- *Vibrio ostreicida*
- *Vibrio owensii*
- *Vibrio pacinii*
- *Vibrio parahaemolyticus*
  Homotypic synonym: *Beneckea parahaemolytica.*
- *Vibrio pectenicida*
- *Vibrio pelagius*
  Junior synonym: *Listonella pelagia, Beneckea pelagia, Vibrio pelagius*
- *Vibrio penaeicida*
- *Vibrio plantisponsor*
- *Vibrio pomeroyi*
- *Vibrio ponticus*
- *Vibrio portersiae*
- *Vibrio proteolyticus*
  Basonym: *Aeromonas hydrophila* subsp. *proteolytica*
- *Vibrio quintilis*
- *Vibrio rarus*
- *Vibrio rhizosphaerae*
- *Vibrio rotiferianus*
- *Vibrio ruber*
- *Vibrio rumoiensis*
- *Vibrio scophthalmi*
- *Vibrio sagamiensis*
- *Vibrio shilonii*
  Later heterotypic synonym of *V. mediterranei.*
- *Vibrio sinaloensis*
- *Vibrio splendidus*
  Basonym: *Beneckea splendida.*
- *Vibrio stylophorae*
- *Vibrio superstes*
- *Vibrio tapetis*
- *Vibrio tasmaniens*
- *Vibrio toranzoniae*
- *Vibrio tubiashii*
- *Vibrio variabilis*
- *Vibrio vulnificus*
  Basonym: *Beneckea vulnifica.*
- *Vibrio xiamenensis*
- *Vibrio xuii*

Genus II. *Photobacterium* Beijerinck 1889

- *Photobacterium phosphoreum* (type species)
- *Photobacterium aestuarii*
- *Photobacterium angustum*
- *Photobacterium aphoticum*
- *Photobacterium aplysiae*
- *Photobacterium aquae*
- *Photobacterium aquimaris*
- *Photobacterium damselae* subsp. *damselae*
  Basonyms: *Vibrio damselae, Listonella damselae.* Junior heterotypic synonym: *Photobacterium histaminum.*
- *Photobacterium damselae* subsp. *piscicida*
  Originally classified as *Pasteurella piscicida.*
- *Photobacterium frigidiphilum*
- *Photobacterium gaetbulicola*
- *Photobacterium ganghwense*
- *Photobacterium halotolerans*
- *Photobacterium iliopiscarium*
  Basonym: *Vibrio iliopiscarius.*
- *Photobacterium indicum*
  Basonym: *Hyphomicrobium indicum.*
- *Photobacterium jeanii*
- *Photobacterium kishitanii*
- *Photobacterium leiognathi*
- *Photobacterium lipolyticum*
- *Photobacterium lutimaris*
- *Photobacterium profundum*
- *Photobacterium rosenbergii*
- *Photobacterium swingsii*

Genus III. Salinivibrio Mellado *et al.* 1996

- *Salinivibrio costicola* subsp. *costicola* (type species)
  Basonym: *Vibrio costicola.*
- *Salinivibrio costicola* subsp. *alcaliphilus*
- *Salinivibrio costicola* subsp. vallismortis
- *Salinivibrio proteolyticus*
- *Salinivibrio sharmensis*
- *Salinivibrio siamensis*

Genus IV. *Enterovibrio* Thompson *et al.* 2002

- *Enterovibrio norvegicus* (type species)
- *Enterovibrio calviensis*
  Basonym: *Vibrio calviensis.*
- *Enterovibrio coralii*
- *Enterovibrio nigricans*

Genus V. *Grimontia* Thompson *et al.* 2003

- *Grimontia hollisae* (type species)
- *Grimontia marina*

Genus VI. *Alitivibrio* Urbanczyk *et al.* 2007

- *Alivibrio fischeri* (type species)
  Homotypic synonym: *Photobacterium fischeri.*
- *Alitivibrio finisterrensis*
- *Alitivibrio logei*
  Basonym: *Vibrio logei, Photobacterium logei.*
- *Alitivibrio salmonicida*
  Basonym: *Vibrio salmonicida.*
- *Alitivibrio sifiae*
- *Alitivibrio wodanis*
  Basonym: *Vibrio wodanis.*

Tab. 1.1 Taxa within *Vibrionaceae* according to the members of *Vibrio* Working Group of the International Committee on Systematics of Prokaryotes (http://www.the-icsp.org/subcoms/*Vibrionaceae*.htm) (last update: March 2011), updated on the List of Prokariotic names with Standing in Nomenclature (last consultation: January 2015).

A comprehensive phylogenetic study of the Vibrionaceae was accomplished by Kita-Tsukamoto and coworkers in 1993, based on a fragment of about 450 nt of 16S rRNA sequences of 50 species, including most common vibrios, species of *Aeromonas*, *Deleya*, *Escherichia*, *Marinomonas*, *Pseudomonas* and *Shewanella*. Outcomes of this study circumscribed the species (with at least 99.3% 16S rRNA similarity), genera (95–96 %) and family (90–91 %) within the Vibrionaceae and highlighted high heterogenity among vibrios.

The most recent phylogenetic trees for *131* Vibrionaceae species and one describing species (*Vibrio tritonius* sp. nov.) based on 16S rRNA gene according to the ALL-Species Living Tree Project (LTP) database (Yarza *et al*., 2010) are shown in Figure1a and Figure1b (Gomez-Gil *et al*., 2014). All species are grouped into a single cluster forming Vibrionaceae on the basis of neighbor-joining algorithm and each genus *Photobacterium*, Enterovibrio, *Grimontia* and *Salinivibrio* seems to form a cluster. However, the genus *Aliivibrio* is nested within the cluster of the genus *Vibrio* (Figure 1b); nevertheless, *V. fischeri*, *V. logei*, *V. salmonicida* and *V. wodanis* have been recently reclassified as *Aliivibrio* gen. nov. (Urbanczyk *et al.*,2007).

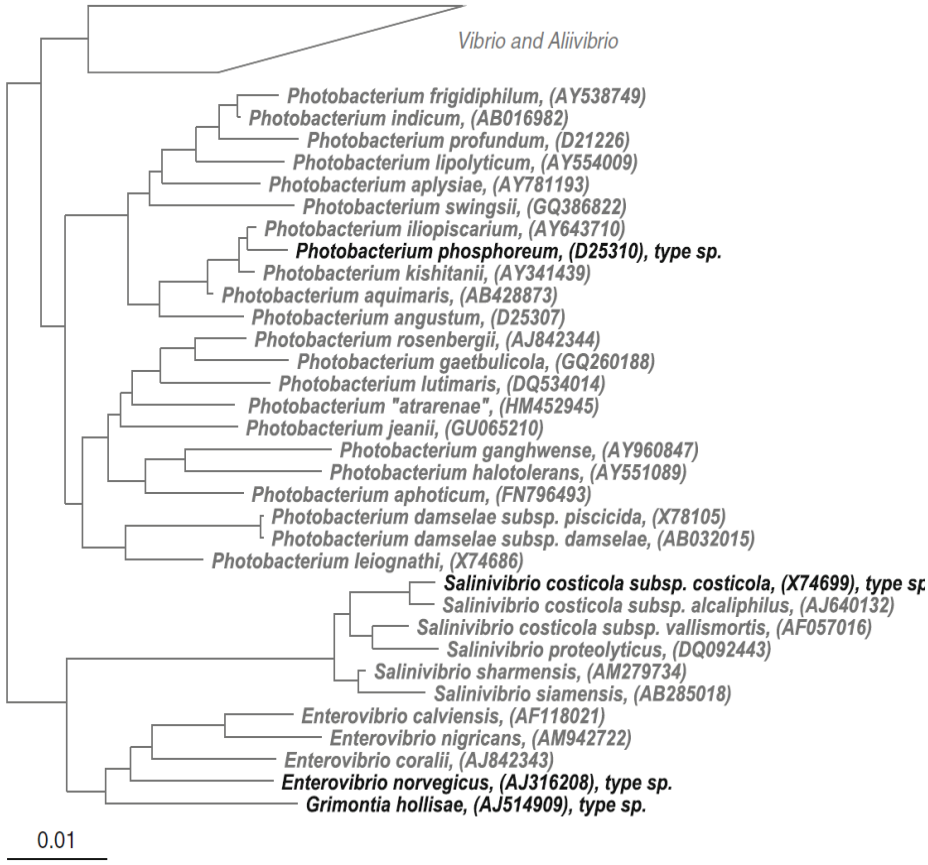


Figure 1.1a: Phylogenetic reconstruction of the genera *Photobacterium*, *Salinivibrio*, Enterovibrio and *Grimontia* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction (Gomez-Gil *et al*., 2014). The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (http://www.arb-silva.de/projects/living-tree) (Yarza *et al*., 2010).

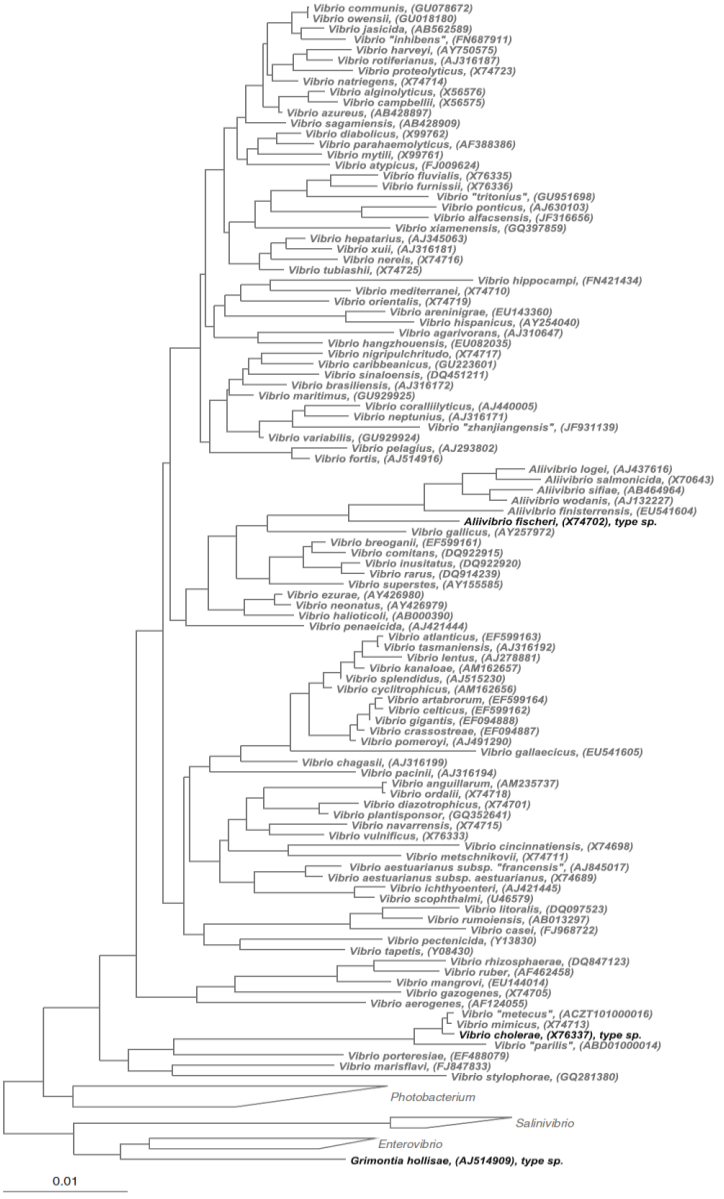


Figure 1.1b. Phylogenetic reconstruction of the genera *Vibrio* and *Aliivibrio* based on 16S rRNA and created using the neighbor-joining algorithm with the Jukes-Cantor correction (Gomez-Gil *et al*., 2014). The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (http://www.arb-silva.de/projects/living-tree) (Yarza *et al*., 2010).

New phylogenetic studies based on other chronometers correlate well with the 16S rRNA tree of vibrios. Concatenated sequence analysis of the *recA*, *rpoA*, 16S rRNA and *pyrH* provide more clear and robust separation of clusters for genera *Vibrio*, *Photobacterium*, Enterovibrio, *Grimontia*, *Salinivibrio*, and *Aliivibrio* (Thompson *et al.*, 2004).

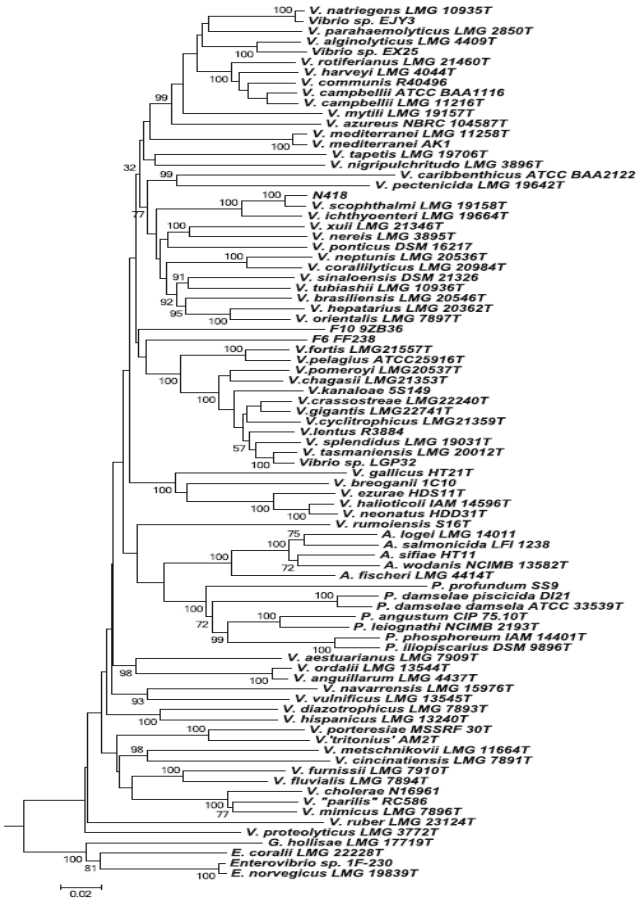


Figure 1.2 Phylogenetic tree for *Vibrionaceae* based on 8 gene multilocus gene sequences. The evolutionary history was inferred using the Neighbor-Joining (NJ) method, the Maximum Likelihood (ML) method and the Maximum Parsimony (MP) method (Gomez-Gil *et al*., 2014).

The most recent MLSA phylogenetic tree based on eight housekeeping genes (*ftsA*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA*, *topA*) is shown in Figure1.2 (Gomez-Gil *et al*., 2014). At least two distinct clades were found; one involved the genera *Photobacterium* and *Aliivibrio* nesting within the large *Vibrio* cluster, and the other was a robust clade formed by the genera *Enterovibrio* and *Grimontia*.

There is still lack of gene data in *Photobacterium* spp., *Salinivibrio* spp. and *V. gazogenes* and the related species, and there are still many orphan species (*V. proteolyticus*, *V. nigripulchritudo*, *mediterranei*, *V. tapetis* and so on).

Whole microbial genome sequence launched microbial taxonomy into a new era, with the possibility of establishing systematics on the basis of complete genomes, called genomic taxonomy (Coenye *et al.*,2005). The main goal of the genomic taxonomy is to extract taxonomic and phylogenetic information that can be used to establish a solid approach for the identification and classification of prokaryote species. The birth of the genomic taxonomy in vibrios occurred with a series of papers that attempted to use multilocus sequence analysis (MLSA) (Sawabe *et al*., 2007; Thompson *et al*., 2005) and allowed the setup of rapid and powerful identification systems through the Internet, even if establishing a universal MLSA is not possible: studies accomplished so far have shown that the resolution of different markers varies according to the taxonomic groups. Clearly, genes have different molecular clocks in different microbes, indicating the need of a multigene approach. Currently, there are 510 Vibrionaceae genomes sequenced (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/sites/genome). With the increase of sequenced Vibrionaceae genomes, it is possible to establish their taxonomy on the basis of complete genomes. Recently, Thompson *et al.*, (2009) utilized genome sequences from 43 vibrio strains, including 14 species, to define vibrio species based on specific genomic criteria, including MLSA. All methods, except 16S rRNA and codon usage, provided taxonomic resolution for differentiation of species and genera of vibrios, while the taxonomic resolution of 16S rRNA is restricted to differentiation of genera.

Based on the genomic taxonomy, a Vibrionaceae species is defined as a group of strains that share >95% DNA identity in MLSA, >96%, average amino acid identity, ≤10 genome signature dissimilarity and >61% proteome identity. Strains of the same species and species of the same genus form monophyletic groups on the basis of MLSA and supertree. This definition may enhance the field of *Vibrio* taxonomy.

1.3. Occurrence and distribution

Recent developments of group- or species-specific identification methods have been providing new insights into the ecology of Vibrionaceae.

Members of the Vibrionaceae are natural components of marine ecosystems worldwide, occurring in a wide range of aquatic environments, including estuaries, marine coastal waters and sediments and aquaculture settings throughout the world, and vibrios are free-living bacteria or may be found in association with particulate organic matter (POM), phytoplankton, zooplankton, and in or on marine organisms, such as corals (Rosenberg and Ben Haim 2002), fish gorgonians (Martin *et al*., 2002), shellfish (Sawabe *et al*., 2003), sea grass (Weidner *et al*., 2000), sponges (Hentschel *et al*., 2001), shrimps (Gomez-Gil *et al*., 1998c), and squids (Nishiguchi 2000). In particular, the association with marine particles (POM and plankton) is an important aspect of vibrio ecology: it has been shown to support the rapid growth of *V. cholerae* in microcosm and mesocosm studies (Worden *et al*., 2006).

As heterotrophic bacteria, vibrios are known to elaborate an extracellular chitinase and can utilize the chitinous exoskeletons of zooplankton and other crustaceans as a source of carbon and nitrogen (Thompson *et al.*, 2004). The association with zooplankton and the degradation of chitin is an important aspect of vibrio ecology and nutrient cycling in the marine environment (Meibom *et al*., 2004). *Vibrio*s are believed to play an important role in the mineralization of the chitinous exoskeletons of zooplankton (Colwell *et al.*, 1977).

Although Vibrionaceae species are ubiquitous around the globe, they do not exhibit spatially or temporally static characteristics. Changes in the density and diversity of the vibrio community can be correlated with fluctuations in the physiochemical parameters such as temperature and salinity (Thompson *et al.*, 2004). Additionally, changes in the community may be correlated with shifts in the abundance or composition of reservoirs such as plankton, sediment and shellfish (Maugeri *et al.*, 2004)

To better understand under what conditions vibrios occur and proliferate, most studies have investigated environmental variables that can be measured from seawater such as temperature, salinity, dissolved oxygen, nitrogen, phosphorus, and chlorophyll a concentrations. They are easily measured and many are observable remotely by satellite so that potential for presence of vibrios might be easily assessed. In addition, several studies have extended measurements to more complex physicochemical and biotic variables, including dissolved organic carbon (DOC) and zoo- and phyto-plankton taxa.

Strongest abiotic parameters correlated to *Vibrio* dynamics are temperature and salinity, at both the genus and species levels. These two variables most often explain the greatest amount of variance in total *Vibrio* abundance in the water column (e.g Heidelberg *et al.*, 2002 ; Oberbeckmann *et al.*, 2012), but do not necessarily capture species-level trends, and thus it is necessary to monitor populations of interest directly to capture their dynamics (Takemura *et al.*, 2014).

It is important to note that a minority of analyses has found temperature and salinity to be non-significant toward explaining *Vibrio* abundance. This inconsistency might be a result of the ranges considered; for instance, temperature may be found non-significant due to a narrow range observed, such that *Vibrio* abundance varies little. In fact, evidence supports this hypothesis; the correlation strength of temperature to vibrios varies by season (Oberbeckmann *et al.*, 2012; Froelich *et al.*, 2013; Takemura *et al.*, 2014), suggesting the magnitude of the correlation may depend on the temperature range examined.

In consequence to fluctuations in a combination of physiochemical parameters, the abundance and diversity of the *Vibrio* population exhibit a complex seasonal variation. It is worth noting that among environmental variables, the increase in sea surface temperature occurred during the last four decades explains 45% of the variance in occurrence and composition of vibrio communities, supporting the hypothesis that ocean warming is now enhancing the spread of vibrios and may be linked to the increasing trend in the related diseases worldwide (Vezzulli *et al*., 2014).

Given that also pathogenic species live in the environment, knowing vibrio ecology is the key for epidemiologic investigations. As an example, *V. cholerae* outbreaks are known to coincide with warming sea surface temperatures associated with large-scale climate events such as the El Niño-Southern Oscillation (Colwell, 1996; Lipp *et al.*, 2002). Filter-feeding shellfish can also accumulate pathogenic *Vibrio* species and serve as a reservoir for seafood-borne infections, especially related to raw shellfish consumption (Lipp and Rose, 1997). Similarly, ecological link between *Vibrio* prevalence and plankton plays a potential role in the epidemiology of these pathogens: plankton, colonized by pathogenic *Vibrio* species, can be a vehicle for the transmission of disease, as in the case of cholera (Huq and Colwell, 1996).

1.3.1. Survival in the environment

Key to the seasonal prevalence of vibrios is the ability of these bacteria to survive and persist in marine environment with fluctuations in temperature, salinity, nutrient concentration, DO and pH. Numerous mechanisms aid in survival and persistence, such as entering into a dormant state characterized by decrease in metabolic rate, loss of the flagellum, conformational change to a small sphere and inability to grow on standard culture media, in response to unfavorable conditions (Huq *et al*., 2000). This quiescent state was first described in *V. cholerae* by Xu *et al.*,(1982) and named as the viable-but-non-culturable state (VBNC). Since its discovery, the

ability to enter a VBNC state has been characterized for many gram-negative bacteria including *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Oliver, 2005). Furthermore, Matz *et al*, (2005) hypothesized that, as pathogenic *Vibrio* survival in the environment is independent of a human host, genes involved in human pathogenesis may have a natural role in the marine environment.

Another mechanism for survival involves the ability to associate with biotic and abiotic surfaces such as POM, plankton and sediment (Heidelberg *et al.*, 2002; Turner *et al.*, 2009). Associations range from a simple sporadic attachment to the development of dense, highly differentiated biofilms that are more resistant to extreme environmental conditions and protozoan predation (Matz *et al*., 2005).

The chitinous exoskeletons of planktonic organisms play an important role in the survival of *Vibrio* species, representing a nutrient-rich microhabitat. Changes in the abundance and diversity of vibrio communities, indeed, may be correlated with shifts in the abundance or composition of the plankton reservoir (Maugeri *et al.*, 2004; Turner *et al.*, 2009).

1.4. Public health aspects and disease spectrum

Although several *Vibrio* spp. are known to be human pathogens, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are considered the predominant ones.

In addition to these and the other major pathogens *V. alginolyticus*, *V. fluvialis*, *V. metschnikovii* and *V. mimicus*, other species including *V. cincinnatiensis*, *P. damselae*, *V. furnissii*, *V. harveyi* and *G. hollisae* have a low incidence of disease in humans.

	Type of infections		
	Gastro-intestinal	External (wounds)	Systemic
Main pathogens			
Vibrio cholerae			
Serogroup O1	Strong gastroenteritis, Rice-water diarrhea		
Serogroup non-O1	Cholerae-like disease, mild diarrhea		Might cause septicemia
Serogroup O139	Similar to the O1		
V. parahaemolyticus	Mild to strong gastroenteritis	Infection of open wounds	–
V. vulnificus			
Biotype 1	Rarely	Infection of open wounds (necrotizing fasciitis)	Primary septicemia
Biotype 3	–	Infection of open wounds	–
Other pathogens			
V. alginolyticus	–	Wounds, ears, sometimes eyes	–
V. cincinnatiensis	–	–	Bacteremia, meningitis
V. fluvialis	"Cholera-like" diarrhea	–	–
V. furnissii	Diarrhea (?)		
V. metschinokovii	Diarrhea (?)	Foot ulcer	Bacteremia
V. mimicus	Similar to V. cholerae	–	–
Photobacterium damselae	–	Wound infections	Bacteremia
Grimontia hollisae	Diarrhea	–	Bacteremia

(?) doubtful

Table 1.2. List of Vibrionaceae of clinical relevance (Gomez-Gil *et al*., 2014).

Their pathogenic action to the human host can be classified into three broad forms (Table 1.2): the gastrointestinal, the external and the systemic one. The external diseases arise from contamination of cuts and other skin lesions with seawater (and to a lesser extent, marine animals) and can remain localized or become systemic and ultimately fatal, depending on the causative species. The gastrointestinal ones arise from oral ingestion of raw, undercooked or cooked but re-contaminated seafood and seawater and lead to various degrees of gastroenteritis and, in some cases (e.g., *V. vulnificus*) life threatening systemic infections.

According to the Centers for Disease Control and Prevention reports (2009 and 2010), laboratory-confirmed infections of *Vibrio* spp. exceed those from *Salmonella*, *Shiga*, toxin-producing *Escherichia coli* O157, *Campylobacter* and *Listeria* in US in late 2000 and this trend has increased through 2008. In 2009, the overall rate of foodborne disease caused by *Vibrio*s was 0.35 per 100,000 population. The relative rates of the other four pathogens have either remained at the same level (*Salmonella*)

or shown a decrease (*Shigella* rates decreased 40% and STEC O157 decreased 25%) while *Vibrio* rates have increased by 47%.

As most of the pathogenic Vibrionaceae are mesophiles, it has been suggested that global warming might increase the incidence of vibrio infection worldwide (Colwell, 1996; Constantin de Magny *et al*., 2008). Clearly, it is beginning to look like increasing ocean temperatures are increasing the incidence of *Vibrio* disease and as more data become available this relation may be substantiated. In the last decade, indeed, scientists are using remotely-sensed satellite data, including temperature and salinity, to predict human health risks from pathogenic vibrios in water (Louis *et al*., 2003; Phillips *et al.*, 2007).

***Vibrio* spp.**

Vibrio cholerae, the causative agent of cholera, is endemic in south Asia and particularly in the Ganges delta, where epidemics have been recorded since ancient times (Sack *et al*., 2004).

It is normally found as a free-living organism in brackish and marine environments or associated to the plankton (Huq *et al., 1*983), although the toxigenic strains are mostly isolated from environments exposed to fecal contamination and the nontoxigenic from less contaminated areas (Faruque *et al.,*1998).

The disease is transmitted primarily through the ingestion of contaminated food or contaminated water supplies by human feces. Descriptions of patients probably with cholera are found in Hippocrates works (Barua1992).

The first documented pandemic occurred in 1817 near Calcutta (India) and spread to almost all southeast and central Asian countries, many African and Middle Eastern countries. During the second pandemic, beginning in 1826, the disease reached many European cities by the early 1830s and the American continent in 1832. Five more pandemics have occurred and the seventh, originated in Indonesia during 1905 and first isolated from Indonesian pilgrims to Mecca in the Egyptian town of El Tor, involved almost the whole world and became endemic in many places.

After more than 40 years into the seventh pandemic, cholera is still causing around 120,000 notified cases every year (WHO, 2003). It has been reported from around 50 countries during each year from 2000 to 2003, with the 96 % of the total cases occurring in Africa. The vast majority of the cases are assigned to the O1 serogroup (WHO, 2004), but in 1992, a non-O1 serogroup, named O139 Bengal, appeared in India and rapidly spread to Bangladesh and neighboring countries.

The ability of emergence, re-emergence and the pandemic potential of V. cholerae pathogenic strains are probably consequences of a combination of bacterial genetic background, physiological conditions and environmental factors such as plankton blooms. Entering in the viable but nonculturable state (VBNC), may allow the organism to persist or be introduced in a new region without being identified by standard bacteriological methods (Colwell, 1993). The, further changes in the metabolic state and/or environmental conditions can lead to the recovery of the bacteria from this stage.

There are two main genetic elements involved in pathogenicity of V. cholerae: CTX, which is the genome of a bacteriophage (CTX Φ) and encodes for the cholera enterotoxin (CT), and the vibrio pathogenicity island (VPI), deriving from the bacteriophage VPI Φ that can be transferred between *V. cholerae* strains (Karaolis and Kaper 1999), which carries genes for the pilus colonization factor (TCP), normally absent in non-epidemic strains (Sack *et al*., 2004), These virulence factors are regulated by the ToxR regulation factor, which is at the top of a regulatory cascade and under the control of environmental factors, such as temperature, pH, and NaCl (Skorupski and Taylor, 1997).

V. parahaemolyticus has been implicated in diarrhea associated with seafood consumption in many parts of the world, mainly in Japan, resenting more than 70 % of the cases due to bacterial food poisoning (Raimondi *et al*., 2000). Symptoms consist in gastroenteritis with nausea, watery diarrhea and less common, bloody diarrhea, vomiting, abdominal cramps, low-grade fever and chills (Farmer *et al.*, 2003), all self-limiting, excluding immunocompromised patients where can be fatal.

The most common cause of infection is by the consumption of raw or undercooked oysters, sea fishes, shrimps, and other shellfish.

V. parahaemolyticus has many strains, but only those producing a thermostable direct hemolysin (TDH) and/or the thermostable-related hemolysin (TRH) have the ability to cause gastroenteritis (Nishibuchi *et al*., 1992), while only 1-2% of environmental isolates harbor the genes encoding for these two haemolysins (DePaola *et al.*,2003).

V.vulnificus is a natural inhabitant of marine environments (Harwood *et al*., 2004), where it is commonly found when water temperature exceeds 18–20 °C and salinity is between 5 and 25 ppt (Pfeffer *et al., 2*003). Below 10 °C, the bacterium enters a viable but nonculturable state (Oliver 1995).

This species can cause primary septicemia and wound infections, with an estimated mortality rate of up to 60% in immunocompromised patients (Linkous and Oliver, 1999). Wounds may be infected with *V. vulnificus* when exposed to seawater, raw fish, or shellfish even in healthy individuals. Inflammation at the wound site can be observed with localized pain, edema, erythema, and necrosis of the surrounding tissue.

Within *V. vulnificus* have been identified three biotypes:

- biotype 1 - typically associated with human infections;
- biotype 2 - primarily pathogen of marine animals, although also an opportunistic pathogen of humans (Amaro and Biosca 1996);
- biotype 3 - found only in Israel and causing wound infections and bacteremia (Bisharat *et al*., 1999b).

Davis *et al.*,(1981) found atypical strains of V.cholerae, sucrose negative, in human stools from several countries and also from environmental samples, oysters and shrimp, and described *V. mimicus* for the first time. *V. mimicus* is hardly found in the environment at salinities above 10 ppt, being the optimal salinity 4 ppt. Infections occur after ingestion of raw or undercooked seafood, with virulence and clinical signs similar to cholera. All V. mimicus strains, either clinical or

environmental, present the hemolysin gene (vmh) (Shinoda *et al*., 2004). Furthermore, the complete lysogenic filamentous bacteriophage (CTX Φ) and the filamentous phage VPI Φ of *V. cholerae* have been found in some strains of *V. mimicus*, evidencing recent horizontal gene transfer between the two species (Boyd *et al.*,2000). These DNA elements harbor the genes for the cholera toxin (CT) and the toxin-coregulated pilus (TCP), while genes encoding a thermostable direct hemolysin (tdh) and the heat-stable enterotoxin (st) are found only in some strains.

Also *V. fluvialis* causes a "cholerae-like" diarrhea with vomiting, abdominal pain, dehydration and fever, primarily in infant, children and young adult, where consumption of raw seafood has been determined as the infection vector (Klontz and Desenclos, 1990).

V.alginolyticus is a common inhabitant of marine and estuarine environments with a worldwide distribution (Thompson *et al*., .2004). Strains have been isolated from wound, ear and eye infections (Schmidt *et al*., 1979), occurring when seawater got into contact with open wounds or other trauma. Another *Vibrio* that has been isolated from wounds and from the ear infection is *V. cincinnatiensis* (Farmer and Hickman-Brenner, 2006), even if the cases are extremely rare and occur only in immunocompromised people.

V. furnissii is commonly found in aquatic environments, especially in estuaries, but also in fresh water (Thompson *et al*., 2004). It was originally known as has been implicated in food-related gastroenteritis, isolated from humans with diarrheic stools (Dalsgaard *et al*., 1997).

V. metschnikovii rarely causes cases of diarrhea (Dalsgaard *et al*., 1996), but has also been isolated from wound infections (Linde *et al*., 2004). All the infections occurred in elderly people or infants, which highlighted its opportunistic nature.

Photobacterium damselae* subs. *damselae

Strains of *P. damselae* have been isolated from wound infected by seawater or caused by marine animals, which can sometimes develop into a fulminant and fatal

septicemia or necrotizing fasciitis in individuals with predisposing conditions (Rivas *et al*., 2013).

Grimontia hollisae

G. hollisae was originally described as *Vibrio hollisae* from patients with diarrhea (Hickman *et al*., 1982), but evidence of its role as pathogen is still weak. Consumption of raw seafood has frequently been reported as the probable route of infection.

A thermostable direct hemolysin (Vh-tdh), has been detected in *G. hollisae* strains isolated from clinical samples and its gene sequence is homologous (>93% similarity) to the hemolysin genes of *V. parahaemolyticus*, *V. cholerae* non-O1 and *V. mimicus* (Nishibuchi *et al*., 1990).

1.5. Interactions with aquatic animals

Most Vibrionaceae species have been isolated from diseased aquatic organisms, but, obviously, this does not mean that all of them are pathogens. Many Vibrionaceae are, indeed, normally isolated in an association with marine animals, as their facultative anaerobic metabolism allows them to grow under limited oxygen environments, such as in gastrointestinal tracts, muscle and organs.

However, species of genera *Vibrio*, *Aliivibrio* and *Photobacterium* are responsible for severe disease in marine wild and reared organisms, including fish, mollusks, crustaceans, rotifers, and corals. The “red pest,” probably a vibriosis, was described in 1718 by Bonaveri (Drouin de Bouville, 1907) and vibriosis have been recognized in fish since the nineteenth century, when *V. anguillarum* was isolated from diseased eels (Canestrini, 1883).

V. anguillarum, *P. damselae* and *A. salmonicida* appear to be primary pathogens, whereas the other species (such as *V. alginolyticus*, *V. harveyi*, *V. ordalii*, *V. splendidus* , *V. vulnificus*, *V. tapetis* and *V. furnissii*) may present virulent strains that affect organisms under stressing factors, e.g., crowded and polluted environments, or advantageous environmental conditions (Table 1.3).

In 2010, Austin suggested a classification of zoonotic vibrios in two classes named "higher-risk" vibrios (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) and "lower-risk" vibrios (*V. alginolyticus*, *V. fluvialis*, *V. furnissii*, *V. harveyi* and *V. mimicus*) in relation to their involvement in zoonoses.

On the other hands, some *Vibrio* spp. (especially *V. alginolyticus*) have been used as potential probiotic strains to combat other pathogenic vibrios that affect marine cultured organisms (Hjelm *et al.*,2004).

Species	Host
V. alginolyticus	Aquarium fishes
	Groupers (*E. malabaricus*)
	Sea bream (*S. aurata*)
	Scallops (*Argopecten ventricosus and Nodipecten subnodosus*), Penshell (*Atrina maura*), Pacific oysters (*Crassostrea gigas*)
	Scallop (*Argopecten purpuratus*)
V. anguillarum	Eels (*Anguilla anguilla*)
	Many species of fish
V. harveyi	Penaeid shrimp
	Packhorse rock lobster (*Jasus verreauxi*) larvae
	Brine shrimp (*Artemia* sp.)
	Abalone (*Haliotis* spp.)
V. harveyi (prev. *V. carchariae*)	Sharks (*Carcharhinus plumbeus, Negaprion brevirostris, Squalus acanthias*)
V. lentus	Octopus (*Octopus vulgaris*)
V. ordallii	Salmonids
V. parahaemolyticus	Penaeid shrimp
	Brine shrimp (*Artemia* sp.)
	Blue crab (*Callinectes sapidus*)
V. penaeicida	Penaeid shrimp
V. pectenicida	Scallop (*Pecten maximus*)
V. proteolyticus	Brine shrimp (*Artemia* sp.)
V. salmonicida	Atlantic salmon (*Salmo salar*)
	Rainbow trout (*Salmo garderi*)
	Cod (*Gadus morhua*)
V. splendidus	Oyster (*Crassostrea gigas*)
	Scallop (*P. maximus*)
	Rainbow trout fingerlings (*Oncorhynchus mykiss*)
	Flounder (*Paralichthys adspersus*)
	Gilt-head sea bream (*Sparus aurata*)
	Turbot larvae (*Scophthalmus maximus*)
	Corkwing wrasse (*Symphodus melops*)
V. tapetis	Manila clam (*Ruditapes philippinarum*)
	Fine clam (R. *decussates*)
V. tubiashii	Hard-clam (*Mercenaria mercenaria*)
	American oyster (*Crassostrea virginica*)
	Pacific oyster (*P. gigas*)
	Flat oyster (*Ostrea edukis*)
V. vulnificus	Eels (*A. anguilla* and *A. japonica*)

Table 1.3. List of Vibrionaceae causing diseases in aquatic animals (Gomez-Gil et al., 2014).

***Vibrio* spp.**

Within members of the genus *Vibrio*, *V. anguillarum* is the major cause of vibriosis in fish. These organisms have a worldwide distribution and may cause disease in marine, estuarine and freshwater fish. Among the 10 serotypes described for *V. anguillarum*, only serotypes 01, 02, and 03 have been associated with mortality in a great variety of farmed and wild fish (Frans *et al*., 2011). It is not an obligate parasite, but warm weather, particularly when crowding of fish is high, high salinities and organic loads, may lead the bacterium to became a primary pathogen for fish, causing an acute hemorrhagic condition known as "red pest".

This species has been reported to be associated not only to fish "red pest", but also to bivalve larvae mortality episodes in Japanese oyster (*Crassostrea gigas*), *Ostrea edulis* and scallop (Jeffries, 1982).

V. ordalii has been isolated from salmonids, especially in the Pacific Ocean, and was first described as *V. anguillarum* biovar II, but later it was recognized as a distinct species (Schiewe and Crosa, 1981). It induces a pathogenesis similar but usually less severe than *V. anguillarum*.

V. alginolyticus has been implicated in vibriosis or gas gut disease of groupers and sea bream (*Sparus aurata*), with septicemia, hemorrhaging, dark skin and sometimes ulcers on the skin surfaces. Penetration of the bacterium into the fish occurs when the mucus layer is removed and the skin is damaged (Balebona *et al*., 1998). Experimental infections with this species have shown mortalities also in mollusks (Luna-Gonzalez *et al*., 2002)

V. splendidus was originally regarded as an environmental organisms with no pathogenic importance, but it has been later implicated in infections in a wide range of fish, with clinical signs similar to those caused by *V.anguillarum* (Nasfi *et al*., 2015). The biovar II has been reported in the disease called "summer mortality" that affects 6- to 12-month-old oysters in France when the water temperature exceeds 16 °C (Lacoste *et al*., 2001).

V. vulnificus serovar E (formerly biovar 2) is a primary pathogen for eels. It has been isolated for the first time from *Anguilla japonica* in Japan between 1975 and 1977, although it is also found in Europe (Amaro and Biosca, 1996).

V. carchariae (a later synonym of *V. harveyi*) causes shark meningitis and was first observed in brown sharks (*Carcharhinus plumbeus*) in captivity, but it has been proven to infect also other species of sharks experimentally (Grimes *et al*., 1984).

Within crustaceans pathogens, *V. harveyi* primarily infects larval stages of penaeid shrimps. Many strains of this species are luminescent and have been associated with the disease named "luminescent vibriosis", because infected larvae become luminescent due to the massive bacterial colonization (Austin and Zhang, 2006).

V. parahaemolyticus is a common inhabitant of estuarine and marine environments and of healthy shrimp, but it has also been proven to be an opportunistic pathogen that can infect crustaceans under certain conditions (Roque *et al*., 2009). *V. penaeicida* causes a disease first observed in Kuruma shrimp (*Penaeus japonicus*) during 1980 in Japan, but it is also isolated from apparently healthy shrimp and from seawater (Ishimaru *et al*., 1995).

Among molluscan pathogens, *V. lentus* has been associated with diseased wild octopuses (*Octopus vulgaris*) in Galicia, Spain (Farto *et al*., 2003), while *V. pectenicida* has been recently identified as responsible for scallop (*Pecten maximus*) larvae mortalities in France (Lambert *et al*., 1998).

V. tapetis causes the disease named brown ring disease (BRD) that affects the Manila clam (*Ruditapes philippinarum*), first observed in introduced clams in the Atlantic affects larvae and juveniles of bivalve mollusks (Allam *et al*., 2000).

Over recent decades, a dramatic deterioration in coral reef have been documented with 1–2% annual declines of coral cover over broad areas of the Indo-Pacific and greater than 50% cover coral loss of in some regions (Selig *et al*., 2010). Factors contributing to the spread of coral diseases include poor water quality due to

anthropogenic impacts, combined with rising seawater temperatures linked to climate change and increased greenhouse gases. Some vibrios have been shown to cause bleaching on corals: *Vibrio coralliilyticus* (isolated from a bleached coral, *Pocillopora damicornis*, present on the Zanzibar coral reef) and *V. shiloi,* later synonym of *V. mediterranei* (the causative agent of an extensive bleaching of the coral *Oculina patagonica* in the eastern Mediterranean Sea) are emerging coral pathogens that have been associated with coral disease from geographically distinct global regions (Rosenberg *et al.*, 2009).

***Aliivibrio* spp.**

An example of successful adaptations to an ecological niche occur in genus *Aliivibrio*, with the particularly host-specific associations of bioluminescent *A. fischeri* to the light organ of temperate and tropical coastal benthic *Monocentridae* fishes and sepiolid squids *Euprymna scolopes*, small, nocturnal squids living in shallow-water reefs in Hawaii, which is believed to use the light emitted by its bacterial symbionts as a camouflaging behavior called counter-illumination (Ruby, 1996).

A. salmonicida is specifically responsible for the Atlantic salmon condition known as cold-water vibriosis (because it is observed only at low water temperature, normally not above 10 °C) (Colquhoun and Sørum, 2001), hemorrhagic syndrome or Hitra disease (from the island of Hitra in Norway, where heavy losses were recorded since the 1970s (Egidius *et al*., 1981). *A. salmonicida* infects not only the Atlantic salmon (*Salmo salar)* and the rainbow trout (*Salmo gairdneri*), but also the Arcto-Norwegian cod (*Gadus morhua*) (Sørum *et al*., 1990). There are two distinct serotypes of *V. salmonicida*: one more prevalent among non-salmonid species such as cod, but both serotypes are more virulent in salmon than in other fishes (Schrøder *et al*., 1992).

***Photobacterium* spp.**

In the *Photobacterium* genus, *P. phosphoreum* and *P. leiognathi* are well-known symbionts of light organs, and the two subspecies of *P. damselae* are human and/or

fish pathogens. Ecology of *P. phosphoreum* and *P. leiognathi*, and the epidemiology of *P. damselae* have been intensely studied, while there are limited studies on the ecology of *P. angustum* and *P. profundum*.

P. phosphoreum can be found in a variety of marine habitats, fish intestine, light organs, and seawater (Urbanczyk *et al*., 2011). The association of this bacterium with deep-sea fishes has been well. It has been isolated from the light organs of three species of the bathyal fish family *Macrouridae* dwelling from 600 to 1,260 m in the Atlantic and Pacific Oceans and from the midwater species *Opisthoproctus grimaldii* (200–600 m) of the Atlantic Ocean (Ruby and Morin, 1978). The seawater temperature where these deep-sea fishes live varies between 2 °C and 10 °C. Nowadays, it is recognized that *P. phosphoreum* is a facultative light-organ symbiont of midwater or benthic fishes. Interestingly, it has been unexpectedly isolated from the skin of the Chum salmon *Oncorhynchus kisutch*, which migrates up 1,228 km along the Yukon River (Alaska) with a glacial water origin (Budsberg *et al*., 2003).

P. leiognathi is a light-organ symbiont of shallow tropical water ponyfishes (*Percichthyidae*, *Apogonidae* and *Leiognathidae*) of the Indo-West Pacific region. The light organ of these ponyfishes is an internal ring tissue located just anterior to the esophagus. Inside the light organ, *P. leiognathi* cells take the shape of coccobacilli and lack storage granules and flagella, while the free-living bacterial cells are elongated and flagellated with large PHB granules (Dunlap and McFall-Ngai, 1987).

Nonluminescent *P. damselae* includes two subspecies, subsp. *damselae* and subsp. *piscicida*, both known as fish pathogens.

P. damselae subsp. *damselae* (formerly *V. damsela*) was first isolated from skin ulcer on a temperate-water damselfish, the Blacksmith (*Chromis punctipinnis*). Strains have later been isolated from a variety of other fishes, mollusks, marine reptiles and marine mammals (Osorio *et al*., 2000).

P. damselae subsp. *piscicida* (formerly *Pasteurella piscicida*) is a fish pathogen causing disease known as pasteurellosis (because the previous name of this pathogen) (Romalde, 2002), or also named as bacterial pseudotuberculosis, responsible for major outbreaks among a wide number of cultured marine fishes. Pasteurellosis was first observed on the east coast of the United States in 1963 affecting the white perch (*Roccus americanus*) and the striped bass (*Morone saxatilis*), then, it appeared for the first time in the Mediterranean coast of Europe in 1990, being diagnosed in cultured population of sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) in France, Italy, Spain, Portugal, Malta, and Croatia, as well as in Turkey and Israel (Thyssen *et al.*,2000). This pathogen has a worldwide distribution with variety of marine fishes as natural hosts. The high degree of sequence similarity (>99 %) of rRNA genes (5S, 16S, and 23S) and the intergenic spacer region (ITS) between the two subspecies of *P. damselae* are unlikely to develop subspecies-specific identification tools.

Grimontia, Salinivibrio* and *Enterovibrio

Except for *Grimontia*, a human pathogen, ecology of genera *Salinivibrio* and *Enterovibrio* is not fully understood.

Grimontia hollisae (formerly *V. hollisae*) has been isolated not only from stool cultures of patients with diarrhea (Hickman *et al*., 1982), but also from the marine environment. Both clinical and environmental isolates produce a thermostable direct hemolysin (TDH) related to the hemolysin of *V. parahaemolyticus* (Lin *et al*., 2013).

Salinivibrio costicola includes subsp. *costicola*, subsp. *vallismortis* and subsp. *alcaliphilus*. All subspecies are probably free-living in saline or hypersaline environments or associated to salt crystals; there are no reports of associations with animal or plant hosts.

S. costicola subsp. *costicola* can be isolated from solar saltern, salted foods and brine as a moderate halophilic vibrio with 3–15 % salt for optimal growth (Mellado *et al*., 1996).

S. costicola subsp. *vallismortis* was isolated from sediments collected at the Death Valley, California (Huang *et al*., 2000), while *S. costicola* subsp. *alcaliphilus* was isolated from a salty spring in the Campania region of Italy (Romano *et al*., 2005). Species-specific identification for *Salinivibrio* has been not fully established.

Members of the *Enterovibrio* genus have been isolated from marine organisms: *E. norvegicus* from healthy turbot larvae in an aquaculture station in Norway (Thompson *et al*., 2002) and *E. coralii* from the bleached coral *Merulina ampliata* (Thompson *et al*., 2005). Ecology of both species of *Enterovibrio* is practically unknown.

1.6. Detection, isolation and identification

The *Vibrio* population exhibits a high degree of inter and intra-species variability, characterized by a diverse range of serovars and genotypes within the same species (Dikow and Smith, 2013) and genetic variation between virulent and avirulent strains (Busschaert *et al*., 2014), that can be used to screen samples for potentially virulent vibrios. For example, the thermolabile direct hemolysin (*tdh*) of *V. parahaemolyticus* is more commonly found in clinical isolates and this gene is often used to mark virulence in this species (Paydar *et al.*, 2013). Indeed, the control and prevention of *Vibrio* infections is largely based on the screening of water and seafood for the presence of pathogenic species and genes responsible for the pathogenesis. Unfortunately, not all clinical isolates elaborate the more common virulence genes and the occurrence of the pathogens is not spatially or temporally static, varying greatly among localities and seasons (Gennari *et al.*, 2012), thus the knowledge of their ecology is the first step for a proper and early surveillance. On the other hand, to achieve a better understanding of the ecology of these species, it is important to determine their phenotypic features.

Traditional microbiology commonly employs two types of media to enrich pathogens and isolate vibrios. Alkaline peptone water (APW) (1% peptone, 1% NaCl, pH 8.5) is a common enrichment broth for vibrios, while thiosulfate citrate

bile salts sucrose (TCBS) agar is routinely used to selectively isolate and enumerate Vibrionaceae. These media use an alkaline pH, bile and a moderate salinity to promote vibrio growth and inhibit non-vibrio species (Harwood *et al.*, 2004). Unfortunately, both APW and TCBS lack selectivity for other gram-negative members of distantly related bacterial groups (including *Flavobacterium*, *Pseudoalteromonas* and *Shewanella*) may present growth as well (Nigro and Steward, 2015).

Even if the plating assay on selective medium is a simple tool to detect presumptive vibrios in aquatic environments, molecular approaches are essential to obtain the most accurate vibrio abundance estimates and identification from environmental samples. Isolation media, indeed, were originally developed to isolate pathogenic *Vibrio* species from clinical sources which are less complex than environmental ones. Recently, more selective media have been developed for the isolation of specific vibrios, especially pathogenic species, from environmental samples (Froelich *et al.*, 2014), but APW and TCBS still remain the preferred choice.

Historically, a combination of biochemical and physiological characteristics has been employed to confirm the identity of a *Vibrio* species isolated both from clinical or environmental samples. Regardless taxonomic aspects, indeed, members of the Vibrionaceae exhibit an extreme diversity, with a wide array of phenotypic traits, versatile metabolism and unique physiological features. Classical biochemical identification and dichotomous keys have been used to try an identification of vibrios (Alsina and Blanch, 1994; Noguerola and Blanch, 2008), despite numerous limitations due to the wide phenotypic variability within the same species. Such phenotypic characterization typically requires long times is expensive and often restricted to a limited number of species, as most of the commercial systems applied for phenotypic characterization are not able to identify the broad spectrum of environmental strains.

O'Hara *et al.,* (2003) evaluated six commercial systems for the ability to identify the 12 species of *Vibrio* and found that commercial kits have an accuracy ranging from

63.9% to 80.9%. It may also depend on the fact that most Vibrionaceae requires the addition of NaCl for their enzymatic activities and it can affect the biochemical profile and lead to erroneous identification with API 20E system (Martinez-Urtaza *et al.*, 2006). It has then become evident that the phenotypic identification of vibrios is problematic, particularly for some sister specie such as *V. cholerae*-*V. mimicus*, *V. coralliilyticus*-*V. tubiashii*-*V. brasiliensis*, *V. alginolyticus*-*V. parahaemolyticus*-*V. natriegens*, and *V. harveyi*-*V. campbellii*, which have nearly indistinguishable phenotypes (Amaral *et al*., 2014).

Alternately, in the last three decades, the use of molecular techniques such as ribotyping, amplified fragment length polymorphism (AFLP), repetitive extragenic palindromes (rep), multilocus enzyme electrophoresis (MLEE) and later on multilocus sequence analysis (MLSA), polymerase chain reaction (PCR) and nucleotide sequence determination, overcame many of the limitations of phenotypic methods and provide an unambiguous identification, especially in environmental samples. In addition, distinction between virulent and avirulent strains can be achieved targeting species-specific genes and/or virulence-associated genes (Lipp *et al.*, 2003). PCR primers, indeed, have now been developed for specific detection of a range of targets (species, serogroup, toxin). Such methods can lead to identification of an isolate within hours and can be used on small quantities of cells, including those that are not viable or are otherwise unculturable.

Environmental detection, indeed, can be complicated when bacteria entre the viable-but-non-culturable state (VBNC), non-detectable by conventional food and water testing methods, which may lead to an underestimation of the environmental *Vibrio* species. The failure to consider VBNC cells could result in an underestimation of risk based on the environmental prevalence of a pathogen. It is worth noting, indeed, that although the infectivity of VBNC cells is still controversial, it has been observed that different pathogenic bacteria display virulence attributes even in the VBNC state. Baffone and coworkers (2003) found that *Vibrio alginolyticus* and *Vibrio parahaemolyticus* VBNC cells lose virulence after their resuscitation in the

mouse, but it could be restored after two consecutive passages of the strains in the rat ileal loop.

Therefore, methods based upon direct detection by PCR are the most accurate, as they have proven a highly sensitive approach to detect both viable and non-culturable cells (Lipp *et al.*, 2003). A rapid and accurate molecular method that identifies multiple species in one assay (such as multiplex PCR) is a useful tool to enhance surveillance and diagnosis of Vibrionaceae.

1.6.1. The multiplex PCR method

PCRs have been used for the detection of pathogen Vibrionaceae for years, but a major limitation of the single PCR assay is that usually only a single species or gene is detected. The multiplex PCR (mPCR) enables simultaneous amplification of many target bacteria in a single polymerase chain reaction, with multiple pairs of primers. Of course, the expense of reagents and preparation time is less in multiplex PCR than in systems of uniplex PCRs; however, mPCRs must be developed with careful consideration for the regions to be amplified, the relative sizes of the amplified fragments, the dynamics of the primers and the optimization of the amplification conditions.

Since its first description in 1988 (Chamberlain *et al.*, 1988), this method has been successfully applied in many areas of DNA analysis and now multiplex PCR is firmly established as a general technique. Its applications in Microbiology include pathogen identification, species and biotypes screening as the end point of analysis, or preliminary to further analyses such as sequencing or hybridization.

Regions selected for multiplex amplification depend on the nature of the analysis; for example, microbial assays may exploit strain- or species-specific variation in marker genes to distinguish similar templates, such as bacteria strains. For identification purposes in Microbiology, conserved housekeeping genes are usually chosen as target gene of the mPCR, as they are present in all isolates of a species.

Detailed sequence information for the selected target gene is important, because nonspecific amplification may occur at other sites with similar sequences, or reduced amplification may occur at primer-template mismatched sites. If the multiplex product is to be resolved electrophoretically, fragment sizes should be selected so that they may be separated easily from each other. At the same time, the range of band sizes should not be so wide that all fragments cannot be resolved well on the same gel.

Primer sequences should be designed so that their predicted hybridization kinetics are similar in all the primers in the multiplex reaction. A G/C content of 40-60% and a length of 18-28 nucleotides are suggested as general guidelines for specific annealing at moderate temperatures (Edwards and Gibbs, 1994). Primer annealing temperatures and concentrations may be calculated *in silico* to some extent, but conditions will have to be refined empirically for each set of primers individually and modified if necessary as primer sets are added. The possibility of nonspecific priming and other artifacts is increased with each additional primer. If equimolar primer concentrations do not yield uniform amplification for all fragments, the concentration of some primer pairs can be reduced. When all primer pairs are not compatible it may be necessary to subgroup them in smaller multiplexes.

It is already reported on a number of studies as a valuable tool for the detection of many different bacteria, but these works usually targeted only specific strains, such as human or animal pathogenic vibrios (Kim *et al.*, 2012; Tsai *et al.*, 2012).

1.7. Current status and future directions

To date, no isolation media can reliably identify and quantify the presence of pathogenic *Vibrio* species without further confirmation using molecular techniques. However, even molecular techniques underestimate the actual number of pathogens in food and environmental samples. Techniques, such as PCR and real-time PCR assays, indeed, can return more accurate data in shorter time, but there may be false

positives due to the presence of dead cells, small sample size and the inhibition of DNA polymerase in complex environmental samples (Lipp and Rose, 1997; Harwood *et al.*, 2004).

Given the growing number of infections caused by *Vibrio* species, a more comprehensive approach is needed to safeguard the public from these pathogens.

Currently, bacteriological standards (fecal coliform bacteria and enterococcus) are used as indicators of marine water quality as a proxy for fecal contamination (Karydis and Kitsiou, 2013). However, such indicators are not predictive of non-enteric microbial threats such as pathogenic Vibrionaceae (Lipp and Rose, 1997). At the same time, the European Community (regulation CE 2073) did not adopt specific microbiological criteria for vibrios in seafood, even if the legislation recommended developing new reliable methods for risk assessment related to Vibrionaceae, especially in shellfish.

Thus, at the moment, public health guidelines for marine waters fail to predict the presence of naturally occurring microbial pathogens, of which Vibrionaceae represent a greatest percentage. The accurate and reliable identification of these bacteria, indeed, is important to assess public health risks and to monitor other potential problems for humans and animals linked to the presence and distribution of this family in the environment,

An improvement of the identification methods may also lead to a deeper knowledge of the ecology of these species. Combining their ecology with environmental conditions and environmental factors would allow to alert risk prior to the outcome, thanks to predictive models. Of course, accurate models will require a clearer understanding of how complex factors such as climate change, anthropogenic disturbances and global transport may result in an changes in the prevalence, diversity and distribution of pathogenic *Vibrio* species.

2. BACKGROUND AND RELATED WORKS

Species diversity, spatial distribution, and virulence-associated genes of culturable vibrios in a brackish coastal Mediterranean environment [1]

As described in sections "Public health aspects and disease spectrum" (1.4) and "Interactions with aquatic animals" (1.5), some species belonging to *Vibrio* genus, including *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, are a recognized cause of even severe human infections. Many other species found in the aquatic environment and defined as halophilic 'non-cholera vibrios' (NCVs), such as: *V. alginolyticus*, *V. anguillarum*, *V. harveyi*, *V. fluvialis*, *V. furnissii*, *V. metschnikovii* and *V. mimicus*, are known as mainly marine animal pathogens.

Although the environmental isolates usually lack the virulence-associated genes found in the clinical ones, recent studies showed that they can also carry such genes, acquired through horizontal transfer events (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. Besides 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). The ability to enter a viable but not culturable state prolongs their survival in the environment, especially during winter (Maugeri *et al*., 2004). Observations collected over a range of environmental

[1] Data, tables and figures presented in this chapter are adapted from Matteucci *et al*. (2015), Annals of Microbiology **65**:1-11 (Annex 1)

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 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 there are limited data on the free-living *Vibrio* populations along the Italian Tyrrhenian coasts, and about their correlation with environmental factors.

Macchiatonda coastal ponds are brackish lakes located in a temperate area of the Italian Tyrrhenian coast and subjected to seasonal changes in temperature and wide fluctuations of salinity. Although brackish environments are regarded as restrictive due to the environmental factors, vibrios are commonly found in these habitats, where salinity and temperature gradients can significantly influence their abundance and distribution.

Therefore, Macchiatonda ponds were considered ideal for evaluating the effects of natural fluctuations of temperature and salinity on *Vibrio* populations, and the study aimed at investigating for the first time the occurrence, diversity and distribution of culturable *Vibrio* species in these habitats, and their correlation with both temperature and salinity variations. Moreover, the occurrence of virulence-associated genes was evaluated.

2.1. Temperature and salinity ranges in the sampled ponds

Macchiatonda wetland (Figure 2.1) offers the unique feature of the coexistence of three ponds with different salinity degrees but substantially similar temperature at each sampling time, making it possible to compare the effect of salinity besides the seasonal temperature shifts. The sampling stations have been chosen to warrant the most varying environments: station 1 was in the artificial pond *Didattico* of constant depth: about 1.5 mt when completely filled. Stations 2 and 3 were placed along the

net of ditches of *Alberobello* (max depth about 60 cm) and station 4 into the major water hole (max depth about 90 cm). Two stations have been established in *Piscinula*: Station 5 in the shallower area (max depth 60 cm) and station 6 in the deeper one (max depth 1 mt). A seventh station has been placed in the costal seawater in front of the ponds, as a control site with seasonal temperature shift and the constant salinity typical of the Mediterranean Sea.

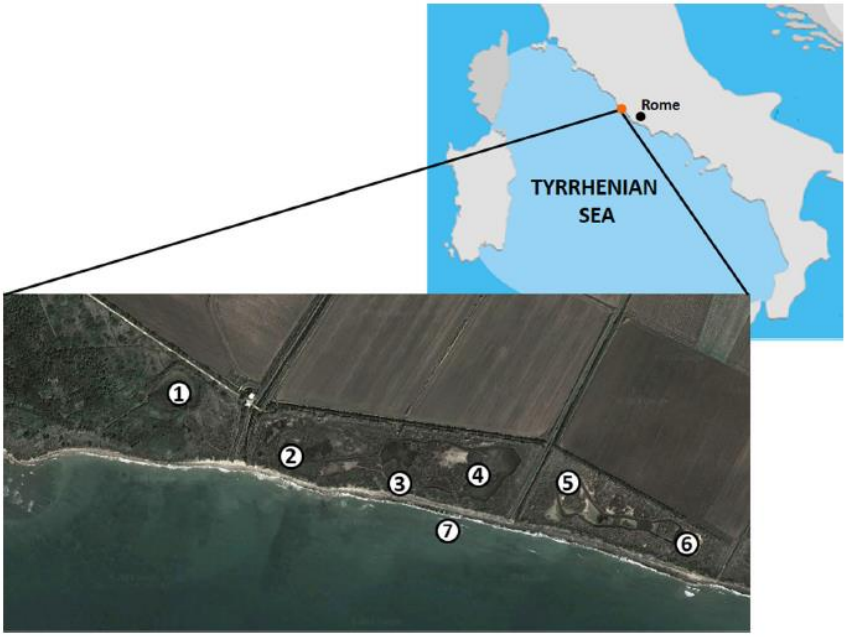


Figure 2.1. Sampling sites: 1, *Didattico* artificial pond; 2, 3, 4, *Alberobello* pond; 5, 6, *Piscinula* pond; 7, coastal seawater in front of the Macchiatonda wetland.

Water temperature ranged from a minimum of 2.8 °C to a maximum of 29.3 °C with a clear seasonal trend, and no significant differences among ponds (Kruskal-Wallis, p=0.99). Seawater was markedly less cold than the ponds in winter, 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 range, that was rather constant in both *Didattico* and *Alberobello. Piscinula*, which is more exposed to dilution in rainy months and evaporation in summer, particularly in the shallow station 5, underwent to wider and abrupt fluctuations (Table 2.1). In September, salinity dramatically raised 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 the station 6 was sampled, as the station 5 was completely dried.

Station	September		November		December		February		March		May		June		July	
	° C	ppt	° C	ppt	° C	ppt	° C	ppt	° C	ppt	° C	ppt	° C	ppt	° C	ppt
1	25.6	22.0	17.6	10.9	4.2	12.1	7.9	11.9	14.3	6.5	22.5	6.6	26.5	6.63	27.7	11.9
2	23.2	42.7	17.5	17.4	4.9	21	7.8	17.0	16.0	13.9	22	16.1	27.9	21.4	28.9	30.0
3	23.2	41.8	17.2	17.6	5.7	25.2	8.1	17.7	15.4	16	23.8	15.6	28.3	21.5	29.1	30.5
4	23.0	47.1	16.9	20.9	4.0	27.3	7.6	18.2	14.3	16.9	21.8	16.3	28.1	19.5	28.8	30.0
5	23.4	51.2	16.5	16.9	3.4	18.5	7.4	12.4	14.5	8.96	21.8	9.36	28.3	9.72	29	25.3
6	22.8	54.5	19.5	13.1	2.8	18.8	7.5	12.2	14.0	9.1	21.0	9.36	29	9.17	29.3	25.4
7	24.1	37.8	17.8	37.5	12.0	37.2	11	36.7	16.2	37.6	19.8	37.6	25.8	38.4	26.6	37.7

Table 2.1. Temperature and salinity values in the sampling stations

2.2 *Vibrio* counts and correlations with temperature and salinity

CFU counts on TCBS ranged from 20 CFU ml^{-1} in *Didattico*-March and *Piscinula*-May, up to $6.92*10^3$ CFU ml^{-1} in *Piscinula*-September, following a general seasonal trend consistent with the ecology of vibrios (Fig. 2). In the sea station the counts ranged from a minimum of 60 CFU*ml^{-1} in December and February, to a maximum of $2.1 *10^3$ CFU ml^{-1}in November.

CFU abundance (Figure 2.2) was high in September and November, minimal during the winter months, and raised 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 (Kruskall-Wallis, p=0.25) were significant.

A significant positive correlation was found between CFU counts and water temperature (Spearman's rs=0.67; p(uncorr)= 0.002) up to 25.6 °C; at higher temperatures the correlation turns to be negative (Spearman's rs = -0.63; p(uncorr)= 0.07).

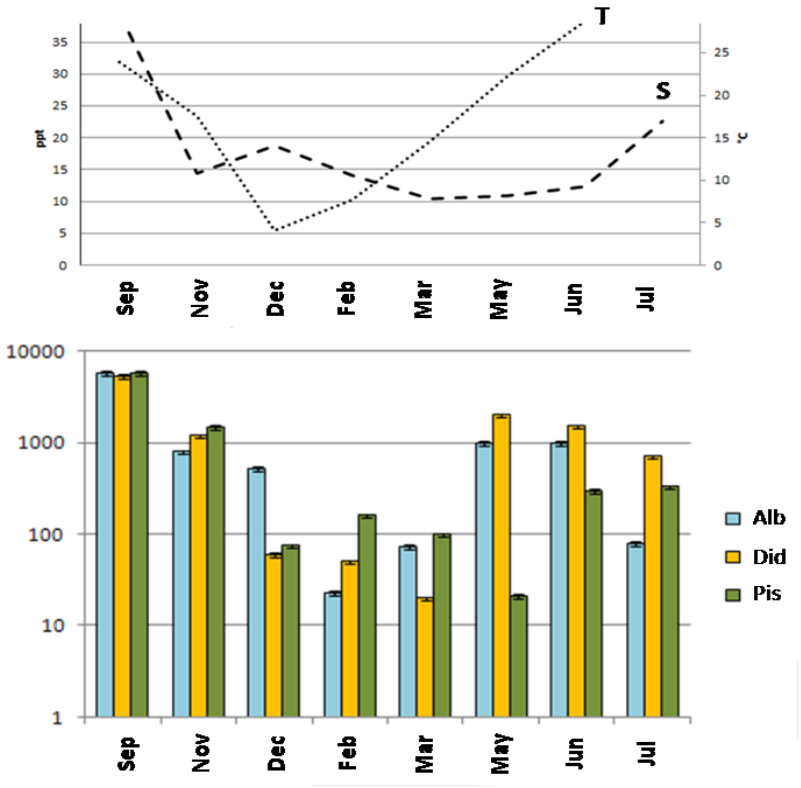


Figure 2.2. A) mean seasonal values of temperature and salinity in the Macchiatonda wetland; B) Mean CFU abundance with confidence intervals 95%
Alb: Alberobello; Did: Didattico; Pis: Piscinula

Both temperature and salinity, however, influenced the CFU number variations. By incorporating CFU number, temperature and salinity values, indeed, 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 2.3).

	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

Table 2.3. Multiple Linear Regression model values.

According to these data, CFU abundance on TCBS in the brackish ponds depends on 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.

2.3. Diversity and distribution of *Vibrio* species

From 56 water samples obtained, a total of 130 Gram-negative, cytochrome-oxidase positive and glucose fermenting isolates were selected: 109 were from the brackish ponds and 21 from seawater. Sequencing of amplicons obtained with genus-specific primers within *recA* gene, allowed to identify them as belonging to 20 named species and four unnamed groups within the genus *Vibrio* (Table 2.4).

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 FAL1533, two unclassified strains from estuarine waters, loosely clustering with *V. lentus* and *V. pacinii*, respectively (Preheim *et al.*, 2011).

Table 2.4. *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

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

Out of the identified species, only *V. alginolyticus, V. anguillarum, V. kanaloae, V. parahaemolyticus* and *V. 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.

Vibrios presence in sea waters is often reported as temperature- (upon/below 15 or 17 °C) and salinity-related.

In some brackish environments as theMacchiatonda 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: to better appreciate them, the temperature and salinity observed values have been categorized within discrete intervals to form environmental classes (T/S classes), as follows.

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;

D) 21–26 °C, the cooler and the warmer intervals within the permissive temperature range, respectively;

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;

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;

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 (Figure 2.3). The distribution of *Vibrio* species, along the T/S actually occurring conditions and yielding *Vibrio* isolates, is also shown in Figure 2.3.

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 *V. 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*, *V. cyclotrophicus*, *V. lentus*, and the unnamed isolate similar to the FALF 1533 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*, *V. diabolicus*, *V. litoralis* and unnamed isolate homologous to FALF 273 were scattered along different salinity ranges, whilst *V. harvey*, *V. diazotrophicus*, *V. cholerae*, *V. vulnificus*, *V. 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.

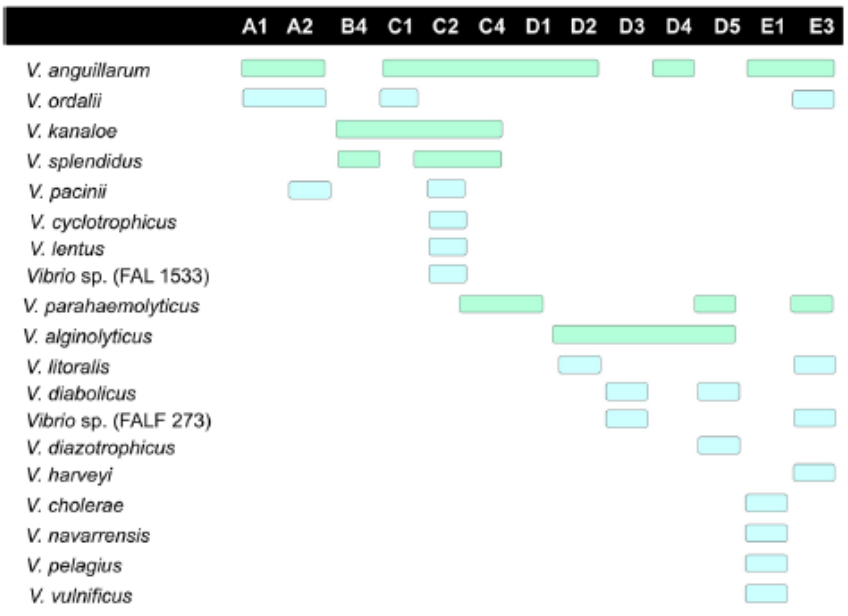
Within the last cluster, the human pathogen *V. cholerae* is well known to prefer low salt and warm conditions (Colwell *et al*., 1977; Takemura *et al.,* 2014) and was indeed isolated only from warm, poorly salted waters (E1 class) in Didattico and in Piscinula, together with *V. vulnificus* that was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

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 CAIM 1833 and MWB 21 strains were found above 20 °C, (D4) in June or May.



		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*	§	§	§	§	§

Figure 2.3. *Vibrio* species diversityalong the Temperature/Salinity (T/S) classes. §: combination of temperature and salinity not occurring in the field. Alb:Alberobello pond; Did: Didattico pond; Pis: Piscinula pond; Tyr: Tyrrhenian Sea. Cyano: Only ponds
Green: Ponds and sea

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

All the isolates from brackish and marine waters were screened for a battery of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* virulence-associated genes. Molecular methods were used to detect pathogenicity genes including *ctx*, *ace*, *tcpA*, *tdh*, *trh*, *vvhA*, *vllY*, and *toxRS*,

A total of 73 isolates, 61 from brackish and 12 from marine environment, were found positive for one or more determinants as shown in Table 2.5.

Table 2.5. 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
Vibrio sp. MWB 21				1			
V. splendidus						1	

The most frequently encountered virulence-associated gene was *toxR*, detected both as a unique determinant and in association with others. The distribution pattern of virulence genes we found in Macchiatonda isolates is consistent with the data by Sechi *et al.*, (2000) on environmental vibrios from Sardinia, with *toxR* and *toxS* genes being the most common virulence determinants, and *ace* and *zot* only rarely found. The search for the genes more strictly associated to *V. cholerae* and *V. vulnificus*, as *ctx, vvha,* and *vllY* did not yield positive results within the Macchiatonda isolates belonging to these species; the amplification of either *tdh* or *trh*, in *V. parahaemolyticus*, was

observed only twice. These data are in agreement with the studies of Nigro *et al.,* (2011) who observed that virulence-associated genes are rarely found in the environmental isolates of the three human pathogenic species.

Although *vvha* has been demonstrated to belong to the core genome of *V. vulnificus* (Morrison *et al.*, 2012) its sequence differs in clinical and environmental strains, dividing the species in a group1, exclusively pathogen for humans, and a group2, pathogen for eels but only occasionally associated to human diseases (Senoh *et al.*, 2005); the failure to amplify the hemolysin-encoding genes with the primers specific for the first group, shows that the Macchiatonda isolates are to be included among the non-pathogenic ones.

The negativity in *vllY* amplification was rather surprising, as both Wong *et al.* (2005) and Bier *et al.* (2013) regarded *vllY* as too frequently found to be discrimination tool among *V. vulnificus* strains. It must be noticed, however, that these authors used a different primer pair. In this work the primers originally proposed by Chang *et al.* (1997) were used; these primers allowed to amplify *vllY* from about 40 strains in the first study and yielded positive results in subsequent ones (e.g. Baffone *et al.*2006). However, a search in the database available sequences (five) revealed three mismatches in the sense primer and two gaps in the antisense one (data not shown). As a consequence, sequence heterogeneity can be speculated in the outer regions of this operon.

In *V. parahaemolyticus*, *tdh* and *trh* were scarcely found in the Macchiatonda isolates so assigning most of the isolates to the environmental ones (Ceccarelli *et al.* 2013; Robert-Pillot *et al.*, 2004); however the frequency of virulence-positive isolates within this species was high and seven different virulence-associated profiles were observed, including ctx, *toxR, toxS, tcpA, tdh, trh* and *ace*, as unique determinants and/or in association. Moreover, one isolate from *Piscinula*, September, was positive for *tdh*, *toxR* and *toxS* genes, the typical genetic markers of the pandemic groups (Ottaviani *et al.*, 2013).

This evidence is in agreement with the reports on the isolation of *V. parahaemolyticus* pandemic strains from marine water sample and on gastrointestinal infections caused by the O3:K6 serotype in Italy (Caburlotto *et al.*, 2008; Ottaviani *et al.*, 2010).

On the other side, we have found virulence determinants typical of the human pathogens, also in the "non-pathogenic" vibrios, which were the majority of the autochthonous culturable Macchiatonda vibrio community; the proportion of virulence-gene positive populations in our samples being consistent with other analysis on the spreading of such genes among environmental free-living vibrios in the Mediterranean area (Gennari *et al.*, 2012; Sechi *et al.*, 2000).

A particular mention should be reserved to the *ctx* positive reactions obtained from one isolate of *V. parahaemolyticus* and one of *V. alginolyticus*: the first a recognized and the second a possible pathogen for humans.

The occurrence of *tdh* and *trh* hemolysin genes, in species other than *V. parahaemoliticus*, was particularly frequent in *V. kanaloae*, being found in six strains isolated in November, all from different stations. As *V. kanaloae* is often associated to 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 hemolysins in this species should be kept in mind whenever considering the risk of sea-food borne diseases.

The *V. vulnificus* hemolysins-encoding genes *vvhA* and *vllYA* were rarely and randomly found *in V. anguillarum, V. diabolicus* and in a MWB21-like isolate. Although we found no horizontally transferred traits in our isolate, the presence of *V. navarrensis* in the same low salted warm waters preferred by *V. cholerae* and *V. vulnificus* suggests to survey its diffusion in the brackish environments and on its possible pathogenic role. *V. navarrensis,* indeed, is endowed of protease, phospholipase, lipase and β-hemolytic activity (Silva *et*

al., 2013) that, according to Zhang and Austin (2005) would suggest a potential virulence.

2.6. Discussion

In this study, we evaluated the correlation between temperature and salinity and the diversity and abundance of *Vibrio* populations in the Macchiatonda Nature Reserve. To our knowledge this is the first report analyzing the vibrios communities in a brackish system in this area, in Italy. Moreover, Macchiatonda is a unique environment in terms of consisting of three shallow coastal basins, very close one another but differing mostly for salinity, and where the environmental factors can sometimes fluctuate suddenly within few days, due to tides, rain and evaporation. Such a peculiar site can help to better understand the role of temperature and salinity variations on the distribution of *Vibrio* spp.in a temperate Mediterranean brackish area with an unprecedented contribution.

As a whole, our findings confirm the studies that cite temperature as a significant driver of free-living *Vibrio* concentration in Mediterranean coastal environments and brackish waters (Maugeri *et al.*, 2000; Baffone *et al.*, 2006; Covazzi-Harriague *et al.*, 2008; Vezzulli *et al*., 2009; Caburlotto *et al.*, 2012). Furthermore, data on the occurrence of culturable *Vibrio* species in 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.

The role of these brackish coastal basins as a possible reservoir for *V. anguillarum* must therefore be kept in mind, as this species 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), in view of the 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.

To this extent, the retrieval of *V. kanaloae* strains endowed of the *V. parahaemolyticus* hemolysin encoding-genes and found mainly in autumn, deserves to be mentioned. 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. In the Macchiatonda reserve such a role could be easily played by *V. anguillarum* as many isolates have been found positive for the presence of some virulence-associated genes typical of the human pathogenic species and could help their circulation even trough environmental conditions unfavorable to the pathogens.

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. As many brackish environments, Macchiatonda is the place for both educational and recreational activities, so that the presence of vibrios regarded as environmental, but endowed of virulence determinants, could be a risk as suggested by the increase of seawater-related infections, mainly wounds, experienced in Europe, even in the Northern regions since the mid-1990s (Baker-Austin *et al.*, 2012; Boer *et al.*, 2013). In addition, as a consequence of the thermo-dependant virulence of most of these bacteria, there is substantial evidence that *Vibrio*-associated diseases are increasing worldwide with climate warming.

The difficult phenotypic and genotypic identification of vibrios greatly limits our understanding of *Vibrio* ecology, the estimation of the incidence of *Vibrio* infections, and the evaluation of the associated risk. Therefore, the development of a suitable and rapid assay to properly detect these microorganisms in the environment

is an important mission in Environmental Microbiology, and studies in this direction are greatly welcome.

3. MATERIALS AND METHODS

3.1. Bacterial cultures and DNA template preparation

A total of 84 vibrio isolates were used to validate the assay, representing 27 named species and including *Vibrio*, *Aliivibrio* and *Photobacterium* genera.

62 strains of *Vibrio*, were obtained from a 2010-2011 sampling campaign in Macchiatonda Nature Reserve coastal ponds, located on the Tyrrhenian coast, 50 km North of Rome (Matteucci *et al*., 2015). All the strains, belonging to various *Vibrio* species, previously underwent complete phenotypic analyses, according to the identification key proposed by Alsina and Blanch (1994), and molecular identification at the species level was obtained by amplifying and sequencing a 739 bp fragment of *recA* gene, (Thompson *et al.*, 2004) and a 1481 bp fragment of *rpoB* gene *(*Ki *et al.*, 2009), highly conserved regions encoding for two housekeeping proteins (DNA recombinase A and beta subunit of the RNA polymerase, respectively) (Table 3.1, Table 3.2).

Gene	Reference	Primers
recA	Thompson *et al*., 2004	FW 5'-TGGACGAGAATAAACAGAAGGC- 3' REV 5'-CCGTTATAGCTGTACCAAGCGCCC-3'
rpoB	Ki *et al*., 2009	FW 5'-AACATCGGTCTGATCAACTC-3' REV 5'-ACACCCTTGTTACCGTGACGACC-3'

Table 3.1: primer used for the molecular identification of Macchiatonda isolates.

Target Gene	PCR conditions Temperature (°C)	Time	N. of cycles
recA	95	5 min	
	95	45 sec	3
	55	2 min	
	72	1 min	
	95	20 sec	30
	55	1 min	
	72	1 min	
	72	5 min	
rpoB	95	3 min	
	95	20 sec	35
	55	30 sec	
	72	1.5 min	
	72	5 min	

Table 3.2: PCR conditions for the molecular identification of Macchiatonda isolates

The amplification products were sequenced by Macrogen Europe (The Netherlands) with an automated capillary sequencing.

22 additional reference strains, including isolates belonging to genus *Photobacterium* and *Aliivibrio*, were kindly provided by H. Sørum, Dept. of Food Safety and Infection Biology of the Norwegian School of Veterinary Sciences, Oslo.

Strains used to validate our multiplex PCR assays are listed in Table 3.3 and detailed in the Supplementary Table 1.

Every strain has been kept at -80°C in Brain Heart Infusion (BHI) medium + 20% glycerol.

From the cryopreserved suspensions, every strain has been streaked for purity on Thiosulfate-Citrate-Bile-Sucrose (TCBS) agar and incubated overnight at species-specific temperature (10 °C, 21 °C, 35 °C). Isolates have then been streaked on non-selective medium Tryptone Soy Agar (TSA) added with 2% NaCl, in order to avoid possible inhibition effects of the bile salts contained in the TCBS agar on DNA amplification.

Species	n. strains	Species	n. strains
A. fischeri	1	*V. fluvialis ATCC 35016*	1
A. salmonicida LFI 1226	1	*V. furnissii*	1
A. salmonicida PT2	1	*V. harveyi*	7
P. damselae subs. *damselae*	1	*V. kanaloe*	9
P. iliopiscarius	1	*V. mediterranei*	1
V. agarivorans	3	*V. metschnikovii ATCC 7708*	1
V. alginolyticus	5	*V. nigripulchritudo*	1
V. anguillarum O1	1	*V. ordalii NCMB2169*	1
V. anguillarum AL 102 (O1)	1	*V. ordalii NCMB2167*	1
V. anguillarum O3	1	*V. ordalii*	5
V. anguillarum	12	*V. pacinii*	2
V. carchariae	1	*V. parahaemolyticus*	6
V. cholerae	6	*V. splendidus*	2
V. cincinnatiensis ATCC 35912	1	*V. tubiashii*	1
V. cyclitrophicus	1	*V. vulnificus*	4
V. diabolicus	2	*V. wodanis*	1
V. diazothrophicus	1		

Table 3.3: list of strains and number of isolates used to test the multiplex PCR method developed in this study.

Template DNA has been extracted through repeated boiling (95 °C/5 min) and freezing (-20 °C/5 min) cycles. Cell debris were discarded by centrifugation for 5 min at $10\,000 \times g$ and 200 µl supernatants containing bacterial DNA extract were transferred to a fresh tube and stored at -20 °C to be used as template for the multiplex PCRs.

3.2. Sequence alignment and primer design for multiplex PCRs

New sets of genus- and species-specific primers were designed based on the comprehensive analyses and alignments of the Vibrionaceae genomes available in GenBank database of Microbial Genomes (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/genomes/MICROBES.html; updated as of March, 2013). A total of 50 Vibrionaceae genomes were available at the moment of the consultation, mainly belonging to *Vibrio* genus (Table 3.4). No genomes or sequences for the selected genes were available for *Salinivibrio* genus.

Strain	GenBank accession n.	Strain	GenBank accession n.
A. fischeri ZF-211	AJYI01000014.1	*V. cyclitrophicus* ZF14	AIDH01000092.1
A. salmonicida LFI1238	NC011312.1	*V. fischeri* MJ11	CP001139
E. calviensis 1F-230	AJYH01000181.1	*V. furnissii* NCTC 11218	CP002377
E. calviensis FF-85	AJYF01000150.1	*V. furnissii* CIP 102972	ACZP01000018.1
E. norvegicus FF-33	AJYD01000004.1	*V. ichhtyoenteri* ATCC 700023	AFWF01000319.1
E. norvegicus FF-454	AJWN01000101.1	*V. harveyi* ATCC BAA-1116	CP000789
G. hollisae CIP 101886	ADAQ01000008.1	*V. kanaloe* 5S-149	AJYX01000006.1
P. angustum S14	AAOJ01000024	*V. metschnikovii* CIP 69.14	ACZO01000005.1
P. damselae subs. damselae CIP 102761	ADBS01000001.1	*V. mimicus* SX-4	ADOO01000009
P. profundum SS9	NC006370.1	*V. nigripulchritudo* ATCC 27043	AFWJ01000013.1
P. profundum 3TCK	AAPH01000056	*V. ordalii* ATCC 33509	AEZC01000085.1
V. alginolyticus 40B	ACZB01000006	*V. orientalis* CIP 102891	ACZV01000003.1
V. anguillarum 775	CP002284	*V. parahaemolyticus* AQ3810	AAWQ01000244
V. anguillarum RV22	AEZB01000076.1	*V. rotiferianus* DAT722	AFAJ01000015.1
V. anguillarum 96F	AEZA01000180.1	*V. rumoiensis* 1S-45	AJYK01000108.1
V. brasiliensis LMG 20546	AEVS01000031.1	*V. scophtalmi* LMG 19158	AFWE01000176.1
V. breoganii ZF-29	AJYM01000056.1	*V. shilonii* AK1	ABCH01000132.1
V. breoganii ZF-55	AJYL01000116.1	*V. sinaloensis* DSM 21326	AEVT01000002.1
V. campbellii DS40M4	AGIE01000044.1	*V. splendidus* LGP32	NC011753
V. carribenticus ATCC BAA-2122	AEIU01000099.1	*V. tasmaniensis* 5F-79	AJZP01000014.1
V. cholerae O395	CP000627	*V. tasmaniensis* ZS-17	AJZQ01000005.1
V. corallilyticus P1	AEQS01000154.1	*V. tasmaniensis* 1F-155	AJZN01000079.1
V. crassostrae 9ZC88	AJZB01000013.1	*V. tasmaniensis* 1F-187	AJZM01000039.1
V. crassostrae 9ZC77	AJZA01000150.1	*V. tubiashii* NCIMB 1337	AHHF01000016.1
V. crassostrae 9ZC13	AJYZ01000064.1	*V. vulnificus* CMCP6	AE016795

Table 3.4: list of strains and GenBank accession numbers of genomes used in this study.

To select the most suitable target genes for the multiplex assay, all the sequences of the genes *rpoD* (RNA polymerase sigma D factor), *recG* (ATP-dependent DNA helicase), *rplB* (50S ribosomal subunit L2), *gyrB* (DNA gyrase, subunit B), *pyrG* (CTP synthase), *dnaE* (DNA polymerase III alpha subunit), *dnaJ* (chaperone Hsp40,

DnaK co-chaperone), *fusA (*elongation factor G), *hlyA* (haemolysin A), *pyrH* (uridylate kinase), *rctB* and *tl* were downloaded from GenBank and aligned using ClustalW2 (www.EBI.ac.uk/tools/msa/clustaw2).

Suitable primer regions were visually searched in order to identify sequences that potentially could differentiate genera and/or group of species and candidate primer sites were identified. Out of the 12 genes analyzed, the analysis allowed choosing *rpoD*, *recG*, *rplB*, *gyrB*, *pyrG* and *dnaE* for primer designing. Based on the availability of eligible regions, indeed, these 6 genes, have been identified as the most suitable for the possibility of designing specific primers and for their resolution.

Primer specificity for the target groups has been firstly evaluated *in silico* through Blast analysis (NCBI-BLAST; http://blast.ncbi.nlm.nih.gov), and a total of 27 primers has been chosen according to a number of features: specificity, lack of primer-primer dimerization, uniform length and GC contents (in order to minimize differences in the Tm of each primer), adequate difference in the obtained amplicon length (in order to easily separate the DNA fragments in agarose gel electrophoresis). The primer sequences and expected amplicon lenghts are listed in Table 3.5.

All the oligonucleotide primers used in this study were synthesized and purchased from SIGMA-ALDRICH.

Due to the good resolution showed *in silico*, the optimization and validation process has been focused on multiplex assays targeting *rpoD, recG* and *rplB* gene.

Target Gene	Primer name	Primer sequence	Expected amplicon	T_m (°C)
rpoD	rpoD FW	5'- AGCTGTGACTGCGGATTTTG- 3'		57.3
	rpoD REV1	5'- GTCGGCTTATCAGTCGAAGA- 3'	161 bp	57.3
	rpoD REV2	5'- AGCGTAGAAAGCGAGATCGG- 3'	249 bp	59.4
	rpoD REV3	5'- ACGTGAGCAACTTGGCAAAA- 3'	673 bp	55.3
	Vib FW	5'- AAGCCTTGGAATGCTACATG- 3'		55.3
	Vib REV	5'- TGAGCCGAAGAAGTTGCGTA- 3'	527 bp	57.3
recG	recG FW	5'- GCCCATGAAGGGCAAAC- 3'		55.2
	recG REV1	5'- AACAAGATGAAAACCAAAGA- 3'	655 bp	49.1
	recG REV3	5'- GAAGATATGGATCAAATGGC- 3'	896 bp	53.2
	recG REV4	5'- TTCCCAGATGAGATTTACGT- 3'	1082 bp	53.2
rplB	rplB FW	5'- CCTACACTTGCTGAAGAAGG- 3'		57.3
	rplB REV 3	5'- GACCAAGCTCTAGAAATCCT- 3'	367 bp	55.3
	rplB REV 4	5'- ATCTTCGTAGATGAAGGCCC- 3'	496 bp	57.3
	rplB REV 5	5'- AAGTAAGCGGTCGTCTAGG- 3'	1086 bp	56.9
	rplB REV 6	5'- CATCACTGTTGTTGTAGCAG- 3'	588 bp	55.3
	rplB REV 7	5'- TACGCGCAGCTGTAGCTAA- 3'	891 bp	56.7
gyrB	gyrB FW	5'- AATTACGATTCATCGAGTAT- 3'		50.9
	gyrB REV1	5'- TCTTTGATGTACTGCTCTTG- 3'	1640 bp	53.2
	gyrB REV4	5'- GCTTCATCAATTGAGTTATC- 3'	143 bp	51.2
pyrG	pyrG FW	5'- CGACCTAGAACTTTAACGAT- 3'		53.2
	pyrG REV 4	5'- AACGTTGCGGGTATGGAA- 3'	584 bp	51.4
	pyrG REV 5	5'- CACGCAGGTCTTAAGAAC- 3'	1081 bp	53.7
	pyrG REV 8	5'- GTGAAAACAAAGTACCTTAC- 3'	661 bp	51.2
	pyrG REV 9	5'- ATGCGTCTTGGTTCACAG- 3'	453 bp	56.0
dnaE	dnaE FW	5'- GGACAGGTCAGCTTTGATG- 3'		56.7
	dnaE REV 4	5'- GCGTCTTTTAGTCGACAACT- 3'	252 bp	55.3
	dnaE REV 6	5'- ATTTTCATCGCCAACTCGCC- 3'	971 bp	57.3

Table 3.5: sequences, expected amplicon length and melting temperature (T_m) of designed primers.

3.3. Monoplex PCR amplifications

Primers for *rpoD*, *recG* and *rplB* genes have been then tested empirically in monoplex PCR amplifications against the above mentioned 84 strains of *Vibrio*, *Aliivibrio* and *Photobacterium*

PCR reactions contained 1 µl (≈ 50 ng) of template DNA, 5 µl 2× TaKaRa PCR Master Mix (Buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs and 0.5 U Taq DNA polymerase), 0.5 µM each of forward and reverse primers and deionized sterile water to a final volume of 10 µl.

The thermal programs consisted of 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 53 °C or 55 °C (depending on the set of primers), 3 min at 72 °C and a final elongation for 7 min at 72 °C. All the runs included a negative control.

When PCR amplification of non-vibrio species was negative, the *rpoB* gene was amplified following the protocol described by Ki *et al*. (2009) to ensure that the quality of bacterial DNA was not responsible for the failed reaction.

PCR products were analyzed by 1% agarose gel electrophoresis against a 100 bp molecular ladder marker to detect amplicons of the expected sizes.

3.4. Multiplex PCR amplifications

DNA, primers, $MgCl_2$ concentration and the PCR protocol including annealing temperatures, extension times and cycling numbers were optimized for the multiplex assay. A typical optimized reaction mixture for a total 10 µL volume contained 1 µl (≈ 50 ng) of template DNA, 1× Promega Master Mix 2× TaKaRa PCR Master Mix (Buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs and 0.5 U Taq DNA polymerase), a variable concentration of each forward and reverse primer and deionized sterile water to the final volume.

The annealing temperatures were optimized for each multiplex PCR and primer concentrations ranged from 0.25 µM to 1 µM as described in Table 3.6.

Target Gene	Primer	Concentration (µM)	Annealing
rpoD	rpoD FW	1.0	55 °C
	rpoD REV1	0.4	
	rpoD REV2	0.4	
	rpoD REV3	0.4	
	Vib FW	0.5	
	Vib REV	0.5	
recG	recG FW	1.0	50 °C
	recG REV1	0.35	
	recG REV3	0.35	
	recG REV4	0.35	
rplB	rplB FW	1.0	53 °C
	rplB REV 3	0.25	
	rplB REV 4	0.25	
	rplB REV 5	0.25	
	rplB REV 6	0.25	
	rplB REV 7	0.25	

Table 3.6: primer concentration and annealing temperature for each multiplex PCR reaction.

The optimized PCR program contained the following steps: initial denaturation of 15 min at 95°C followed by 35 cycles of denaturation at 95°C for 30 s, primer annealing at 50 °C, 53 °C or 55 °C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were subjected to electrophoresis on 1.5% agarose gels.

The developed multiplex PCR has been also performed on non-target bacteria, such as *Escherichia coli*, *Citrobacter freundii*, *Shewanella haliotis*, *Shewanella algae*, and *Pseudomonas aeruginosa* to validate the specificity of the assay for Vibrionaceae.

3.5. Sensitivity of multiplex PCR

To test the sensitivity of the multiplex PCR assay for *rpoD* gene, serial dilutions of bacteria DNA templates were amplified for 5 major species (*A. fischeri, A. salmonicida, P. damselae, V. alginolyticus, V. parahaemolyticus*) to evaluate the detection limits of the multiplex PCR assay developed in this study.

Briefly, bacterial cultures of each selected strain were suspended in sterile saline to a density corresponding to a 1.0 McFarland turbidity standard (10^8 CFU/ml). The suspensions were serially diluted tenfold (from 10^6 to 10 CFU/ml) and plated to count the CFU/ml.

1 ml portion from each serial dilution was centrifuged and the DNA was extracted from each cell pellet as described in the section 'Bacterial cultures and DNA template preparation', in order to perform the sensitivity test of the multiplex PCR and determine the lower limits of detection (minimal numbers of CFU detectable) of the target gene *rpoD*.

3.6. Application of the developed multiplex PCR on a sets of samples containing various vibrio-like bacteria representing different environments

rpoD multiplex PCR has been tested for a species-specific identification within a mixture of Vibrionaceae. Different samples containing an environmental vibrio population were investigated for the presence of *Vibrio*, *Aliivibrio* and *Photobacterium* genera, to evaluate the ability of *rpoD* primers to monitor vibrios in various environmental systems.

Briefly, different dilutions of bacteria DNA templates (10^4, 10^5, 10^6) for 3 major genera (represented by *A. fischeri, A. salmonicida, P. damselae, V. anguillarum 01, V. parahaemolyticus*) have been mixed in different combinations to evaluate the sensitivity of the multiplex PCR assay developed in this study with environmental samples.

Each strain was grown separately in 10 ml of APW (Alkaline Peptone Water) to an optical density of 0.140 at 600 nm. The cultures were then 10-fold serially diluted,

and viable plate counts were determined. Aliquots of 10^4, 10^5or 10^6 cultures of different genera were mixed together. 1 ml portion from each sample was centrifuged and the DNA was extracted from each cell pellet as described in the section 'Bacterial cultures and DNA template preparation'. A 5 µl aliquot of each sample was used for 50 µl final volume PCR amplification.

Each sample included the 3 genera in different concentration:

1) *Aliivibrio* spp. 10^4 + *Photobacterium* spp. 10^5 + *Vibrio* spp. 10^6
2) *Aliivibrio* spp. 10^5 + *Photobacterium* spp. 10^6 + *Vibrio* spp. 10^4
3) *Aliivibrio* spp. 10^6 + *Photobacterium* spp. 10^4 + *Vibrio* spp. 10^5

3.7. Application of the developed multiplex PCR on samples representing various environmental settings.

During this three years study, a number of environmental samplings have been done in collaboration with several foreign partners, in order to improve the collection of Vibrionaceae strains for a wider validation and implementation of the designed Multiplex PCRs.

3.7.1. Oban, Scotland (UK), North East Atlantic

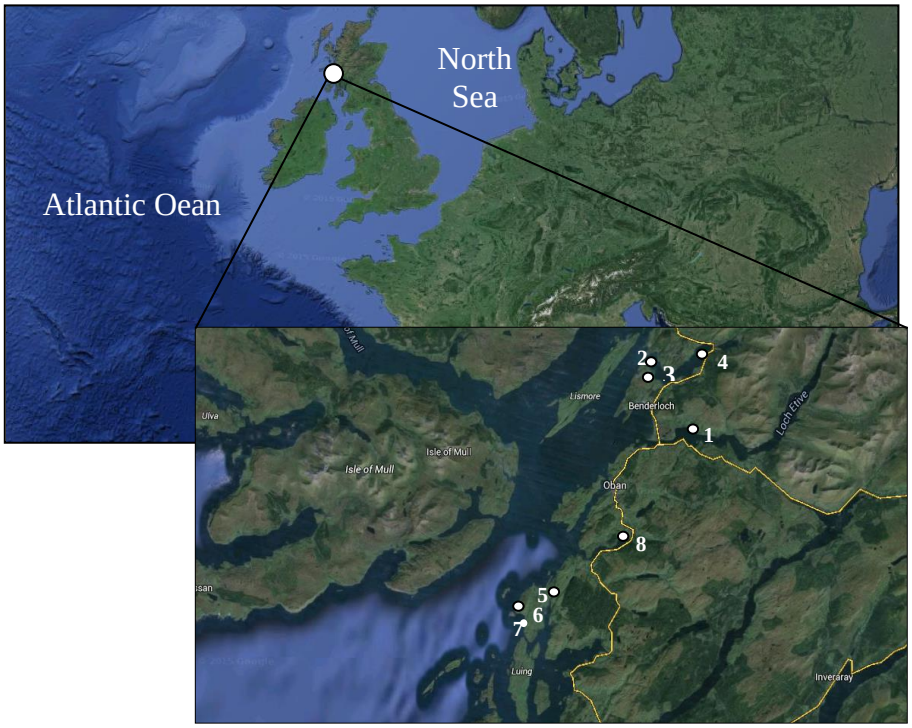


Figure 3.1. Surface water and sediment sampling sites along North East Atlantic coast of UK.

A total of 19 different water and sediment samples were collected from September to October 2013 along the West coast of Scotland, in the Argyll and Bute region (UK). Samples were collected at different locations and on different days:

- four water and sediment samples were collected from the Loch Etive, Ardchattan Priory (site 1);
- three water sample from Loch Creran estuary (site 2 and 3);
- one water sample from inner Loch Creran, Barcaldine (site 4);
- one water sample each from Atlantic Ocean, Clachan Bridge (site 5);

- two seawater sample from two different sites on the Atlantic Ocean, Easdale (site 6 and 7);
- two water and sediment samples from Loch Feochan (site 8);
- six aquaculture water samples from oyster racks, aquaculture facility and salmon farm corresponding to site 1, 2 and 3.

Surface water temperature and salinity were determined at each site.

Within 3 h from collection, samples were centrifuged at 5000 g for 15 min, pellet was resuspendend on 5 ml of sterile artificial seawater and a 100 µl aliquot was spread on TCBS agar (Difco) in 3 replicates and on TSA (Difco) + 2% NaCl plates for control. The TCBS plates were incubated for 24 h at 14 °C, 21 °C and 35 °C. Colonies grown on TCBS plates were enumerated to quantify culturable vibrios and those on the TSA were counted to quantify total bacteria.

Each presumptive vibrio colony was streaked for purity onto TCBS agar, then transferred onto Sheep Blood Agar and grown overnight at 14 °C, 21 °C and 35 °C to test the haemolytic activity and the susceptibility to O129 vibriostatic (150µg). A total of 326 presumptive Vibrio strains has been isolated and transferred into marine agar slant to be sent to the Microbiology Laboratory of the University of Rome "Tor Vergata" for further analysis. Presumptive Vibrio strains were identified by colony shape and pigmentation on TCBS, Gram staining, cytochromeoxidase activity, and glucose fermentation. The oxidase-positive, gram-negative, glucose acidifying isolates were selected for molecular analysis by multiplex PCR.

3.7.2. Gulf of Aqaba, Israel, North-East Red Sea

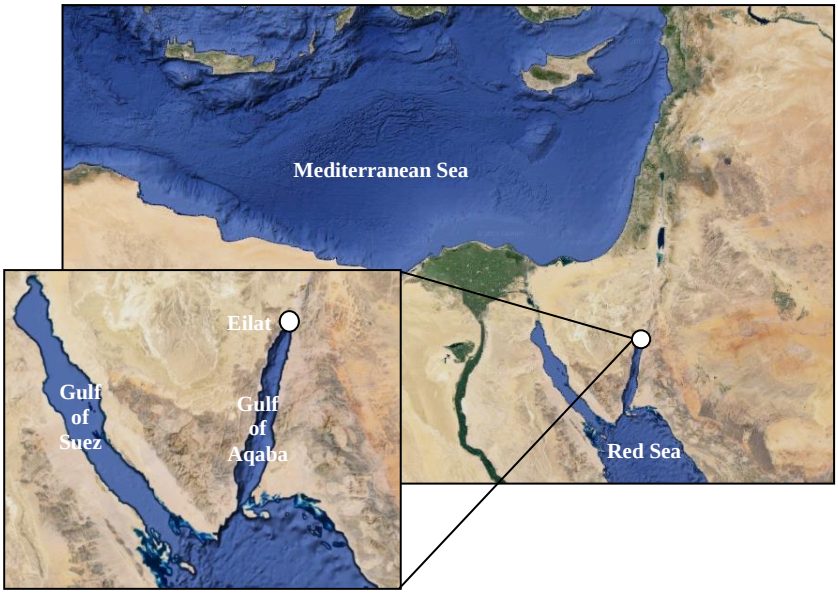


Figure 3.2. Surface water and sediment sampling area in the Gulf of Aqaba.

A total of 23 different water and sediment samples were collected from 5 to 13 May 2013 on the Israel coast of the Gulf of Aqaba, the far northern end of the eastern branch of the Red Sea. Samples were collected at different locations from boat and in the Interuniversity Institute for Marine Sciences (Eilat, Israel):

- nine water and sediment samples were collected in in different sites in the Gulf of Aqaba from boat (table 3.7);
- nine water sample from the coral reef in front of the Interuniversity Institute for Marine Sciences (Eilat);
- five water sample from mesocosms set at the Interuniversity Institute for Marine Sciences (Eilat).

Table 3.7. Coordinates of the nine sampling sites in the Gulf of Aqaba.

Site	Coordinates	Depth
1	2932.6177 N 03458.200 E	10.0 m
2	2932.5021 N 03458.4177 E	19.0 m
3	2932.6810 N 3457. 8456 E	16.0 m
4	2932.759 N 03457.626 E	17.5 m
5	2932.522 N 03456.955 E	18.6 m
6	2931.587 N 03456.296 E	18.4 m
7	2930.937 N 03455.671 E	17.3 m
8	2929.710 N 03454.583 E	22.4 m
9	2930.166 N 03456.653 E	397.6 m

Within 3 h from collection, samples were processed as described before. Colonies grown on TCBS plates were enumerated to quantify culturable vibrios and those on the TSA were counted to quantify total bacteria.

Each presumptive vibrio colony was streaked for purity onto TCBS agar. A total of 238 presumptive Vibrio strains has been isolated and transferred into marine agar slant to be stored and sent to the Microbiology Laboratory of the University of Rome "Tor Vergata" for further biochemical and molecular analysis. Presumptive Vibrio strains were identified by colony shape and pigmentation on TCBS, Gram staining, cytochromeoxidase activity, and glucose fermentation. The oxidase-positive, gram-negative, glucose acidifying isolates were selected for being submitted to multiplex PCR.

4. RESULTS

4.1. Sequence alignment and primer design for multiplex PCR

In this investigation, 27 oligonucleotide primers targeting *rpoD, recG, rplB, gyrB, pyrG* and *dnaE* were designed to simultaneously detect and differentiate Vibrionaceae by multiplex PCR (table 4.5). To facilitate PCR product detection, the primers were designed such that the predicted sizes of the amplification products of each target gene would be different to permit size discrimination by gel electrophoresis.

Analysis of primers with respect to self-complementarity, inter-primer annealing and optimum annealing temperatures were accomplished and all primer sequences were compared against each other and homology searches performed against the GenBank database for sequence similarity using the BLAST program. *In silico* analyses indicated that all oligonucleotide primer pairs showed significant affinities only for their target genes and group of species.

Based on their good resolution, the empirical validation process has been focused on multiplex assays targeting *rpoD, recG* and *rplB*.

4.2. *In silico* results and expected amplicons of the multiplex PCR

Overall, *in-silico* PCR results showed 100% specificity for all oligonucleotides designed. Blast analyses showed that the 27 primers designed in this study were specific for their corresponding target organisms only and were suitable for the detection and identification of genera *Photobacterium, Grimontia* and *Aliivibrio,* so as the cluster including *A. fischeri* and *Vibrio* species.

Among the six sets of multiplex PCR primers, the *rpoD* set targeted genus-specific regions that specifically differentiate Vibrionaceae genera: *Photobacterium*, *Grimontia, Aliivibrio* and *Vibrio*. The remaining five sets, directed at the *recG*, *rplB*, *gyrB*, *pyrG* and *dnaE* genes produced species-specific amplicon patterns allowing to differentiate species within genus *Vibrio*. Sequence alignments, indeed, highlighted

typical and conserved regions of these genes that allowed designing *Vibrio*-specific primer and exclude other genera from amplification. A representation of all steps of the identification key developed in this study is shown in Figure 4.1.

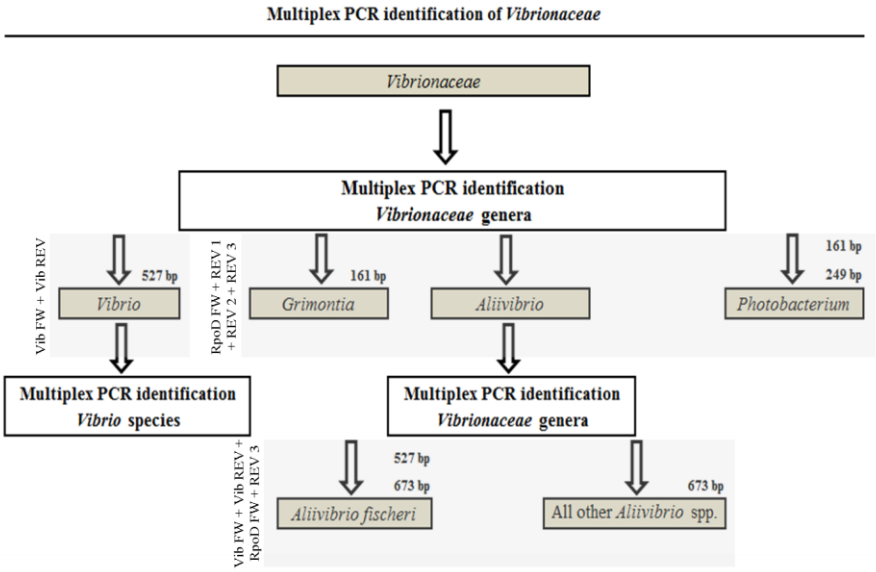


Figure 4.1 Scheme of all steps of the identification key based on a set of multiplex PCR developed in this study.

4.2.1. Profiles expected by *rpoD* multiplex amplification.

Six oligonucleotides (2 forward + 4 reverse) were designed based on the sequence alignment of *rpoD* and targeting different genera of Vibrionaceae. Both the oligonucleotides and the expected sizes of amplicons are summarized in Table 4.1. They are targeted at a genus-specific region of the *rpoD* gene.

Primer	Target group	Expected amplicon	*A. salmonicida*	*A. fischeri*	*A. logei*	*P. leiognathi*	*P. profundum*	*P. damselae* subs. *damselae*	*P. damselae* subs. *piscicida*	*G. hollisae*	*Vibrio* spp.
rpoD FW	ALL		+	+	+	+	+	+	+	+	+
rpoD REV1	*Photobacterium, Grimontia*	161 bp				+	+	+	+	+	
rpoD REV2	*Photobacterium*	249 bp				+	+	+	+		
rpoD REV3	*Aliivibrio*	673 bp	+	+	+						
Vib FW	*Vibrio, A. fischeri*			+							+
Vib REV	*Vibrio, A. fischeri*	527 bp		+							+

Table 4.1. designed primers, target groups and expected sizes of amplification products for *rpoD* multiplex PCR.

A total of 5 patterns were expected by *in silico* study (Table 4.1). *In silico* analysis of *rpoD* amplification profiles revealed the presence of five distinct patterns (*Photobacterium* - P, *Grimontia* - G, *Aliivibrio* - A, *Vibrio* – V, and *A. fischeri* - Af pattern). One cluster consisted of 3 patterns (P, G and A) for rpoD FW + REV1, REV2 or REV3 positive strains (*Photobacterium*, *Grimontia*, *Aliivibrio).* The other cluster consisted of 2 patterns (V and Af) for strains negative to rpoD FW + REV1,2 or 3 amplification, and positive to the amplification with Vib FW+REV primer pair (*Vibrio* and *A. fischeri*). *A. fischeri* strains resulted to be distinguishable from other *Aliivibrio* species thanks to their peculiar pattern (527 bp + 673 bp fragments).

4.2.2. Profiles expected by *recG* multiplex amplification.

According to *in silico* analysis, the 5 *recG* primers designed in this study (recG FW, recG REV1, REV3, REV4 and REV5) were expected to produce 665 bp, 896 bp, 1082 bp and 1352 bp amplicons in 23 of the 30 species examined. *In silico*

evaluation, indeed, showed that recG FW primer does not anneal with *V. breoganii*, *V. carribenticus*, *V. coralliilyticus*, *V. metschnikovii*, *V. rotiferianus*, *V. rumoiensis* and *V. shilonii* sequences. Thus, these 7 species were not expected to amplify any of the described amplicons for *recG* gene.

Only *V. scophtalmi* was expected to amplify all the due amplicons.

V. harveyi cluster (*V. alginolyticus*, *V. campbellii*, *V. harveyi*, *V. ichtyoenteri*, V. *parahaemolyticus*) showed *in silico* a predicted amplification of 665 bp, 896 bp and 1082 bp fragments.

Another group of species, including *V. anguillarum* and members of *V. splendidus* cluster (*V. kanaloe*, *V. sinaloensis*, *V. splendidus*, *V. tasmaniensis* and *V. tasmaniensis* 1F) were expected to show a peculiar amplification pattern for *recG* with the 665 bp band only.

V. cholerae cluster (comprising *V. cholerae* and *V. mimicus*) together with *V. furnissii* lack of the 896 bp fragment amplification.

V. orientalis and *V. tubiashii* were expected to amplify the 896 bp and 1082 bp *recG* fragments, while *V. brasiliensis* and *V. nigripulchritudo* were expected to give only the 1352 bp amplification product.

According to *in silico* analysis, no reverse primers anneal with *V. ordalii* and *V. crassostrae recG* sequence, thus these 2 species were not expected to give any product for the amplification of *recG* fragments.

Vibrio species examined in the *in silico* analysis and expected products of the multiplex PCR for *recG* are shown in table 4.2.

Species	recG FW	recG REV1 (665 bp)	recG REV3 (896 bp)	recG REV4 (1082 bp)	recG REV5 (1352 bp)
V. breoganii					
V. carribbenticus					
V. coralliilyticus					
V. metschnikovii					
V. rotiferianus					
V. rumoiensis					
V. shilonii					
V. alginolyticus	+	+	+	+	
V. anguillarum	+	+			
V. brasiliensis	+				+
V. cambpellii	+	+	+	+	
V. cholerae	+	+		+	
V. crassostreae	+				
V. cyclotrophicus	+	+	+		
V. furnissii	+	+		+	
V. harveyi	+	+	+	+	
V. ichtyoenteri	+	+	+	+	
V. kanaloe	+	+			
V. mimicus	+	+		+	
V. nigripulchritudo	+				+
V. ordalii	+				
V. orientalis	+		+	+	
V. parahaemolyticus	+	+	+	+	
V. scophtalmi	+	+	+	+	+
V. sinaloensis	+	+			
V. splendidus	+	+			
V. tasmaniensis	+	+			
V. tasmaniensis 1F	+	+			
V. tubiashii	+		+	+	
V. vulnificus	+	+			+

Table 4.2 *Vibrio* species examined in the *in silico* analysis and expected products of the multiplex PCR amplification of *recG*.

4.2.3. Profiles expected by *rplB* multiplex amplification.

Based on *in silico* analysis, the designed 6 *rplB* primers (rplB FW, rplB REV 3, REV 4, REV 5, REV 6 and REV 7) were found to produce specific 367 bp, 496 bp, 588 bp, 891 bp and 1086 bp amplicons in all the 31 *Vibrio* species examined. Our *in silico* results therefore, indicated that this set of primers was suitable for the differentiation of the analyzed *Vibrio* species as showed in Table 4.3. The amplification of 496 bp fragment is expected for several species. Among these, *V. scophtalmi* and *V. ichtyoenteri* should amplify only this fragment. Besides to this fragment, *V. carribenticus* is expected to show the 588 bp amplicon and *V. orientalis* of the 1086 bp one.

V. nigripulchritudo, *V. sinaloensis*, *V. shilonii* and *V. tubiashii* were expected to be positive for the amplification of the 496 bp, 588bp and 1086 bp fragments; *V. brasiliensis* and *V. breoganii* for the 496 bp, 588 bp and 891 bp fragments; *V. coralliilyticus* for the 367 bp, 496 bp and 588 bp fragments.

According to *in silico* simulations, *V. parahaemolyticus* cluster, comprising also *V. alginolyticus* and *V. rotiferianus*, should amplify all the expected fragments except the 496 bp one, while *V. harveyi* should lack also of the 1086 bp fragment.

V. anguillarum together with the closely related *V. ordalii* were characterized by the strict homology to the primers amplifying the 588 bp, 891 bp and 1086 bp fragment, respectively.

The whole *V. splendidus* cluster (*V. splendidus*, *V. tasmaniensis*, *V. cyclitrophicus*) was expected to be characterized by the amplification of the 891 bp fragment only.

The *V. cholerae* and *V. mimicus* cluster showed no homologies with any of the *rplB* REV designed primer, thus, according to the *in silico* analysis no *rplB* amplicons were expected.

Species	rplB FW	rplB REV 3 (367 bp)	rplB REV 4 (496 bp)	rplB REV 6 (588 bp)	rplB REV 7 (891 bp)	rplB REV 5 (1086 bp)
V. alginolyticus	+	+		+	+	+
V. anguillarum	+			+	+	+
V. brasiliensis	+		+	+	+	
V. breoganii	+		+	+	+	
V. campbellii	+	+		+		
V. carribbenticus	+		+	+		
V. cholerae	+					
V. coralliilyticus	+	+	+	+		
V. crassostreae	+	+			+	
V. cyclotrophicus	+				+	
V. furnissii	+	+				+
V. furnissii CIP	+					+
V. harveyi	+	+		+	+	+
V. ichtyoenteri	+		+			
V. kanaloe	+	+				
V. metschnikovii	+	+				
V. mimicus	+					
V. nigripulchritudo	+		+	+		+
V. ordalii	+			+	+	+
V. orientalis	+		+			+
V. parahaemolyticus	+	+		+	+	+
V. rotiferianus	+	+		+	+	+
V. rumoiensis	+			+	+	
V. scophtalmi	+		+			
V. shilonii	+		+	+		+
V. sinaloensis	+		+	+		+
V. splendidus	+				+	
V. tasmaniensis	+				+	
V. tasmaniensis 1F	+				+	
V. tubiashii	+		+	+		+
V. vulnificus	+			+		+

Table 4.3 *Vibrio* species examined in the *in silico* analysis and expected products of the multiplex PCR amplification of *rplB.*

4.2.4. Profiles expected by *gyrB, pyrG* and *dnaE* multiplex amplification

As described in the section 2.2 ‘Sequence alignment and primer design for multiplex PCRs’, to differentiate species within *Vibrio* genus, genes have been selected together with the above-described *recG* and *rplB* genes. Nevertheless these 3 genes, when analyzed *in silico*, showed less discriminative power and resolution for *Vibrio* spp. Thus, we decided to validate the *recG* and *rplB* multiplex PCRs first.

Expected products of the multiplex PCR amplification of *gyrB*, *pyrG* and *dnaE* according to *in silico* studies are listed in Table 4.4.

Species	gyrB FW	gyrB REV4 (143 bp)	gyrB REV1 (1640 bp)	pyrG FW	pyrG REV9 (453 bp)	pyrG REV4 (584 bp)	pyrG REV8 (661 bp)	pyrG REV5 (1081 bp)	dnaE REV	dnaE FW4 (252 bp)	dnaE FW6 (971 bp)
V. alginolyticus	+	+	+	+	+				+	+	+
V. anguillarum	+			+					+		
V. brasiliensis	+	+	+	+	+	+	+	+	+	+	
V. breoganii	+		+	+					+		
V. cambpellii	+	+	+	+					+	+	
V. carribbenticus	+	+		+		+			+		
V. cholerae	+	+	+	+					+	+	
V. coralliilyticus	+	+	+	+		+			+		+
V. crassostreae	+		+	+	+				+		
V. cyclotrophicus	+			+			+		+		
V. furnissii	+		+	+		+		+	+		
V. harveyii	+	+	+	+	+		+		+	+	
V. ichtyoenteri	+	+	+	+	+	+	+	+	+		+
V. kanaloe	+			+	+		+	+	+		
V. metschnikovii	+			+					+		+
V. mimicus	+	+	+	+		+			+		
V. nigripulchritudo	+		+	+	+	+		+	+		
V. ordalii	+	+	+	+			+		+		
V. orientalis	+	+	+	+	+	+	+	+	+	+	
V. parahaemolyticus	+	+	+	+		+			+		+
V. rotiferianus	+	+	+	+	+		+		+	+	
V. rumoiensis	+	+		+			+		+		
V. scophtalmi	+	+	+	+	+		+	+	+		
V. shilonii	+	+		+		+			+		
V. sinaloensis	+	+	+	+		+	+	+	+		
V. splendidus	+		+	+	+		+		+		
V. tasmaniensis	+		+	+	+		+		+		
V. tubiashii	+	+	+	+	+	+	+	+	+		
V. vulnificus	+	+	+	+		+			+		+

Table 4.2 *Vibrio* species examined in the *in silico* analysis and expected products of the multiplex PCR amplifications of *gyrB, pyrG* and *dnaE* genes.

4.2.5 Multiplex PCR identification for *Vibrio* spp.: scheme of combined profiles

Combining the patterns expected by the multiplex amplification of *rpoD*, *recG* and *rplB* genes, we obtained 24 profiles univocally identifying 20 *Vibrio* species and 4 groups of species that share common profiles. These groups are:

1) species of the *V. harveyi* cluster (*V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus*);
2) species of the *V. splendidus* cluster *(V. splendidus* and *V. tasmaniensis)*;
3) members of the *V. cholerae* cluster (*V. cholerae* and *V. mimicus*);
4) *V. brasiliensis* and *V. rotiferianus*;

Species	recG REV1 (665 bp)	recG REV3 (896 bp)	recG REV4 (1082 bp)	recG REV5 (1352 bp)	rplB REV 3 (367 bp)	rplB REV 4 (496 bp)	rplB REV 6 (588 bp)	rplB REV 7 (891 bp)	rplB REV 5 (1086 bp)
V. alginolyticus	+	+	+		+		+	+	+
V. anguillarum	+						+	+	+
V. brasiliensis				+		+	+	+	
V. breoganii						+	+	+	
V. cambpellii	+	+	+		+		+		
V. carribbenticus					+	+			
V. cholerae	+		+						
V. coralliilyticus				+	+	+			
V. crassostreae				+			+		
V. cyclotrophicus	+	+						+	
V. furnissii	+		+		+				+
V. harveyi	+	+	+		+		+	+	+
V. ichtyoenteri	+	+	+			+			
V. kanaloe	+				+				
V. metschnikovii				+					
V. mimicus	+		+						
V. nigripulchritudo				+		+	+		+
V. ordalii							+	+	+
V. orientalis		+	+			+			+
V. parahaemolyticus	+	+	+		+		+	+	+
V. rotiferianus				+		+	+	+	
V. rumoiensis							+	+	
V. scophtalmi	+	+	+	+		+			
V. shilonii						+	+		+
V. sinaloensis	+					+	+		+
V. splendidus	+							+	
V. tasmaniensis	+							+	
V. tasmaniensis 1F	+							+	
V. tubiashii		+	+			+	+		+
V. vulnificus	+			+			+		+

Table 4.5 Scheme of the amplification profiles obtained combining expected *recG* end *rplB* multiplex results.

4.3. Validation of the multiplex PCR

To validate the designed multiplex PCR key, we compared the empiric results of the experimental assay with the identification scheme previously obtained from *in silico* analysis including *rpoD*, *recG* and *rplB* genes. In order to evaluate and verify the specificity of the primers in this study, each multiplex reaction was tested on DNA templates extracted from a collection of 84 well-characterized vibrio strains including genera *Vibrio* (*n* = 79), *Aliivibrio* (*n* = 3) and *Photobacterium* (*n* = 2).

4.3.1. Validation of the multiplex PCR for *rpoD* gene

We found a 100% concordance in identifying Vibrionaceae genera between the *in silico* study and the results obtained with our multiplex PCR (Table 4.6). Our assay correctly identified the isolates as *Vibrio*, *Aliivibrio* or *Photobacterium*, and amplicons corresponded to the expected genus-specific product (Figure 4.2).

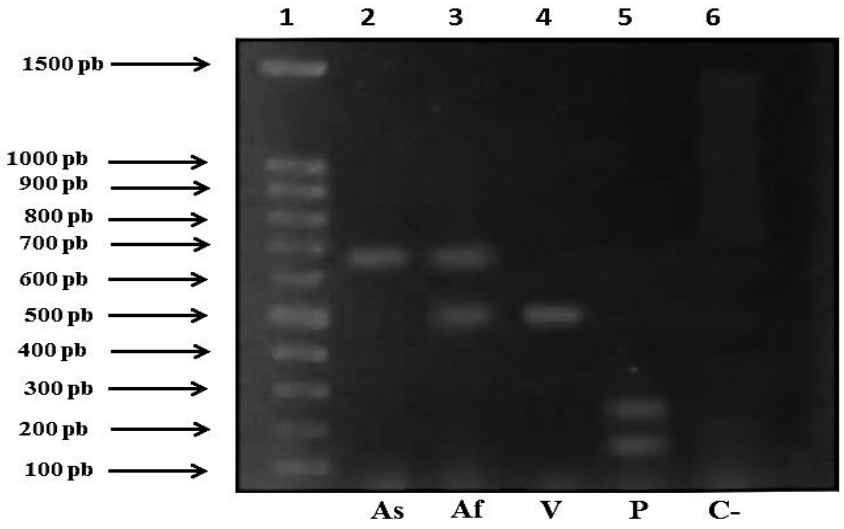


Figure 4.2. Representative gel picture showing the multiplex amplification of *rpoD* gene for Vibrionaceae genera detection. Lanes 1: 100 bp DNA marker; lane 2: *Aliivibrio salmonicida PT2* (673 bp); lane 3: *Aliivibrio fischeri* (527/673 bp); lane 4: *Vibrio parahaemolyticus* (527 bp); lane 5: *Photobacterium damselae* subs. *damselae* (161/249 bp)*;* lane 6: negative control (*E. coli*).

The assay showed further discriminatory power in differentiating the 3 *Aliivibrio* strains available into *A. fischeri* (n = 1) and *A. salmonicida* (n=2), as expected according to preliminary *in silico* study. No *Grimontia* strains were available in the collection, so it was not possible to evaluate the ability of the multiplex in identifying this genus.

An additional weak band (1000 bp) was observed in 22 *Vibrio* isolates, but did not interfere with the sensitivity or specificity of the PCR. No other aspecific amplification products were observed.

Multiplex PCR identification of Vibrionaceae **genera (*rpoD*)**

	rpoD FW + REV1	rpoD FW + REV2	rpoD FW + REV3	Vib FW + Vib REV
A. salmonicida LFI 1226			+	
A. salmonicida PT2			+	
A. fischeri			+	+
P. damselae subs. *damselae*	+	+		
P. iliopiscarius	+	+		
V. agarivorans				+
V. alginolyticus				+
V. anguillarum O1				+
V. anguillarum AL 102 (O1)				+
V. anguillarum O3				+
V. anguillarum				+
V. carchariae				+
V. cholerae				+
V. cincinnatiensis ATCC 35912				+
V. cyclitrophicus				+
V. diabolicus				+
V. diazothrophicus				+
V. fluvialis ATCC 35016				+
V. furnissii				+
V. harveyi				+
V. kanaloe				+
V. mediterranei				+
V. metschnikovii ATCC 7708				+
V. nigripulchritudo				+
V. ordalii NCMB2169				+
V. ordalii NCMB2167				+
V. ordalii				+
V. pacinii				+
V. parahaemolyticus				+
V. splendidus				+
V. tubiashii				+
V. vulnificus				+
V. woodanis				+

Table 4.6 Results for the multiplex amplification of *rpoD* gene.

4.3.2. Validation of the multiplex PCR for *recG* gene

When multiplex PCR was conducted using specific primers designed for *recG* gene, we obtained the products of the amplification for recG FW + REV1 (665 bp), REV3 (896 bp) and REV4 (1082 bp). After the optimization process described in section 2.4. 'Multiplex PCR amplifications', REV5 primer (amplifying a 1352 bp fragment) has been excluded from the *recG* multiplex reactions because it has been proven to compete with the other primers.

As expected, species belonging to *Aliivibrio* and *Photobacterium* genera did not give any amplification product, except for *A. fischeri* that yielded the 896 bp amplicon.

All the 5 strains of *V. alginolyticus* analyzed showed the same profile on agarose gel, in agreement with *in silico* prediction.

On the other hand, some species did not yield the expected results. This occurred for the *V. agarivorans* (n = 2), *V. cholerae* (n = 6) and *V. harveyi* (n = 7) isolates examined in this study, which showed the positive amplification of the 665 bp amplicon, corresponding to recG FW + REV 1 primers pair. Also the common amplification pattern for the 6 *V. parahaemolyticus* and the 2 *V. splendidus* strains examined was not consistent with *in silico* prediction (Table 4.7).

Both isolates of *V. diabolicus* examined gave the same amplification profile for *recG*. However, it was not possible to compare the obtained pattern with an expected one as *V. diabolicus recG* sequences were not available in online database.

Considering all the other species represented in this study by ≥ 2 isolates (e.g.: *V. anguillarum*, *V. kanaloe*, *V. ordalii*, *V. pacini*, *V. vulnificus*), it was not possible to individuate other species-specific amplification pattern that allowed to univocally assign an isolates to the corresponding species.

Non-specific products were observed in 4 strains belonging to *V. anguillarum*, *V. kanaloe* (n=2) and *V. vulnificus*.

Multiplex PCR identification of *Vibrio* species (*recG)*

	recG FW + REV 1	recG FW + REV 3	recG FW + REV 4	Aspecific
A. fischeri		+		
A. salmonicida LFI 1226				
A. salmonicida PTP2				
P. damselae subs. *damselae*				
P. iliopiscarius				
V. agarivorans (3)	+			
V. alginolyticus	+	+	+	
V. alginolyticus (5)	+	+	+	
V. anguillarum	+			
V. anguillarum (9)	+			
V. anguillarum (1)	+			700
V. anguillarum (4)				
V. anguillarum AL 102 (O1) (1)	+			
V. cholerae	+		+	
V. cholerae (6)	+			
V. cyclitrophicus	+	+		
V. cyclitrophicus	+	+		
V. diabolicus (2)	+		+	
V. diazothrophicus	+		+	
V. fluvialis	+			
V. harveyi	+	+	+	
V. harveyi (7)	+			
V. kanaloe	+			
V. kanaloe (3)	+			1400
V. kanaloe (3)	+	+		
V. kanaloe (3)				
V. mediterranei				
V. metschnikovii		+	+	
V. nigripulchritudo				
V. nigripulchritudo	+	+		
V. ordalii				
V. ordalii (1)	+	+		
V. ordalii (1)				
V. ordalii (5)	+			
V. pacini (1)				
V. pacini (1)	+			
V. parahaemolyticus	+	+	+	
V. parahaemolyticus (6)	+	+		
V. splendidus	+			
V. splendidus (2)	+	+		
V. vulnificus	+			
V. vulnificus (1)	+			450
V. vulnificus (3)	+			

Table 4.7 Results obtained for the multiplex amplification of *recG* gene. Rows in red represent the expected results, where present. Numbers in brackets represent the number of isolates yielding the corresponding profile. Where not shown, the number of isolate per species is 1.

10 isolates, belonging to *V. anguillarum* (n = 4/15), *V. kanaloe* (n = 3/9), *V. mediterranei* (n = 1/1), *V. ordalii* (1/7) and *V. pacini* (n = 1/2), gave no positive amplification products for *recG* multiplex.

4.3.3. Validation of the multiplex PCR for *rplB* gene

A total of 9 amplification patterns were experimentally observed for the 79 *Vibrio* strains examined (Table 4.8):

1. pattern type REV3: *V. alginolyticus* (n = 2/5), *V. carchariae* (n = 1/1), *V. cholerae* (n = 1/6), *V. metschnikovii* (n = 1/1), *V. pacini* (n = 1/1), *V. parahaemolyticus* (n = 2/6), *V. vulnificus* (n = 1/3);
2. pattern type REV4: *V. cholerae* (n = 3/6)
3. pattern type REV3 + REV4: *V. furnissii* (n = 1/1);
4. pattern type REV4 + REV5: *V. kanaloe* (n = 1/9);
5. pattern type REV6 + REV5: V. wodanis (n=1/1);
6. pattern type REV3 + REV4 + REV5: *V. alginolyticus* (n = 1/5), *V. fluvialis* (n = 1/1) and *V. ordalii* (n = 3/7);
7. pattern type REV3 + REV4 + REV6: *V. harveyi* (n = 3/7), *V. ordalii* (n = 2/7), V. *splendidus* (n = 1/2) and *V. tubiashii* (n = 1/1);
8. pattern type REV3 + REV6 + REV5: *V. parahaemolyiticus* (n = 2/6);
9. pattern type REV3 + REV4 + REV6 + REV5: *V. agarivorans* (n =3/3), *V. alginolyticus* (n = 2/5), *V. anguillarum* (n = 10/15), *V. cyclitrophicus* (n = 1/1), *V. diabolicus* (n = 2/2), *V. diazothrophicus* (n = 1/1), *V. harveyi* (n = 2/7), *V. kanaloe* (n = 4/9), *V. nigripulchritudo* (n = 1/1), *V. ordalii* (n = 2/7), *V. pacini* (n = 1/2), V. *parahaemolyti*cus (n = 2/6) and *V. splendidus* (n = 1/2);

A total of 16 strains belonging to *V. anguillarum* (n = 5/15), *V. cholerae* (n = 2/6), *V. cincinnatiensis* (n = 1/1), *V. harveyi* (n = 1/7), *V. kanaloe* (n = 4/9), *V. mediterranei* (n = 1/1) and *V. vulnificus* (n = 2/3) were not positive to *rplB* multiplex

amplification. As expected, species belonging to *Aliivibrio* and *Photobacterium* genera did not give any amplification product.

Only the isolates belonging to *V. agarivorans* and to *V. diabolicus* showed a coherent species-specific amplification pattern (REV3 + REV4 + REV6 + REV5). Unfortunately, it was not possible to compare the obtained pattern with expected results as there were no *V. agarivorans* and *V. diabolicus rplB* sequences available in online database.

Only the single *V. metschnikovii* strain analyzed showed a profile (REV3 amplification) in agreement with *in silico* prediction.

130 bp, 150 bp, 500 bp, 600 bp and 1250 bp non-specific products were yielded by 44 isolates (55.7%), despite several trials and optimizations, varying DNA, primers and $MgCl_2$ concentration, and annealing temperature.

Multiplex PCR identification of *Vibrio* species (*rplB*)

	rplB FW + REV 3	rplB FW + REV 4	rplB FW + REV 6	rplB FW + REV 7	rplB FW + REV 5	Aspecific
A. fischeri						
A. salmonicida LFI 1226						
A. salmonicida PTP2						
P. damselae subs. *damselae*						
P. iliopiscarius						
V. agarivorans (3)	+	+	+		+	1250
V. alginolyticus	+		+	+	+	
V. alginolyticus (2)	+					
V. alginolyticus (1)	+	+			+	
V. alginolyticus (2)	+	+	+		+	1250
V. anguillarum			+	+	+	
V. anguillarum (5)						
V. anguillarum (10)	+	+	+		+	1250
V. carchariae	+					
V. cholerae						
V. cholerae (1)	+					
V. cholerae (2)						
V. cholerae (3)		+				500
V. cincinnatiensis						
V. cyclitrophicus				+		
V. cyclitrophicus	+	+	+		+	1250
V. diabolicus(2)	+	+	+		+	1250
V. diazothrophicus	+	+	+		+	1250
V. fluvialis	+	+			+	1250
V. furnissii	+				+	
V. furnissii	+	+				
V. harveyi	+		+	+	+	
V. harveyi (2)	+	+	+		+	130; 1250
V. harveyi (1)						
V. harveyi (4)	+	+	+			1300
V. kanaloe	+					
V. kanaloe (4)	+	+	+		+	1250
V. kanaloe (4)						130; 150
V. kanaloe (1)		+			+	130; 150; 1250
V. mediterranei						
V. metschnikovii	+					
V. metschnikovii	+					
V. nigripulchritudo		+	+		+	
V. nigripulchritudo	+	+	+		+	
V. ordalii			+	+	+	
V. ordalii (2)	+	+	+		+	1250
V. ordalii (3)	+	+			+	
V. ordalii (2)	+	+	+			
V. pacini (1)	+	+	+		+	1250
V. pacini (1)	+					
V. parahaemolyticus	+		+	+	+	
V. parahaemolyticus (2)	+					
V. parahaemolyticus (2)	+		+		+	1250
V. parahaemolyticus (2)	+	+	+		+	1250
V. splendidus				+		
V. splendidus (1)	+	+	+		+	1250
V. splendidus (1)	+	+	+			
V. tubiashii		+	+		+	
V. tubiashii	+	+	+			
V. vulnificus			+		+	
V. vulnificus (1)	+					
V. vulnificus (2)						
V. woodanis			+		+	600

Table 4.8 Results for the multiplex amplification of *rplB* gene. Rows in red represent the expected results, where present. Numbers in brackets represent the number of isolates yielding the corresponding profile. Where not shown, the number of isolate per species is 1.

4.4. Comparison of the specificity of *recG* and *rplB* multiplex amplification

Multiplex PCR using primers targeting *rplB* gene failed to univocally identify most of the *Vibrio* species examined in this study, except for *V. agarivorans* and *V. diabolicus*, and showed 55.7% of isolates yielding non-specific amplification products.

The *recG* primers were 100% specific for 7 species, as they gave the same amplification profile for all the strains of the same species, for *V. alginolyticus* (n = 5), *V. agarivorans* (n = 2), *V. cholerae* (n = 6), *V. harveyi* (n = 7), *V. parahaemolyticus* (n = 6), *V. splendidus* (n = 2) and *V. diabolicus* (n = 2). However, it failed to distinguish among species showing the same amplicon pattern:

1) recG FW + REV 1: *V. agarivorans*, *V. cholerae* and *V. harveyi*;
2) recG FW + REV 1, recG FW + REV 3*: V. parahaemolyticus* and *V. splendidus*.

The overall results showed that the *recG* primers were more specific than *rplB* in *Vibrio* species differentiation.

4.5 Sensitivity of the multiplex PCR

The sensitivity of our multiplex PCR assay for *rpoD* gene was tested in 5 Vibrionaceae control strains for genera *Photobacterium* (*P. damselae* subs. *damselae*), *Aliivibrio* (*A. fischeri* and *A. salmonicida* PT2) and *Vibrio* (*V. alginolyticus* and *V. parahaemolyticus*).

The assay was capable of detecting, with reproducibility, a band in ethidium bromide-stained gels at dilutions slightly depending on the genus examined, being 4×10^5 CFU per PCR for the *Photobacterium* and *Aliivibrio* strains, and 4×10^4 CFU per PCR for both *Vibrio* strains (Figure 4.3).

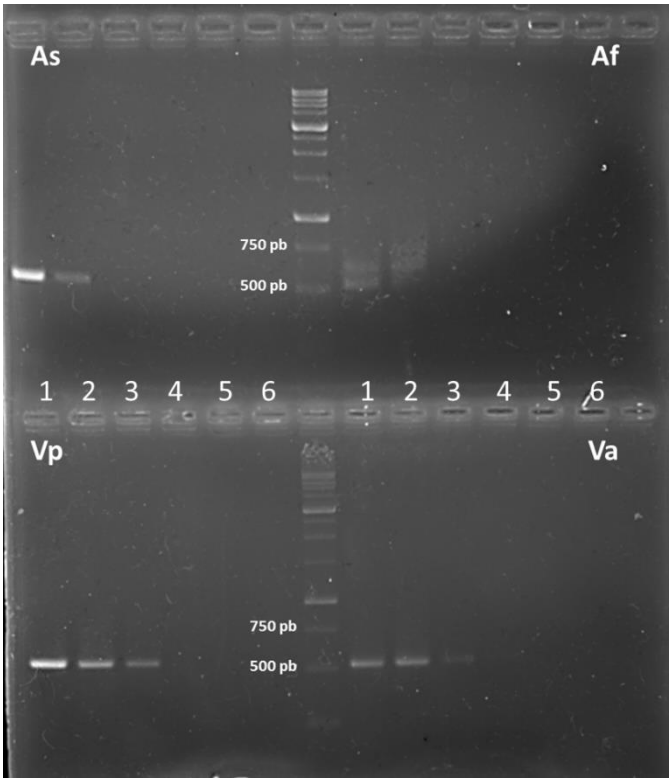


Figure 4.3. Sensitivity of multiplex PCR for *rpoD* gene for detection of serial dilutions of: As: *A. salmonicida* PT2; Af*: A. fischeri*; Vp: *V. parahaemolyticus;* Va: *V. alginolyticus.* Lane 1 to 6: DNA extracted from bacterial suspensions of 10^6 to 10 CFU/ml per reaction.
.

4.6. Application of the developed multiplex PCR on samples representing various environmental settings.

The specificity and sensitivity of the multiplex PCR from samples representing environmental setting with different concentration of *Vibrio*, *Aliivibrio* and *Photobacterium* was confirmed by electrophoretic results (Figure 4.4). Concentrations ranged from 10^4 to 10^6 CFU/ml in the starting aliquot, which correspond to 1 to 100 CFU after centrifugation and dilution in the PCR final

volume. All the samples were found to be positive for the genus-specific amplicons due for *rpoD* multiplex PCR.

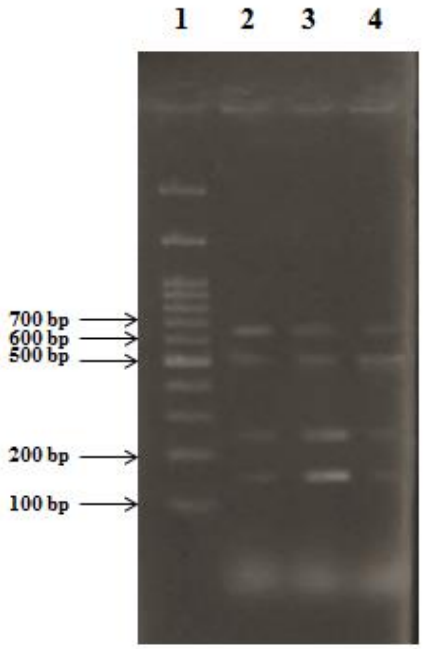


Figure 4.4. Sensitivity of multiplex PCR for *rpoD* gene for detection of different vibrio dilutions mixed as follows: lane 2: *Aliivibrio* spp. 10^4 + *Photobacterium* spp. 10^5 + *Vibrio* spp. 10^6; *lane 2:Aliivibrio* spp. 10^5 + *Photobacterium* spp. 10^6 + *Vibrio* spp. 10^4; lane 3: *Aliivibrio* spp. 10^6 + *Photobacterium* spp. 10^4 + *Vibrio* spp. 10^5

4.7. Applicability and accuracy of the multiplex PCR on unidentified presumptive vibrio strains

The multiplex PCR protocol was tested on isolates collected from various estuarine and coastal areas of different geographical locations as an example of how the protocol can be used for monitoring and research purposes.

To validate our assay's ability in differentiating unidentified vibrio strains, we tested it on 464 randomly selected environmental strains from the collection of the presumptive vibrio isolated along the Scottish West Coast (n=240) and in the Red Sea (n=224).

4.7.1. Application of the method to additional isolates from different environments:

A) isolates from Oban, Scotland (UK), North East Atlantic

Multiplex PCR amplification for Vibrionaceae genera (*rpoD* gene)

Among the 240 analyzed isolates, we found the *rpoD* band profile in 224 isolates, in agreement with preliminary phenotypic results. Among them, 212 (94.7%) showed the typical *Vibrio* amplicon (527 bp band), 7 (3.1%) showed the typical *Aliivibrio* amplification profile (both 673 bp and 527 bp bands), while 5 (2.2%) showed an unexpected profile with only one of the typical *Aliivibrio* amplicon (673 pb band).

No *Photobacterium* and *Grimontia* profile types were found among the isolates tested. To confirm the multiplex identification, a total of 96 isolates were randomly selected to be identified by amplification and sequencing of the *rpoB* gene as described in section 2.1 'Bacterial cultures and DNA template preparation'.

According to *rpoB* sequencing, all the environmental isolates were univocally assigned to the proper species by the multiplex amplification of *rpoD*.

However, there were 16 (6.7%) isolates that were not typeable using the first step of our assay, showing no amplification of *rpoD* gene. These isolates were later characterized and identified by amplification and sequencing of the *rpoB* gene fragment. Of the 16 not typeable strains, 12 grew at 14 °C:

- 8 were isolated from the same seawater sample from site 2 (Loch Creran estuary); among these, 4 isolates showing no amplification of *rpoB* marker gene were classified as non-Vibrionaceae, while other 4 were identified as *P. profundum* (n=3) and *V. brasiliensis* (n=1);

- 3 were isolated from seawater sample from site 5 and 6 (Atlantic Ocean, Clachan Bridge and Easdale, respectively) and were identified as *V. splendidus*;
- 1 was isolated from seawater sample from site 6 (Atlantic Ocean, Easdale) and was identified as *V. natriegens*.

Other 4 non typeable strains, isolated from sediment samples, were able to grow at 21 °C but not at 10 or 35 °C and did not yield any amplification with the universal *rpoB* primers, so that they will need to be further analysed in order to ascertain their identity.

The 5 isolates harboring the 673 bp amplicon (corresponding to only one of the two bands expected for *Aliivibrio* spp. by our identification key) were:
- 2 isolates grew at 10 °C and were isolated from sediment collected in site 1 (Loch Etive, Ardchattan Priory) and seawater sampled in site 2 (Loch Creran estuary);
- 3 isolates grew at 20 °C and were all isolated from sediments and seawater collected in site 1.

They were submitted to the amplification of the *rpoB* gene fragment and none of them showed amplification of *rpoB* marker gene. So that they will need to be further analysed in order to ascertain their identity.

B) isolates from Gulf of Aqaba, Israel, North-East Red Sea

Multiplex PCR amplification for Vibrionaceae genera (*rpoD* gene)

Among the 238 analyzed isolates, we found 197 isolates positive for the amplification of *rpoD* fragments.

Among them, 183 (92.9%) showed the typical *Vibrio* amplicon (527 bp band), 4 (2.0%) showed the typical *Aliivibrio* amplification profile (both 673 bp and 527 bp bands), 2 (1.0%) showed the typical *Photobacterium* amplification profile (both 161 bp and 249 bp bands), while 8 (4.1%) showed an unexpected profile with only one of the typical *Photobacterium* amplicon (249 pb band).

No *Grimontia* profile type were found among the isolates tested. These strains were not further identified by amplification and sequencing of the *rpoB* gene.

There were 41 (17.2%) isolates that were not typeable using this step of the multiplex, showing no amplification of *rpoD* gene. These isolates will be later characterized and identified by amplification and sequencing of the *rpoB* gene fragment.

5. DISCUSSION

This study presents a rapid and reliable method for the simultaneous differentiation of *Aliivibrio*, *Photobacterium* and *Vibrio* genera and a number of *Vibrio* species using an identification key based on multiplex PCRs. Rapid detection and quantification of Vibrionaceae becomes more crucial due to the increase of vibrio infections, even at high latitudes (Baker-Austin *et al*., 2013; Vezzulli *et al*., 2013). A particularly outstanding example is the case of *Vibrio parahaemolyticus*, which is considered an indicator of climate change as it proliferates only in waters with $T \geq 15$ °C. The State of Alaska began screening oysters for V. parahaemolyticus in 1995, but did not detect it until summer 2004 when the first human outbreak of V. parahaemolyticus -associated gastroenteritis was documented, involving 62 people who consumed raw oysters (Goertz *et al*., 2013).

The tracking of vibrios in the environment is a very important challenge for both animals and human health and is closely related to their ecology and epidemiology. However, common phenotypic approaches for vibrios present a number of limitations, such as the time required to obtain confirmatory results, or the great variability in the biochemical characteristics, that is a peculiar trait of Vibrionaceae and renders their phenotypic identification particularly difficult. Commonly used molecular techniques may provide an efficient means of identifying Vibrionaceae (Ki *et al.*, 2009). However, these typing approaches do not have the ability to discriminate the whole range of Vibrionaceae and simultaneously identify these species.

We therefore combined in a multiplex PCR-based identification key one genus-specific and two species-specific primer sets.

This molecular identification key has a hierarchical and sequential structure and not all steps are obligatory. Once the first step of the multiplex PCR (amplification of *rpoD* gene) has been realized and positive results have been obtained for the

detection of *Vibrio* genus in the samples, the characterization of the detected *Vibrio* isolates to the species level may be achieved with the following steps (*recG* and/or *rplB* gene amplification).

Critical parameters of such an assay rely on the choice of proper target genes and the design of corresponding primers. The combination of several genes for the key proposed in this work was pursued to give the most robust identification (Thompson *et al.*, 2005). Unfortunately, there is no consensus among the genes examined across species, with the selection of loci often specific to a particular study.

For instance, the 16S rRNA gene, widely used for species definition and identification among bacteria, has limited power for *Vibrio* spp. because of its low taxonomic resolution. Apart from the fact the 16S rDNA spans a very small portion of the genome, the lack of informative characters and its slow evolutionary rate can complicate the differentiation of closely related strains of bacteria. As alternative markers to 16S rDNA to assess genetic variation within individual species, we therefore selected and validated *rpoD*, *recG* and *rplB*, three highly conserved housekeeping genes previously demonstrated to possess the discriminating potential for the development of specific primers for bacterial identification (Pascual *et al.*, 2010; Santos and Ochman, 2004). Because of lateral gene transfers and chromosomal rearrangements, indeed, common phylogenetic markers for the identification of bacteria may fail in identifying vibrios (Thompson *et al*., 2004).

Compared to 16S rRNA, which shows more than 98 % homology within *Vibrio*-core species (Dorsch *et al*., 1992), *rpoD* gene of Vibrionaceae shows a minimum of 96% intraspecific sequence similarity and 79.1% interspecific sequence similarity (Pascual *et al.*, 2010). This gene was thus considered available for identification of Vibrionaceae genera by genetic differences.

The method for the differentiation of *Vibrio* spp. was based on the multiplex amplification of *recG* and *rplB* genes, selected thanks to numerous primer binding sites conserved across group of species, even if they had never been used before to

detect inter-specific diversity among *Vibrio* spp. Both *recG* and *rplB* genes were selected for the validation of the specific *Vibrio* identification assay.

Although non-specific reactions by primers specifically designed in this study to target *recG* gene occurred in 4 test strains (4.8%), it did not interfere with the specificity of the PCR, as these strains belonged to species not resolved by *recG* multiplex PCR (*V. anguillarum*, *V. kanaloe* and *V. vulnificus*).

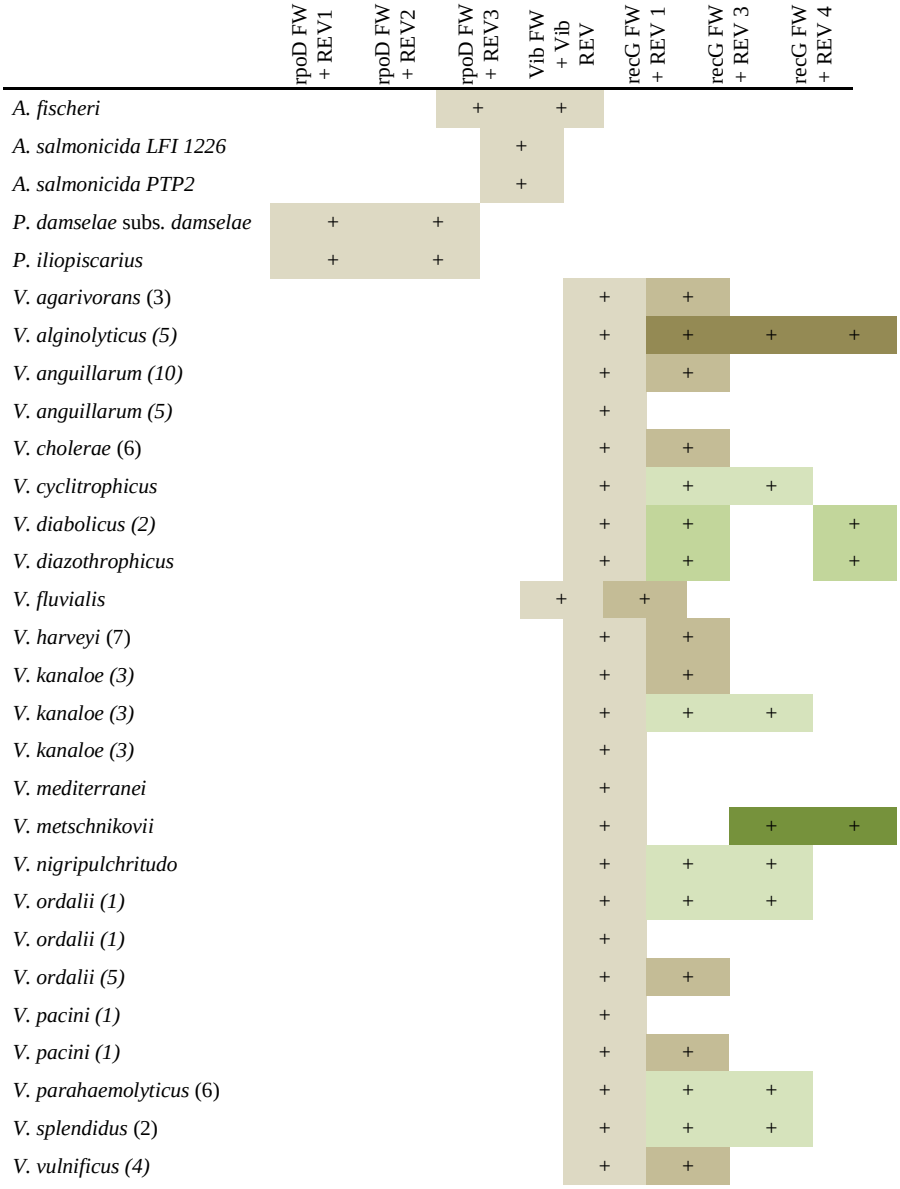
Unfortunately, *rplB* primer combinations resulted in high levels of non-specific amplification (52.4 %) among the tested isolates, despite the optimization of PCR conditions and the high specificity of the primers highlighted by *in silico* studies. No previous studies are available to investigate the usefulness of *rplB* gene as a genetic marker specific for vibrios. However, our results show that *rplB* is unable to resolve *Vibrio* spp., therefore it is not recommended for differentiate them within the identification key.

It is likely that the other specific primers designed for *gyrB*, *pyrG* and *dnaE* multiplex amplification will be validated in the future with the possibility of extending the protocol described here to a 6 steps identification key.

Thus, at this stage, the work presents a validated multiplex PCR assay capable of simultaneous detection and differentiation of genus *Aliivibrio*, *Vibrio* and *Photobacterium*, in the first step, and of *V. alginolyticus*, *V. diabolicus*, a group of species comprising *V. agarivorans*, *V. cholerae* and *V. harveyi*, and a group comprising *V. parahaemolyticus* and *V. splendidus*, thanks to the concatenated multiplex amplification of *rpoD* + *recG* genes. Our protocol achieves reliable identification of isolates in 3 h (multiplex PCR) and is more reliable and faster than biochemical tests and faster than sequencing of genetic markers.

Multiplex amplification of *rpoD* + *recG* genes split the examined strains in 2 main groups of *Vibrio* isolates: one positive to the amplification with recG FW + REV 1 primer and the other positive to the amplification with recG FW + REV 1 and recG FW + REV 3 primers (Table 5.1).

Table 5.1. Identificative patterns of amplication obtained from the examined isolates for *rpoD* and *recG* genes.



However, it is important to note that this step allows to resolve the *V. harveyi* cluster (*V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus* e *V. diabolicus*): despite the *in silico* simulation, *recG* multiplex PCR is able to discriminate this group of species. All *V. harveyi*, *V. parahaemolyticus* and *V. diabolicus*, indeed, showed species-specific amplification patterns. The discriminative power showed by this assay is of particular importance, considering that *Vibrio* species currently documented as members of the Harveyi clade are known pathogens of fishes, corals, mollusks and humans (Lin *et al.*, 2010), but often undergo to inconsistencies regarding their identification within the clade (Hoffmann *et al.*, 2012). Variability within *V. alginolyticus* has already been highlighted by George *et al*. (2005) and the four strains analyzed effectively showed 3 different amplifications profile.

V. cholerae, that showed a specie-specific profile for *recG*, should be distinguishable from the closely related *V. mimicus*, according to *in silico* evaluations. However, unfortunately *V. mimicus* was not present in the strain collection at the time of the study, thus we could not validate the expected result.

V. splendidus cluster (*V. splendidus*, *V. kanaloe* and *V. cyclitrophicus*) underwent to the identification key, except for *V. tasmaniensis*, whose strains are not available in the collection. Multiplex amplification of *recG* gave the same amplicon pattern for *V. splendidus* and *V. cyclitrophicus* and does not allow the differentiation among these two related species, while strains belonging to *V. kanaloe* are not univocally characterized by the same pattern. However, this step of the multiplex PCR allowed to characterize *V. splendidus* and *V. cyclitrophicus*.

Among most important human pathogen, *V. alginolyticus* is clearly identified through the multiplex by a peculiar profile. On the contrary, at this stage, it is not possible to differentiate *V. cholerae* from *V. vulnificus*. However several primers are available in literature for the species-specific identification of these well studied species (Nandi *et al*., 2000; Nhung *et al*., 2007).

Beside the well-studied *V. alginolyticus*, *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*, known to be severe human pathogens, *Aliivibrio* spp.,

Photobacterium spp., *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *V. splendidus* are all relevant pathogens of marine animals. This is one of the reasons why it is important to know whether a sample contains certain vibrios, since their presence is linked to potential pathogenicity towards humans or animals.

Among the main animal pathogens, strains of *V. anguillarum* did not give a coherent amplification pattern through the identification key. The specificity of this multiplex PCR assay for detection of *V. anguillarum* would have to be further tested with multiple biotypes of this species and close relatives (*V. ordalii*), given its well-known intraspecific heterogeneity and the importance of this pathogen for a wide range of aquatic organisms.

In this work, 3 *V. anguillarum* and 2 *V. ordalii* biotypes from the collection of the Norwegian School of Veterinary Sciences have been tested. Amplification of *recG* allowed the identification of one group showing peculiar profiles within the *V. anguillarum* isolates and one group negative for the amplification. It is possible that these differences are due to the presence of different serotypes among the tested isolates: the O1 serotype, indeed, is mainly related to vibriosis in fish, while the other nine are commonly environmental (Sørensen and Larsen, 1986). Thus, further investigation are planned to individuate *V. anguillarum* biotypes involved in this study and eventually select genes and primes able to resolve them.

To test the usefulness of our method to this purpose, we eventually used the identification key based on multiplex PCR to evaluate the occurrence of these species using environmental samples collected from Oban (Scotland) and Eilat (Israel) coastal waters. The assay was able to correctly identify 88.1 % of the isolates as *Aliivibrio*, *Photobacterium* or *Vibrio*. The percentage of non-typeable isolates at this stage was higher for Eilat isolates (17.2 %). This is probably due to the different composition of the Red Sea microbial community, particularly in sediments, that may harbor mesophilic bacteria capable of growing on TCBS selective medium even if not belonging to Vibrionaceae.

Previously, several authors carried out a similar work based on multiplex PCR the simultaneous detection or identification of vibrios in clinical samples or in seafood (Espiñeira *et al.*, 2010; Panicker *et al*., 2004; Tarr *et al*., 2007). To our knowledge, this is the first study which aimed to develop a multiplex PCR-based identification key for simultaneously differentiate environmental *Aliivibrio*, *Photobacterium* and *Vibrio*. Beside the well-studied *Vibrio*, such genera are of growing interest due to the presence of human and animal pathogenic species (*Photobacterium damselae* and *Aliivibrio salmonicida*). During this study, the identification key has been validated only on *A. salmonicida*, *A. fischeri*, *P. damselae subs. damselae* and *P. iliopiscarius*, other than *Vibrio* spp. giving a 100% concordance with sequencing results. The assay has been able to promptly detect the presence of *Photobacterium* spp. and *Aliivibrio* spp. with the possibility of excluding *A. fischeri* (a non-pathogenic species) from the results, as it has a species-specific profile. This feature makes the assay particularly useful for monitoring and tracking species related to vibriosis outbreaks in marine environment.

Through our experimentation, the lower detection limit of the multiplex PCR for Vibrionaceae genera (amplification of *rpoD* gene) was established to be as low as between 10^5 and 10^4 cfu/ml. Similar results for PCR detection limit were reported for the detection of *V. vulnificus* in environmental and clinical isolates through multiplex PCR (Panicker *et al*., 2004), suggesting that our assay is sufficiently robust. In terms of sensitivity, specificity and analysis time, our multiplex PCR is clearly a powerful, rapid and robust methodology. The sensitivity of the multiplex PCR could be further increased with a preliminary concentration step, particularly useful in case of screening directly performed on field or food samples.

Main limitations of the assay are mostly related to the impossibility of detected viable non culturable strains without using enrichment methods and to the requirement of working with culturable bacteria, unless the method is applied in combination with Real-Time multiplex PCR. The application of this protocol in the routine practice requires extensive optimization that will be the subject for further

studies in the next future: new target genes will be tested and vibrio identification will be implemented.

Vibrios are among the most important emerging waterborne and food pathogens both in developing and developed countries. Therefore, the development of a suitable methodology permitting sufficiently rapid, sensitive and correct detection of these microoganisms in the environment is of great interest as it would facilitate greatly the monitoring of Vibrionaceae and associated foodborne infections. Such a tool not only would help epidemiologists to estimate the incidence of vibrio infections and prevent the associated risk, but also provide an efficient method for ecologists to study seasonal and geographical distribution of this group of bacteria.

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SUPPLEMENTAL DATA

Table 1: Detailed list of the isolates used in the study.

	NAME	IDENTIFICATION	COLLECTION
1	giu m2	*V. agarivorans*	Sampling campaign Riserva Regionale di Macchiatonda
2	giu m3	*V. agarivorans*	Sampling campaign Riserva Regionale di Macchiatonda
3	giu m6	*V. agarivorans*	Sampling campaign Riserva Regionale di Macchiatonda
4	sett 1.1	*V. alginolyticus*	Sampling campaign Riserva Regionale di Macchiatonda
5	giu m4	*V. alginolyticus*	Sampling campaign Riserva Regionale di Macchiatonda
6	sett 4.2	*V. alginolyticus*	Sampling campaign Riserva Regionale di Macchiatonda
7	sett 6.1	*V. alginolyticus*	Sampling campaign Riserva Regionale di Macchiatonda
8	feb 2.1	*V. anguillarum*	Sampling campaign Riserva Regionale di Macchiatonda
9	feb 3.1	*V. anguillarum*	Sampling campaign Riserva Regionale di Macchiatonda
10	feb 6.3	*V. anguillarum*	Sampling campaign Riserva Regionale di Macchiatonda
11	giu 5.2	*V. anguillarum*	Sampling campaign Riserva Regionale di Macchiatonda
12	lug pis 2	*V. anguillarum*	Sampling campaign Riserva Regionale di Macchiatonda
13	mag 3a-5	*V. anguillarum*	Sampling campaign Riserva Regionale di Macchiatonda
14	mag 6b-1	*V. anguillarum*	Sampling campaign Riserva Regionale di Macchiatonda
15	mar 2.2	*V. anguillarum*	Sampling campaign Riserva Regionale di Macchiatonda
16	mar 5.2	*V. anguillarum*	Sampling campaign Riserva Regionale di Macchiatonda
17	mar 5.7	*V. anguillarum*	Sampling campaign Riserva Regionale di Macchiatonda
18	nov 3b 3	*V. anguillarum*	Sampling campaign Riserva Regionale di Macchiatonda
19	nov 5a 3-2	*V. anguillarum*	Sampling campaign Riserva Regionale di Macchiatonda
20	giu 1.2	*V. cholerae*	Sampling campaign Riserva Regionale di Macchiatonda
21	giu 5.3	*V. cholerae*	Sampling campaign Riserva Regionale di Macchiatonda
22	giu 6.3	*V. cholerae*	Sampling campaign Riserva Regionale di Macchiatonda
23	lugl did 1	*V. cholerae*	Sampling campaign Riserva Regionale di Macchiatonda
24	lugl did 2	*V. cholerae*	Sampling campaign Riserva Regionale di Macchiatonda
25	lugl did 6	*V. cholerae*	Sampling campaign Riserva Regionale di Macchiatonda
26	mar 2.1	*V. cyclotrophicus*	Sampling campaign Riserva Regionale di Macchiatonda
27	sett 1.2	*V. diabolicus*	Sampling campaign Riserva Regionale di Macchiatonda
28	sett 2.3	*V. diabolicus*	Sampling campaign Riserva Regionale di Macchiatonda
29	sett 2010 did 2.2	*V. diazothrophicus*	Sampling campaign Riserva Regionale di Macchiatonda
30	giu 2.4	*V. harveyi*	Sampling campaign Riserva Regionale di Macchiatonda
31	giu 2.6	*V. harveyi*	Sampling campaign Riserva Regionale di Macchiatonda
32	giu 2.7	*V. harveyi*	Sampling campaign Riserva Regionale di Macchiatonda
33	giu 3.5	*V. harveyi*	Sampling campaign Riserva Regionale di Macchiatonda
34	giu 4.1	*V. harveyi*	Sampling campaign Riserva Regionale di Macchiatonda
35	giu 4.3	*V. harveyi*	Sampling campaign Riserva Regionale di Macchiatonda
36	giu 4.4	*V. harveyi*	Sampling campaign Riserva Regionale di Macchiatonda
37	feb m2	*V. kanaloe*	Sampling campaign Riserva Regionale di Macchiatonda
38	feb m2-2	*V. kanaloe*	Sampling campaign Riserva Regionale di Macchiatonda
39	feb m8	*V. kanaloe*	Sampling campaign Riserva Regionale di Macchiatonda
40	feb m8 F	*V. kanaloe*	Sampling campaign Riserva Regionale di Macchiatonda

41	mar m1	*V. kanaloe*	Sampling campaign Riserva Regionale di Macchiatonda
42	mar m1-2	*V. kanaloe*	Sampling campaign Riserva Regionale di Macchiatonda
43	nov 1a-2	*V. kanaloe*	Sampling campaign Riserva Regionale di Macchiatonda
44	nov 5a 3-1	*V. kanaloe*	Sampling campaign Riserva Regionale di Macchiatonda
45	nov 5a 3-2	*V. kanaloe*	Sampling campaign Riserva Regionale di Macchiatonda
46	giu m10	*V. mediterranei*	Sampling campaign Riserva Regionale di Macchiatonda
47	feb 1.1	*V. ordalii*	Sampling campaign Riserva Regionale di Macchiatonda
48	feb 5.3	*V. ordalii*	Sampling campaign Riserva Regionale di Macchiatonda
49	feb 6.1	*V. ordalii*	Sampling campaign Riserva Regionale di Macchiatonda
50	giu 3.1	*V. ordalii*	Sampling campaign Riserva Regionale di Macchiatonda
51	mag 3a-2	*V. ordalii*	Sampling campaign Riserva Regionale di Macchiatonda
52	feb 4.2	*V. pacini*	Sampling campaign Riserva Regionale di Macchiatonda
53	nov 3b-2	*V. pacini*	Sampling campaign Riserva Regionale di Macchiatonda
54	giu 2.3	*V. parahaemolyticus*	Sampling campaign Riserva Regionale di Macchiatonda
55	giu 3.2	*V. parahaemolyticus*	Sampling campaign Riserva Regionale di Macchiatonda
56	mar 4.1	*V. parahaemolyticus*	Sampling campaign Riserva Regionale di Macchiatonda
57	sett 6.2	*V. parahaemolyticus*	Sampling campaign Riserva Regionale di Macchiatonda
58	sett did 1.2	*V. parahaemolyticus*	Sampling campaign Riserva Regionale di Macchiatonda
59	feb m11	*V. splendidus*	Sampling campaign Riserva Regionale di Macchiatonda
60	giu 1.1	*V. vulnificus*	Sampling campaign Riserva Regionale di Macchiatonda
61	giu 5.1	*V. vulnificus*	Sampling campaign Riserva Regionale di Macchiatonda
62	giu 6.1	*V. vulnificus*	Sampling campaign Riserva Regionale di Macchiatonda

	STRAIN	COLLECTION
63	*A. fischeri*	Norwegian School of Veterinary Science
64	*A. salmonicida LFI 1226*	Norwegian School of Veterinary Science
65	*A. salmonicida PT2T*	Norwegian School of Veterinary Science
66	*P. damselae*	Norwegian School of Veterinary Science
67	*P. iliopiscarius*	Norwegian School of Veterinary Science
68	*V. alginolyticus*	Norwegian School of Veterinary Science
69	*V. anguillarum AL 102 (O1)*	Norwegian School of Veterinary Science
70	*V. anguillarum O1*	Norwegian School of Veterinary Science
71	*V. anguillarum O3*	Norwegian School of Veterinary Science
72	*V. carchariae*	Norwegian School of Veterinary Science
73	*V. cincinnatiensis*	Norwegian School of Veterinary Science
74	*V. fluvialis*	Norwegian School of Veterinary Science
75	*V. furnisii*	Norwegian School of Veterinary Science
76	*V. metschnikovii*	Norwegian School of Veterinary Science
77	*V. nigripulchritudo*	Norwegian School of Veterinary Science
78	*V. ordalii NCMB2167*	Norwegian School of Veterinary Science
79	*V. ordalii NCMB2169*	Norwegian School of Veterinary Science
80	*V. parahaemolyticus*	Norwegian School of Veterinary Science
81	*V. splendidus*	Norwegian School of Veterinary Science
82	*V. tubiashi*	Norwegian School of Veterinary Science
83	*V. vulnificus*	Norwegian School of Veterinary Science
84	*V. wodanis*	Norwegian School of Veterinary Science

ANNEX 1

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

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Matteucci G., Schippa S., Di Lallo G., Migliore L., and Thaller M. C.

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ORIGINAL ARTICLE

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

Giorgia Matteucci[1] · Serena Schippa[2] · Gustavo Di Lallo[1] · Luciana Migliore[1] · Maria Cristina Thaller[1]

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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*, *V. parahaemolyticus*, and *V. 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

✉ Maria Cristina Thaller
mcthaller@gmail.com; thaller@uniroma2.it

[1] Biology Department, University of Rome, Tor Vergata, Viale della Ricerca Scientifica snc, 00133 Rome, Italy

[2] Department of Public Health and Infectious Diseases, University of Rome La Sapienza, Piazzale A. Moro 5, 00185 Rome, Italy

Introduction

The family *Vibrionaceae* is a large group within the Gammaproteobacteria, 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 *Vibrio 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*, *V. anguillarum*, *V. harveyi*, *V. fluvialis*, *V. furnissii*, *V. metschnikovii*, and *V. 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, *V. 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*

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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 and salinity 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.

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).

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;

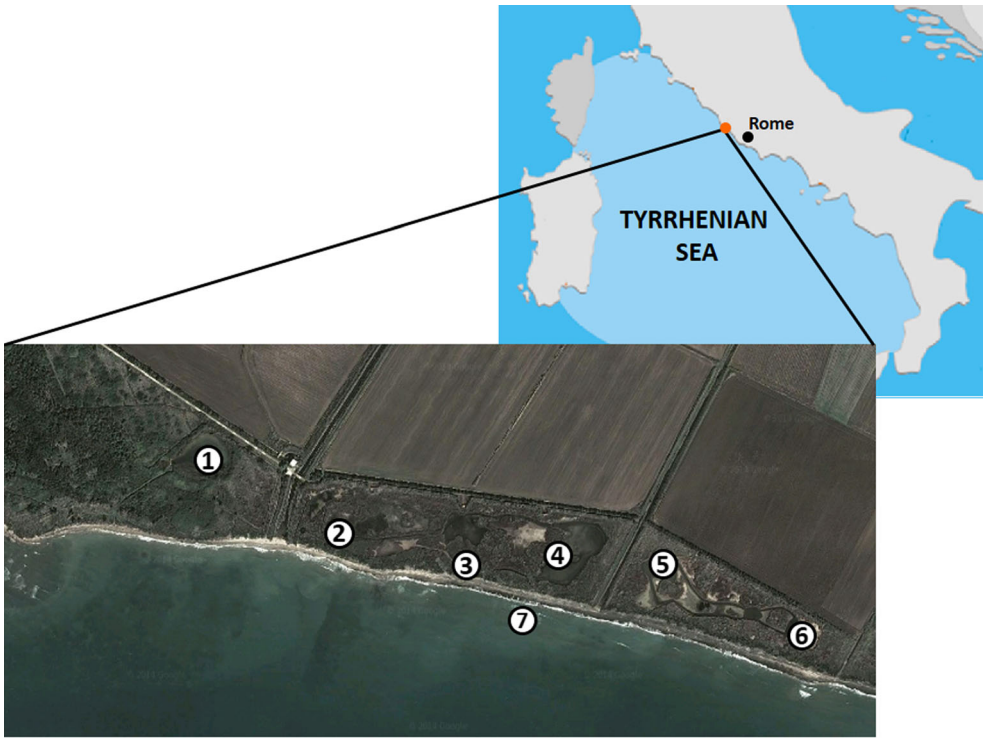


Fig. 1 Sampling sites: 1, *Didattico* artificial pond; 2, 3, 4, *Alberobello* pond; 5, 6, *Piscinula* pond; 7, coastal seawater in front of the Macchiatonda wetland

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

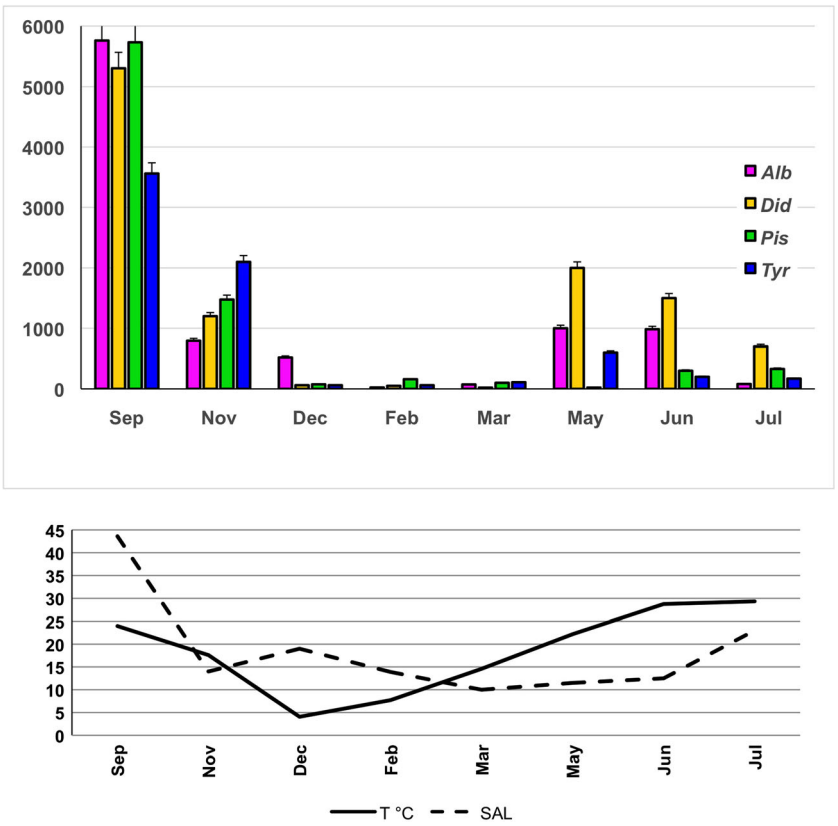


Fig. 2 Monthly variation, over the investigated period, of temperature, salinity, and *Vibrio* spp. abundance in Macchiatonda wetland. **a** Mean CFU ml^{-1} abundance with confidence intervals 95 %; **b** Mean seasonal values of temperature and salinity in the Macchiatonda wetland. *Alb*: Alberobello pond; *Did*: Didattico pond; *Pis*: Piscinula pond; *Tyr*: Tyrrhenian Sea; T °C: Temperature; SAL: Salinity

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.

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).

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*; MWB 21 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 *V. pacinii*, respectively (Preheim et al. 2011). Out of the identified species, only *Vibrio alginolyticus*, *V. anguillarum*, *V. kanaloae*, *V. parahaemolyticus*, and *V. 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.

Table 1 Multiple Linear Regression model values

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

Vibrio species distribution *vs* temperature-salinity combinations

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.

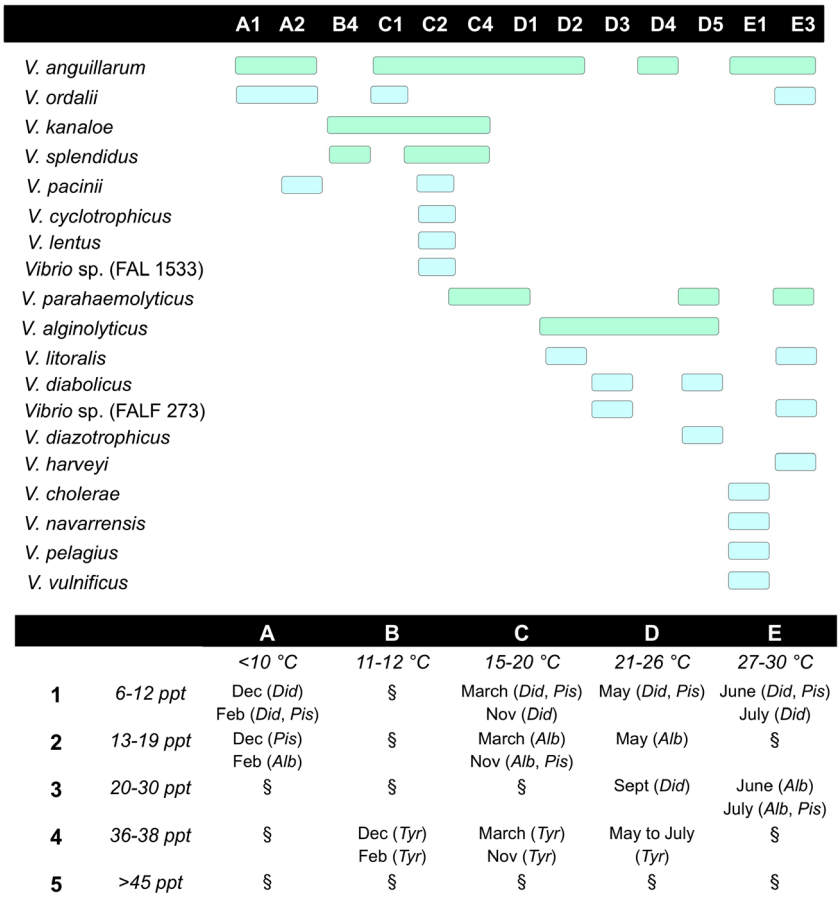
As to salinity, we have divided the wide range 0.5 to 30 ppt, usually referred to as brackish, in three sections: 1) 0.5–10 ppt -"Baltic-like"; 2) 11–20 ppt "Black-sea-like," and 3) 20–30 ppt, the higher salinity still to be regarded as truly brackish. The further intervals were 4) 31–40 typical of euhaline 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. 3 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 also shown in Fig. 3. *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 *V. 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*, *V. cyclotrophicus*, *V. lentus*, and the unnamed isolate similar to the FAL 1533 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

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. CAIM 1833	1	SW
V. kanaloae	14	BP, SW	*Vibrio* sp. FAL 1533	1	BP
V. lentus	1	BP	*Vibrio* sp. FALF 273	4	BP
V. litoralis	2	BP	*Vibrio* sp. MWB 21	2	SW

[a] Non O1/O139

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, V. diabolicus*, *V. litoralis* and unnamed isolate homologous to FALF 273 were scattered along different salinity ranges, whilst *V. harvey*, *V. diazotrophicus*, *V. cholerae*, *V. vulnificus*, *V. 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



		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*	§	§	§	§	§

Fig. 3 *Vibrio* species diversity along the Temperature/Salinity (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

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.

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 CAIM 1833 and MWB 21 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 *V. 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

Table 3 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
Vibrio sp. MWB 21				1			
V. splendidus						1	

belong to one rare and one recognized pathogenic species for humans, respectively.

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 MWB 21-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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