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Specific detection and quantification of the marine flavobacterial genus *Zobellia* on macroalgae using novel qPCR and CARD-FISH assays

Maéva Brunet [a], Nolwen Le Duff [a], Bernhard M. Fuchs [b], Rudolf Amann [b], Tristan Barbeyron [a], François Thomas [a,*]

[a] Sorbonne Université, CNRS, Integrative Biology of Marine Models (LBI2M), Station Biologique de Roscoff (SBR), 29680 Roscoff, France
[b] Department of Molecular Ecology, Max Planck Institute for Marine Microbiology, Bremen, Germany

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ABSTRACT

The flavobacterial genus *Zobellia* is considered as a model to study macroalgal polysaccharide degradation. The lack of data regarding its prevalence and abundance in coastal habitats constitutes a bottleneck to assess its ecological strategies. To overcome this issue, real-time quantitative PCR (qPCR) and fluorescence *in situ* hybridization (FISH) methods targeting the 16S rRNA gene were optimized to specifically detect and quantify *Zobellia* on the surface of diverse macroalgae. The newly designed qPCR primers and FISH probes targeted 98 and 100% of the *Zobellia* strains *in silico* and their specificity was confirmed using pure bacterial cultures. The dynamic range of the qPCR assay spanned 8 orders of magnitude from 10 to 10^8 16S rRNA gene copies and the detection limit was 0.01% relative abundance of *Zobellia* in environmental samples. *Zobellia*-16S rRNA gene copies were detected on all surveyed brown, green and red macroalgae, in proportion varying between 0.1 and 0.9% of the total bacterial copies. The absolute and relative abundance of *Zobellia* varied with tissue aging on the kelp *Laminaria digitata*. *Zobellia* cells were successfully visualized in *Ulva lactuca* and stranded *Palmaria palmata* surface biofilm using CARD-FISH, representing in the latter 10^5 *Zobellia* cells·cm^{-2} and 0.43% of total bacterial cells. Overall, qPCR and CARD-FISH assays enabled robust detection, quantification and localization of *Zobellia* representatives in complex samples, underlining their ecological relevance as primary biomass degraders potentially cross-feeding other microorganisms.

Introduction

Macroalgae are dominant primary producers in coastal ecosystems and constitute a large reservoir of organic matter worldwide. A wide range of microorganisms colonize macroalgal surfaces [20]. These biofilms contain diverse bacteria at densities ranging between 10^6 and 10^9 cells·cm^{-2} [35], which contribute to the remineralization of algal biomass [33]. Among those are the members of the genus *Zobellia* (phylum *Bacteroidetes,* class *Flavobacteriia*), first described by Barbeyron et al. [8]. To date, seven valid species compose the genus, namely *Z. amurskyensis, Z. galactanivorans, Z. laminariae, Z. nedashkovskayae, Z. russellii*, *Z. roscoffensis* and *Z. uliginosa.* They were isolated from marine environments throughout the world, notably from the surface of brown [9,37], red [40] and green macroalgae [37]. Additionally, the NCBI database references 65 unclassified *Zobellia* strains, mostly isolated from seaweeds. This genus comprises highly potent degraders of macroalgal polysaccharides. Described members of the genus *Zobellia* can grow on algal polysaccharides such as agar, porphyran and carrageenans from red algae and laminarin, alginate and fucoidans from brown algae, in line with the high number of carbohydrate-active enzymes (CAZymes) encoded in their genomes [10,16]. A recent survey of the twelve available *Zobellia* genomes revealed that CAZyme-encoding genes accounted for more than 6% of the genomic content, from 263 genes in *Z. nedashkovskayae* Asnod3-E08-A to 336 genes in *Z. galactanivorans* Dsij^T [15], illustrating the specialization of *Zobellia* representatives in the degradation of algal polysaccharides. Accordingly, *Z. galactanivorans* Dsij^T has become a model for algal polysaccharide breakdown and assimilation [32], allowing the discovery of many novel polysaccharidases and Polysaccharide Utilization Loci (PULs) targeting algal compounds [21,28,45]. Despite the potential of *Zobellia* strains for algal biomass recycling, little is known regarding their prevalence and abundance in macroalgae-dominated habitats, hindering our understanding of their role in coastal biogeochemistry. Molecular studies revealed the presence of *Zobellia* members in diverse coastal ecosystems [1,19,29,34]. Searches in marine metagenomes

* Corresponding author.
E-mail address: francois.thomas@sb-roscoff.fr (F. Thomas).

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show numerous hits with high sequence identities (>90%) to *Zobellia* genes in a variety of environments rich in organic carbon, including algal microbiomes, coastal sediments and seawater from the Atlantic and Pacific Oceans as well as the North Sea and the English Channel (Supplementary Table 1). Overall, this suggests a widespread distribution and ecological relevance of *Zobellia* for carbon cycling in coastal ecosystems. However, metagenomic sequencing does not allow a precise quantification of taxon abundance in the environment and can be biased by promiscuous taxonomic sequence assignation. This prompted us to develop new tools targeting the genus *Zobellia*, which would allow specific detection in complex samples, cell localization and both absolute and relative quantification of its abundance.

Real-time quantitative polymerase chain reaction (qPCR) and fluorescence *in situ* hybridization (FISH) are two well-established molecular methods for specific bacterial taxa detection. qPCR is a fast, highly sensitive and specific tool allowing absolute quantification of DNA gene copies and is suitable to detect minor amounts of target DNA [24]. qPCR assays were previously developed to quantify microbial taxa associated with macroalgae, such as endophytic eukaryotic pathogens in brown seaweeds [13,25], bacterial pathogens on *Saccharina latissima* [6] or the genus *Pseudoalteromonas* at the surface of green macroalgae [44]. FISH is a widely used method in microbial ecology for cell identification and enumeration in complex environments. Its principle is based on the use of rRNA-targeted oligonucleotide probes labeled with fluorescent dyes, which allow the detection of a defined group of microorganisms [3]. The use of horseradish peroxidase-labeled probes in combination with catalyzed reported deposition (CARD) of fluorescently labeled tyramides was then developed to enhance the fluorescence and improve the detection signal [38]. FISH combined or not with catalyzed reporter deposition (CARD-FISH) was successfully applied previously to enumerate seaweed-associated epiphytic or endophytic bacteria [12,31,46,48,49]. Here, we describe new qPCR and FISH protocols specifically targeting the genus *Zobellia*, allowing its detection, absolute quantification and localization at the surface of macroalgae. qPCR primers and FISH probes were designed and validated *in silico*. Both methods were optimized on pure bacterial cultures before being applied on environmental samples.

Material and methods

Bacterial strains

All bacterial strains used in this study are listed in Supplementary Table 2 and were grown in ZoBell 2216 medium [53].

Environmental samples

Surface microbiota were sampled in February 2020 using sterile swabs on healthy specimens of *Laminaria digitata* (Ldig), *Ascophyllum nodosum* (Anod), *Ulva lactuca* (Ulac) and *Palmaria palmata* (Ppal) at the Bloscon site (48°43′29.982″ N, 03°58′8.27″ W) in Roscoff (France). Three individuals of each species were sampled. One additional sample was obtained from a stranded specimen of *P. palmata* at the same time (PpalS). The swabbed surface was 25 cm^2 on both sides of the algal thallus. Three different regions of the kelp *L. digitata* were sampled: the basal meristem (young tissue, hereafter LdigB), the medium frond (ca. 20 cm away from the meristem, hereafter LdigM) and the old frond (the blade tip, hereafter LdigO). Swabs were immersed in DNA/RNA Shield reagent (ZymoBiomics), kept on ice during transport (<2h) and stored at −20 °C until DNA extraction. Algal pieces from the same individuals were collected with sterile punchers (1.3 cm diameter), rinsed with autoclaved seawater and placed directly in 2% PFA in PBS overnight at 4 °C. All fixed samples were washed twice in PBS and stored in PBS:EtOH (1:1 v/v) at −20 °C before FISH analysis.

DNA extraction

Environmental DNA from swabs was extracted using the DNA/RNA Miniprep kit (ZymoBiomics) within 1 month after sampling. Genomic DNA (gDNA) was extracted from *Z. galactanivorans* Dsij^T and *Z. russellii* KMM 3677 ^{T} pure cultures as described in Gobet et al. [26].

Primer design

Zobellia-specific PCR primers were designed using DECIPHER (http://www2.decipher.codes/DesignPrimers.html) with 139 aligned 16S rRNA gene sequences from flavobacteria, including 52 *Zobellia* strains and 87 sequences representing 23 close genera (Supplementary File 1). Primers were searched to amplify 75–200 bp products, with DECIPHER default parameters. The designed sets of primers were tested for specificity against the reference database SILVA 138.1 SSU Ref using SILVA TestPrime with 0 mismatch allowed (Table 1).

qPCR assays

qPCR was carried out in 384-multiwell plates on a LightCycler 480 Instrument II (Roche). Each 5 µl reaction contained 2.5 µl of LightCycler 480 SYBR Green I Master mix 2X (Roche Applied Science), 0.5 µl of each primer and 1.5 µl of template DNA. Each reaction was prepared in technical triplicates. The amplification program consisted of an initial hold at 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 20 s at the chosen annealing temperature (T_a), and 72 °C for 10 s. After the amplification step, dissociation curves were generated by increasing the temperature from 65 °C to 97 °C.

The qPCR protocol targeting *Zobellia* was first optimized on gDNA purified from cultured *Zobellia* strains, by testing the efficiency of three different primer pairs and varying T_a (55–65 °C) and final primer concentration (100–500 nM). Strains *Cellulophaga baltica* NN015840^T, *Cellulophaga* sp. Asnod2-G02 [34] and *Maribacter forsetii* KT02ds18-6 ^{T} were used as negative controls.

The optimum parameters were then used for qPCR amplifications on environmental DNA as described below, using *Zobellia*-specific primers 142F/289R. The amplicon size was checked by 2% agarose gel electrophoresis.

Total and *Zobellia* 16S rRNA gene copies were quantified in environmental samples using the universal bacterial primers 926F/1062R [5] and the *Zobellia*-specific primers 142F/289R, respectively. Reactions were prepared with 1.5 µl of template DNA normalized at 0.5 ng.µl^{-1} with T_a set to 60 °C for universal primers and 64 °C for *Zobellia*-specific primers and a primer concentration in the reaction of 300 nM for both pairs. Serial dilutions of purified *Z. galactanivorans* Dsij^T gDNA ranging from 10 to 10^8 16S rRNA gene copies were used as a standard curve and were amplified in triplicate in the same run as the environmental samples. No-Template Controls (NTC) were included in the run. The LightCycler 480 Software v1.5 was used to determine the threshold cycle (C_t) for each sample. Linear standard curves were obtained by plotting C_t as a function of the logarithm of the initial 16S rRNA gene copies. PCR efficiency was calculated as $10^{-1/slope} - 1$ [14]. One-way ANOVA analyses followed by pairwise post-hoc Tukey HSD were conducted in R v3.6.2 [41]. Details on qPCR assays are given in Supplementary File 2, following the MIQE guidelines [14].

Table 1
Oligonucleotides used in this study. Specificity was tested *in silico* using the SILVA tools TestPrime and TestProbe for qPCR primers and FISH probes, respectively. Searches were performed against the reference database SILVA 138.1 SSU Ref with 0 mismatch allowed.

	Target group	Name	Sequence (5′–3′)	Size of target group	Number of hits in target group	Group coverage (%)	Outgroup hits	Reference
qPCR primer pairs	*Zobellia*	142F/285R	CCTACTGTGGGATAGCCCAG/ GCGGTCTTGGTGAGCCG	54	52	96.3	0	This study
	Zobellia	142F/289R	CCTACTGTGGGATAGCCCAG/ CATCGCGGTCTTGGTGA	54	52	96.3	0	This study
	Zobellia	142F/294R	CCTACTGTGGGATAGCCCAG/ CTACCCATCGCGGTCTT	54	52	96.3	0	This study
	Bacteria	926F/1062R	AAACTCAAAKGAATTGACGG/ CTCACRRCACGAGCTGAC	1 916 523	1 565 274	81.7	0	De Gregoris et al. 2011 [5]
FISH probes	*Zobellia*	ZOB137	GGCTATCCCACAGTAGGG	54	54	100	3	This study
	Bacteria	EUB338, EUB338-II and EUB338-III	GCTGCCTCCCGTAGGAGT, GCAGCCACCCGTAGGTGT and GCTGCCACCCGTAGGTGT	1 916 512	1 794 219	93.6	0	Amann et al. 1990 [2] Daims et al. 1999 [18]
	None	NON338	ACTCCTACGGGAGGCAGC	-	-	-	-	Wallner et al. 1993 [51]
FISH helpers	*Zobellia*	H116	GGYAGATYGTATACGCSTTGC	-	-	-	-	This study
	Zobellia	H155	GTMTTAATCCAAATTTCTCTG	-	-	-	-	This study
	Zobellia	H176	CACATGGTACCATTTTACGGC	-	-	-	-	This study

Sequencing of qPCR products

qPCR products obtained from two selected environmental samples (LdigO and PpalS) were re-amplified using a Taq DNA polymerase to add 3′ A-overhangs and ligated into pCR 2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen) before transformation into *Escherichia coli* NEB5α cells (New England Biolabs). Clones were grown overnight on LB-agar plates containing 50 $\mu g \cdot ml^{-1}$ kanamycin, with addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) following the manufacturer's instructions. For each environmental sample, 10 white or light-blue clones were randomly picked and grown separately in LB medium (50 $\mu g \cdot ml^{-1}$ kanamycin) overnight. Plasmids were purified using the Wizard Plus SV Minipreps DNA Purification System (Promega) and sequenced with the M13 Forward (-20) primer. Insert DNA sequences were analyzed by blastn against the NCBI 16S rRNA gene database.

FISH probe and helper design

The ZOB137 FISH probe specific to the genus *Zobellia* (Table 1) was designed using the ARB software (http://www.arb-home.de). Available *Zobellia* 16S rRNA gene sequences were aligned using SINA v1.6.1 and added to the Silva reference database (SILVA132_SSURef_12.12.17) in ARB to be included in the Silva tree. All *Zobellia* sequences were selected for probe search using the PROBE DESIGN tool with the following parameters: Max. non-group hits: 2, Min group hits (%): 100, Length of probe: 18, Temperature: 30–100, G + C content: 50–100. The newly designed ZOB137 probe, as well as the EUB338 I-III [2,18] and the NON338 [51] probes, were ordered from Biomers (www.biomers.net), labeled either with the fluorescent dye Atto488 (FISH protocol) or with the horseradish peroxidase (HRP) enzyme (CARD-FISH protocol). Three non-labeled helper oligonucleotides (21 nt) were designed to bind adjacent to the ZOB137-target site (Fig. S1B) in order to enhance the probe signal (see H116, H155 and H176 in Table 1). All probes and helpers were resuspended in nuclease-free water and diluted to a working stock concentration of 8.4 $pmol \cdot \mu l^{-1}$.

Optimization of hybridization conditions

FISH formamide melting curves were performed with the ZOB137-Atto488 probe on pure cultures of *Z. galactanivorans* Dsij^T and *Z. russellii* KMM 3677 ^{T}. Cells were grown at room temperature in ZoBell medium for 3 days and fixed in 1% paraformaldehyde (PFA) in PBS [2]. Fixed cells were spotted on glass slides, air-dried and dehydrated in successive short EtOH-baths. Hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH8, 0.01% SDS) with increasing formamide concentration (10–70%) was mixed (9:1) with the ZOB137-Atto488 probe and spotted on each well. After incubation for 2 h at 46 °C, slides were washed 15 min in the corresponding preheated washing buffer (0.45 to 0 M NaCl, 1 M Tris-HCl pH8, 0 to 5 mM EDTA pH8, 0.01% SDS) at 48 °C. Counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI, 1 $\mu g \cdot mg^{-1}$) and slides were mounted using Citifluor:Vectashield (3:1). Fluorescence was observed on a Nikon 50i epifluorescence microscope (filter ET-EGFP (LP)) equipped with an AxioCam MRc camera (Carl Zeiss, Germany). Signal intensity was quantified using ImageJ [43] on three random images per hybridization. Only the green channel was analyzed and the background signal was removed. For each hybridization, the maximum intensity of 50 randomly selected cells was measured.

CARD-FISH

CARD-FISH was performed using a method adapted from Pernthaler et al. [38] and optimized for the detection of alga-attached bacteria [49]. Fixed *Zobellia* cells from pure cultures were harvested onto a 0.2 µm polycarbonate membrane. Membrane portions or fixed algal samples (ca. 25–30 mm^2) were embedded in 0.1% LE agarose and bleached in ethanol with increasing concentration. Cells were permeabilized with lysozyme (10 $mg \cdot ml^{-1}$, 15 min, 37 °C). Endogenous peroxidases were inactivated by short incubation in 0.1 M HCl followed by 10 min in 3% H_2O_2 for membrane portions or by incubation in 0.15% H_2O_2 in methanol (30 min) for algal pieces. Samples were covered with the hybridization mix (0.9 M NaCl, 20 mM Tris-HCl pH8, 35% formamide, 1% blocking reagent, 10% dextran sulfate, 0.02% SDS, 28 nM probe and each helper if needed) for 2.5 h at 46 °C. Samples were washed 15 min in a preheated washing buffer (70 mM NaCl, 1 M Tris-HCl

pH8, 5 mM EDTA pH8, 0.01% SDS) at 48 °C and 15 min in 1 × PBS before being covered with a detection solution (1 × PBS, 2 M NaCl, 0.1% blocking reagent, 10% dextran sulfate, 0.0015% H_2O_2 and 1 $\mu g \cdot ml^{-1}$ of $Alexa_{546}$-labeled tyramides) for amplification (45 min, 46 °C). DAPI (1 $\mu g \cdot mg^{-1}$) counter-staining was performed and slides were mounted once samples were completely dry with Citifluor:Vectashield (3:1). Membrane portions were visualized with a Leica DMi8 epifluorescent microscope equipped with an oil objective 100X and a Leica DFC3000 G camera (Wetzlar, Germany). Cells on algal tissues were visualized with a confocal microscope Leica TCS SP8 equipped with HC PL APO 63x/1.4 oil objective and K-Cube filters A UV and N2.1 using the 405 et 552 nm lasers to detect DAPI and $Alexa_{546}$ signal, respectively. Z-stack images were collected due to the uneven algal surface (between 21 and 75 single layers of 0.15 or 0.3 µm thickness, depending on the observed field). Total and *Zobellia* cell counts were processed on maximum intensity images with Imaris v9.5.1. A total of 21 000 cells were counted automatically from 7 different fields.

Results

Optimization of qPCR parameters for specific detection of the genus Zobellia

One possible forward primer (142F) and three possible reverse primers (285R, 289R and 294R) were designed to target specifically the V2 region of the 16S rRNA gene in the genus *Zobellia* (Fig. S1A). The target regions were identical in multiple rRNA operons found in available *Zobellia* genomes (Supplementary Table 3). *In silico* tests confirmed that the three primer pairs match only *Zobellia* sequences in the entire 16S rRNA database (Table 1). Out of the 54 *Zobellia* strains available in SILVA SSU r138.1, only the two strains *Zobellia* sp. SED8 and *Zobellia* sp. M-2 were missed by a stringent *in silico* test (no mismatch allowed), the former being rescued when relaxing the stringency (1 mismatch allowed with a 3-bp perfect match zone at 3′ end of primers).

We assessed the optimal annealing temperature (T_a) and primer concentration for qPCR using 10^4 16S rRNA gene copies from two *Zobellia* strains. Since the 16S rRNA gene sequences of all valid *Zobellia* species were identical in the region targeted by primers, we focused our efforts on *Z. galactanivorans* Dsij^T and *Z. russellii* KMM 3677 ^{T}, which belong to two separate subclades based on core genome phylogeny [15]. Similar results were obtained with the three qPCR primer pairs and only those obtained with 142F/289R are shown in Fig. 1. Amplification was successful for the two *Zobellia* species with primer concentration ranging between 100–500 nM (Fig. 1A) and T_a ranging between 55–65 °C (Fig. 1B). Agarose gel electrophoresis of qPCR products showed a single band at the expected amplicon size (148 nt) with *Zobellia* gDNA (Fig. S2A). A slight increase of C_t was observed on *Zobellia* gDNA for primer concentration below 200 nM (Fig. 1A). Therefore, primer concentration was set at 300 nM for further experiments. C_t remained stable irrespective of the temperature (around 24 and 21 for *Z. galactanivorans* and *Z. russellii* respectively).

Ten-fold serial dilutions of *Z. galactanivorans* Dsij^T gDNA were amplified (T_a = 60 °C, [primers] = 300 nM) to evaluate the qPCR dynamic range and efficiency of each primer pair. The highest efficiency (88%) was observed for the pair 142F/289R, compared to 142F/285R and 142F/294R (86% and 81%, respectively) (Fig. 1C). The linear dynamic range spanned 8 orders of magnitude, from 10 to 10^8 16S rRNA gene copies. To ensure high efficiency and specificity of qPCR assays on environmental samples, we further used the pair 142F/289R and a high annealing temperature (T_a = 64 °C). With these conditions, the strains *Cellulophaga baltica* NN015840^T and *Cellulophaga* sp. Asnod2-G02 were used as negative controls to check the assay specificity, since the target regions of their 16S rRNA gene sequences were the closest to that of *Zobellia* based on SILVA TestPrime results (3 mismatches with 142F, 3 mismatches with 289R). We also tested amplification from *Maribacter forsetii* KT02ds18-6 ^{T} (5 and 4 mismatches to 142F and 289R, respectively), since *Maribacter* is the closest described genus to *Zobellia* [7]. No amplification was detected with any of these negative controls (Fig. S2A). In addition, amplification from environmental DNA yielded a single product at the expected size (Fig. S2B), with melting curves showing a single dissociation peak (Fig. S2D) similar to that obtained on pure *Z. galactanivorans* Dsij^T gDNA (Fig. S2C). qPCR products obtained with two selected environmental samples (LdigO and PpalS, see below) were sequenced (Supplementary File 3). All retrieved sequences (20/20) had their best blastn hit against 16S rRNA gene sequences from *Zobellia* spp (Supplementary Table 4), confirming the specificity of the assay on natural samples. Sensitivity and specificity were further assessed using environmental DNA extracted from natural seawater devoid of *Zobellia* (no amplification detected after 45 cycles of qPCR with primers 142F/289R) mixed with 10-fold serial dilutions of *Z. galactanivorans* Dsij^T gDNA. The proportion of *Zobellia* was calculated by dividing *Zobellia* 16S rRNA gene copies (amplification with primers 142F/289R) by total 16S rRNA gene copies (amplification with primers 926F/1062R) (Fig. 1D). Measured and theoretical proportions were highly congruent, confirming the specificity of the method toward *Zobellia* spp. The assay shows a strong sensitivity, as it can detect minor proportions of *Zobellia* DNA (approximately 0.01%, i.e., 5 16S rRNA genes copies) when combined with environmental DNA. Detection was saturated when the proportion of *Zobellia* was close to 100% (Fig. 1D, top-right corner), a situation unlikely to happen in natural samples.

Characterization of the newly-designed FISH probe ZOB137

Probe design yielded one candidate, ZOB137 (Table 1) covering 100% of the target group. ZOB137 target site was similar to that of primer 142F (Fig. S1B). *In silico* analysis showed ZOB137 matches the 54 *Zobellia* sequences available in the SILVA SSU r138.1 database. Three outgroup hits were found (uncultured members of the genera *Aquibacter* and *Euzebyella*).

We optimized the hybridization stringency for FISH with ZOB137, i.e. the highest formamide (FA) concentration in the hybridization buffer that does not result in loss of fluorescence intensity of the target cells [39]. Melting curves were obtained from hybridizations of *Z. galactanivorans* Dsij^T and *Z. russellii* KMM 3677 ^{T} with ZOB137-Atto488 at increasing FA concentrations (Fig. 2). For both strains, cell fluorescence intensity was stable until 35% FA, slightly decreased using 40% FA and dropped using 50–70% FA. Therefore, the optimal FA concentration for ZOB137 was set to 35% for further experiments. ZOB137 was tested using these conditions with two additional *Zobellia* strains, Z. *amurskyensis* KMM 3526 ^{T} and *Z. roscoffensis* Asnod1-F08^T, and a fluorescent signal was observed (data not shown).

The CARD-FISH signal obtained after hybridization of *Z. galactanivorans* remained weak and might not be detectable in environmental samples (Fig. 3E). The addition of three helper oligonucleotides in the hybridization mix (H116, H155 and H176) increased the signal intensity ca. 3-fold under the same exposure time (Fig. 3F). CARD-FISH specificity was tested using *Lewinella marina* MKG-38 ^{T} and *Marinirhabdus citrea* MEBiC09412^T that feature only one mismatch with the ZOB137 target sequence (respectively C and A instead of T at position 145). No CARD-FISH signal was observed after hybridization of both strains with ZOB137 and helpers (Fig. 3G–H), confirming the absence of hybridization to non-target organisms using 35% FA.

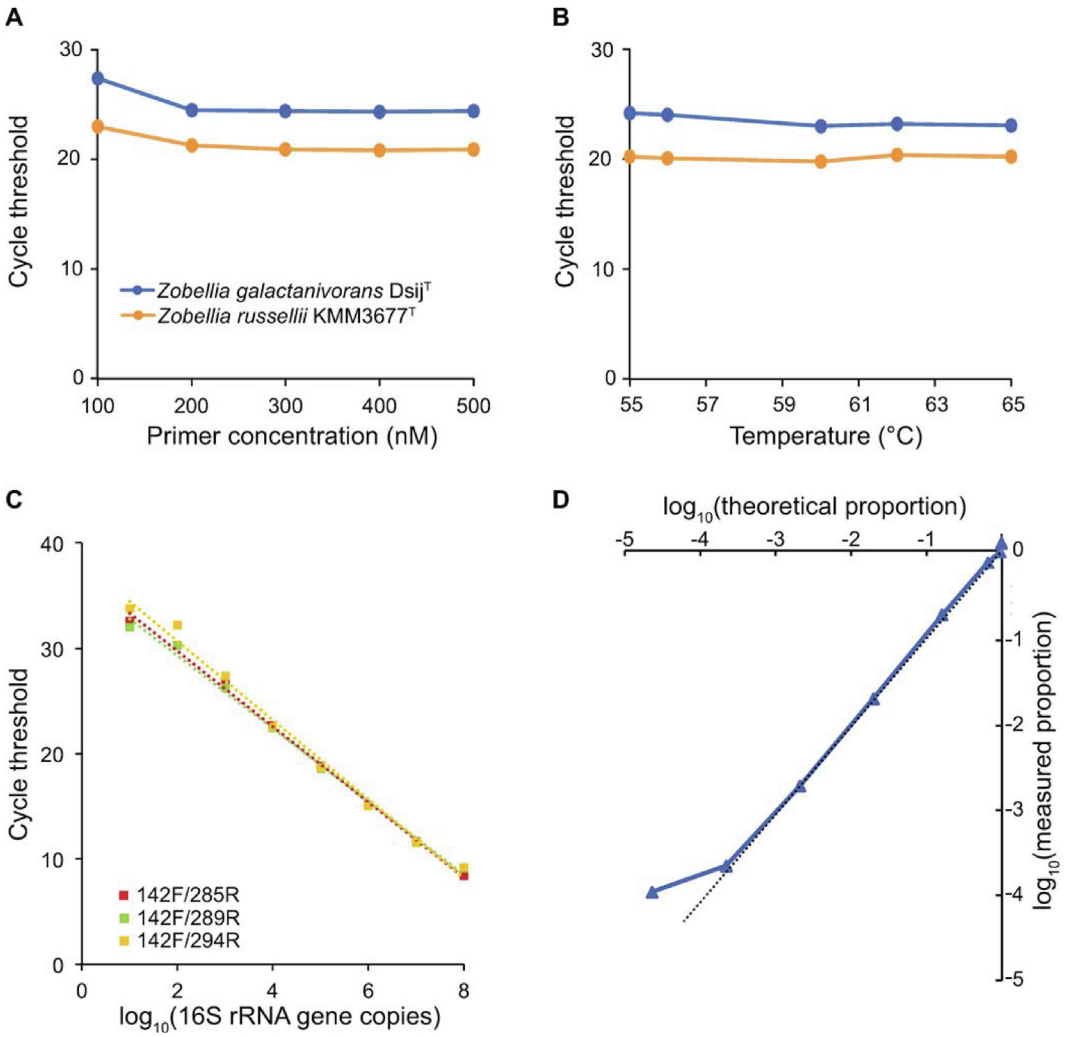

Fig. 1. Optimization of qPCR parameters. A-B: Effect of primer concentration (A) and annealing temperature (T_a) (B) on the cycle threshold (C_t) was determined using the primer pair 142F/289R and 10^4 16S rRNA gene copies. T_a was set to 60 °C in A and primer concentration to 300 nM in B. C: Standard curves obtained in qPCR with the three sets of primers using *Z. galactanivorans* DsijT gDNA diluted from 10^8 to 10^1 16S rRNA gene copies. D: Comparison of measured and theoretical proportion of *Zobellia* 16S rRNA gene copies in environmental DNA with increasing load of *Zobellia* gDNA. The 1:1 line was drawn in light grey as a comparison. Values are mean ± s.d (n = 3). In C and D, T_a = 64 °C and [primer] = 300 nM. In all panels (A–D), measurements were performed in technical triplicates.

Application of Zobellia-specific qPCR and CARD-FISH assays on natural macroalgal microbiota

These newly-designed qPCR and CARD-FISH assays were tested on environmental samples, to detect and quantify the abundance of the genus *Zobellia* on the surface of macroalgae. Total and *Zobellia* 16S rRNA gene copies were quantified on the surface of two fresh brown macroalgae *Laminaria digitata* (base, medium and old part) and *Ascophyllum nodosum*, a fresh green macroalga *Ulva lactuca* and a red macroalga *Palmaria palmata* (fresh and stranded individuals) (Fig. 4).

No significant difference was observed in the average total copies·cm^{-2} between the basal (0.27×10^6 copies·cm^{-2}) and medium (1.8×10^6 copies·cm^{-2}) part of *L. digitata* (Tukey HSD post-hoc pairwise comparisons, P = 0.8). However, the total number of copies was significantly higher on the old blade where 14.6×10^6 copies·cm^{-2} were quantified (P = 0.003 and P = 0.005 in comparison to LdigB and LdigM, respectively). The average total copy number on *A. nodosum*, *U. lactuca* and *P. palmata* fresh and stranded was estimated at 7.2, 5.7, 5.1 and 14.6×10^6 copies·cm^{-2}, respectively.

16S rRNA gene copies were also detected on the four macroalgae with the *Zobellia*-specific primers (Fig. 4B). The number of *Zobellia*-16S rRNA gene copies·cm^{-2} differed along the *L. digitata* blade (ANOVA, $F_{2,6}$ = 31.8, P = 0.001). It was higher on the old blade (10.5×10^3 copies·cm^{-2}) compared to the basal (1.3×10^3 copies·cm^{-2}, Tukey HSD P = 0.001) and the medium (2.5×10^3 copies·cm^{-2}, P = 0.002) part. The proportion of *Zobellia* cells within the epiphytic communities (Fig. 4C) was significantly higher on the basal part (0.54%) than on the old blade of *L. digitata* (0.07%) (P = 0.01). Fresh *A. nodosum*, *U. lactuca* and *P. palmata* displayed 9.7, 6.1 and 11.3×10^3 *Zobellia* copies·cm^{-2} representing 0.14, 0.12 and 0.21% of the total number of copies, respectively. On the surface of the stranded *P. palmata* sample, 1.3×10^5 *Zobellia* copies·cm^{-2} were estimated, ie. 0.87% relative abundance.

The genus *Zobellia* was successfully detected on the surface of the stranded *P. palmata* individual and on a fresh individual from *U. lactuca* using CARD-FISH (Fig. 5). ZOB137-hybridized cells were well visible among the epiphytic bacterial communities. A 2–3 µm thick space without fluorescence was observable between the biofilm and the external algal cells, likely due to the algal extracellular matrix. ZOB137-hybridized cells did not seem to form aggregates

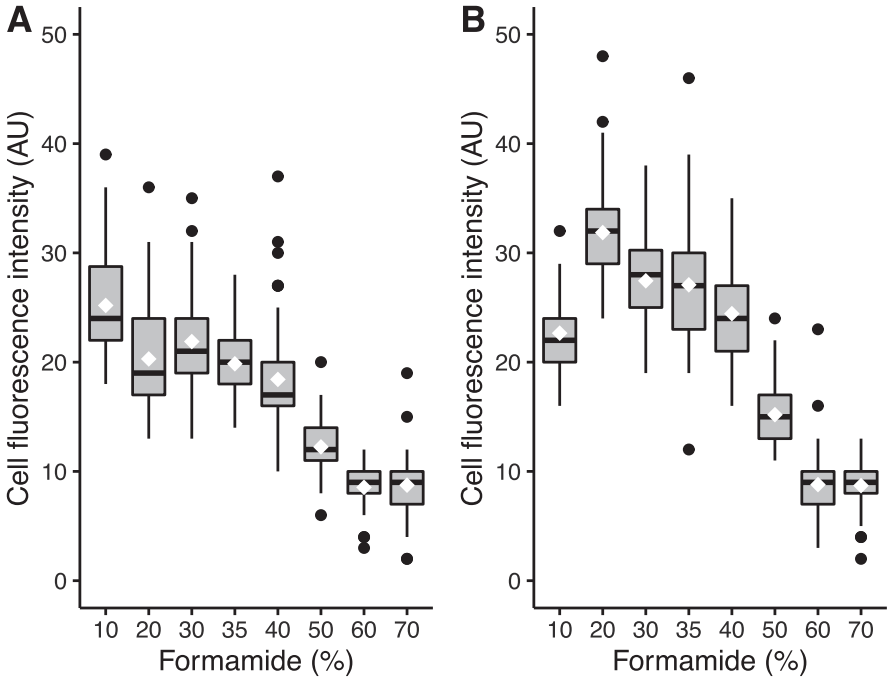

Fig. 2. Dissociation curves of the probe ZOB137 determined using the strains *Zobellia galactanivorans* Dsij^T (A) and *Zobellia russellii* KMM 3677 ^{T} (B) under increasing formamide concentration. For each formamide concentration, the maximum fluorescence intensity was assessed for cells on five different microscopic fields (between 30 and 70 counted cells in total for each concentration). White diamonds represent the mean intensity.

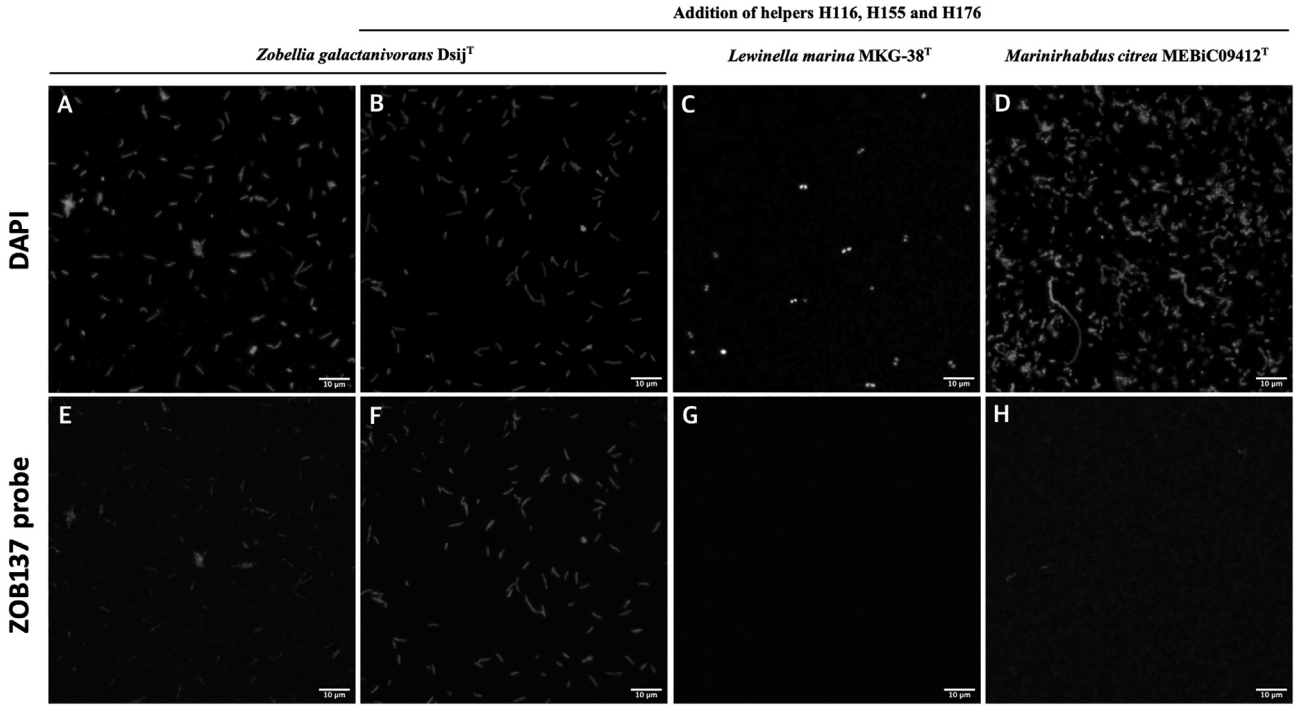

Fig. 3. Epifluorescence microscopy images of bacterial cells hybridized with the CARD-FISH probe ZOB137 (E–H) and counterstained with DAPI (A–D). *Lewinella marina* MKG-38^T and *Marinirhabdus citrea* MEBiC09412^T were used as negative controls to assess the specificity of the newly-designed probe ZOB137. Addition of the three helpers in the hybridization mix increased the fluorescence of the *Zobellia galactanivorans* cells (F) without affecting the probe specificity (G, H). Upper (DAPI-stained cells) and lower (CARD-FISH signal) epifluorescent micrographs show identical fields. Exposure time was 100 ms for CARD-FISH. FA = 35%.

on both macroalgae. *Zobellia* cells observed on algal tissues (Fig. 5) were shorter than those observed in pure cultures in rich medium (Fig. 3F), indicating different morphologies depending on environmental conditions. After image analysis on *P. palmata,* 1.0×10^5 *Zobellia* cells were counted per cm^2 of algal surface, representing ca. 0.43% of detected bacterial cells. *Zobellia* cells displayed a homogeneous distribution on stranded *P. palmata* but not on the surface of *U. lactuca* where *Zobellia*-specific signal was visible only

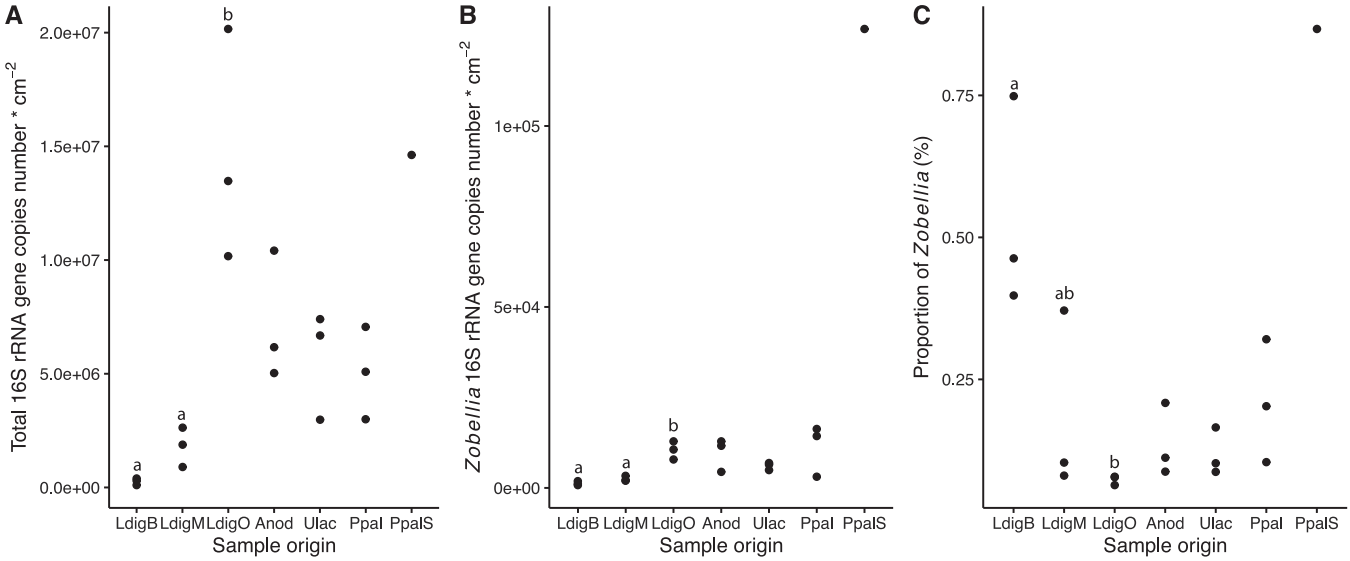

Fig. 4. Number of 16S rRNA gene copies per cm^2 of algal surface detected either with the universal bacterial primers (A) or with the *Zobellia*-specific primers 142F/289R (B). Proportion of *Zobellia* (C) was obtained by dividing the number of *Zobellia* 16S rRNA gene copies by the number of 16S rRNA gene copies detected with the universal primers for each sample. Measurements were performed in technical triplicates. LdigB, LdigM and LdigO are basal, median and apical parts of *Laminaria digitata*, respectively. Anod, *Ascophyllum nodosum*; Ulac, *Ulva lactuca*; Ppal, healthy *Palmaria palmata;* PpalS, stranded *P. palmata*. For *L. digitata* samples, within-blade variations were tested using one-way ANOVA analyses followed by pairwise post-hoc Tukey HSD. Different letters denote significant difference ($P < 0.05$).

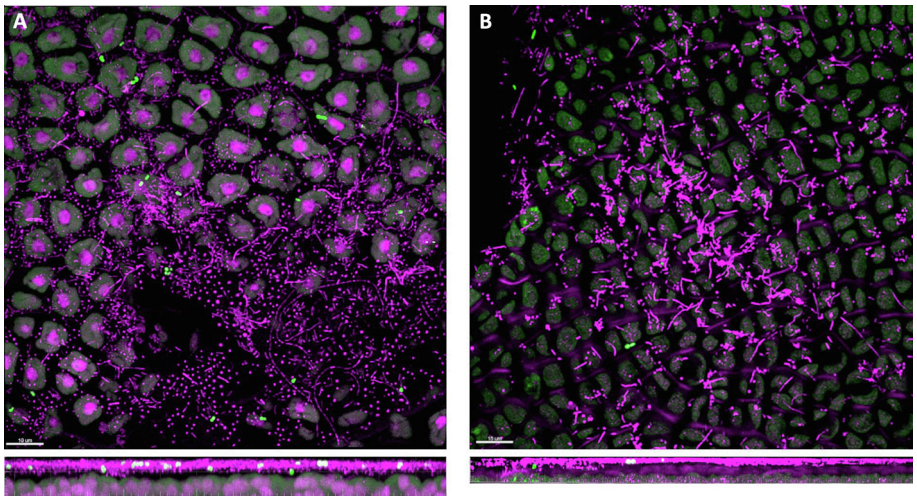

Fig. 5. *Zobellia* cells visualization in the stained biofilm of *Palmaria palmata* (A) and *Ulva lactuca* (B). Micrographs are overlay of the CARD–FISH signal (green cells, ZOB137-HRP probe with $Alexa_{546}$ as the reporter signal) and the DAPI signal (magenta cells) and represent the maximum intensity projection of Z-stack. Transversal view is shown at the bottom. Bars: 10 μm (A) and 15 μm (B).

in a few areas. Hence, *Zobellia* cell count was not performed on *U. lactuca* as it would not have been reliable.

Discussion

qPCR and CARD-FISH are widely used, complementary molecular methods to examine microbial abundance in environmental samples. qPCR is a fast, highly sensitive and specific tool allowing absolute quantification of gene copies, suitable to detect minor amounts of target DNA [24]. CARD-FISH is more time-consuming but is not subject to DNA extraction or PCR biases and enables direct visualization and localization of single bacterial cells associated with the host.

qPCR using universal bacterial primers estimated around 10^6-10^7 total 16S rRNA gene copies·cm^{-2} on the different macroalgal species. Considering an average of 4 copies of 16S rRNA gene per bacterial cell [50], these results concur with previous studies showing 10^6-10^9 cells per cm^2 on algal surfaces [17,35]. qPCR assays on *L. digitata* samples showed that total bacterial abundance increased with kelp tissue age, corroborating observations made on different kelp species [36,42].

Newly designed qPCR and CARD-FISH assays specifically targeting the genus *Zobellia* were tested and optimized on pure cultures before application on macroalgal samples. qPCR assays using the novel *Zobellia*-specific primers 142F/289R were shown to be a robust, fast, high-throughput and sensitive way to quantify the abundance of *Zobellia* spp. in environmental samples. The detection limits were determined by pooling targeted with untargeted DNA, a relevant approach as we aim to detect *Zobellia* in natural samples (as discussed in Skovhus et al. [44]). This method allows

the detection of minor proportions of *Zobellia* in environmental samples (0.01%), which would be technically difficult using CARD-FISH.

CARD-FISH detection of non-abundant taxa directly on macroalgae is difficult due to the high background autofluorescence of algal pigments. Here, algal bleaching was performed with extended ethanol and methanol baths to reduce autofluorescence. Moreover, the addition of three unlabeled helper oligonucleotides improved the CARD-FISH signal, facilitating cell visualization. The secondary structure of the 16S rRNA target region for probe ZOB137 might partly hinder hybridization [11,23]. The combined hybridization of multiple adjacent helper oligonucleotides assisted to open the targeted region, facilitating probe binding. The use of helpers for FISH was first described by Fuchs et al. [22] who recommended to test experimentally their influence on the probe specificity. In our study, no hybridization signal was detected using ZOB137 combined with helpers on two strains with single-mismatch, confirming helpers did not impact probe specificity. A specific CARD-FISH signal was detected with ZOB137 at the surface of macroalgae with a flat thin blade, such as the red seaweed *Palmaria palmata* or the green seaweed *Ulva lactuca*. This new protocol represents a powerful tool to (i) determine the spatial localization of *Zobellia* cells within macroalgal surface microbiota, (ii) estimate their physical interactions with other taxa or the host and (iii) assess whether they are structured into patches or evenly distributed on the surface, as it seems to be the case on *P. palmata* thallus. Visualization was more complex with thicker algae such as *Laminaria*, since multiple cell layers caused a strong autofluorescence masking the bacterial CARD-FISH signal. Moreover, thick algal pieces sometimes decomposed during the CARD-FISH process as their structure became weaker, more viscous and tissues tended to disintegrate during washing steps. Hence, specific taxa visualization on algal surfaces remains a challenge depending on algal species. A way to overcome these issues would be to embed algae in resin and cut thin thallus sections using a microtome prior to CARD-FISH, as it was successfully done in Ramirez-Puebla et al. [42] and Tourneroche et al. [46].

Altogether, our analyses revealed that the genus *Zobellia* was part of the microbiota of all tested macroalgae, including *U. lactuca*, *P. palmata*, *A. nodosum* and *L. digitata* on both young or old regions of the blade. This confirms its widespread distribution in algal microbiomes from temperate ecosystems, in line with *Zobellia* numerous adaptive traits to live on macroalgae such as the ability to degrade polysaccharides and to counteract antibacterial algal defense [10].

Previous studies already reported the presence of *Zobellia* spp. on *A. nodosum* (3% of the cultivable microbiota) [9,34] and *L. digitata* [47] in Roscoff. Since macroalgal microbiomes are diverse and can host hundreds of taxa [20], the observed frequency of 0.1–1% for *Zobellia* shows the quantitative relevance of this genus in macroalgae-dominated habitats. The highest *Zobellia* proportion was observed on the stranded decaying *P. palmata* individual, highlighting its putative important activity in macroalgal biomass recycling. Although not dominant in the bacterial communities, *Zobellia* spp. might act as primary degraders of algal polysaccharides due to their outstanding CAZyme repertoire, releasing degradation products that could fuel other taxa via cross-feeding interactions. In line with this, *Z. galactanivorans* Dsij^T was recently shown to degrade intact kelp tissue, releasing soluble algal compounds that become accessible to opportunistic taxa [52]. The absolute number of *Zobellia* 16S rRNA gene copies was positively correlated with the age of the *L. digitata* tissues. Kelp decay is more pronounced at the tip of the blade, which might result in more available substrates for diverse bacterial algal degraders, including *Zobellia* spp. By contrast, the proportion of *Zobellia* 16S rRNA gene copies over total copies was highest in the youngest region of *L. digitata* (i.e. the basal meristem). The high proportion of the genus *Zobellia* on young algal tissues might thus reflect its metabolic specialization towards complex polysaccharides found in the meristem, while it could be outcompeted by opportunistic taxa on the decaying old blade. *Zobellia* spp. might also be early colonizers of newly-produced meristematic tissues, reflecting their adaptation to attach to and live on algal surfaces. Moreover, intra-thallus variability of algal defense metabolites, such as iodine, reactive oxygen and nitrogen species and phlorotannins, might create different ecological micro-niches [4,27,30] selecting for specific taxa.

Using *P. palmata* surface microbiota as a test case, we showed that both the absolute number and proportion of ZOB137-hybridized cells concur with the qPCR-estimated *Zobellia* 16S rRNA gene copy number. However, it should be interpreted carefully as available *Zobellia* genomes feature between 1 to 4 copies of 16S rRNA genes (Supplementary Table 3), so qPCR results cannot be directly converted into a number of cells. Another bias might lie in the size of the sampled algal area, as bacteria were quantified from a 50-cm^2 surface with qPCR but CARD-FISH was performed only on 30-mm^2 algal pieces. Tourneroche and co-workers recently drew attention to the potential within-tissue spatial variability of kelp-associated bacterial communities [46]. Finally, CARD-FISH analyses detect metabolically active cells as the probe targets 16S rRNA, while here qPCR was applied on DNA samples and therefore could detect resting or dead cells. The close proximity of values obtained on stranded *P. palmata* with qPCR (1.3×10^5 copies·cm^{-2} for *Zobellia* 16S rRNA gene) and CARD-FISH (1.0×10^5 *Zobellia* cells·cm^{-2}) suggests that most *Zobellia* cells in this sample were active. In the future, the newly-designed primer pair 142F/289R could be used for reverse-transcription qPCR on RNA extracts to better estimate *Zobellia* transcriptional activity.

To conclude, we validated novel qPCR and CARD-FISH protocols to specifically target the marine flavobacterial genus *Zobellia*. We provide the first quantitative estimates of *Zobellia* absolute and relative abundance on different macroalgae, suggesting a widespread distribution. This work paves the way for further studies of the spatiotemporal dynamics of *Zobellia* in marine environments.

CRediT authorship contribution statement

Maéva Brunet: Formal analysis, Investigation, Visualization, Writing – original draft, Writing - review & editing. **Nolwen Le Duff:** Formal analysis, Investigation, Visualization. **Bernhard M. Fuchs:** Supervision, Writing - review & editing. **Rudolf Amann:** Supervision, Writing - review & editing. **Tristan Barbeyron:** Supervision, Writing - review & editing. **François Thomas:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Visualization, Writing – original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2021.126269.

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