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Revisiting CARD-FISH for detection of *Vibrio* spp. in natural seawater

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Abstract: *Vibrio* is a bacterial genus widely distributed in natural aquatic systems. Some *Vibrio* species are pathogenic and can cause severe diseases in both marine organisms and humans. Previous studies revealed a link between the current climate change and increased incidence of the *Vibrio*-associated diseases recently causing sanitary, economic and/or ecological problems worldwide. The conventional culture-based methods (e.g. cultivation of TCBS agar) used to monitor the presence of *Vibrio* spp. in environmental samples are not always straightforward and can underestimate the number of cells, especially if the microbial population contains a fraction of 'dormant' cells (e.g. cells in Viable but not Culturable (VBNC) state). However, this problem can be overcome by using alternative culture-free approaches such as Catalyzed Reporter Deposition-Fluorescence In situ Hybridization (CARD-FISH). To optimize CARD-FISH for efficient and reliable detection of *Vibrio* spp. in environmental samples, we have used both computer-assisted and experimental approaches. Our results demonstrate that the use of the optimized protocol along with a very specific probe, ViB572a, can offer the high sensitivity and selectivity of CARD-FISH detection of marine vibrios in natural seawater.

Keywords: *Vibrio* enumeration; CARD-FISH; seawater

1. Introduction

Global warming has lately emerged as a major concern for research and healthcare communities. The recent increase in the average temperature of earth resulted in melting the polar ice caps and rise of the sea level [1]. In turn, an increase in temperature of aquatic systems was shown to affect the balance and survival of organisms [2], thereby altering their diversity, structure and interaction in ecosystems [3]. Thompson et al. [4] suggested that the observed climate change has a direct impact on the current spread of invasive pathogenic species, including those of the *Vibrio* genus. As a result, there is an increase in the incidence of diseases caused by the use of water contaminated by these microorganisms [2,5,6], even in European countries where these diseases were not common [2]. Moreover, the increased incidence of vibriosis in non-endemic regions [6] has already led to great economic loss [6-8], thus causing public concerns around the globe.

The ubiquity and spread of *Vibrio* species indicate that these bacteria can easily adapt to the changing environment caused by the current climate change [9,10]. Although there are numerous studies addressing the impact of global warming on adaptation and survival of *Vibrio* spp. [5,6,9,11], still little is unknown about how these environmental changes can alter, directly or indirectly, the structure and abundance of *Vibrio* communities and their pathogenicity in various aquatic ecosystems [6].

The ever increasing presence of vibrios in aquatic systems as well as their appearance in areas that previously lacked the detectable levels of these microorganisms indicate the urgent need to

monitor the abundance and distribution of *Vibrio* spp. worldwide. However, the direct detection of vibrios run into the same general problems associated with enumeration of bacteria in natural environments. To some extent, these problems are caused by the continuous revision of bacterial taxonomy [4,12], the inability to culture some environmental microorganisms and/or their entry into the Viable But Non Culturable (VBNC) state (nonculturable but metabolically active cells) [13-15], the use of culture media often formulated for clinical diagnostics [16,17], etc. To overcome the limitations of culture-based methods, different culture-independent techniques have been developed for detection and enumeration of microbial species [14,18-20]. Among these techniques, Fluorescence In situ Hybridization (FISH) became one of the methods frequently used to detect and quantify microbial species in complex environmental samples [21,22]. The original FISH approach consists in hybridization of fluorescently-labeled oligonucleotide probes with 16S rRNA of microbial cells fixed on a solid support (e.g. membrane) followed by detection of fluorescent signals by epifluorescence microscopy [21,23,24]. However, the sensitivity of standard FISH protocol is not always enough to readily detect microorganisms that are grown in adverse environments (e.g. limitation of nutrients) and therefore have a reduced number of ribosomes [21]. To improve the sensitivity of conventional FISH, a variant of this technique, CARD-FISH (Catalyzed Reporter Deposition Fluorescence In situ Hybridization) was developed [25,26]. The use of CARD-FISH can increase the fluorescence emitted by cells up to 20 times in comparison to the standard FISH [27], thus making it possible to detect bacteria that could not be visualized by the conventional FISH technique [28]. CARD-FISH seems to be an ideal method for the study of marine microorganisms such as *Vibrio* spp., which usually causes detection problems [28]. Although the ability of this method to distinguish microorganisms depends on the specificity of the probe used, the adequate and specific probe design is often difficult to achieve. This is particularly challenging in the case of the *Vibrio* genus, known for its enormous diversity [4,29]. A number of *Vibrio*-specific probes reported previously [28,30-36 and others] show different specificity and have not been scrutinized for their ability to reliably detect and enumerate *Vibrio* species in complex environmental samples. The main objective of this study was to adapt the CARD-FISH technique for monitoring the presence of *Vibrio* spp. in seawater and demonstrate the efficiency of the optimized protocol and probes in detection of marine vibrios.

2. Materials and Methods

2.1. Selection of a specific probe for the detection and enumeration of Vibrio spp.

To retrieve the most updated articles (primarily published during the last 20 years) dealing with the use of CARD-FISH for detection of *Vibrio* spp., several databases (i.e. Science Direct, Springer, Google Scholar, NCBI and others) were systematically searched by using combinations of keywords including "Vibrio", "detection", "specific probe", "CARD-FISH" and "environmental".

Based on the information obtained, 12 oligonucleotide probes targeting specific sequences within *Vibrio* 16S rRNA genes were pre-selected. As the preference was given to those probes that could potentially hybridize with most of the species belonging to the genus *Vibrio*, only three probes were selected (i.e. GV841 [30-32,34,36], Vib-16S-1 [35] and VIB57a [31]), whereas the remaining 9 probes were discarded.

The coverage and specificity of the selected probes were further evaluated using the online tools such as TestProbe (https://www.arb-silva.de/search/testprobe/) [37], which is part of the SILVA rRNA database project (hereafter SILVA); as well as the computer Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Since *in silico* analysis (SILVA and BLAST) has shown that the Vib-16S-1 probe did not meet the above criteria, it was discarded too and only probes GV841 and VIB572a were further used in this study.

2.2. Bacterial strains and natural seawater samples

To test and optimize CARD-FISH detection of *Vibrio* spp., a number of Gram negative (including *Vibrio* species) and Gram positive bacteria along with one eukaryotic microorganism were

used as positive and negative controls. *Vibrio* spp. included *V. harveyi* ATCC 14126 and 4 environmental isolates of *V. harveyi*, *V. cyclitrophicus*, *V. kanaloae* and *V. tubiashii*. The latter were isolated from seawater samples collected from Port of Armintza in the North of Spain (43º 26' 24" N; 2º 54' 24" W) by selection on Thiosulfate Citrate Bile salts Sucrose Agar plates (TCBS, Fluka). Gram negative bacteria that were oxidase-positive, were further characterized by using API 20E test (Biomerieux). The nature of *Vibrio* strains identified in phenotypical and biochemical assays was further verified by genotyping. Namely, multilocus sequence analysis (MLSA) was performed by analyzing the sequence of 8 genes including the 16S rRNA gene and 7 housekeeping genes (*gapA*, *ftsZ*, *topA*, *mreB*, *gyrB*, *pyrH* and *recA*). The corresponding fragments of these genes were PCR amplified and sequenced at the Genomics and Proteomics Core Facility-SGIKER (University of the Basque Country). The resulting sequences were subjected to BLAST search against the NCBI database to complete the assignment of each *Vibrio* isolate to a particular taxon as described in previous studies [38-40].

Apart from *Vibrio* spp., other Gram-negative (*Escherichia coli* ATCC 27325, *Citrobacter freundii* ATCC 13316, *Pseudomonas aeruginosa* ATCC 27853 and *Serratia marcescens* ATCC 274) and some Gram-positive (*Bacillus subtilis* ATCC 21770, *Micrococcus luteus* ATCC 10240 and *Staphylococcus aureus* ATCC 6538P) bacteria along with *Saccharomyces cerevisiae* ATCC 9763 were included as negative controls. The strains used were stored at -80ºC using the Pro Lab Diagnostics™ Microbank™ system. Recovery and growth conditions were adjusted to the specific microorganism. Namely, *Vibrio* species were grown in Marine Broth (MB, AppliChem) at 26ºC; whereas *B. subtilis*, *M. luteus* and *S. marcescens* in Tryptone Soy Broth (TSB, Oxoid Ltd.) at 30ºC; *E. coli*, *C. freundii*, *P. aeruginosa* and *St. aureus* in TSB at 37ºC and *S. cerevisiae* in Potato Dextrose Broth (Oxoid Ltd.) at 20ºC. Cells grown for 24 h were harvested by centrifugation (4,000 g, 20 min) and then suspended in sterile seawater (collected from the Armintza port sampling station and treated as explained below) to obtain cell suspensions A to G listed in Table 1. The final microbial density for these suspensions was 10^6 cells ml^{-1}. To perform the microorganism mixtures (Table 1; suspensions C, D, E and F), 25 ml of seawater were inoculated with equal volumes of each individual microorganism suspension. The final density of each of them was approximately 2 10^5 cells ml^{-1}. These suspensions were further used to test the efficiency and specificity of the probes selected for detection and quantification of *Vibrio* spp. To prepare sterile seawater for cell suspensions, it was consecutively filtrated through ester membranes (Merck Millipore) with a pore size of 8, 0.8, 0.45 and 0.22 μm, respectively, and then autoclaved (121ºC, 20 min).

Besides testing CARD-FISH on pure cultures and their defined mixtures, detection of *Vibrio* spp. was also carried out with seawater samples collected from three sampling sites of the Monitoring Network of the Ecological Status of Transitional and Coastal Waters of the Autonomous Community of the Basque Country (AZTI Tecnalia). The collection sites are located in coastal (43º 23′ 1″ N; 3º 4′ 50″ W), open sea (43º 24′ 0″ N; 2º 18′ 0″ W) and estuarine (43º 22′ 15″ N; 11º 47′ 20″ W) areas. The seawater samples were kept refrigerated (4ºC) immediately after collection and the analysis was carried out within a 24 h period. Namely, the samples were processed to determine the total number of bacteria (Total Direct Count, TDC) according to Hobbie et al. [41], whereas vibrios were enumerated by using CARD-FISH (see below). The number of culturable chemoorganotrophic bacteria and the number of culturable presumptive *Vibrio* spp. were estimated by plating on Marine Agar (MA, AppliChem) and TCBS Agar, respectively. The plates were incubated at 26ºC for 48-96 h. If necessary, the cell suspensions were diluted with sterile saline solution (NaCl, 1.94% [w/v]) prior plating.

Table 1. Suspensions of microorganisms test and purpose.

Suspensions	Microorganisms	Objective
A	*V. harveyi* ATCC 14126	Positive control (collection)
B	*V. harveyi* environmental	Positive control (environmental)
C	*E. coli* ATCC 27325 *C. freundii* ATCC 13316 *P. aeruginosa* ATCC 27853 *S. marcescens* ATCC 274	Negative control (Gram negative bacteria)
D	*V. harveyi* ATCC 14126 *E. coli* ATCC 27325 *B. subtilis* ATCC 21770 *M. luteus* ATCC 10240 *St. aureus* ATCC 6538P	Selective detection of *Vibrio*
E	*V. harveyi* ATCC 14126 *V. harveyi* environmental *V. cyclitrophicus* environmental *V. kanaloae* environmental *V. tubiashii* environmental	Detection of *Vibrio*
F	*V. tubiashii* environmental *V. kanaloae* environmental *V. cyclitrophicus* environmental	Detection of *Vibrio*
G	*S. cerevisiae* ATCC 9763	Negative control

2.3. CARD-FISH protocol *for detection of Vibrio spp.*

The quantification of total *Vibrio* spp. was performed using the CARD-FISH protocol described by Pernthaler et al. [26] with some modifications. 25 ml of cell suspensions and 200 ml of seawater samples were fixed with 25 ml or 100 ml of 4% [v/v] paraformaldehyde and kept refrigerated at 4ºC for 24 h. Then, each sample was filtered onto polycarbonate filters (type GTTP; 0.2 μm pore size; 47 mm diameter, Merck Millipore), and the filters were kept frozen at -20°C until further analyses.

To avoid cell loss during the permeabilization and hybridization, the filters were impregnated from both sides in 0.1% agarose solution at 35-40ºC. The endogenous peroxidases were deactivated with HCl (0.01 M, for 10 min). The filters were washed with sterile 1X Phosphate Buffered Saline (PBS, Sigma-Aldrich) and then with MilliQ water.

The permeabilization was carried out for 1 h at 37ºC in 2 ml of lysozyme solution (10 g l^{-1}). This concentration of lysozyme solution double that recommended by Pernthaler et al. [26] and was selected based on the results of pilot experiments (high intensity of green fluorescence emitted). The filters were then washed with sterile MilliQ water and absolute ethanol and were afterwards, kept frozen at -20°C until further analyses. The samples containing Gram-positive bacteria were processed differently. Namely, following treatment with lysozyme, the filters were immersed in acromopeptidase solution (final concentration 60 U ml^{-1}) with NaCl 0.01 M and Tris-HCl 0.01 M, pH 8.0 (for 30 min at 37ºC) and then washed twice with sterile MilliQ water and absolute ethanol.

The hybridization was carried out for 2 h in 900 μl of hybridization buffer containing 30% formamide (FM), at 46ºC with VIB572a probe [31] and at 49ºC with GV 841 probe [30]. The HPR-labeled probes were purchased from Biomers.net GmbH. They were diluted with sterile MilliQ water to a final concentration of 50 ng μl^{-1}, aliquoted into 30 μl aliquots and stored at -20ºC until further use. The specific hybridization conditions for each probe are indicated in Table 2. The HPR-labeled probes (50 ng μl^{-1}) were used at dilutions 1:300 and 1:100 for filters containing cells from axenic cultures and for mixed cultures (or seawater), respectively. Following hybridization with VIB572a or GV 841 probes, the filters were washed with 640 μl of 5 M NaCl for 5 min at 48ºC or 42ºC, respectively, and then, with 1X PBS for 10-15 min at room temperature.

Table 2. Specific hybridization conditions for CARD-FISH with GV841 and VIB572a probes. The hybridization and washing temperatures volumes and percentages of formamide (FA) indicated in the bibliography (T, theoretical) and those finally used (U) in this study are shown, as well as the MilliQ water and NaCl solution used.

Probe	Temperature (ºC)		Hybridization				Washing			
			%FA		FA (ml)		MilliQ (ml)		NaCl 5M (µl)	
	Hybridization	Washing	T	U	T	U	T	U	T	U
GV841	49	42	30	30	6	6	8	8	640	640
VIB572a	46	48	40	30	8	6	6	8	270	640

The signal amplification and all subsequent steps were performed in dark. To amplify the signal, the filters were overlaid with solution containing 1 ml of Amplification Buffer, 10 µl of 100X H_2O_2 stock (30% [v/v]) and 4 µl of reconstituted tyramid labeled with Alexa Fluor 488 (0.5 mg ml^{-1}) (Thermo Fisher Scientific) followed by incubation in dark at 42ºC for 15 min. Then, the filters were consecutively washed with 1X PBS for 10 min, running sterile distilled water (6 min), sterile MilliQ water and absolute ethanol.

To observe microorganisms, filters were stained with DAPI-Mix (1 µg ml^{-1}, Thermo Fisher Scientific) and examined with an Eclipse E-400 epifluorescence microscope (Nikon), equipped with filter blocks consisting of a B-2A filter (EX 450-490 excitation filter, DM 505 dichroic mirror and BA 520 barrier filter) and of a UV-2A filter (excitation filter EX 330-380, dichroic mirror DM 400 and barrier filter BA 420). The cells hybridized with the CARD-FISH probes and microorganisms stained with DAPI emitted green and blue fluorescence, respectively, detected by using B-2A and UV-2A filters. 50 fields were routinely used to enumerate each type microbial cells and calculate the number of bacteria ml^{-1}.

2.4. Statistical analysis

All the results obtained in this study were managed with Microsoft Excel. In the experiments performed to test the suitability of the probes selected for the CARD-FISH protocol tested here, cell densities were transformed into percentages. To determine the presence of the *Vibrio* genus in various seawater samples, the results obtained (cell densities) were transformed to decimal logarithms. In both cases, the experiments were carried out in triplicate, and the variation coefficients between replicates were found to be lower than 12%. Differences between means were determined by analysis of variance. Any variables with $p \leq 0.05$ were assumed to be significant.

3. Results

According to the procedure described in Materials and Methods and results obtained by SILVA, three perspective CARD-FISH probes (i.e. Vib-16S-1, GV841 and VIB572a) were selected. Although the specificity of all 3 probes was similar towards *Vibrio* spp., the Vib-16S-1 probe, unlike GV841 and VIB572a, additionally showed the complete coverage and high specificity (99.3%) towards genomic regions of other species (e.g. *Gayadomonas*, *Gallaecimonas* and *Candidatus Photodesmus* and enterosymbionts 10 group). Furthermore, the data obtained by BLAST search indicated that the Vib-16S-1 probe could also be complementary to non-16S rRNA regions present in the genomes of some *Vibrio* species and non-related genera (e.g. *Oceanisphoera*, *Avibacterium* and *Catenovulum*) occasionally with complete coverage and identity. Therefore, given its broad cross-reactivity extended to non-*Vibrio* species revealed *in silico* (SILVA analysis and BLAST searches), the Vib-16S-1 probe was discarded and only GV841 and VIB572a probes were selected for further assays.

The latter were performed with different microbial populations including both positive (group A and B) and negative (group C and D) controls. Table 3 summarizes the results of this analysis. As seen in Table 3, the efficiency of hybridization obtained with both probes (GV841 and VIB572a) towards *V. harveyi* strains in suspensions A and B (positive controls) was close to 100% (Figure 1). In other words, the numbers of cells counted using the UV-2A filter (i.e. the total number of cells detected after staining with DAPI) and those that had hybridized with the CARD-FISH probes were nearly identical. Furthermore, the intensity of green fluorescence emitted by the probe fluorophore (i.e. ALEXA Fluor 488) was very high.

Table 3. Percentage (± standard deviation) of cells that hybridized with the probes for the tested microorganism suspensions. The data are mean values from three independent experiments.

| Suspensions	Microorganisms	Percentage of cells that hybridized	
		GV841 probe	**VIB572a probe**
A	*V. harveyi* ATCC 14126	100	100
B	*V. harveyi* environmental	100	100
C	*E. coli* ATCC 27325 *C. freundii* ATCC 13316 *P. aeruginosa* ATCC 27853 *S. marcescens* ATCC 274	ND*	ND*
D	*V. harveyi* ATCC 14126 *E. coli* ATCC 27325 *B. subtilis* ATCC 21770 *M. luteus* ATCC 10240 *St. aureus* ATCC 6538P	7.5 (± 0.11)	21.0 (± 0.92)
E	*V. harveyi* ATCC 14126 *V. harveyi* environmental *V. cyclitrophicus* environmental *V. kanaloae* environmental *V. tubiashii* environmental	18.2 (± 0.25)	100
F	*V. tubiashii* environmental *V. kanaloae* environmental *V. cyclitrophicus* environmental	**ND***	**100**
G	*S. cerevisiae* ATCC 9763	**ND***	**ND***

* ND, not detected.

The specificity of the probes was verified using suspensions C and D (Table 1). For suspension C, composed of non-*Vibrio* bacteria (negative control), cells did not hybridized with GV841 or VIB572a and did not produce any fluorescence (data not shown). For suspension D (a mixture of non-*Vibrio* bacteria and *V. harveyi* ATCC 14126), 7.5% (± 0.1) and 21% (± 0.9) yielded fluorescence with probes GV841 and VIB572a, respectively (Table 3, Figure 2). The morphological characteristics of the fluorescent cells corresponded to those of *V. harveyi* ATCC 14126 and their percentage corresponded to that in the initial mix. As anticipated, epifluorescence microscopy using the UV-2A filter confirmed the presence of cells with different morphologies (coccoid, bacilar and coccobacilar) in C and D suspensions stained with DAPI. In addition, the CARD-FISH protocol was also tested on suspensions E and F (Table 1), composed of different *Vibrio* species. While the results obtained with suspension E, composed of two species previously tested (*V. harveyi* ATCC 14126 and environmental *V. harveyi*) and three new isolates (*V. cyclitrophicus*, *V. kanaloae* and *V. tubiashii*) demonstrated the high detection rate (nearly 100%) with VIB572a, the detection efficiency was unexpectedly low (18.2%) with GV841 (Table 3). Likewise, VIB572a have demonstrated high efficiency (nearly 100%) in detection of three environmental *Vibrio* isolates (*V. cyclitrophicus*, *V. kanaloae* and *V. tubiashii*)

present in suspension F, whereas the result obtained with GV841 was entirely negative (Table 3, Figure 2).
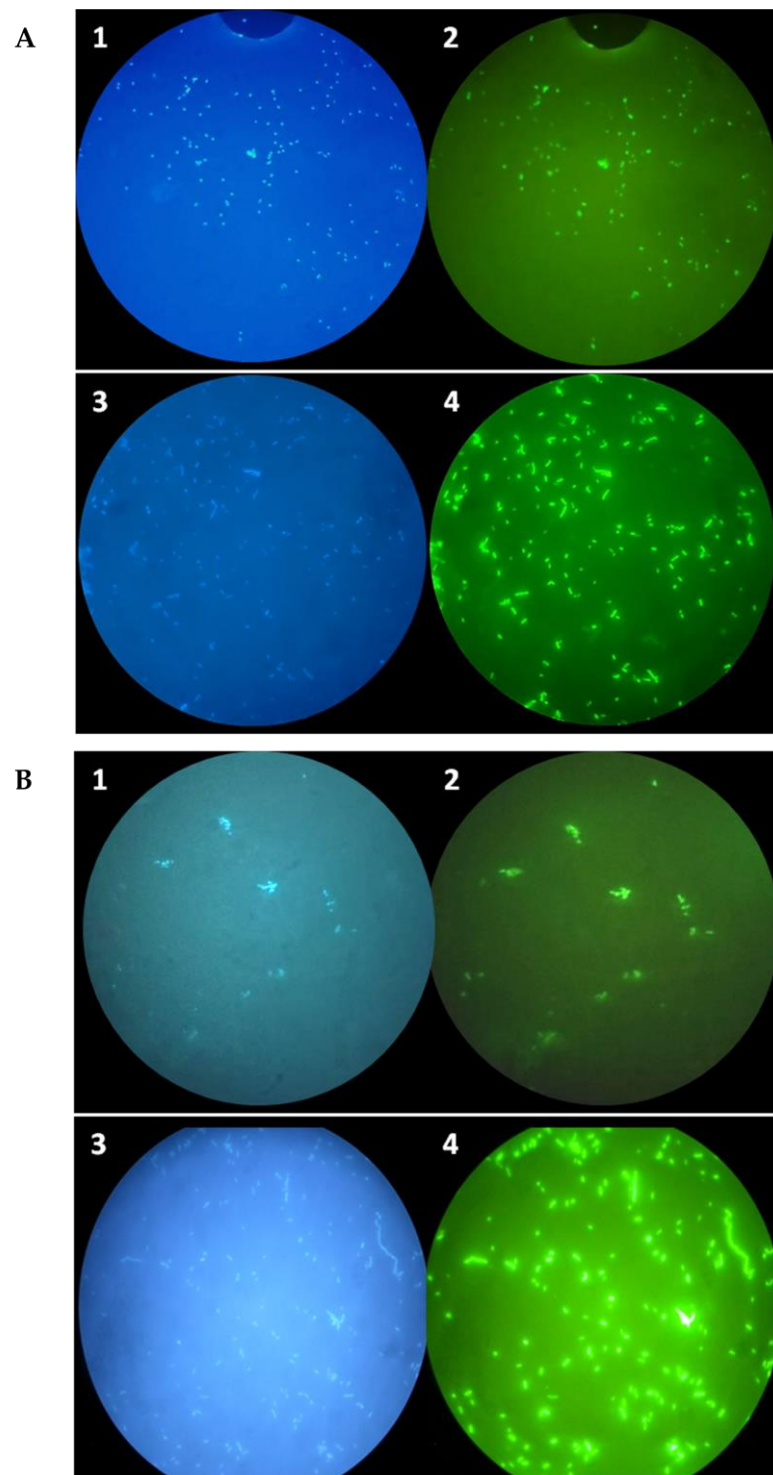
Figure 1. Epifluorescence microscopy images obtained from *V. harveyi* suspensions A (ATCC 14126) and B (environmental strain) by CARD-FISH using GV841 (1 and 2) and VIB572a (3 and 4) probes. Images 1 and 3, total cells stained with DAPI (UV-2A filter); images 2 and 4, cells that have hybridized with the probes used (B-2A filter). Magnification x1000.
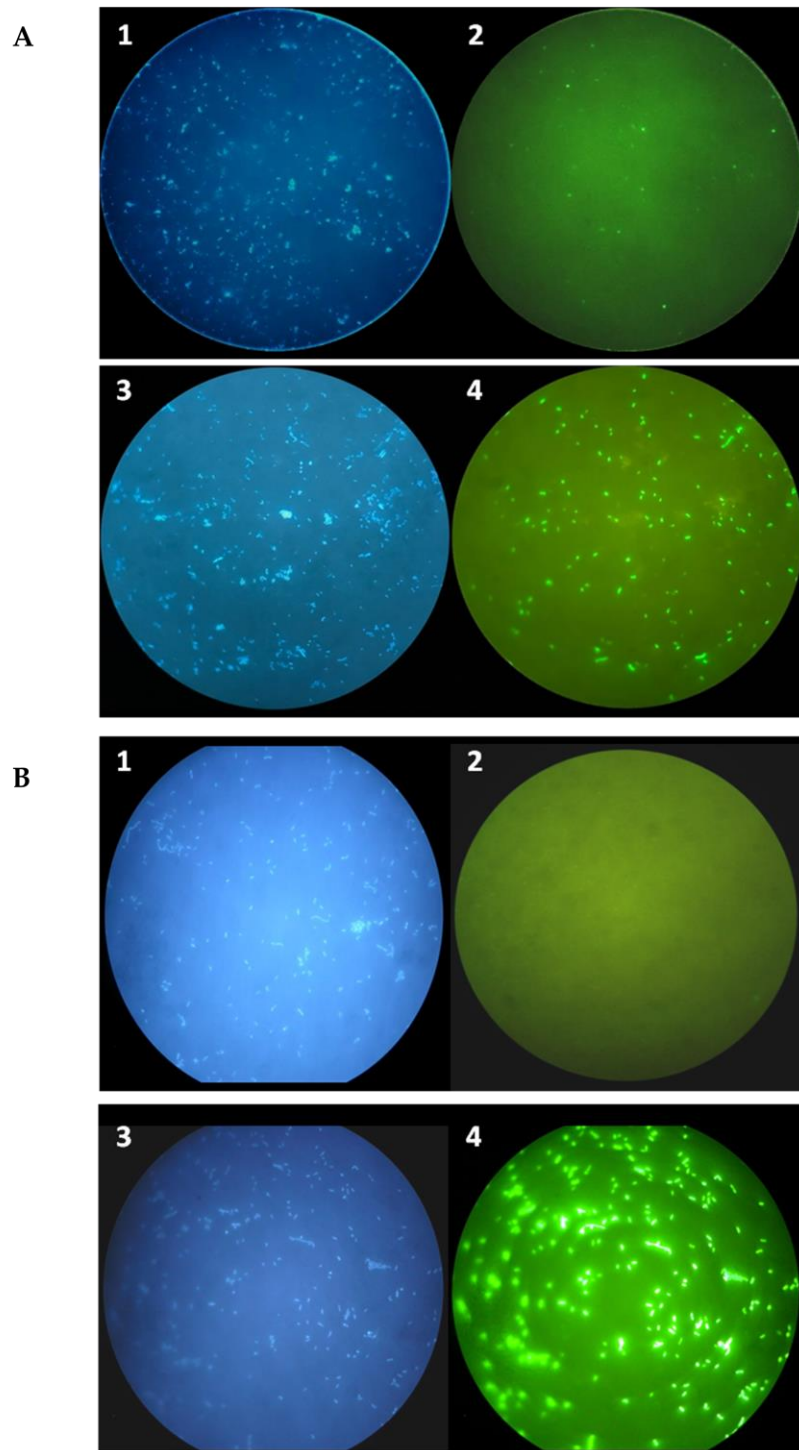
Figure 2. Epifluorescence microscopy images obtained from bacterial mixed suspensions after CARD-FISH method using GV841 (1 and 2) and VIB572a (3 and 4) probes. Images 1 and 3, total cells stained with DAPI (UV-2A filter); images 2 and 4, cells that have hybridized with the probes used (B-2A filter). Images A: suspension D (*V. harveyi* ATCC 14126, *E. coli* ATCC 27325, *B. subtilis* ATCC 21770, *M. luteus* ATCC 10240, *St. aureus* ATCC 6538P); and B: suspension F (environmental *V. cyclitrophicus*, *V. kanaloae*, *V. tubiashi*). Magnification x1000.

Finally, the experiments carried out with suspension G containing a eukaryotic microorganism, *Saccharomyces cerevisiae* ATCC 9763, showed that both *Vibrio*–specific probes could not hybridize with eukaryotic nucleic acids. In all cases where hybridization was

detected, the green fluorescence emitted by the probe fluorophore (i.e. ALEXA Fluor 488) was more intense using VIB572a than that obtained with GV841.

Besides testing the sensitivity and selectivity of CARD-FISH probes, this method was also used along with other techniques for detection and enumeration of microorganisms and *Vibrio spp.* in seawater samples collected in 3 different locations (coastal, open sea or estuarine areas) within the Biscay Bay at 11 to 14ºC according to AZTI Tecnalia. In these experiments, the cultivation on Marine Agar and TCBS agar were respectively used to determine the number of culturable chemoorganotrophic and putative *Vibrio* spp., whereas the total number of bacteria and *Vibrio* spp. were enumerated by epifluorescence microscopy using either acridine orange staining or CARD-FISH with VIB572a, respectively. The results of this analysis are summarized in Table 4. While the total number of bacteria in seawater samples was found to be between $1.05 \cdot 10^5$ and $4.92 \cdot 10^6$ cells ml^{-1}, the percentages of chemoorganotrophic bacteria were highly variable (<1-62.4%), with a number of chemoorganotrophic bacteria in the range of $7.5 \cdot 10^1$ to $3.25 \cdot 10^5$ CFU ml^{-1} depending on the place and time of sampling. The *Vibrio* counts determined by CARD-FISH were less than $1.3 \cdot 10^3$ *Vibrio* ml^{-1}, thus representing a very small fraction (less than 1%) of the entire bacterial population. The number of culturable putative *Vibrio* spp. ranged from 7 to $1.66 \cdot 10^2$ CFU ml^{-1} and its fraction was very dynamic (from less than 1% to nearly 81%) among the entire *Vibrio* spp. The higher percentages of culturable putative *Vibrio* spp. were obtained for estuarine water samples.

The vibrios density was low and not enough to be detected by CARD-FISH using GV841 probe. Occasionally, some cells were detected with this probe, but the green fluorescence emission was weak.

4. Discussion

In this study, CARD-FISH technique and probes cited in literature for *Vibrio* spp. detection and enumeration have been compared and tested. CARD-FISH technique corrects the problems attributed to the conventional FISH technique [23,42] that possesses insufficient sensitivity for detection of microorganisms in environmental samples. The latter often originate from nutrient-limited environments, in which microorganisms contain a low number of ribosomes and therefore have a limited number of sites for hybridization with the 16S rRNA-specific probes [21,43]. In present study, the protocol described by Pernthaler et al. [26] has been modified, increasing (up to twice) the amount of lysozyme used for the cell permeabilization [44]. This modification improves the accessibility of fluorochrome, and therefore provides a greater emission of fluorescence in turn, contributing to better detection of *Vibrio* spp.

Since the accuracy of detection depends on the specificity of the probe towards the target cells, we initially pre-selected three probes, GV841 [30], Vib-16S-1 [35] and VIB572a [31] and evaluated their specificity using *in silico* tools (SILVA and BLAST search). Although according to the results obtained with SILVA, the specificity of all 3 probes was similar with respect to *Vibrio* spp., further examination of Vib-16S-1 by BLAST showed that it has wider coverage and specificity and should efficiently hybridize with 16S rRNA of some non-*Vibrio* genera. Namely, unlike GV841 and VIB572a, Vib-16S-1 showed a complete coverage of and perfect complementarities to the target 16S rRNA regions present *Oceanisphoera*, *Avibacterium* and *Catenovulum*, and therefore, this probe was discarded due to its lower specificity for the *Vibrio* genus. Likewise, the BLAST search results obtained for the GV841 probe [30] indicated that it was capable of hybridizing not only with the target regions of *Vibrio* spp. but also with those present in other genera of the *Vibrionaceae* family (*Candidatus Photodesmus*, *Aliivibrio* and *Catenococcus*). An example of *Candidatus Photodesmus* species includes the symbiotic bacterium of *Anomalops katoptron* [45]. As to *Catenococcus* genus, a single species, *C. thiocycli* isolated from a hydrothermal marine sulfuric environment in New Guinea has been described [46]. Considering the habitats of *Candidatus Photodesmus* and *Catenococcus* species, these genera are likely absent in regular water samples and therefore should not interfere with detection of *Vibrio* spp. using GV841. In contrast, the genera *Aliivibrio* and *Vibrio* share the same habitats and their 16S rRNAs have a high sequence identity (ca. 95.5%), which makes it difficult to

distinguish these genera with GV841 [47]. Moreover, further analysis of GV841 revealed that it is not capable of hybridizing with all the species of the *Vibrio* genus. Apparently, the coverage of the target region was incomplete [31] and not specific enough to detect all *Vibrio* species that may be present in seawater [38]. Indeed, Oberbeckmann et al. [34] previously pointed out that this probe failed to detect *V. cholerae* and *V. mimicus* [48].

Concerning the VIB572a probe [31], *in silico* analysis revealed that this probe should provide a comprehensive coverage and high specificity in detection of different *Vibrio* strains, including *V. cholerae* and *V. mimicus*. Although, Huggett et al. [31] also suggested its ability to hybridize with 16S rRNA of *Photobacterium* and *Listonella*; our *in silico* data ruled out this scenario. *In silico* analysis has also revealed that, similar to GV841, VIB572a can potentially hybridize with 16S rRNA of other genera such as *Photodesmus*, *Aliivibrio* or *Catenococcus.*

Although *in silico* results suggest that both GV841 and VIB572a probes are potentially suitable for detection of nearly all *Vibrio* spp., the results obtained with different bacterial suspensions showed that, in contrast to VIB572a, GV841 was not capable of hybridizing with all the species of the *Vibrio* genus tested in this study. This different hybridization capacity could be attributed to an imperfect binding of the GV841 probe to the target site. Indeed, previous studies demonstrated that, some areas in 16S rRNA are less accessible than others; and their poor accessibility can strongly impede hybridization [49,50]. According to the existing classification, class I and class VI regions are the most (81-100%) and least (0-5%) accessible ones, respectively. Thus, GV841 apparently hybridizes to the class IV and V regions with an approximate accessibility of 6-40%, and therefore this probe is less suitable for the detection of *Vibrio* spp. In contrast, the hybridization sites of VIB572a [31] are within class I and class II areas with an accessibility of 61-100%, which makes this probe nearly ideal for CARD-FISH detection of *Vibrio* spp. Similar results have been obtained in studies dealing with the accessibility of hybridization sites within 16S rRNA [51] and 23S rRNA in *E. coli* [52], thus indicating that they can be extrapolated to other microorganisms.

Further analysis of seawater samples led to several observations. In agreement with the results obtained in other studies [14,41,53] including those carried within the Bay of Biscay [54-56], we found that the number of total bacteria was significantly higher than that of culturable chemoorganotrophic bacteria. Although the total number of vibrios enumerated with CARD-FISH was dependent on the probe used, the VIB572a probe provided the most consistent results, provided that the percentage of vibrios among the total bacteria was similar to that obtained for their culturable fractions (i.e. vibrios *vs* chemoorganotrophic bacteria). Moreover, the CARD-FISH protocol we tested here is robust yielding no difference in the intensity of the fluorescence signals emitted by vibrios from oligotrophic environments and by *Vibrio* spp. grown under laboratory conditions. Therefore, our findings demonstrate that the CARD-FISH technique that employs the VIB572a probe [31] could offer a nice tool to detect *Vibrio* spp. in seawater samples, even when vibrios are present at low concentrations.

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