Journal of Applied Microbiology
June 2013, Volume 114, Issue 6, pages 1713–1724
http://dx.doi.org/10.1111/jam.12181

Diversity of *Vibrio* spp. isolated at ambient environmental temperature in the Eastern English Channel as determined by *pyrH* sequencing

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Abstract :

Aims

To describe the diversity of the culturable mesophilic and potentially pathogenic vibrios isolated at 22 and 37°C on TCBS medium, in September 2009 from seawater and surface sediments.

Methods and Results

q-PCR assays previously selected for the identification of bacterial strains isolated at 37°C were used in combination with the partial sequencing of two housekeeping genes, *pyrH* and *toxR*, to identify 315 strains isolated at 22°C. The great majority of the 37°C strains was identified by q-PCR assays, (five of the six species) with the predominance of *Vibrio alginolyticus* (85·9%) and *V. harveyi* (10·7%). The human pathogens *V. parahaemolyticus* and *V. cholerae* were rarely detected (two strains each). The 22°C strains were successfully identified by the phylogeny analysis of *pyrH* and *toxR* genes, revealing 20 *Vibrio* species, with the predominance of the clam pathogen *V. celticus* (36·8%). The Splendidus and the Harveyi groups represented the main *Vibrio* group at 22°C (80%) and 37°C (99·5%), respectively.

Conclusions

The combination of q-PCR assays and the sequencing of *pyrH* and *toxR* genes highlighted two different *Vibrio* communities at 22 and 37°C both dominated by pathogenic species for marine organisms.

Significance and Impact of the Study

The sequencing of the *pyrH* gene revealed to be a valuable tool to identify environmental *Vibrio* spp. strains isolated at 22°C, as 92·3% of them were identified in this study.

Keywords : coastal environment ; culturable *Vibrio* spp. ; diversity ; *pyrH* and *toxR* genes partial sequencing

Introduction

The vibrios are widespread worldwide and ubiquitous in the aquatic environments, occupying various habitats including marine, freshwater and estuarine ecosystems as well as aquaculture facilities (Dalsgaard 1998; Sobecky *et al.* 1998; Urakawa *et al.* 1999; Heidelberg *et al.* 2002; Thompson *et al.* 2004b). To date, if we refer to the various banks of genomic sequences and to the on-line sources of *Vibrio* species inventory (Association of *Vibrio* Biologists, www.vibriobiology.net, last consultation August 20, 2012), the genus *Vibrio* includes 135 species (Dawyndt *et al.* 2005). Numerous vibrios are pathogenic for marine organisms and 12 have been described as pathogenic for humans (Dalsgaard 1998; Paillard *et al.* 2004; Thompson *et al.* 2004a; Austin 2010). The sanitary and economic losses due to these pathogens, particularly to the species *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* have been widely described (Colwell *et al.* 1977; DePaola *et al.* 2003; Oliver 2005). In the context of global climate changes, human infections caused by vibrios are expected to increase with the increase of Sea Surface Temperature (Tantillo *et al.* 2004; Baker-Austin *et al.* 2010; Philippart *et al.* 2011; Vezzulli *et al.* 2012). Thus, a better understanding of the natural diversity of *Vibrio* species and the environmental drivers of their spatial and temporal evolution, are a prerequisite to address public health issues or the abnormal mortalities in hatcheries and aquaculture industries.

Today, powerful molecular techniques such as pyrosequencing or direct cell enumeration by solid or flow cytometry combined to Fluorescent *In Situ* Hybridization (FISH), allow to evaluate the microbial global diversity in natural samples (Sogin *et al.* 2006; Alonso-Saez *et al.* 2007; Brown *et al.* 2009; Kirchman *et al.* 2010; Lucas *et al.* 2010). However, due to the low discriminatory power of *16S rRNA* gene among the *Vibrio* genus

(Thompson *et al.* 2004a), these methods are not applicable to an environmental study of these bacteria. In contrast, direct quantification methods like real-time PCR (q-PCR) are valuable tools to specifically detect genomic units of different *Vibrio* species in natural or seafood samples (Gubala and Proll 2006; Nordstrom *et al.* 2007). In addition to these techniques, isolation of strains remains an essential step to fully characterize the environmental strains and their potential virulence and to compare environmental monitoring and epidemiological data (mainly based on culture standards). Furthermore, establishing environmental strain collections is very useful and valuable to further study the presence and emergence of some *Vibrio* spp., in relation to environmental variables such as physicochemical and biological parameters.

Until the 1990's, the taxonomy of the vibrios was mainly based on morphological traits description on agar selective media and on biochemical tests. These techniques suitable for type or clinical strains are not reliable for the identification of most environmental strains due to the diversity of the metabolism expressed by these bacteria (Croci *et al.* 2007). The molecular biology provided decisive tools such as the PCR and DNA-DNA hybridization or more recently, MultiLocus Sequence Analysis (MLSA), which improved the taxonomy within this group. Numerous phylogenetic markers, ubiquitous within the genus, were proposed for a more precise delineation of the *Vibrio* species (Thompson *et al.* 2005; Sawabe *et al.* 2007; Pascual *et al.* 2010). A multilocus sequence analysis comparing the species resolution level of *rpoA*, *recA* and *pyrH* genes, performed by Thompson *et al.* (2005), described the *pyrH* gene as a good discriminant marker at the species level. They showed a stability of this locus due to high proportions of synonymous mutations leading to the conservation of the amino acid sequence. Their work was based on a previous study from Zeigler (2003) who proposed that a single protein-encoding gene, carefully selected, could be used to assign strains to a species

with an acceptable precision. He defined that the selected gene must be characterized by a wide distribution among bacteria, must be conserved long enough to be informative to have an acceptable precision in the prediction of the whole-genome. Thus, the *pyrH* gene, fitting these criteria, constitutes a good predictor of the whole *Vibrio* genome and also a good discriminatory target at the species level. This has been validated by other authors for the identification of environmental strains (Chimetto *et al.* 2009; Alves *et al.* 2010; Gregoracci *et al.* 2012). Pascual *et al.* (2010) performed the same analysis on the *16S rRNA* gene plus six protein-encoding genes including two of those tested by Thompson *et al.* (2005) (*recA*, *pyrH*, *rpoD*, *gyrB*, *rctB*, and *toxR*) and determined the *toxR* gene as showing the most powerful taxonomic resolution within the *Vibrio* genus.

In a context of a 2-year study regarding the ecology of *Vibrio* populations and the presence of pathogenic species in French coastal waters, we previously described a strategy based on the isolation/identification of *Vibrio* strains at 37°C and 22°C (Tall *et al.* 2012). We optimized q-PCR assays and we were able to identify *V. alginolyticus*, *V. parahaemolyticus, V. vulnificus*, *V. cholerae* and *V. harveyi* species. This strategy was efficient to determine the culturable diversity at 37°C as 97.2% of the strains were well identified, with *V. alginolyticus*, the major species, representing 85.9% of the strains. At this temperature, only 10 strains remained to be identified. However, only 2.2% of the strains isolated at 22°C were identified by q-PCR and as *V. alginolyticus*, leading to select the sequencing of housekeeping genes as an alternative. The present study evaluates the diversity of culturable *Vibrio* isolated at two temperatures of isolation (22°C and 37°C) on TCBS agar medium from environmental samples in a coastal area. This was performed by combining q-PCR assays and the sequencing of the *pyrH* and the *toxR* genes of a large number of environmental *Vibrio* spp.

strains isolated from seawater and surface sediments during the first sampling campaign (September 2009) of a 2-year study.

Materials and methods

Study area and sample collection

The study area, located in Northern France (English Channel, Pas de Calais, 59, France), has been chosen for its contrasting coastal characteristics and water uses (Fig. 1). The first sub-area is located around industrial and human recreational activities (bathing, water sports and fishing) near the cities of Gravelines and Grand-Fort Philippe. The second sub-area is located around a natural restricted area (Platier d'Oye). The mean seawater temperature in the area at the time of sampling (September 23rd and 24th, 2009) was 18.6°C (±0.2°C) and the salinity ranged from 33.5 to 34.4 ppt. Seawater and surface sediments samples were collected during an ebb tide and along two transects. The Transect 1 (T1) is located in front of the coastal segment including human and industrial activities and the Transect 2 (T2) is located in front of the natural restricted area. To study the coast-off distribution of *Vibrio* abundance and diversity, at the two temperatures of isolation, water and sediment samples were collected along both transects including four different sampling points, one at the coast and the three others at 300, 1,300 and 3,000 m from the coast with mean depths of 4, 6, 9 and 23 m, respectively (Fig. 1). To study a depth gradient, surface and bottom seawater and surface sediment were sampled at each point (except for the first point at the coast, only surface water was sampled). Two liters of surface and bottom seawater were sampled at each point using a Van Dorn bottle and conditioned into 2-liter sterile flasks to perform bacteriological analyses. The surface sediments were sampled at each point using a metallic grab sampler and

conditioned into 1-liter sterile flasks. The samples (14 water samples and 8 sediment samples) were transported in isothermal condition to the laboratory for bacterial strains isolation and analyzed within 5 to 6 hours.

Isolation and conservation of the strains

The isolation and conservation of the *Vibrio* strains were performed according to Tall *et al*. (2012). Briefly, seawater samples were filtered (0.1, 1, 10, 50 and 100 ml) onto 0.45μm-pore-size nitrocellulose filters (Millipore™ SA, ST Quentin Les Yvelines, France). Ten gram of sediment samples were diluted in 90 ml of tryptone salt, mixed during 10 min and filtered (10 to 25 ml) through similar membranes or the dilutions were directly plated. Membranes and dilutions were plated onto thiosulphate–citrate-bile-salts–sucrose agar plates (TCBS, Difco™, Becton Dickinson and Company, Pontde Claix, France) and incubated at 22 ± 0.2°C or 37 ± 0.2°C for 48h and 24h, respectively. Twenty presumptive *Vibrio* single colonies (yellow and green colonies) from each point were streaked onto TCBS agar and then isolated on Bacto™ Heart Infusion (HI) 2% NaCl agar medium pH 7.4 (HI; Difco™, Becton Dickinson and Company, Le Pont de Claix, France) to control purity. They were enriched in 1.5 ml HI 2% NaCl liquid medium at 22 ± 0.2°C or 37 ± 0.2°C for 24h and the cultures were conserved on 96-well plates with 10% glycerol and at -80°C for further analyses.

DNA extraction

The strains DNA extracts were obtained according to Tall *et al.* (2012). Briefly, the strains stored at -80°C were newly cultured in a 2 ml 96-well plate containing 1.5 ml of Heart Infusion 2% NaCl liquid medium per well and incubated 24h at 22°C ± 0.2°C or 37°C ± 0.2°C, depending on the previous isolation temperature. Bacterial cultures were centrifuged

and pellets were washed twice with sterile buffered saline at 2000 g for 10 min and suspended in 100 µl of sterile distilled water and transferred in a 96-well microplate. After heating at 100°C for 15 min, the supernatants containing the DNA extracts were obtained by centrifugation at 3500 g for 10 min. The DNA extracts were diluted to obtain a final concentration of 50 ng μl^{-1} and stored at -20°C until use.

Identification of strains

All the DNA extracts from presumptive *Vibrio* strains, showing phenotypic features of *Vibrio* on TCBS agar (yellow or green colonies), were tested by q-PCR SYBR Green (Invitrogen Kit, Fischer Scientific SAS, Ilkirch Graffenstaden, France) using primers selected in the *16S rRNA* region specific for the *Vibrio* genus (Thompson *et al.* 2004b). All the strains identified as belonging to the genus *Vibrio* spp. were then identified at the species level, by specific q-PCR concerning the 37°C-strains or by partial sequencing in the cases where the q-PCR could not provide identification and for the 22°C-strains. *Vibrio cholerae*, *V. vulnificus* and *V. alginolyticus* were identified using SYBR Green q-PCR systems targeting the *dnaJ* gene (Tall *et al.* 2012). *Vibrio harveyi* was identified using a SYBR Green q-PCR system targeting the *toxR* gene (unpublished data) designed with primers previously used by Pang *et al.* (2006).

***pyrH* and *toxR* genes amplification and sequencing**

The conditions of the following conventional PCR protocols were slightly modified from those described previously by Chimetto *et al.* (2009) and Pascual *et al.* (2010). A region of the *pyrH* gene (440 nt) was amplified by conventional PCR using the forward primer pyrH80F (5'GAT CGT ATG GCT CAA GAA G3') and the reverse primer pyrH530R

(5'TAG GCA TTT TGT GGT CAC G3') as previously described by Chimetto *et al.* (2009). The amplification reactions contained 1 X PCR buffer, 0.2 mmol l^{-1} of each deoxyribonucleotide triphosphate, 2 U Taq polymerase (Roche Diagnostics, Meylan, France), 0.48 μmol l^{-1} of each primer (Eurogentec, Seraing, Belgium) and 2 μl of the template (50 ng μl^{-1}) or of sterile water (negative control) in a final reaction volume of 50 μl. The thermal program consisted of a 5 min initial denaturation step at 95°C, followed by 30 cycles at 95°C for 1 min, 65°C for 2 min 15 s and 72°C for 1 min 15 s, and a final elongation step of 7 min at 72°C. A region of the *toxR* gene (477 nt) was amplified by conventional PCR using the forward primer toxRs (5'-GANCARGGNTTYGARGTNGAYGAYTC-3') and the reverse primer toxRas (5'-TTDKKTTGNCCNCYNGTVGCDATNAC-3') as previously described by Pascual *et al.* (2010). The thermal program consisted of a 5 min initial denaturation step at 95°C, followed by 30 cycles at 95°C for 1 min, 61°C for 2 min 15 s and 72°C for 1 min 15 s, and a final elongation step of 7 min at 72°C. The quality of the amplified products was examined after an electrophoresis on 1.5% (w/v) agarose gel (Eurobio, Courtaboeuf, France) with 500 μg ml^{-1} ethidium bromide (SIGMA-ALDRICH®, Saint-Quentin Fallavier, France) staining in 1X Tris-Borate-EDTA (TBE) electrophoresis buffer (SIGMA-ALDRICH®, Saint-Quentin Fallavier, France). The PCR products were then sent to the genomic platform of Biogenouest® (Station Biologique de Roscoff, Roscoff, France, www.sb-roscoff.fr) for purification and double strand sequencing. The fragments purified with the illustra™ ExoStar 1-Step (Dominique DUTSCHER SAS, Brumath, France) were used directly for DNA sequencing with the dideoxy chain termination method (BigDye® terminator cycle sequencing kit v3.1, Applied Biosystems, Life Technologies, Carlsbad, CA, USA) using the above mentioned *pyrH* or *toxR* primers. The reaction products were analyzed with the ABI Sequence Scanner version 1.0 (Applied Biosystems, Life Technologies, Carlsbad, CA, USA).

Phylogenetic analysis

Sequence nucleotide alignments were first revised visually to identify positions with uncertain results, mainly at the 3' and 5' ends of the sequences, to be corrected or omitted for further analyses. Multiple sequence alignments were obtained using CLUSTALX (Thompson *et al.* 1997) with the Bioedit v7.0.9.0. software (Hall 1999), taking into account the corresponding amino acid alignment for protein-coding genes with the Seaview4 software (Gouy *et al.* 2010). The phylogenetic constructions of both genes were inferred using the Neighbor-Joining (NJ) method (Saitou and Nei 1987) using Kimura-2-parameters distance estimations (Kimura 1980) and conducted with the MEGA 5 software (Tamura *et al.* 2011). The maximum intraspecies and interspecies distances were determined through distance matrix computed with the Kimura 2-parameter model which involved 105 sequences for *pyrH* and 71 sequences for *toxR*. No gap or missing data were observed for any of the *pyrH* sequences. All positions containing alignment gaps and missing data were eliminated by pairwise deletions for *toxR* gene. Clades were drawn based on a bootstrap analysis with the FigTree v1.3.1 program. The sequences from the strains isolated in this study were aligned with a wide range of *pyrH* and *toxR* sequences from reference or type strains of *Vibrio* species available on GenBank/EMBL, TAXVIBRIO (Thompson *et al.* 2009b) and StrainInfo websites (Dawyndt *et al.* 2005). These sequence data have been submitted to the GenBank database under accession No. JX401575 - JX401899 and No. JX401900 - JX401924 for *pyrH* and *toxR*, respectively.

Statistical and diversity analyses

Rarefaction curves were calculated in order to evaluate the representativeness of the number of colonies isolated per point, to picture the culturable *Vibrio* diversity at 22°C and

37°C. A good representativeness is characterized when a plateau is reached, meaning that no increase in the number of species was detected when isolating more colonies. These analyses were performed using the PAST v1.68 software (Hammer *et al.* 2001).

Results

Diversity of *Vibrio* spp. revealed by sequencing

Three hundred and fifteen strains were isolated at 22°C and they were all confirmed as belonging to the *Vibrio* genus by q-PCR identification. Among the strains isolated at 22°C, 291 (92.3%) were well identified through the partial sequencing of *pyrH* and could be assigned to twenty *Vibrio* species (Fig. 2). *Vibrio celticus* was the most frequently detected *Vibrio* species, with *V. atlanticus* and *V. chagasii* (36.8%, 14% and 10.7% of the strains, respectively) (Fig. 3B). *Vibrio crassostreae*, *V. kanaloae*, *V. cyclitrophicus*, *V. splendidus*, *V. rotiferianus*, *V. harveyi* and *V. fischeri* species represented 2.2% to 6% of the strains, with more than ten strains per cluster (Fig. 3B). Among the 24 non-identified strains after *pyrH* partial sequencing, one strain (W2 3G12 – GenBank accession number JX401717 and JX401908 for *pyrH* and *toxR*, respectively) showed 94% *pyrH* sequence similarity with *Vibrio* L2 sp. nov. strain R-77 (Chimetto *et al.* 2009). The *toxR* partial sequencing for this strain showed 98% sequence similarity with *V. cyclitrophicus* LMG 21580. The partial sequencing of *pyrH* and *toxR* did not allow the identification at the species level of the remaining 23 strains (7.3% of the total strains). These 23 strains were distributed among 9 groups showing different percentages of *pyrH* sequence similarity (85-95%) with known *Vibrio* species (Table

1). The majority of those strains were related to the Splendidus group (7 strains) and to the Photobacterium group (12 strains).

Compared culturable *Vibrio* spp. diversity, 37°C versus 22°C

After the q-PCR assays, only ten of the 37°C strains (n=356) remained not identified. The partial sequencing of *pyrH* gene led to the assignment of 2 strains to the *V. parahaemolyticus* species (97-100% sequence similarities with *V. parahaemolyticus pyrH* sequences) (see in supplementary materials Fig. S1). Eight strains remained non-identified by the partial sequencing of *pyrH*. The phylogenetic construction on the basis of the *toxR* gene led to assign 7 strains to the *V. campbelli* species and a single strain (10G9) to the *V. communis* species (see in supplementary materials Fig. S2). Thus, twenty three species were detected among the 22°C- and 37°C- strains (20 species at 22°C, 6 species at 37°C and 3 species in common). The analysis of the spatial distribution of the strains, performed on the phylogenetic trees obtained at 22°C and according to the origin of isolation (seawater or surface sediments, Transect 1 or Transect 2), revealed that the species were not specifically allocated to any of the compartments (seawater or sediment, data not shown). The diversity observed at 22°C and 37°C revealed that, if *V. alginolyticus* was predominant among the strains isolated at 37°C (85.9%), it became a minor species at 22°C, and represented only 1.3% of the strains (Fig. 3A and B). Moreover, the number of *Vibrio* species isolated at 22°C (20 species) was higher than at 37°C (5 species). Rarefaction curves for the different sampling points according to the number of species detected were calculated for the two transects and for the two temperatures of isolation (Fig. 4). The estimates of species numbers indicated that the sampling effort performed in this study (20 colonies per point) contributed to a good

picture of the culturable *Vibrio* diversity at 37°C but to an underestimation of the culturable *Vibrio* diversity at 22°C.

Discussion

The objective to determine the culturable *Vibrio* diversity at 37°C (potential human pathogens) but also at 22°C (potential animal pathogens), was reached by combining q-PCR assays and the partial sequencing of *pyrH* and *toxR* genes. Moreover, rarefaction analyses showed that the diversity of culturable *Vibrio* at 37°C was well represented. However, the culturable diversity at 22°C was underestimated at this temperature, according to the rarefaction curves analyses, and suggesting that more bacterial colonies should be isolated (above 40 colonies per point at least) to better represent the diversity at this temperature. Consequently, q-PCR assays were not adapted for the identification of the 22°C strains, the dominant species at 37°C (*V. alginolyticus* and *V. harveyi*) and the potential human pathogens being under- or not represented at 22°C. The sequencing of *pyrH* and *toxR* were more suitable approaches for the determination of the 22°C culturable *Vibrio* diversity.

The *pyrH* threshold of at least 94% sequence similarity for strains of the same species (Thompson *et al.* 2005) could lead, in some cases, to misidentifications in *Vibrio* groups as it was previously noticed by Pascual *et al.* (2010). These authors showed that the taxonomic resolution of *pyrH* was lower for some of the species of the Harveyi group, such as *V. campbellii* and *V. rotiferianus*, since they formed a polyphyletic group. In our study, the *pyrH* range of intraspecific and interspecific sequence similarities for the Harveyi group was 91.9%-100% and 81.3%-100%, respectively. This species delineation for the *pyrH* gene was determined through the analysis of 105 *Vibrio* sequences, from reference and environmental strains, of the Harveyi group. It revealed a low discriminatory level between the species *V.*

campbelli and *V. rotiferianus* but also with the species *V. harveyi, V. communis* and *V. owensii* (up to 100% interspecific sequence similarities). Recently, Hoffmann *et al.* (2011) pointed *Vibrio* species misidentification within the Harveyi clade, particularly for *V. communis* and *V. owensii* which would belong to the same species. The second gene selected for partial sequencing, the *toxR* gene, led to overcome the limit of *pyrH* observed within the Harveyi group. Indeed, the partial sequencing of the *toxR* gene for the 8 remaining non-identified strains isolated at 37°C, showed a better taxonomic resolution, as confirmed by the gap observed between the maximum interspecies (31%-70.2%) and the minimum intraspecies distances (77.4%-100%). In this phylogenetic analysis, the position of the *V. communis* strains (LMG 25430 = R40496) within the *V. campbelli* group would suggest that *V. communis* and *V. campbelli* belong to the same species and thus, this raises doubts about the identification of the 10G9 strain as *V. communis*. Even if previously described as the best phylogenetic marker, the *toxR* gene did not allow the identification of 23 of the 24 non-identified strains within the 22°C *pyrH* phylogenetic tree. This could probably be explained by the lack of available *Vibrio* species sequences in the genomic banks for the *toxR* gene, which is still used seldomly as an identification tool for *Vibrio*. Another hypothesis is the presence of novel species among the non-identified strains since sequence similarities were recorded below the minimum intraspecies similarities of the known species. Other targets could be selected to confirm this hypothesis. For example, the *rpoD* gene which proved to be a better phylogenetic marker than *pyrH* (Pascual *et al.* 2010) and which was already used to describe novel species (Le Roux *et al.* 2005; Dieguez *et al.* 2011). Indeed, the relatedness of many *Vibrio* species led authors to find numerous phylogenetic markers to better discriminate closely related species (Thompson *et al.* 2007; Thompson *et al.* 2008).

During this past decade, the multilocus sequences analysis (MLSA) raised the question of the notion of species or groups of species within the *Vibrio* genus (Sawabe *et al.* 2007; Thompson *et al.* 2007; Thompson *et al.* 2009a; Pascual *et al.* 2010). Thus, Sawabe et *al.* (2007) described 14 groups or clades with MLSA and other phylogenetic and genetic markers (DNA-DNA hybridization, GC%, Amino Acid Identity, Phi test, radiation time and Habitat): the groups Anguillarum, Cholerae, Coralliilyticus, Diazotrophicus, Gazogenes, Fischeri, Halioticoli, Harveyi, Nereis, Nigripulchritudo, Orientalis, Scopthalmi, Splendidus and Vulnificus. Considering the taxonomic clustering applied to the 22 species identified in this study, the Splendidus group, including *Vibrio celticus*, *V. crassostreae*, *V. chagasii*, *V. cyclitrophicus*, *V. gallaecicus*, *V. gigantis*, *V. kanaloae*, *V. lentus*, *V. pomeroyi*, *V. splendidus* and *V. tasmaniensis* species, constituted the main group of the culturable *Vibrio* detected at 22°C (80% of the strains). It is also the dominant group of *Vibrio* in the coastal marine seawater, sediments (Sobecky *et al.* 1998; Urakawa *et al.* 1999; Urakawa *et al.* 2000; Radjasa *et al.* 2001) and bivalve molluscs (Le Roux and Austin 2006) worldwide. It comprises the highest number of species (above 10) with some pathogenic members for fish, bivalve mollusks or crustaceans (Beaz-Hidalgo *et al.* 2010). *Vibrio celticus*, the main species isolated in this study at 22°C (36.8%), and *V. atlanticus*, (14%), were previously described as potentially pathogenic for clams (Beaz-Hidalgo *et al.* 2010; Dieguez *et al.* 2011). The third main species detected, *V. chagasii* (10.7%), has been isolated from fish larvae, rotifer and seawater (Thompson *et al.* 2003). The Harveyi group, including *Vibrio alginolyticus*, *V. campabllii*, *V. harveyi*, *V. natriegens*, *V. parahaemolyticus*, and *V. rotiferianus*, represented the main group at 37°C (99.5% of the strains), the Cholera group being the second one.

Among the 37°C strains, the predominance of *V. alginolyticus* in the area is consistent with previous studies performed in Northern European waters (Hervio-Heath *et al.* 2002;

Oberbeckmann *et al.* 2010). This species is considered as an opportunistic pathogen, rarely involved in human infections and not at the origin of severe cases. The clinical cases associated with *V. alginolyticus* are very rare and if any, the virulence factors triggering the illnesses are not clearly established for this species yet (Tantillo *et al.* 2004; Schets *et al.* 2006; Campanelli *et al.* 2008). *V. alginolyticus* is part of the natural microflora of marine organisms but it could constitute a threat as this species has been reported in diseased corals, fishes, crustaceans and bivalves mollusks (Balebona *et al.* 1998; Kumar *et al.* 2007; Wang *et al.* 2007; Yan *et al.* 2007; Cervino *et al.* 2008; Harriague *et al.* 2008; Lafisca *et al.* 2008; Austin B. 2010). Moreover, it has been also associated with mortalities in aquaculture (Darbas *et al.* 1992; Snoussi *et al.* 2008). Unlike others studies, the second main species detected at 37°C is *V. harveyi*. This species is a luminescent bacterium associated with surface tissues, luminescent organs and digestive system of numerous marine organisms (Liu and Lee 1999). It was also associated with shrimp and fish mortalities (Alvarez *et al.* 1998; Liu and Lee 1999; Zhang and Austin 2000; Austin and Zhang 2006).

The comparison of the relative proportion of species between the two isolation temperatures showed that the potentially human pathogenic *Vibrio* detected at 37°C represented a very small fraction of the culturable diversity observed at 22°C. As an illustration, *V. alginolyticus*, representing 86% of the strains isolated in September 2009 at 37°C, represented only 1.3% of the strains isolated at 22°C. In a perspective to better evaluate the environmental risk linked to the presence of human and animal pathogenic vibrios, the simultaneous isolation at two temperatures could improve the understanding of the dynamics of their diversity and their ecology. Moreover, the dominance of pathogenic species for marine organisms belonging mainly to the Splendidus group, raised the interest to explore the presence and the expression of virulence markers among these species. The determination of

the environmental features associated with the analysis of the virulence genes distribution of these species could improve the risk assessment for aquaculture industries (Cano-Gomez A. *et al.* 2009).

In this study, *pyrH* and *toxR* genes proved to be appropriate targets for the screening of the culturable *Vibrio* diversity in the environment, particularly for the strains isolated at 22°C. In addition, the diversity analysis allowed picturing part of the potentially human pathogens among the total *Vibrio* population isolated at both temperatures. Moreover, the isolation of strains remains necessary to study pathogenicity mechanisms and simply the opportunity to identify new species. In a context of a routine monitoring and in the perspective of the evaluation of the potential risk of *Vibrio* for the marine organisms and for humans, the isolation at two temperatures could constitute a relevant transition step before the development of reliable direct detection and quantification methods of pathogenic *Vibrio* species in environmental samples.

Acknowledgments

This work was funded by the Vibrio Manche program of EDF R&D, the French Institute of research on marine exploration (Ifremer) and Eurofins IPL Nord. We would like to thank Dr Jean Louis Nicolas (Ifremer), Dr Marie-Anne Cambon-Bonavita (Ifremer) and Valérie Cueff-Gauchard (Ifremer) for providing *Vibrio* strains useful for this study. We also would like to thank Edith Delesmont (Eurofins IPL Nord), Olivier Mabille (EDF R&D), Lamine Barbach (EDF R&D), Joffrey Cholet, Margot Legoff, David Richard (Ifremer), and Gérard Morel (EDF R&D) for technical assistance. Finally, we would like to thanks Alexandre Cebeillac (Ifremer) for his assistance for the Fig. 1 (map of the study area). We are most grateful to the

Biogenouest® Genomics core facility and to the ABiMS® bioinformatic core facility for their technical support.

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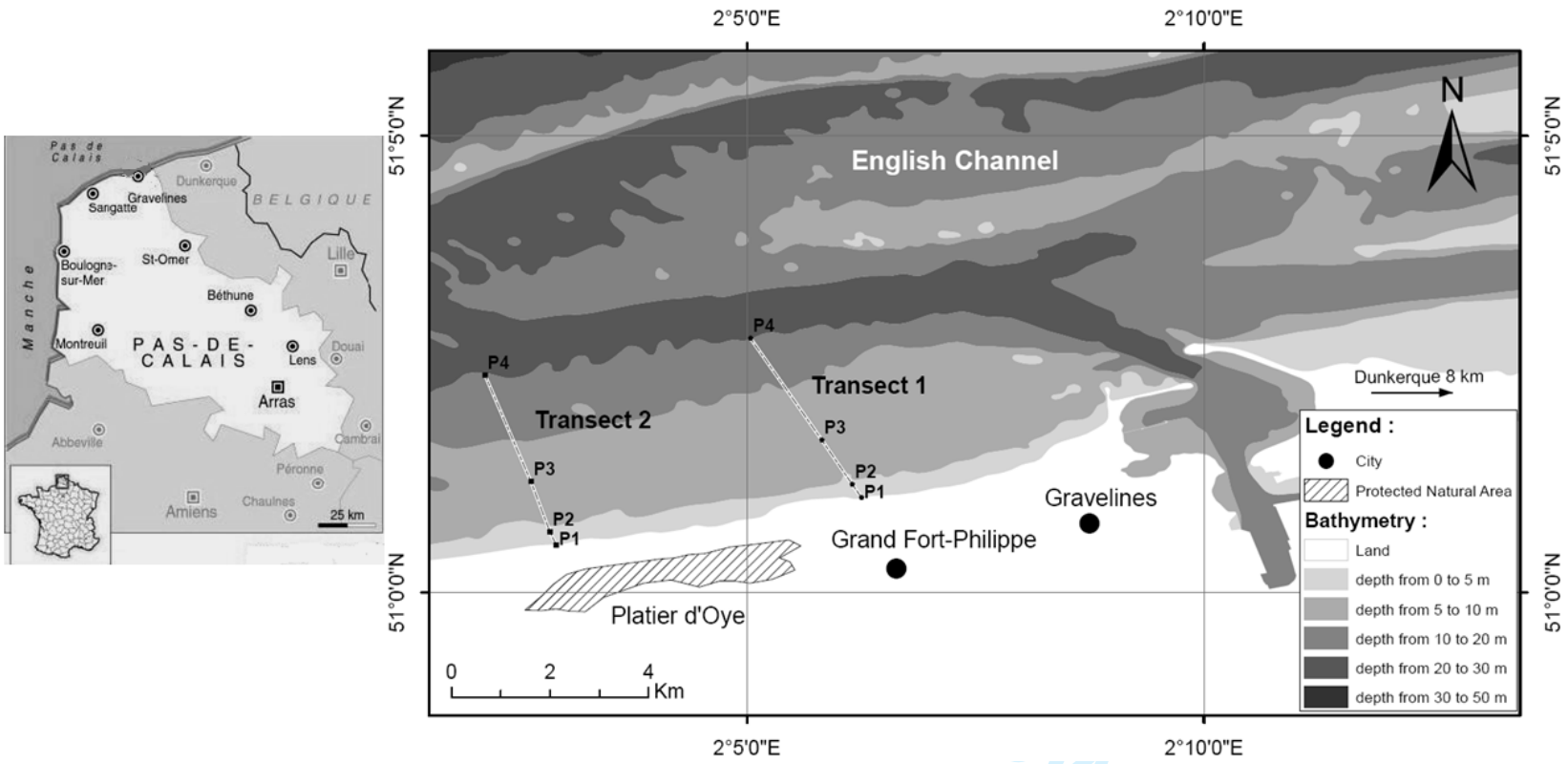

Figure 1 : Study area and sampling points along Transects 1 and 2 off the coast.

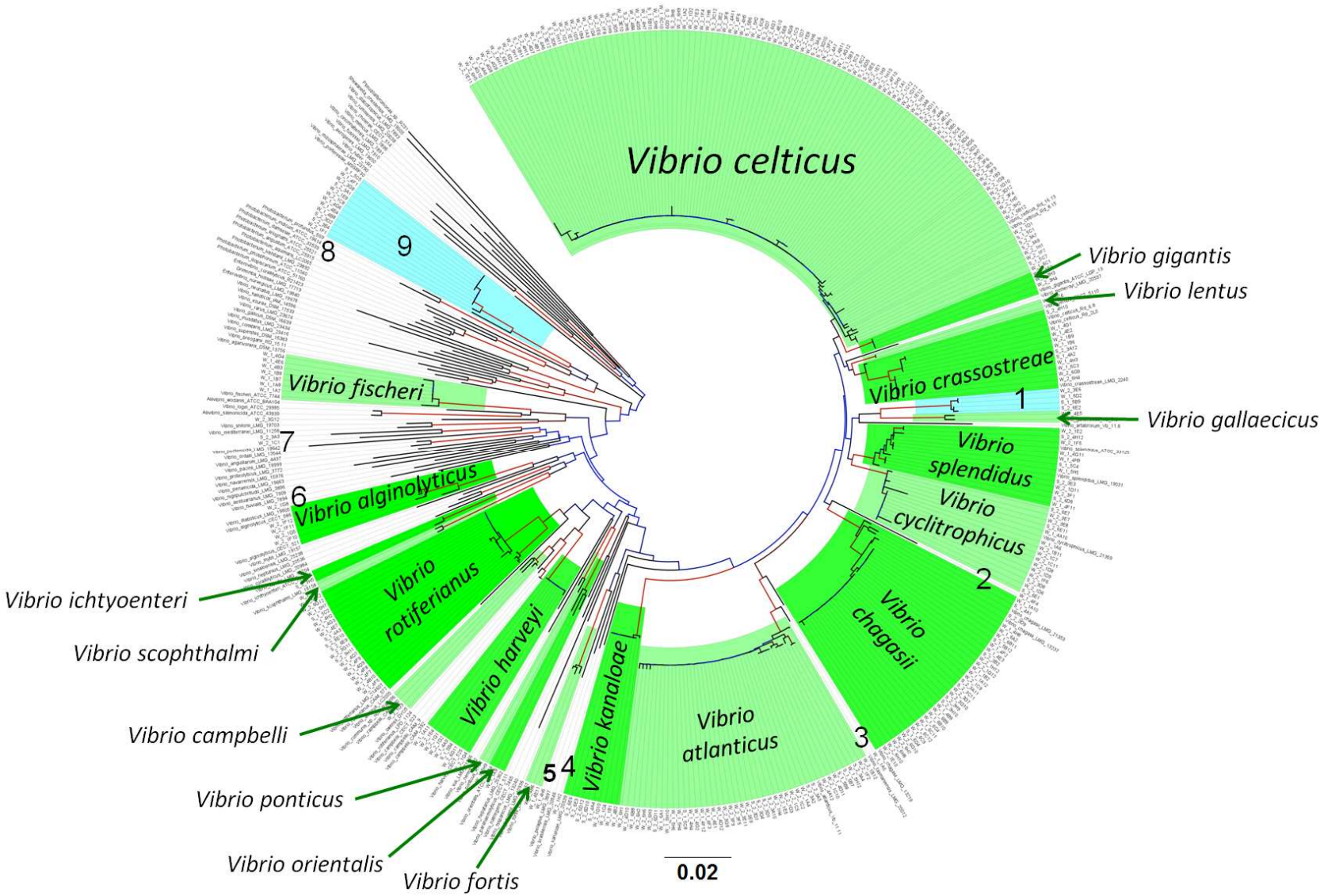
Vibrio celticus
Vibrio gigantis
Vibrio lentus
Vibrio crassostreae
1
Vibrio gallaecicus
Vibrio splendidus
Vibrio cyclitrophicus
2
Vibrio chagasii
3
Vibrio atlanticus
4
Vibrio kanaloae
5
Vibrio harveyi
Vibrio rotiferianus
6
Vibrio alginolyticus
7
Vibrio fischeri
8
9
Vibrio ichtyoenteri
Vibrio scophthalmi
Vibrio campbelli
Vibrio ponticus
Vibrio orientalis
Vibrio fortis
0.02

Figure 2 : Phylogenetic tree based on NJ method using 315 *pyrH* partial gene sequences of the strains isolated at 22°C and of 102 reference strains sequences. The thickness and colour of the lines reflect the bootstrap values (higher value (> 95%) = thicker and red line). Clusters highlighted in green comprise the well-identified strains. The non-identified strains (designed as group 1 to 9, description in Table 1) were highlighted in blue when more than two strains belonged to a same cluster. They were not highlighted when unique. Bar, 2% estimated sequence divergence.

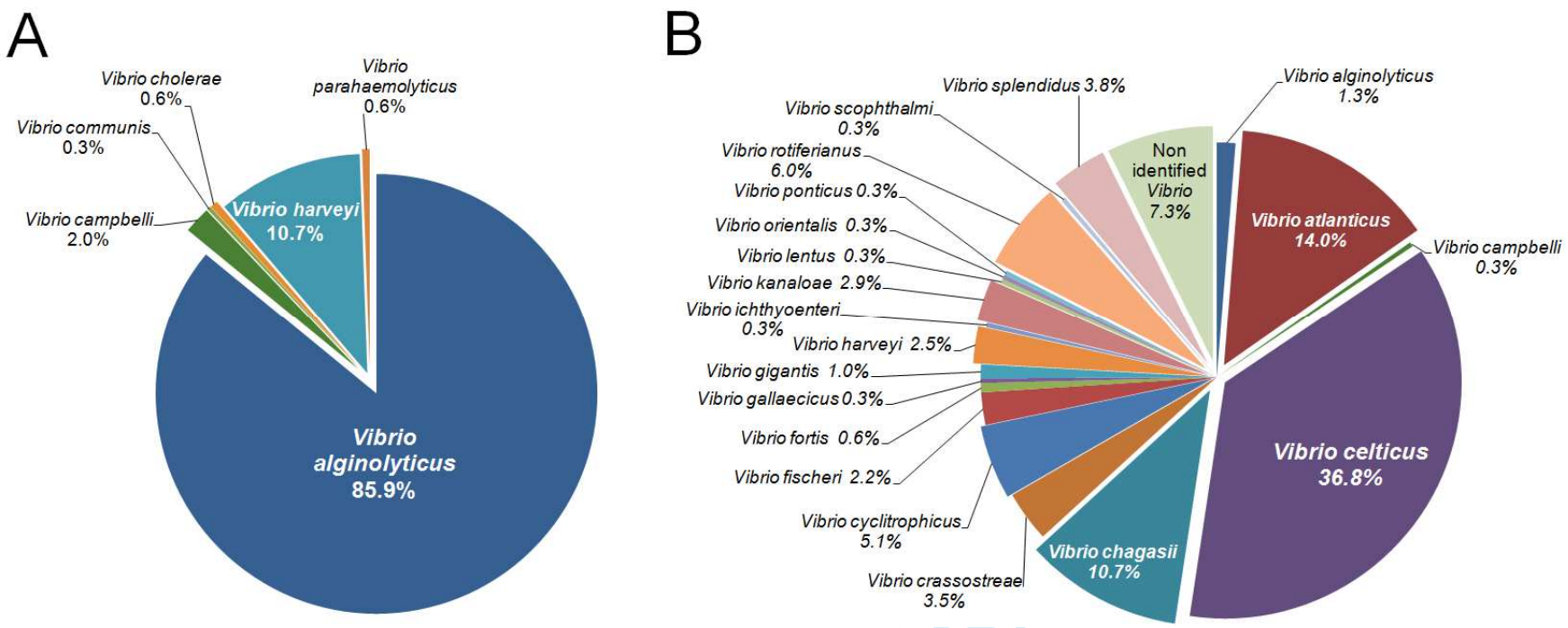

Figure 3 : Comparison of the culturable *Vibrio* population at two temperatures of isolation. The species representing more than 10% of the strains are highlighted in white. (A) Species identities of the strains isolated at 37°C determined by real-time PCR assays, and *pyrH* and *toxR* partial sequencing (356 strains), (B) Species identities of the strains isolated at 22°C determined through *pyrH* and *toxR* partial sequencing (315 strains).

Table 1: Groups (order of appearance in the *pyrH* tree) of the non-identified strains isolated at 22°C and of the closest *Vibrio* species.

Group	Strains	Bootstrap	*pyrH* sequence similarities (%)
1	W 2 3E6 S 1 5B9 W 1 6D2 S 2 6E2	99	92-93% *V. splendidus* related species
2	W 1 4F4		93-94% *V. chagasii*
3	W 1 1A5		93-95% *V. splendidus* related species
4	W 1 4H6		91% *V. orientalis* related species and *V. atypicus*
5	W 2 1H2		92-93% *V. splendidus* related species
6	W 2 1G8		87-88% *V. orientalis*/*V. splendidus* related species and *V. tapetis*
7	W 2 1C1 S 2 3A3	97	85-86% *V. anguillarum* and *V. campbelli*
8	W 2 1F1		85-86% *Photobacterium* related species
9	W 2 1C8 W 2 1E9 S 2 3A1 S 2 3D2 W 2 3G8 S 2 3E4 W 1 4E4 W 1 4F1 W 1 4B8 W 1 4G6 S 1 5C5	98	85-87% *Photobacterium* related species

W: Water; S: Surface sediments; 1-2: Transect 1 or 2

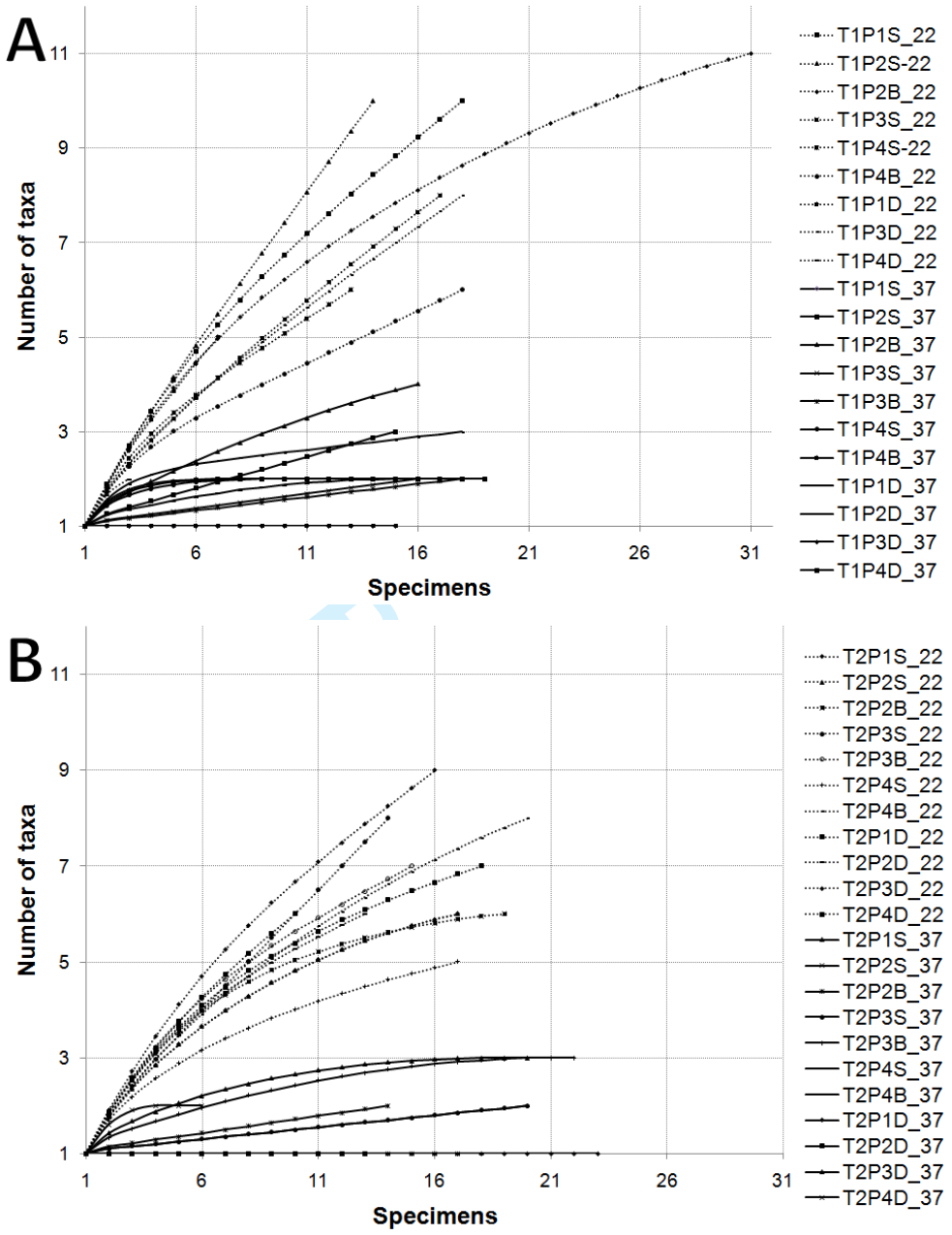

Figure 4 : Rarefaction curves obtained for each sample using the PAST software v1.68 (Hammer et al. 2001) for Transect 1 (A) and Transect 2 (B). Samples are named as follows: T1 or T2 indicate respectively the Transect 1 or 2, P1 to P4 indicate the sampled points off coast, S: sub-Surface of the seawater column, B: Bottom of the seawater column, D:

Sediment, _22: strain isolated at 22°C (in dotted lines), _37: strain isolated at 37°C (in solid lines).