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New 16S rRNA primers to uncover *Bdellovibrio* and like organisms diversity and abundance

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ABSTRACT

Appropriate use and specific primers are important in assessing the diversity and abundance of microbial groups of interest. *Bdellovibrio* and like organisms (BALOs), that refer to obligate Gram-negative bacterial predators of other Gram-negative bacteria, evolved in terms of taxonomy and classification over the past two decades. Hence, some former primers have become inadequate while others are yet to be designed, for both PCR (especially with the advent of NGS) and qPCR approaches. Thus, to study BALOs' abundance and diversity in a variety of aquatic ecosystems, we designed *in silico* specific primer sets for each BALO genera and tested them *in vitro* on a variety of cultures and environmental samples. Also, we performed Sanger and Nano Miseq sequencing to reveal the exact degree of specificity of the most promising primers set. Here we report our success in designing specific primers for some BALOs genera, *i.e. Bdellovibrio* (PCR), *Bacteriovorax* (qPCR), *Peredibacter* (PCR).

1. Introduction

Among predatory bacteria, some are facultative and others are obligate predators (Jurkevitch and Davidov, 2006). The only known obligate predators belong to a group referred to as the *Bdellovibrio* and like organisms (BALOs). These Gram-negative bacterial cells are relatively small, rod-shaped and motile, and solely survive in natural ecosystems by predating other bacteria, here again Gram-negative bacteria (Davidov et al., 2006; Jurkevitch, 2006). Owing to their way of life and their ubiquitous distribution (Williams and Piñeiro, 2006), BALOs are suggested to act as an important “ecological balancer” on microbial communities (Iebba et al., 2013), sometimes comparable to the action of bacteriophages and/or protozoan grazers. It is noteworthy, however, that not all BALOs strains thrive in the same habitat; some may be excluded from some ecosystems (Williams and Piñeiro, 2006) such as the halo-tolerant family of *Halobacteriovoraceae* that have never been reported in freshwaters.

To study BALOs' diversity and abundance, molecular biology tools are required such as PCR-sequencing and quantitative PCR (qPCR). The application of these technics required the use of primers that can target specifically the different representatives of the BALOs. Indeed, the use of universal primers is not adapted since they cannot detect all prokaryotes (Baker et al., 2003; Ben-Dov et al., 2006; Klindworth et al., 2013). Besides, most BALOs are known to be cryptic rather than numerous in natural ecosystems (Kandel et al., 2014; Williams et al., 2016). Therefore, the use of universal primers is likely to overlook the diversity and abundance of less abundant bacteria such as the BALOs group (Ezzedine et al., 2020b).

Previous studies from eminent colleagues reported the design and use of primers targeting some BALOs (Davidov et al., 2006; Jurkevitch and Ramati, 2000; Van Essche et al., 2009). During the recent years, however, the reclassification of BALOs and the development of novel sequencing technologies have rendered difficult the use of “old” primers and we attempted the challenge of designing a new generation of primers. Briefly, since 2000's the classification of BALOs evolved to encompass today two classes (Baer et al., 2004, 2000; Davidov and Jurkevitch, 2004; Hahn et al., 2017; Koval et al., 2015). The first class corresponds to the Oligoflexia (formerly δ-proteobacteria) and includes 5 genera: *Bdellovibrio, Bacteriovorax, Halobacteriovorax, Pseudobacteriovorax* and *Peredibacter*. The second class, the α-proteobacteria, holds a single genus, *i.e. Micavibrio*. Regarding the sequencing technologies, we moved from the Sanger method to the era of high-throughput methods such as the Illumina sequencing approaches, capable of generating a huge amount of sequence data (Bleidorn, 2017).

When looking into the bibliography to search for an adequate set of primers for BALOs, more specifically those targeting the 16S rRNA gene, three findings can be reported. Firstly, there are no qPCR primers for each BALO genus. Secondly, one BALO primer can amplify multiple other BALOs. This is probably due to the old classification where multiple BALO species have been encompassed in the same genus. For

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example, *Bacteriovorax* primers 519F and 677R (Zheng et al., 2008) amplify also *Halobacteriovorax in silico*. Thirdly, the amplicon obtained from available PCR primers exceeds 400 bp (Supplementary Table 1). For instance, the pair of primers Per 676F and Per 1443R targeting *Peredibacter* yield amplicons of 770 bp (Davidov et al., 2006). Hence, these primers cannot be used for Illumina MiSeq 2 × 250 bp sequencing.

Here, we report the design of new primers compatible with qPCR and Illumina MiSeq sequencing approaches for a more in-depth analysis of a functional bacterial group still largely unknown in a variety of ecosystems.

2. Materials and methods

2.1. BALOs primer design workflow for Illumina sequencing technology

The workflow explained below was applied to all primers designed for each BALO genus and subsequent PCR/MiSeq sequencing. However, for the sake of clarity, primers designed for *Halobacteriovorax* are given as an example thereafter. Firstly, the software Primer-Blast (Ye et al., 2012) was used to design primer pairs. *Halobacteriovorax* type species sequence of the 16S rRNA gene, *i.e. H. marinus* strain SJ (Supplementary Table 2), was used as template to generate primers. The parameters set for the design were as follows: PCR product size between 350 and 400 bp to be compatible with a Miseq 2 × 250 bp run; primer melting temperature (Tm) with a minimum of 59 °C, an optimal of 62 °C and a maximum of 65 °C; primer specificity stringency with at least 2 total mismatches to unintended targets, including at least 2 mismatches within the 5 base pairs at 3′ ends; primer size between 18 and 24 bp with an optimal of 20 bp; primer GC content ranging from 40 to 60%; max poly-x set to 4 and the software asked to output 20 pairs of primers. The other parameters were left unchanged. In the next step, primers were placed in 3 sets of alignment made with Geneious 11.1.5 (https://www.geneious.com) to verify their specificity to the *Halobacteriovorax* genus. To get the first set of aligned sequences, the R Primer-Miner package (Elbrecht and Leese, 2017) was used to download all sequences of *Halobacteriovorax* from NCBI (Benson et al., 2013). For instance, *Halobacteriovorax* were downloaded using keywords such as "16 s", "16S", "*Halobacteriovorax*", "*Halobacteriovoraceae*". The script downloaded the sequences that met the requirements and then the sequences were dereplicated and clustered at a 97% identity threshold using Vsearch (Rognes et al., 2016). Clustered sequences were then verified by reassigning them with Mothur (Schloss et al., 2009) to arb-SILVA (Quast et al., 2013) database release r132. Unassigned or miss-assigned sequences were removed. The second set of alignment was composed of 30 bacteria containing BALO type species and non-BALO sequences (Supplementary Table 3), also downloaded from NCBI (Benson et al., 2013). These sequences were used to check if the designed primers amplify other bacteria than the targeted BALO genus. The third and final set of alignment was composed of BALO type species only. For example, *Halobacteriovorax* type species *H. marinus* SJ and *H. litoralis* JS5 were aligned together. A consensus sequence with 25% variability was created with these type species. Then the consensus sequence was mapped to the verified sequence of *Halobacteriovorax* and the bacterial alignment to see better where nucleotides were different. The 20 primers generated by Primer-Blast (Ye et al., 2012) were mapped to the three alignments as follows. Firstly, the primers were mapped to the sequences of the 30 bacteria found in the Supplementary Table 3. If the primer pairs matched regions of other bacteria than the targeted BALO, these primers were removed from the candidates. If the primer was specific enough (*i.e.* both the forward and reverse sequences were very specific or, at minima, the forward or the reverse was highly specific), they were mapped to the type species alignment to see whether the primer could be degenerated to target all type species. For instance, one primer matched completely a region of *H. marinus* but needed to degenerate in the 6th position (M for A or C) to target also *H. litoralis*. Then, the primer was mapped to the *Halobacteriovorax* clustered and verified sequences to see how many other sequences the primer could target and if the primer needed further degeneration but without altering its specificity. The primer that seemed to be specific was tested to a bigger database containing bacterial sequences *via* the online tool TestPrime (Klindworth et al., 2013) of arb-SILVA (Quast et al., 2013). The "maximum number of mismatches" was set to 0. Furthermore, these primers were tested for their secondary structure. When possible the following rules were respected for hairpins, self-dimer and cross dimer: Hairpins, 3′ end with a ΔG of −2 kcal/mol and an internal value with a ΔG of −3 kcal/mol; Self Dimer, 3′ end with a ΔG of −5 kcal/mol and an internal with a ΔG of −6 kcal/mol; Cross Dimer, 3′ end with a ΔG of −5 kcal/mol and an internal with a ΔG of −6 kcal/mol. Hairpin and self-dimer were checked using the online tool OligoAnalyzer (https://www.idtdna.com/OligoAnalyzer/). As for cross dimer, they were checked using NetPrimer from Biosoft (http://www.premierbiosoft.com/netprimer/). As a final step, an *in silico* PCR was performed with the primers on the type species sequence using the program SerialCloner 2.6.1 (http://serialbasics.free.fr/Serial_Cloner.html). Among the 20 primers selected for each BALO genus, we selected the best 5 primer pairs for each BALO and ordered 3 of them from GATC/Eurofins. The list of the selected primers can be found in Table 2 and Supplementary Table 1.

2.2. BALOs primer design workflow for quantitative PCR

The design of qPCR (SYBR Green) primers for *Bacteriovorax*, *Halobacteriovorax*, *Peredibacter* and *Micavibrio* was inspired by Thornton and Basu (2011). Primer3 web 4.1.0 (Untergasser et al., 2012) was used to design the primers. A type sequence of a BALO genus (Supplementary Table 2) was used as template with the following parameters: product size range from 80 to 150 bp (shorter amplicons lengths gives higher PCR efficiencies (Thornton and Basu, 2011)); number of primers to return set to 20; primer size from 18 to 24 bp with an optimal size of 20 bp; primer Tm with a minimum of 60 °C, a maximum of 65 °C and an optimal of 62 °C; maximum Tm difference set to 2 °C; SantaLucia 1998 for table of thermodynamic; product Tm with an optimal of 50 °C; primer GC% from 40 to 65% with an optimal of 60%; max self-complementarity set to 4; 3 for max 3' self-complementarity; 4 for max pair complementarity; 3 for max 3' pair complementarity; 3 for max poly-X; concentration of divalent cations set to 3.5; 0.2 for dNTPs concentration; objective function penalty weights for primers with Tm Lt = 1, GT = 1; Size Lt = 1, Gt = 1, self-complementary = 3, 3′ self-complementary = 3, #N's = 2; and finally objective function penalty weights for primer pairs are product Tm Lt = 1, Gt = 1, Tm difference = 2, any complementary = 3 and 3′ complementary = 3. Then, the generated 20 primers were checked for similarity and mapped on an alignment of 30 non-BALO bacteria and BALOs (Supplementary Table 3) to verify their specificity (as detailed in the section above). Next, the suitable primers were mapped on the sequences of the targeted BALOs as detailed above. *A contrario* to the PCR primers, the qPCR primers were not degenerated to keep their specificity as much as possible. Primers were also checked with TestPrime (Klindworth et al., 2013) from arb-SILVA for specificity. Appropriate primers had their secondary structure verified with the online tool "Beacon Designer Free Edition" (http://www.premierbiosoft.com/qpcr/). When possible, primers with hairpin, cross dimer and self-dimer were discarded if their ΔGs were < −3.5 kcal/mol and/or if they tended to have 3 bp matched at the 3′ end. At last, selected primers were verified *in silico* with Serial Cloner 2.6.1 (http://serialbasics.free.fr/Serial_Cloner.html) and the generated amplicon was copied/pasted to UNAFold for secondary structure check (https://eu.idtdna.com/UNAFold). The temperature was set to 60 °C and Mg concentration to 3 mM. Once again, when possible, amplicons with Tm superior to 60 °C (temperature of hybridization) were discarded. Same as before, we ordered 3 primers out of 5 to test them in the laboratory. The final list of the designed primers

Table 1
BALOs potential primer sets designed for quantitative PCR (qPCR).

Primer qPCR	*E. coli* location (bp)	Sequence (5′-3′)	Target BALOs	Product length (bp)	No. of target BALOs detected	Specificity (%)[a]
Bx qP5 F	421	Fw CGGTCTGTAAAGCTCTGTTAATGT	*Bacteriovorax*	~ 84	19 (from 19 clones)	100
Bx qP5 R	482	Rv GGTGCTTCCTCTATGTGTACCA				
Hbx qP4 F	220	Fw CCAAATGATGAGCCTGCGTAG	*Halobacteriovorax*	~ 80	15 (from 18 clones)	83.3
Hbx qP4 R	279	Rv TCTCAGACCAGCTAAGCATCG				
Per qP5 F	627	Fw AAACTGCGTCTGAAACTGCT	*Peredibacter*	~ 91	20 (from 20 clones)	100*
Per qP5 R	696	Rv TGTTCCTTCACATCTCTACGGA				
Mica qP1 F	737	Fw ACTGGACTGGTATTGACGCT	*Micavibrio*	~ 91	2 (from 19 clones)	10.5
Mica qP1 R	808	Rv TAGCACACATCGTTTACGGC				
Mica qP4 F	1301	Fw TCAGATTGTCCTCTGCAACTC	*Micavibrio*	~ 132	0 (from 19 clones)	0
Mica qP4 R	1413	Rv TCAGGTAGAACCAACTCCCA				

[a] Specificity = (Number of non-target strains undetected / Total number of clones) × 100.
* The BLASTn assigned all the clones as *Peredibacter*, however when constructing phylogenetic tree only one clone seems to be closely related to *Peredibacter* species. The other clones could not be resolved correctly on the tree.

can be found in Table 1 and Supplementary Table 1.

2.3. Bdellovibrio and like organisms (positive control) strains and culture

To test the primers *in vitro* we tried to acquire some BALOs strains to serve as positive control. *Bdellovibrio bacteriovorus* HD100 and 109 J as well as *B. exovorus*, *Micavibrio aeruginosavorus* ARL-13 and *Peredibacter* sp. were courtesy obtained from Prof. Jurkevitch Edouard laboratory. *Halobacteriovorax* sp. was kindly sent by Prof. Williams Henry N. laboratory. Except for *Halobacteriovorax* sp. all strains were cultured and multiplied using the double-layer agar method with suitable prey as recommended by Jurkevitch (2012). We were not able to acquire *Bacteriovorax* strains. We also used a *Bdellovibrio* sp. previously obtained from Lake Geneva (Ezzedine et al., 2020a). At last, a mock community sample was also constituted by pooling all available BALOs DNA, latter referred to as BALOs mix (Supplementary Table 4).

2.4. Negative control of bacteria strain and culture

In order to test primers' specificity, negative controls were made with some bacterial strains (*i.e. Citrobacter freundii* ATCC 8090, *Escherichia coli* ATCC 10536, *Hafnia alvei* ATCC 13, *Pseudomonas fluorescens* ATCC 13525 and *P. putida* ATCC 12633) purchased from the "Centre International de Ressources Microbiennes" (CIRM) (https://www6.inra.fr/cirm_eng/). *P. aeruginosa* was kindly sent by Prof. Jurkevitch. These bacteria were cultured on liquid LB medium (Trypton 10 g, Yeast extract 5 g, NaCl 10 g) and incubated at 25 °C under low shaking conditions (200 rpm). *Vibrio parahaemolyticus* was kindly sent by Prof. Williams but not cultured. We also used *Pseudomonas* sp. previously isolated from Lake Geneva (Ezzedine et al., 2020a). At last, a mix of all the (negative control) bacterial strain DNA was also prepared (Supplementary Table 4).

2.5. Environmental samples for PCR and qPCR tests

Four types of samples were used in amplifying the designed primers for PCR. A first sample corresponded to a pool of filtered water (*i.e.* water filtered on 0.2 µm PC filter) from Lake Geneva taken at 2.5, 50, 200 m in February, May, July and November. A second mixed sample of water originated from Lake Annecy, also filtered on 0.2 µm PC filter, was taken at 3 and 45 m, in February, May, July and November. The third sample was a mixture of filtered water samples from the MOLA station in NW coastal Mediterranean Sea sampled offshore Banyuls-sur-mer (France) at 2, 80 and 200 m in March, April, July and November. The last sample was taken at another reference station in Banyuls bay, *i.e.* SOLA, and was a mix of filtered waters taken at 2 and 24 m, in February, May, July and November. Samples used for qPCR tests were also from different locations covering a range of salinities (< 1, ~15 and > 35 g/L): Lake Geneva (taken at 2.5, 50 and 200 m on June 30th and July 30th 2019 and mixed to obtain a unique pool), the estuary of Arcachon bay (France) near Audenge (taken in April as a single sample), and in the English Channel close to the marine biological station of Roscoff.

2.6. DNA extraction

Before DNA extraction, BALOs pure cultures were filtered through 0.45 µm pore filter to remove other cells *i.e.* prey. The other cultures (negative bacteria control) were not filtered, since they do not require a co-culture with other microorganisms for growth. Environmental samples were all filtered at 0.2 µm as mentioned before. Then < 0.45 µm BALOs filtrates, culture of "negative control bacteria" and environmental samples filtrates were subjected to DNA extraction using a homemade protocol with GenEluteTM-LPA (Sigma-Aldrich) solution. Firstly, all samples were centrifuged for 3 min at 6000 *g* and 4 °C and the supernatant was discarded. Then, 300 µL of TE buffer (TRIS: 1 M – pH 8, EDTA: 0.5 M – pH 8) were added to the pellet. Next, a lysis step was performed by adding 200 µL of lysis solution (TRIS: 1 M – pH 8, EDTA: 0.5 M – pH 8 and sucrose: 0.7 M). After a thermic shock at −80 °C for 15 min and at 55 °C for 2 min, 50 µL of 10% sodium dodecyl sulfate (SDS) as well as 10 µL of proteinase K (20 mg/mL) were added. Samples were then incubated at 37 °C for 1 h with gentle stirring, and placed in a heating block at 55 °C for 20 min. After a quick centrifugation step (13,000 rpm at 4 °C for 3 min), the supernatant was collected. Then, 50 µL of sodium acetate (3 M – pH 5.2) and 1.5 µL of GenEluteTM-LPA (Sigma-Aldrich, 25 µg/µL) was added. Next, one volume of isopropanol was added and the tubes were centrifuged for 10 min at 12,000 g and 4 °C. Following this step, two rounds of ethanol (80%) washing was carried out to clean the DNA pellet. The remaining ethanol was evaporated using a SpeedVac for 20 min. Finally, 30 µL of TE was added and samples were incubated at 37 °C for 1 h to let the pellet gently dissolve into the TE buffer. DNA concentration was measured using NanoDrop 1000 spectrophotometer. For DNA concentration superior to 25 ng/µL, a dilution was performed. All DNA preparations were stored at −20 °C until analysis.

2.7. PCR amplification (primers optimization and Nano MiSeq run preparation)

We used a gradient of temperature for PCR conditions and determined that the optimum annealing temperature for BALO DNA amplification was around 58–60 °C. The chosen protocol for BALOs amplification consisted of a PCR mixture volume set at 25 µL with reagent final concentration as follows: 1× buffer, 0.2 mM dNTP, 3 mM $MgCl_2$, 0.3 mg/mL bovine serum albumin (BSA), 0.2 mM of Forward and Reverse primer, 0.625 U Biotaq DNA polymerase (Bioline) and 1 µL of DNA template concentrated at 25 ng/µL. Negative control and when possible a positive control (BALO isolates) were included in the

Table 2
BALOs potential primer sets designed for Illumina sequencing MiSeq (or PCR).

Primer PCR	*E. coli* location (bp)	Sequence (5′-3′)	Target BALOs	Product length (bp)	No. of target BALOs detected	Specificity (%)[a]
Bd pP2 F	186	Fw TGCGGMTCTAGGGGTYAAAG	*Bdellovibrio*	~ 291	121 (from 121 OTUs)	100
Bd pP2 R	481	Rv CGATCCTTTCTTRCAKGGTACMTT				
Per pP1 F	1024	Fw TGCCCGCAAGGGAATGTAGT	*Peredibacter*	~ 346	91 (from 91 OTUs)	100
Per pP1 R	1349	Rv GGAGCGTGCTGATCTCCGAT				
Bx pP3 F	584	Fw GCGGACCTGCAAGTCAGATG	*Bacteriovorax*	~ 311	69 (from 148 OTUs)	46.6
Bx pP3 R	874	Rv CGTACTTCCCAGGCGGAACA				
Hbx pP2 F	253	Fw GGTGGGGTAAYGGCCTACCA	*Halobacteriovorax*	~ 375	7 (from 157 OTUs)	4.5
Hbx pP2 R	607	Rv CGRGGTTGAGCCCCGAGATT				
Mica pP5 F	132	Fw TGCCCTTAGGTGCGGAACAA	*Micavibrio*	~ 349	27 (from 224 OTUs)	12.1
Mica pP5 R	500	Rv GGCACGAAGTTAGCCGGAG				

[a] Specificity = (Number of non-target strains undetected / Total number of OTUs) × 100.

protocol. The PCR program adapted from Davidov et al. (2006) was as follows: 94 °C – 5 min, 35 × (94 °C – 1 min, 58 °C – 1 min, 72 °C – 3 min) and with a final extension step at 72 °C for 5 min. Primers Bd pP2, Mica pP5, Bx pP3, Per pP1 and Hbx pP2 (Table 2) were thought to be the most promising primers for BALOs specific amplification. Since these primers are meant for sequencing *i.e.* Miseq, we chose to test them directly *in situ via* a Nano Miseq run. Thus, following the instructions of the sequencing platform, the samples were prepared and sent to GenoToul (GeT-PlaGe, INRAE, Toulouse, France). In brief, 25 samples were sent to GenoToul but only 18 were successfully sequenced. The successful samples were: Bd pP2 amplification of Lake Geneva, Annecy, SOLA and mock community for *Bdellovibrio*; Per pP1 amplification of Lake Geneva, Annecy, MOLA and SOLA for *Peredibacter*; Bx pP3 amplification of Lake Geneva and Annecy for *Bacteriovorax*; Hbx pP2 amplification of Lake Geneva, MOLA and SOLA for *Halobacteriovorax* and finally Mica pP5 amplification of Lake Geneva, Annecy, MOLA, SOLA and mock community for *Micavibrio* (see Results).

2.8. qPCR amplification, cloning-sequencing and taxonomic assignation

The Rotor-Gene Q machine (Qiagen) and a SYBR Green PCR kit (Qiagen) were used to test BALO qPCR primers. After optimization, the volume of the reaction was set to 25 µL and the reagent final concentration was 1 × for SYBR Green master mix, 0.2 µM for forward and reverse primers, 0.3 mg/mL for BSA and 1 µL of template DNA. The qPCR program was 95 °C – 15 min, 40 × (95 °C – 45 s, 62 °C – 45 s, 72 °C – 45 s) and 60 to 95 °C with +1 °C every 5 s. After many tests, qPCR primers Bx qP5, Hbx qP4, Per qP5, Mica qP1 and Mica qP4 (Table 1) were selected as the best candidates to get the specific amplification of dedicated BALOs. The qPCR products generated using these primers were purified with GE healthcare illustra GFX according to the manufacturer's instructions and then cloned using TOPO TA Cloning kit (Thermo Fisher Scientific) following the manufacturer's recommendation. The cloning here is meant to reveal the specificity of each set of primers. For primer Per qP5 targeting *Peredibacter*, 20 clones were selected. As for primers Bx qP5, Hbx qP4, Mica qP1 and qP4 targeting *Bacteriovorax, Halobacteriovorax* and *Micavibrio* respectively, 19 clones were chosen. The inserts were sequenced by Sanger technology at GATC/Eurofins. The obtained sequences were dereplicated using Vsearch (Rognes et al., 2016) and then the taxonomic assignment was carried using NCBI BLASTn (Altschup et al., 1990).

2.9. Bioinformatics pipeline

The Nano-Miseq paired-end sequencing of the 18 samples from section 2.7 resulted in two files R1 and R2, each contained 400,519 reads in fastq format. The files were processed using the Frederic Mahé pipeline found at https://github.com/frederic-mahe/swarm/wiki/Fred's-metabarcoding-pipeline. The Text box number 1 in the Supplementary data describe briefly the workflow of the pipeline. The OTU tables (available on the Zenodo repository) were filtered by removing chimera sequences, singletons, sequences with less than 90% identity to the database and sequences with a < 0.0002 quality score. The figures used for the analysis of the reads were drawn on R version 3.5.0 (R Core Team, 2018) with ggplot2 (Wickham et al., 2018).

2.10. Phylogeny

The assigned qPCR clones sequences for each set of primer were phylogenetically related to BALO reference sequences. Supplementary Table 5 shows the reference BALOs sequences used in building these trees. All the alignment files are available in the Zenodo repository cited in the "Data accessibility" statement. Assigned qPCR clones and reference sequences were first aligned together using MUSCLE algorithms *via* MEGA7 (Kumar et al., 2016). The ends of all sequences were trimmed at 5′ and 3′ to make the aligned sequences of equal length. The alignment was only improved for *Bacteriovorax* qPCR amplicons using Gblocks 0.91b program (Castresana, 2000), where it kept 43 positions from 87, with "Minimum Length of a block" set to 5. Gblocks were not used on *Peredibacter* (1297 positions) and *Halobacteriovorax* (1103 positions) alignment, since alignment contained too many gaps, which made Gblocks discard many positions. Next, ModelGenerator v.85 (Keane et al., 2006) was used to select the best nucleotide substitution model with discrete gamma categories set to 4. Corrected Akaike information criterion (AICc) (Akaike, 1973) defined our model selection. The models used for *Bacteriovorax*, *Halobacteriovorax* and *Peredibacter* trees were K80 + G (0.46), GTR + G (0.29) and TrN + G (0.44), respectively. The constructed trees were built using the Maximum likelihood method (100 bootstrap replicates) with PhyML 3.1 (Guindon et al., 2010) and Bayesian method (500,000 generations and 25% burn-in value) using MrBayes 3.2.7a (Ronquist et al., 2012). The same workflow was applied to the assigned OTUs of the Nano MiSeq sequencing run. For the OTUs assigned to *Bdellovibrio*, *Bacteriovorax*, *Halobacteriovorax* and *Micavibrionales,* Gblocks kept respectively 192 out of 342, 251 out of 277, 197 out of 343 and 224 out of 376 positions. Gblocks was not used on *Peredibacter* alignment because it removed too many positions. The best nucleotide substitution model for *Bdellovibrio*, *Bacteriovorax*, *Halobacteriovorax, Micavibrionales* and *Peredibacter* selected under AICc were GTR + G (0.67), GTR + G (0.41), TrN + I (0.36) + G (0.54), TrN + I (0.33) + G (0.39) and GTR + G (0.40), respectively. The ML phylogeny was constructed with 100 bootstraps and the Bayesian phylogeny was run with 2 million generations and a 25% burn-in value.

3. Results

3.1. qPCR primers specificity check and sanger sequencing results

Primers for qPCR were designed to grasp the abundances of BALOs in environmental ecosystems. The amplicons were chosen to be short in order to get high PCR efficiencies. All the selected primers were first checked for specificity *in vitro* on targeted BALOs, not-targeted BALOs

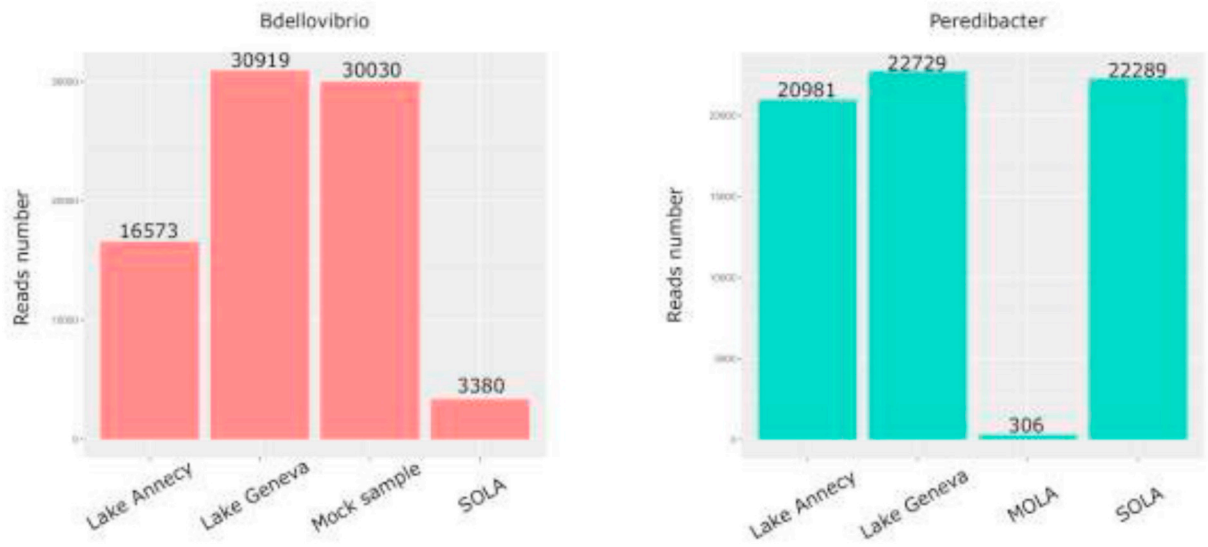


Fig. 1. Number of *Bdellovibrio* (left) and *Peredibacter* (right) raw reads in different environments amplified respectively by the designed primer Bd pP2 (Bd p186F – p481R) and Per pP1 (Per p1024F – p1349R). *Bdellovibrio* is highly detected in Lake Geneva as in the mock sample (pool of BALOs DNA). However, SOLA is characterized by a lower number of *Bdellovibrio* raw reads compared to other ecosystems. Since no other species were detected the *Bdellovibrio* primer set is specific. As for *Peredibacter,* the number of raw reads is almost equivalent in the 3 ecosystems *i.e.* Lake Annecy, Lake Geneva and SOLA, while MOLA yielded fewer reads. The disparity in the number of reads could be from three origins: sequencing depth, primers behavior and rarity of the targeted DNA. Here again, the primer set used in detecting *Peredibacter* is specific.

and other bacterial strains using qPCR amplification *i.e.* melting-curves. Then, the most specific among them were chosen to have their amplicons sequenced using the Sanger method (Table 1). The assignment of the sequences of the clones confirmed whether these primers are perfectly specific or not to the targeted BALOs as first observed with qPCR amplification.

For *Bacteriovorax*, *in vitro* amplification of the primer Bx qP5 showed no amplification of bacterial strains (negative control) and other BALOs that were not *Bacteriovorax* (not shown). In addition, Lake Geneva sample was amplified by Bx qP5. Despite not having a positive control, the *in vitro* result seemed encouraging. Indeed, after sequencing the Lake Geneva sample and assigning the clones, the amplification proved to be very specific to *Bacteriovorax*. *In fine*, all clones were assigned to *Bacteriovorax* sp. according to NCBI BLASTn (not shown). Therefore, the specificity of Bx qP5 primers toward *Bacteriovorax* is 100% (Table 1). Moreover, the phylogenetic tree (Supplementary Fig. 1) confirmed that the assigned clones were phylogenetically close to *Bacteriovorax stolpii* Uki2 and *Bacteriovorax.* sp. F2.

For *Peredibacter,* although Per qP5 primer did not amplify the positive control, a good specificity was also observed (Supplementary Fig. 2). Typically, there was no non-specific amplification and all clones were assigned to *Peredibacter* sp. (not shown). Therefore, the specificity value of this set of primer is 100% (Table 1). The phylogenetic tree (Supplementary Fig. 3) shows that clone 9 is phylogenetically related to *Peredibacter* species. However, the other clones were not resolved on the tree. In this case, the phylogenetic tree did not confirm the result as seen previously with primer Bx qP5.

The exact *in vitro* results can be reported for *Halobacteriovorax* when using the primer Hbx qP4 (not shown). Amplicons from the Bay of Arcachon were sequenced and resulted in 83% of specificity toward *Halobacteriovorax* (Table 1). Briefly, 1 clone was empty, 15, 2 and 1 clone was assigned to *Halobacteriovorax* sp., Alteromonadales and *Bacteriovorax* sp. respectively. Here, the assigned clones of *Halobacteriovorax* were phylogenetically closer to *Peredibacter* species rather than *Halobacteriovorax* (Supplementary Fig. 4).

Finally, the Mica qP1 primer for *Micavibrio* showed high specificity for the positive control and Lake Geneva water sample after 12–17 cycles, but non-specific amplification appeared after approximately 30 cycles (not shown). The non-specific amplification was at the same temperature when observing the melt curve (not shown). The assignation result of the 19 clones was as follows: 3, 2 and 14 clones matching with *Inquilinus*, *Micavibrio* and "Uncultured bacterium", respectively. Said differently, only 10.5% of the clones were identified as *Micavibrio* (Table 1). The second tested primer, Mica qP4 also amplified *Micavibrio* positive control after 12 cycles but, here again, non-specific amplification occurred with a larger number of cycles. The results of the assignment of the generated amplicons were all identified as *Brevundimonas sp* and none as *Micavibrio*. Hence, Mica qP4 had 0% specificity toward *Micavibrio* (Table 1).

3.2. PCR primers specificity check and 2 × 250 NanoV2 MiSeq results

To study BALOs diversity, we designed primer sets compatible with PCR/Miseq 2 × 250 bp. The selected primers (Table 2 and Supplementary Table 1) were first tested in PCR conditions with targeted BALOs (positive control, when available), other BALOs and non-BALOs bacteria to select the most specific and promising primer pairs (Table 2). Then, since these promising primers are intended for sequencing, the amplified amplicons were sequenced with Nano-Miseq to check how they perform and to validate or not their specificity toward the targeted BALOs. The sequencing of the 18 samples generated 400,519 reads. The reads were analyzed per primer set and OTUs tables were generated.

Among the selected primers for *Bdellovibrio*, primer set Bd pP5 amplified not only *in vitro* some BALOs but also other organisms such as *Pseudomonas* sp. (Supplementary Fig. 5). *A contrario*, primer set Bd pP2 amplified only BALOs especially *Bdellovibrio* spp. Disregarding of amplifying the *Micavibrio* DNA and not seeing any band appearing when environmental DNA was tested, Bd pP2 was retained for Nano MiSeq 2 × 250 sequencing. It is noteworthy that this primer did not amplify DNA from the MOLA sample so that no sequences were obtained for this site. However, it successfully amplified DNA from Lakes Geneva and Annecy, the Mock sample (mix of BALO DNA) and SOLA. The bioinformatics analysis resulted in 121 OTUs, all assigned without exception to the *Bdellovibrio* genus. Hence, Bd pP2 specificity value is 100% toward *Bdellovibrio* (Table 2). Also, the phylogenetic tree (Supplementary Fig. 6) confirmed the assignment results with a majority of OTUs closely related to *Bdellovibrio*. Furthermore, the bar plot in Fig. 1 (left) shows

that the *Bdellovibrio* individuals in the mock community were well detected. In addition, Lake Geneva holds the highest number of raw reads for the *Bdellovibrio*. Lake Annecy classified as second and the SOLA station displayed fewer reads.

Despite not amplifying the *in vitro* positive control (*Peredibacter* sp. DNA) and showing amplification to the isolated *Bdellovibrio* sp. from Lake Geneva (Ezzedine et al., 2020a), the *Peredibacter* primer Per pP1 was the most specific to BALOs DNA in comparison to the two other ordered primers (Supplementary Fig. 1). *In silico*, the amplicon size obtained was around 346 bp. In some samples, non-specific bands on the agarose gel were observed (not shown) above or below the targeted size. However, the environmental samples from Lake Geneva and the Bay of Arcachon were amplified around 350 bp. Therefore, the primer set Per pP1 was tested by sequencing. The mock sample was not sequenced since no amplification was visible when constructing the libraries. The results of the sequencing and the bioinformatics analysis gave 91 OTUs which were all assigned to the *Peredibacter* genus. Thus, the specificity of Per pP1 toward *Peredibacter* is 100% (Table 2). Once more, the phylogenetic tree (Supplementary Fig. 7) agreed with the assignment results. However, some OTUs revealed to be phylogenetically related to *Halobacteriovorax* and *Bacteriovorax* sp. EPA and EPC3. Furthermore, when analyzing the number of reads generated upon the detection of *Peredibacter* DNA (Fig. 1 right), the results showed that Lake Geneva, Lake Annecy and the marine SOLA station had high and approximately equal number of raw reads. On the contrary, only a small amount of reads were amplified for MOLA sample.

For the *Batceriovorax* primers set we did not test them on a positive control since we could not obtain one. However, after analyzing the agarose gel of each primer set (Supplementary Fig. 1), primer Bx pP3 revealed itself as the most specific primer set toward the tested BALOs strains. Despite amplifying BALOs DNA other than *Bacteriovorax* the primer Bx pP3 was considered as promising. Also we noted, that some non-specific bands were visible but as previously explained for *Peredibacter*, they were above or below the targeted size (not shown). The sequencing was carried and the results showed that the primer amplified not only *Bacteriovorax* but also *Peredibacter* at high quantity, especially for the sample from Lake Annecy (Fig. 2 left). On the other hand, for Lake Geneva, *Bacteriovorax* was amplified almost equally as *Peredibacter* in terms of raw reads (Fig. 2 right) and OTUs. Aside from amplifying *Bacteriovorax* and *Peredibacter*, we noted that two sequences were assigned to *Bdellovibrio* and 6 to other bacteria. In summary, the specificity of Bx pP3 toward *Bacteriovorax* is at 46.6% (Table 2). The phylogenetic tree (Supplementary Fig. 8) was in accordance with these results. The assigned OTUs clustered either with *Peredibacter* or *Bacteriovorax*. However, some OTUs seemed to be related more to *Halobacteriovorax* than to *Bacteriovorax* or *Peredibacter*.

On one hand, the *Halobacteriovorax* primer set Hbx pP2 did not amplify non-BALO DNA (not shown). In another hand, Hbx pP2 did not amplify the positive control sample containing *Halobacteriovorax* sp. DNA. Also, it amplified were it should not *i.e.* Lake Geneva. Results from sequencing revealed that it mainly amplified other bacteria and BALOs than *Halobacteriovorax* (Fig. 3 left). Among the few detected BALOs, Fig. 3 (right) revealed that *Bacteriovoracaceae*, *Peredibacter* and *Bacteriovorax* were overall more detected than the *Halobacteriovorax* itself. Again, few reads and OTUs (7 out of 157) were assigned to *Halobacteriovorax*. Besides, some OTUs were not assigned to *Halobacteriovorax* but to "Marine BALOs" (6 OTUs). In short, the specificity of Hbx pP2 is only at 4.5% toward *Halobacteriovorax* (Table 2). A phylogenetic tree (Supplementary Fig. 9) was constructed with both *Halobacteriovorax* and "Marine BALOs" reads. In majority, *Halobacteriovorax* reads clustered with *Halobacteriovorax marinus* SJ but two were placed next to *Micavibrio*. Furthermore, the "Marine BALOs" were not phylogenetically related to *Halobacteriovorax* but *Bacteriovorax* and *Peredibacter*.

At last, the *Micavibrio* primer Mica pP5 revealed *in vitro* a specific amplification for the *Micavibrio* DNA (*i.e.* the positive control). However, results from the sequencing revealed that other bacteria than *Micavivibrio* were also amplified. The *Micavibrio* DNA was exclusively amplified in the mock sample as shown in Fig. 4 (left). However, only a few *Micavibrio* were detected in the 4 natural environmental samples. Among the 224 obtained OTUs, only 27 were assigned to *Micavibrionales*. Besides, unlike previous assignments, *Micavibrio* OTUs were only assigned to the order level *i.e. Micavibrionales* except of the first OTU which represented the *Micavibrio* used in the mock sample. Also, this OTU was poorly detected in natural samples. However, other *Micavibrionales* seemed to be more detected. According to Fig. 4 (right), the SOLA station revealed a higher number of reads for *Micavibrionales* than the other ecosystems. Overall, the specificity of Mica pP5 toward *Micavibrio* in environmental samples is 12.1%. The phylogenetic tree (Supplementary Fig. 10) showed that the majority of *Micavibrionales* detected OTUs were closely related to *M. aeruginosavorus* ARL-13 and

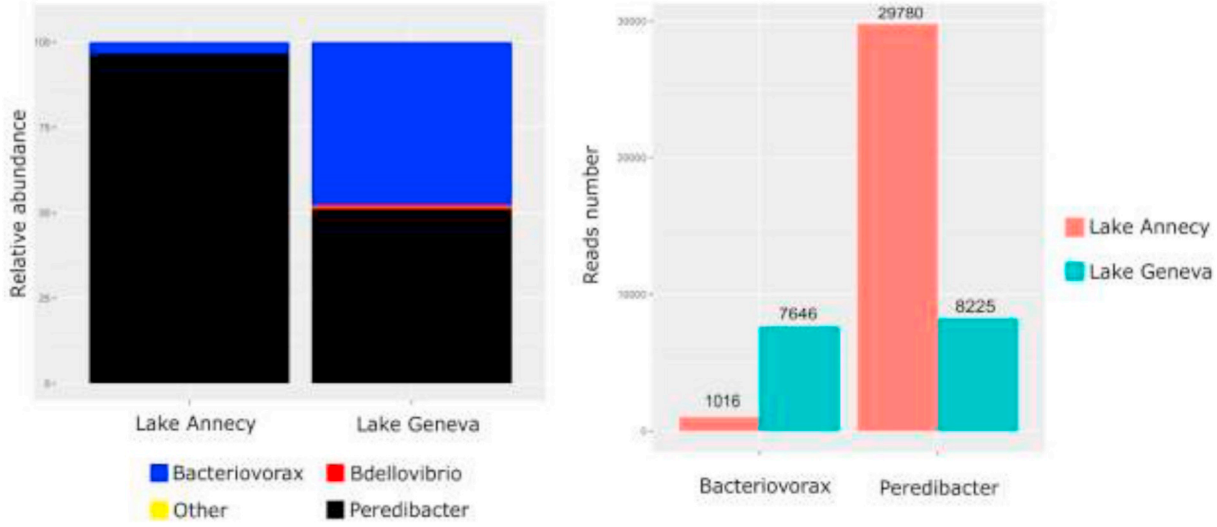


Fig. 2. Specificity of the designed primer Bx pP3 (Bx p584F – p874R) toward *Bacteriovorax* in different environments (left) illustrated with relative abundance. We can note that Bx pP3 amplified not only *Bacteriovorax* but also *Peredibacter* especially in Lake Annecy. Other bacteria and other BALOs are not significantly amplified. The bar plot on the right shows the distribution of raw reads using Bx pP3. *Bacteriovorax* is more amplified in Lake Geneva than in Lake Annecy. However, *Peredibacter* reads are overall more abundant and especially in Lake Annecy. In conclusion, the primer set Bx pP3 is only half specific toward *Bacteriovorax*.

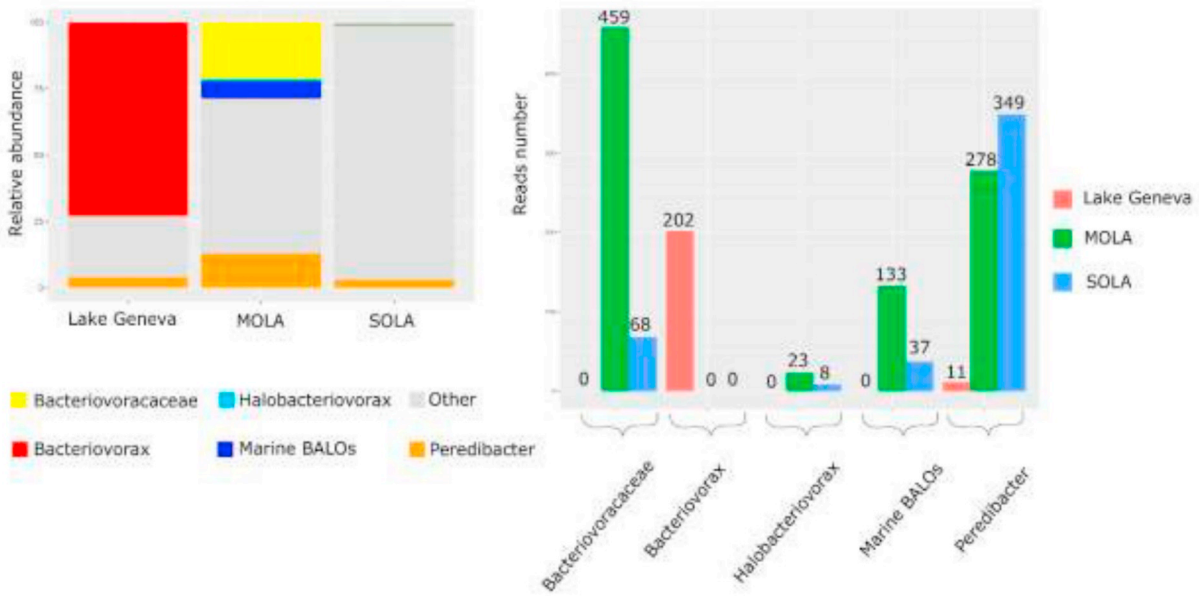


Fig. 3. Specificity of the designed primer Hbx pP2 (Hbx p253F – p607R) toward *Halobacteriovorax* or/and marine BALOs in different environments (left) with relative abundance. *Halobacteriovorax* reads are majority detected at MOLA. Few sequences are detected at SOLA and none in Lake Geneva. Unassigned marine BALOs were also detected in MOLA and SOLA. Overall, the primer amplifies more other bacteria than *Halobacteriovorax*. The bar plot on the right shows the distribution of raw reads number of detected BALOs in the three selected ecosystems. *Halobacteriovorax* and marine BALOs DNA are poorly detected. *Bacteriovoracaceae* and *Peredibacter* are more detected in the three different environments than *Halobacteriovorax*. In conclusion, this primer set is not specific to *Halobacteriovorax*.

Micavibrio sp. EPB, especially OTUs 372, 340, 440, 400, 145 and 200. However, some OTUs were shown to be phylogenetically closer to *Bdellovibrio exovorus* MPR11 than *Micavibrio*.

4. Discussion

To study BALOs diversity and abundance with the current classification of the *Bdellovibrio* and like organisms group and the new technological advances, we designed and tested *in silico* and *in vitro* new specific primers for both qPCR and PCR/MiSeq sequencing. Most importantly, we used Sanger method (qPCR) and Nano Miseq (PCR) sequencing to reveal what exact taxa hide behind the obtained amplicons. Hence, attributing a specificity value for each promising set of primers. By designing specific primers, amplification biases were minimized and the recovery of BALOs taxa maximized (Elbrecht et al., 2019). Globally, some of our primers worked properly for the different tested ecosystems and we are confident they could be applied successfully to other systems, even if further testing and optimization may be required.

We successfully designed qPCR primers for *Bacteriovorax* as the taxonomic assignment and phylogenetic results showed. For *Halobacteriovorax* and *Peredibacter* their specificity is as good as the NCBI taxonomic assignment indicated since a large number of the sequences of the clones could not be resolved phylogenetically. Therefore, the use of *Halobacteriovorax* and *Peredibacter* set of primers should be handled with utmost caution. On the other hand, *Micavibrio* qPCR set of primer Mica qP1 and Mica qP4 showed very poor and no specificity at

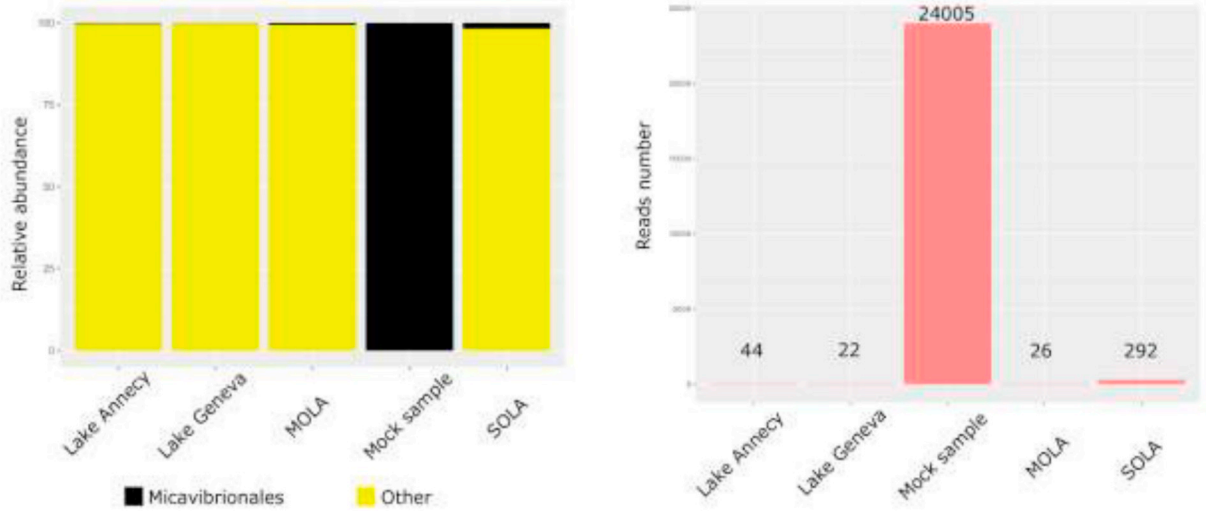


Fig. 4. Primer Mica pP5 (Mica p132F – p500R) significantly amplify *Micavibrionales* when they are abundant in the mock sample. The primer set is very specific toward the *Micavibrionales* despite the presence of other BALOs. *A contrario*, we can see that in the environmental samples *Micavibrionales* are not abundantly amplified. The bar plot on the right shows that *Micavibrionales* raw reads numbers are more numerous at SOLA than in other natural ecosystems. The detection of *Micavibrio* is poorly undertaken by this set of primers.

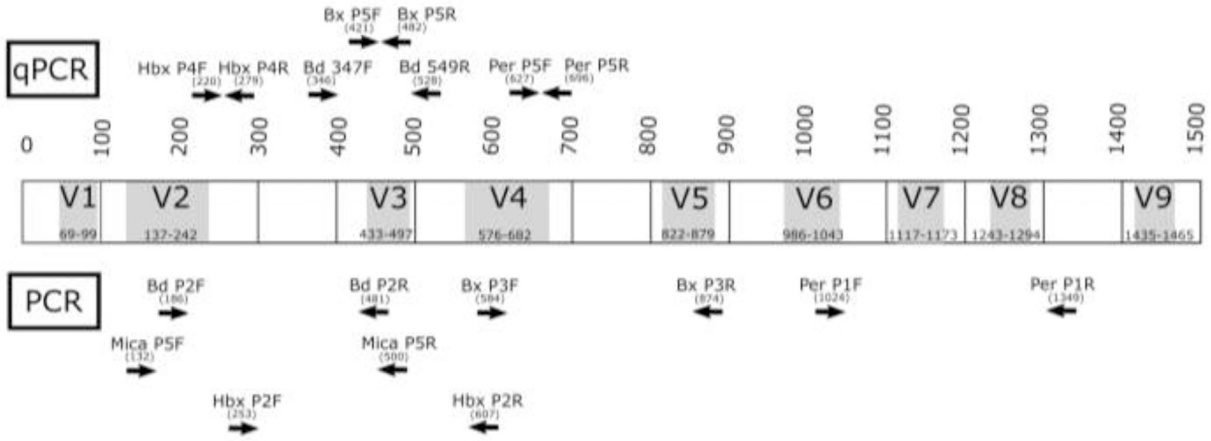


Fig. 5. Primer positions on the 16S rRNA gene of *E. coli*. F and R symbols represent forward and reverse primer respectively. Under each primer name, the position based on the 16S of *E. coli* is given according to the TestPrime tool from arb-SILVA (Quast et al., 2013). For example, the forward qPCR primer of *Bacteriovorax* named Bx qP5F is positioned at 421 bp of *E. coli* 16S rRNA gene. The hypervariable regions V1 to V9 (gray rectangle) are positioned according to Chakravorty et al. (2007).

all toward *Micavibrio*. Thus, these primers should not be used to study *Micavibrio* abundance. Finally, we did not design qPCR primers for *Bdellovibrio* because of the primer pair Bd347–Bd549, made by Van Essche et al., 2009 (Supplementary Table 1), is already specific for *Bdellovibrio*. Also, the group of *Pseudobacteriovorax* was not considered here since, to the best of our knowledge, this group was only reported to be associated with the octocoral *Antillogorgia elisabethae* that inhabit tropical waters (McCauley et al., 2015). It is noteworthy that no standard curves were used or optimized since the main aim of the study is to design primers and not yet reveal the abundances of BALOs in the considered ecosystems. On the other hand, as an explanation as to why some clones sequences for *Peredibacter* and *Halobacteriovorax* could not be resolved on the phylogenetic trees is that the length of the sequences obtained from Sanger sequencing is short, typically around 80 bp. The sequences could be also of poor quality. Most importantly, the alignment of the sequenced clones, with the reference sequences generated too many gaps. The alignment files were deposited in the Zenodo repository so that the reader can independently judge the quality of the alignment. Lastly, the location of these primers according to TestPrimer from arb-SILVA on the 16S sRNA gene of *E. coli* is represented in Fig. 5. *In fine*, Bx qP5 primers are named Bx q421F and Bx q482R, Hbx qP4 primers are named Hbx q220F and Hbx q279R, and Per qP5 primers are named Per q627F and Per q696R.

Some primers were also successfully designed for Illumina Miseq. Here again we did not consider *Pseudobacteriovorax*. For OTUs clustering we used the program Swarm with "d = 1" in order to get a better taxonomic resolution to detect larger genetic diversity among BALOs. *Bdellovibrio* (Bd pP2) and *Peredibacter* (Per pP1) primers were very successful according to the OTU assignments and the phylogenetic trees. Indeed, 100% of OTUs amplified by Bd pP2 and Per pP1 were assigned to *Bdellovibrio* and *Peredibacter*, respectively. *Bacteriovorax* primer (Bx pP3) was only half-good since it could also detect *Peredibacter*. That being said, *Bacteriovorax* DNA is well present and detected in the environmental samples. On the other hand, the *Halobacteriovorax* primer (Hbx pP2) was not solely specific to *Halobacteriovorax* but also to other BALOs and bacteria. Although this set of primers should not be used to grasp the diversity of *Halobacteriovorax*, these primers most likely can reveal more *Halobacteriovorax* related sequences than using a universal set of primer. The *Micavibrio* primer (Mica pP5) amplified 100% *Micavibrio* in the mock sample containing all available BALOs strains in our laboratory. This suggests that the primer specifically amplified *Micavibrio* and not other BALO strains. However, in a natural ecosystem *Micavibrio* was less amplified compared to the tremendous amplification of other bacteria. This either can suggest that this primer is not that specific and that the bacterial strains that we used as negative controls were not enough to demonstrate that the primer is not specific as we thought. It can also suggest that *Micavibrio* is weakly present in the natural environment so that the primer may amplify another target. In the light of these elements, the behavior of this primer set is instable, thus the repeatability of the results from an environment to another can be compromised. Again, how that set of primer can perform in comparison to a universal primer set toward the detection of *Micavibrio* need an answer. Fig. 5 represents the position of the primers on *E. coli* 16S rRNA gene. *In fine*, we renamed Bd pP2, Bx pP3, Per pP1, Hbx pP2, Mica pP5 as Bd p186F and Bd p481R, Bx p584F and Bx p874R, Per p1024F and Per p1349R, Hbx p253F and Hbx p607R, Mica p132F and Mica p500R, respectively.

Our work was challenging for several aspects and we faced a variety of issues. First, we could not get all BALOs strains (positive control) for *in vitro* tests *e.g. Bacteriovorax stolpii*. We also acquired only one positive strain as a positive control, thus not very representative of the diversity of a BALO genus. As we are aware that this is not sufficient to predict *in vitro* specificity, we then selected the best set of primers and sequenced their amplification. Therefore, not having a positive control or even failing to amplify it does not discredit or invalid the experiment since the analysis of primers specificity was carried on environmental samples *via* sequencing. To prove even more, that positive control was only used to get a general idea about *in vitro* specificity, two examples can be given. The first is that we managed to design a very specific qPCR set of primers for *Bacteriovorax* without a positive control. The second is that despite showing a very promising specificity toward *Micavibrio*'s positive control *in vitro*, the designed primers set for qPCR and PCR failed as demonstrated by the sequencing results to specifically amplify *Micavibrio*. On the other hand, difficulties lied in using uncurated databases (uncertain or wrong taxonomic assignments) with sequences originating from various environments and from different sequencing technologies. This last main problem was recently highlighted by Lydon and Lipp (2018) who reported that sequences of *Pseudoalteromonadaceae* were wrongly placed in the order of the *Vibrionales* and *vice versa* in Greengenes (v13_5 and 13_8; DeSantis et al., 2006) database. 68 published articles have however based their results on these erroneously assigned sequences. The same issue can be reported here since many BALO sequences found at NCBI were/are still under the *Bdellovibrio* name. Also, a confusion between *Bacteriovorax, Peredibacter* and *Halobacteriovorax* is still visible, and some sequences are assigned to the wrong species. For example, *B. exovorus* MPR11 does not phylogenetically relate to *Bdellovibrio* but to *Micavibrio* as also observed in

all our phylogenetic trees. The same problem was also observed for sequences assigned to *Bacteriovorax* sp. EPC3 and EPA. These sequences clustered better with *Peredibacter* than *Bacteriovorax*. Furthermore, OTUs assignment *via* our pipeline was also challenging since we observed a different taxonomic assignment in the arb-SILVA new release version 138 from the agreed BALOs classification reported by Baer et al. (2000, 2004), Koval et al. (2015), and Hahn et al. (2017). Currently, Arb-SILVA follows this taxonomic assignment where BALOs class is Bdellovibrionia and not Oligoflexia (*e.g.* Bacteria |Bdellovibrionota | Bdellovibrionia | Bacteriovoracales). Additionally, the last level in the taxonomic assignment does not always belong to the correct genus of BALOs (*e.g.* |Bacteriovorax|Bdellovibrio_sp._SD1 and Peredibacter|Bacteriovorax_sp._EPC3).

Our results highlighted other important issues. Firstly, samples issued from the marine SOLA and MOLA sites were difficult to amplify with the different primers. For instance, we had to use a sample from the Bay of Arcachon for *Halobacteriovorax* qPCR primers tests since no or low amplification was obtained at MOLA and SOLA. In addition, the Nano MiSeq sequencing showed that fewer reads were detected for these sites, especially for MOLA. We first believed that the problem was associated with PCR inhibitors present in the sample. However, after making a dilution test with qPCR (not shown) we observed that the diluted samples (1/2, 1/4, 1/10, 1/20, 1/40) appeared after the undiluted sample, suggest that the problem was elsewhere. Most likely BALOs are less abundant in the Bay of Banyuls than in Lakes Geneva and Annecy, and more globally in marine than in freshwaters. If so, the relatively low abundance of BALOs and their DNA may have biased the amplification. This could explain the result observed for Mica p132F – p500R that amplified the *Micavibrio* DNA in the mock sample but not in the environmental samples. Furthermore, *Bacteriovorax* primer p584F - p874R might also be concerned with most likely *Peredibacter* being more present than *Bacteriovorax* as reported by Paix et al. (2019). Finally, primers' performance could be related to other reasons that we did not explore here such as DNA polymerase (Śpibida et al., 2017), the number of PCR cycles or the cell GC content (Elbrecht et al., 2019).

The Nano Miseq revealed interesting results regarding the presence of some BALOs in certain ecosystems. To begin, as expected *Halobacteriovorax* DNA was not detected in the freshwater system *i.e.* Lake Geneva (Fig. 3). However, *Peredibacter* DNA was detected in the marine ecosystem. Indeed, in SOLA with the primer set Per pP1 (Fig. 1 right), 22,289 reads assigned to *Peredibacter* were detected. This result is surprising, according to Piñeiro et al. (2008) the only recognized species of *Peredibacteraceae* are found in freshwater and soil environments. The SOLA site located in Banyuls bay is influenced by fluvial contributions from the Rhone, coastal rivers such as the Tech or Têt and locally episodic floods from the Baillaury in Banyuls Bay. Therefore, *Peredibacter* detected DNA could be from allochthonous origin. Alternatively, this could be some freshwater resistant representative that managed to adapt to salinity. The same logic could be applied to *Bdellovibrio* since we detected 3380 reads of the latter at the SOLA site (Fig. 1 left). Furthermore, with Hbx pP2 we found for some sequences the assignment “uncultured marine bacterium” at the last level of the taxonomic assignment. Historically, marine BALOs are initially referred to as “marine *Bdellovbirio*” before being named *Halobacteriovorax*. However, the levels before that assignment indicated that these OTUs belong most probably to *Bacteriovoracaceae* or *Peredibacter*. We also tested these sequences *via* NCBI BLASTn (Altschup et al., 1990) and approximately found the same assignment. These results of taxonomic assignment might be due to erroneous affiliation of some sequences in the database. Alternatively, it can also bring other elements to the existence of probable other halo-tolerant BALOs, which could be proposed as new members of a halo-*Peredibacter*, halo-*Bdellovibrio* group. In fact, in the classification of BALOs, there is no distinction between fresh and saltwater *Bdellovibrio, Peredibacter* and *Micavibrio*. Finally, these results are very interesting and open doors for further research for determining whether other halo-tolerant BALOs can thrive in saltwater.

A final word is that we purposely listed all designed primers set in the Supplementary Table 1 so that other researchers could avoid losing time in testing such sequences or could eventually improve them.

5. Conclusion

Our study aims to participate in the study of the diversity and abundance of BALOs genera except *Pseudobacteriovorax* in natural ecosystems. Therefore, we designed primers for qPCR and PCR/Miseq. We propose new validated primers to detect specifically *Bacteriovorax* abundance and *Bdellovibio* and *Peredibacter* diversity. As discussed, the qPCR primer set for *Peredibacter* and *Halobacteriovorax* is to be used with caution since they are as good as the taxonomic assignment could tell. Also, a word of caution is appropriate to the use of primer set Bx pP3 to unravel *Bacteriovorax* diversity since it can also detect *Peredibacter*. Finally, primer pairs for *Halobacteriovorax* and *Micavibrio* failed to be specific. *In fine*, these validated tools will be very useful to better assess the distribution, dynamics and diversity of this functional bacterial group and highlight the ecology of the BALOs in a variety of ecosystems.

Data accessibility

The majority of the OTUs sequences are available at NCBI under the accession numbers: MT177341 to MT177658. Clones sequence from qPCR are shorter than 150 bp and were not admitted on NCBI. Regardless, these sequences along with all alignment files, OTU tables obtained from the different tested primers are available on the Zenodo repository website (doi:https://doi.org/10.5281/zenodo.3706495).

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2020.105996.

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