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1 **Specific detection and quantification of the marine flavobacterial genus**

2 ***Zobellia* on macroalgae using novel qPCR and CARD-FISH assays**

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10

11 Running head: *Zobellia* quantification on macroalgae

12

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14 Summary

15 Abstract

16 The flavobacterial genus *Zobellia* is considered as a model to study macroalgal polysaccharide
17 degradation. The lack of data regarding its prevalence and abundance in coastal habitats
18 constitutes a bottleneck to assess its ecological strategies. To overcome this issue, real-time
19 quantitative PCR (qPCR) and fluorescence *in situ* hybridization (FISH) methods targeting the
20 16S rRNA gene were optimized to specifically detect and quantify *Zobellia* on the surface of
21 diverse macroalgae. The newly designed qPCR primers and FISH probes targeted 98 and 100%
22 of the *Zobellia* strains *in silico* and their specificity was confirmed using pure bacterial cultures.
23 The dynamic range of the qPCR assay spanned 8 orders of magnitude from 10 to 10^8 16S rRNA
24 gene copies and the detection limit was 0.01% relative abundance of *Zobellia* in environmental
25 samples. *Zobellia*-16S rRNA gene copies were detected on all surveyed brown, green and red
26 macroalgae, in proportion varying between 0.1 and 0.9% of the total bacterial copies. The
27 absolute and relative abundance of *Zobellia* varied with tissue aging on the kelp *Laminaria*
28 *digitata*. *Zobellia* cells were successfully visualized in *Ulva lactuca* and stranded *Palmaria*
29 *palmata* surface biofilm using CARD-FISH, representing in the latter 10^5 *Zobellia* cells.cm⁻² and
30 0.43% of total bacterial cells. Overall, qPCR and CARD-FISH assays enabled robust detection,
31 quantification and localization of *Zobellia* representatives in complex samples, underlining their
32 ecological relevance as primary biomass degraders potentially cross-feeding other
33 microorganisms.

34

35 Keywords: *Zobellia*, qPCR, CARD-FISH, macroalgae, marine flavobacteria

36

37 **Highlights**

- 38 • *Flavobacteriia* are key players of carbon recycling in coastal ecosystems
- 39 • Among them, the genus *Zobellia* is considered as specialist of macroalgae degradation
- 40 • We developed qPCR and CARD-FISH assays allowing robust detection, quantification
41 and localization of *Zobellia* on macroalgae
- 42 • These tools may help unveil the interactions between *Zobellia* spp., macroalgae and other
43 microbial taxa

44 **Introduction**

45 Macroalgae are dominant primary producers in coastal ecosystems and constitute a large
46 reservoir of organic matter worldwide. A wide range of microorganisms colonize macroalgal
47 surfaces [20]. These biofilms contain diverse bacteria at densities ranging between 10^6 and 10^9
48 cells.cm⁻² [35], which contribute to the remineralization of algal biomass [33]. Among those are
49 the members of the genus *Zobellia* (phylum *Bacteroidetes*, class *Flavobacteriia*), first described
50 by Barbeyron et al. [8]. To date, seven valid species compose the genus, namely *Z. amurskyensis*,
51 *Z. galactanivorans*, *Z. laminariae*, *Z. nedashkovskayae*, *Z. russellii*, *Z. roscoffensis* and *Z.*
52 *uliginosa*. They were isolated from marine environments throughout the world, notably from the
53 surface of brown [9,37], red [40] and green macroalgae [37]. Additionally, the NCBI database
54 references 65 unclassified *Zobellia* strains, mostly isolated from seaweeds. This genus comprises
55 highly potent degraders of macroalgal polysaccharides. Described members of the genus *Zobellia*
56 can grow on algal polysaccharides such as agar, porphyran and carrageenans from red algae and

57 laminarin, alginate and fucoidans from brown algae, in line with the high number of
58 carbohydrate-active enzymes (CAZyme) encoded in their genomes [10,16]. A recent survey of
59 the twelve available *Zobellia* genomes revealed that CAZyme-encoding genes accounted for
60 more than 6% of the genomic content, from 263 genes in *Z. nedashkovskayae* Asnod3-E08-A to
61 336 genes in *Z. galactanivorans* Dsij^T [15], illustrating the specialization of *Zobellia*
62 representatives in the degradation of algal polysaccharides. Accordingly, *Z. galactanivorans*
63 Dsij^T has become a model for algal polysaccharide breakdown and assimilation [32], allowing the
64 discovery of many novel polysaccharidases and Polysaccharide Utilization Loci (PULs) targeting
65 algal compounds [21,28,45]. Despite the potential of *Zobellia* strains for algal biomass recycling,
66 little is known regarding their prevalence and abundance in macroalgae-dominated habitats,
67 hindering our understanding of their role in coastal biogeochemistry. Molecular studies revealed
68 the presence of *Zobellia* members in diverse coastal ecosystems [1,19,29,34]. Searches in marine
69 metagenomes show numerous hits with high sequence identities (>90%) to *Zobellia* genes in a
70 variety of environments rich in organic carbon, including algal microbiomes, coastal sediments
71 and seawater from the Atlantic and Pacific Oceans as well as the North Sea and the English
72 Channel (**Supplementary Table 1**). Overall, this suggests a widespread distribution and
73 ecological relevance of *Zobellia* for carbon cycling in coastal ecosystems. However,
74 metagenomic sequencing does not allow a precise quantification of taxon abundance in the
75 environment and can be biased by promiscuous taxonomic sequence assignment. This prompted
76 us to develop new tools targeting the genus *Zobellia*, which would allow specific detection in
77 complex samples, cell localization and both absolute and relative quantification of its abundance.
78 Real-time quantitative polymerase chain reaction (qPCR) and fluorescence *in situ* hybridization
79 (FISH) are two well-established molecular methods for specific bacterial taxa detection. qPCR is
80 a fast, highly sensitive and specific tool allowing absolute quantification of DNA gene copies and

81 is suitable to detect minor amounts of target DNA [24]. qPCR assays were previously developed
82 to quantify microbial taxa associated with macroalgae, such as endophytic eukaryotic pathogens
83 in brown seaweeds [13,25], bacterial pathogens on *Saccharina latissima* [6] or the genus
84 *Pseudoalteromonas* at the surface of green macroalgae [44]. FISH is a widely used method in
85 microbial ecology for cell identification and enumeration in complex environments. Its principle
86 is based on the use of rRNA-targeted oligonucleotide probes labeled with fluorescent dyes, which
87 allow the detection of a defined group of microorganisms [3]. The use of horseradish peroxidase-
88 labeled probes in combination with catalyzed reported deposition (CARD) of fluorescently
89 labeled tyramides was then developed to enhance the fluorescence and improve the detection
90 signal [38]. FISH combined or not with catalyzed reporter deposition (CARD-FISH) was
91 successfully applied previously to enumerate seaweed-associated epiphytic or endophytic
92 bacteria [12,31,46,48,49]. Here, we describe new qPCR and FISH protocols specifically targeting
93 the genus *Zobellia*, allowing its detection, absolute quantification and localization at the surface
94 of macroalgae. qPCR primers and FISH probes were designed and validated *in silico*. Both
95 methods were optimized on pure bacterial cultures before being applied on environmental
96 samples.

97 Material and Methods

98 **Bacterial strains**

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

101 **Environmental samples**

102 Surface microbiota were sampled in February 2020 using sterile swabs on healthy specimens of

103 *Laminaria digitata* (Ldig), *Ascophyllum nodosum* (Anod), *Ulva lactuca* (Ulac) and *Palmaria*

104 *palmata* (Ppal) at the Blosson site (48°43'29.982'' N, 03°58'8.27'' W) in Roscoff (France).

105 Three individuals of each species were sampled. One additional sample was obtained from a

106 stranded specimen of *P. palmata* at the same time (PpalS). The swabbed surface was 25 cm² on

107 both sides of the algal thallus. Three different regions of the kelp *L. digitata* were sampled: the

108 basal meristem (young tissue, hereafter LdigB), the medium frond (ca. 20 cm away from the

109 meristem, hereafter LdigM) and the old frond (the blade tip, hereafter LdigO). Swabs were

110 immersed in DNA/RNA Shield reagent (ZymoBiomics), kept on ice during transport (< 2 h) and

111 stored at -20°C until DNA extraction. Algal pieces from the same individuals were collected with

112 sterile punchers (1.3 cm diameter), rinsed with autoclaved seawater and placed directly in 2%

113 PFA in PBS overnight at 4°C. All fixed samples were washed twice in PBS and stored in

114 PBS:EtOH (1:1 v/v) at -20°C before FISH analysis.

115 **DNA extraction**

116 Environmental DNA from swabs was extracted using the DNA/RNA Miniprep kit

117 (ZymoBiomics) within 1 month after sampling. Genomic DNA (gDNA) was extracted from *Z.*

118 *galactanivorans* Dsij^T and *Z. russellii* KMM 3677^T pure cultures as described in Gobet *et al.* [26].

119 **Primer design**

120 *Zobellia*-specific PCR primers were designed using DECIPHER

121 (<http://www2.decipher.codes/DesignPrimers.html>) with 139 aligned 16S rRNA gene sequences

122 from flavobacteria, including 52 *Zobellia* strains and 87 sequences representing 23 close genera

123 **(Supplementary File 1)**. Primers were searched to amplify 75-200 bp products, with
124 DECIPHER default parameters. The designed sets of primers were tested for specificity against
125 the reference database SILVA 138.1 SSU Ref using SILVA TestPrime with 0 mismatch allowed
126 **(Table 1)**.

127 **qPCR assays**

128 qPCR was carried out in 384-multiwell plates on a LightCycler 480 Instrument II (Roche). Each
129 5 µl reaction contained 2.5 µl of LightCycler 480 SYBR Green I Master mix 2X (Roche Applied
130 Science), 0.5 µl of each primer and 1.5 µl of template DNA. Each reaction was prepared in
131 technical triplicates. The amplification program consisted of an initial hold at 95°C for 10 min
132 followed by 45 cycles of 95°C for 10 s, 20 s at the chosen annealing temperature (T_a), and 72°C
133 for 10 s. After the amplification step, dissociation curves were generated by increasing the
134 temperature from 65°C to 97°C.

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

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

143 Total and *Zobellia* 16S rRNA gene copies were quantified in environmental samples using the
144 universal bacterial primers 926F/1062R [5] and the *Zobellia*-specific primers 142F/289R,
145 respectively. Reactions were prepared with 1.5 µl of template DNA normalized at 0.5 ng.µl⁻¹ with

146 T_a set to 60°C for universal primers and 64°C for *Zobellia*-specific primers and a primer
147 concentration in the reaction of 300 nM for both pairs. Serial dilutions of purified *Z.*
148 *galactanivorans* Dsjj^T gDNA ranging from 10 to 10⁸ 16S rRNA gene copies were used as a
149 standard curve and were amplified in triplicate in the same run as the environmental samples. No-
150 Template Controls (NTC) were included in the run. The LightCycler 480 Software v1.5 was used
151 to determine the threshold cycle (C_t) for each sample. Linear standard curves were obtained by
152 plotting C_t as a function of the logarithm of the initial 16S rRNA gene copies. PCR efficiency
153 was calculated as $10^{-1/\text{slope}} - 1$ [14]. One-way ANOVA analyses followed by pairwise post-hoc
154 Tukey HSD were conducted in R v3.6.2 [41]. Details on qPCR assays are given in
155 **Supplementary File 2**, following the MIQE guidelines [14].

156

157 **Sequencing of qPCR products**

158 qPCR products obtained from two selected environmental samples (LdigO and PpalS) were re-
159 amplified using a Taq DNA polymerase to add 3' A-overhangs and ligated into pCR 2.1-TOPO
160 vector using the TOPO TA cloning kit (Invitrogen) before transformation into *Escherichia coli*
161 NEB5 α cells (New England Biolabs). Clones were grown overnight on LB-agar plates containing
162 50 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin, with addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) and 5-
163 bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) following the manufacturer's
164 instructions. For each environmental sample, 10 white or light-blue clones were randomly picked
165 and grown separately in LB medium (50 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin) overnight. Plasmids were purified
166 using the Wizard Plus SV Minipreps DNA Purification System (Promega) and sequenced with
167 the M13 Forward (-20) primer. Insert DNA sequences were analyzed by blastn against the NCBI
168 16S rRNA gene database.

169 **FISH probe and helper design**

170 The ZOB137 FISH probe specific to the genus *Zobellia* (**Table 1**) was designed using the ARB
171 software (<http://www.arb-home.de>). Available *Zobellia* 16S rRNA gene sequences were aligned
172 using SINA v1.6.1 and added to the Silva reference database (SILVA132_SSURef_12.12.17) in
173 ARB to be included in the Silva tree. All *Zobellia* sequences were selected for probe search using
174 the PROBE DESIGN tool with the following parameters: Max. non-group hits: 2, Min group hits
175 (%): 100, Length of probe: 18, Temperature: 30-100, G+C content: 50-100. The newly designed
176 ZOB137 probe, as well as the EUB338 I-III [2,18] and the NON338 [51] probes, were ordered
177 from Biomers (www.biomers.net), labeled either with the fluorescent dye Atto488 (FISH
178 protocol) or with the horseradish peroxidase (HRP) enzyme (CARD-FISH protocol). Three non-
179 labeled helper oligonucleotides (21 nt) were designed to bind adjacent to the ZOB137-target site
180 (**Fig. S1B**) in order to enhance the probe signal (see H116, H155 and H176 in **Table 1**). All
181 probes and helpers were resuspended in nuclease-free water and diluted to a working stock
182 concentration of 8.4 pmol.µl⁻¹.

183 **Optimization of hybridization conditions**

184 FISH formamide melting curves were performed with the ZOB137-Atto488 probe on pure
185 cultures of *Z. galactanivorans* Dsij^T and *Z. russellii* KMM 3677^T. Cells were grown at room
186 temperature in ZoBell medium for 3 days and fixed in 1% paraformaldehyde (PFA) in PBS [2].
187 Fixed cells were spotted on glass slides, air-dried and dehydrated in successive short EtOH-baths.
188 Hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH8, 0.01% SDS) with increasing
189 formamide concentration (10 to 70%) was mixed (9:1) with the ZOB137-Atto488 probe and
190 spotted on each well. After incubation for 2 h at 46°C, slides were washed 15 min in the
191 corresponding preheated washing buffer (0.45 to 0 M NaCl, 1 M Tris-HCl pH8, 0 to 5 mM

192 EDTA pH8, 0.01% SDS) at 48°C. Counter-staining was performed with 4',6-diamidino-2-
193 phenylindole (DAPI, 1 $\mu\text{g}\cdot\text{mg}^{-1}$) and slides were mounted using Citifluor:Vectashield (3:1).
194 Fluorescence was observed on a Nikon 50i epifluorescence microscope (filter ET-EGFP (LP))
195 equipped with an AxioCam MRc camera (Carl Zeiss, Germany). Signal intensity was quantified
196 using ImageJ [43] on three random images per hybridization. Only the green channel was
197 analyzed and the background signal was removed. For each hybridization, the maximum intensity
198 of 50 randomly selected cells was measured.

199 **CARD-FISH**

200 CARD-FISH was performed using a method adapted from Pernthaler *et al.* [38] and optimized
201 for the detection of alga-attached bacteria [49]. Fixed *Zobellia* cells from pure cultures were
202 harvested onto a 0.2 μm polycarbonate membrane. Membrane portions or fixed algal samples (ca.
203 25-30 mm^2) were embedded in 0.1% LE agarose and bleached in ethanol with increasing
204 concentration. Cells were permeabilized with lysozyme (10 $\text{mg}\cdot\text{ml}^{-1}$, 15 min, 37°C). Endogenous
205 peroxidases were inactivated by short incubation in 0.1 M HCl followed by 10 min in 3% H_2O_2
206 for membrane portions or by incubation in 0.15% H_2O_2 in methanol (30 min) for algal pieces.
207 Samples were covered with the hybridization mix (0.9 M NaCl, 20 mM Tris-HCl pH8, 35%
208 formamide, 1% blocking reagent, 10% dextran sulfate, 0.02% SDS, 28 nM probe and each helper
209 if needed) for 2.5 h at 46°C. Samples were washed 15 min in a preheated washing buffer (70 mM
210 NaCl, 1 M Tris-HCl pH8, 5 mM EDTA pH8, 0.01% SDS) at 48°C and 15 min in 1 \times PBS before
211 being covered with a detection solution (1 \times PBS, 2 M NaCl, 0.1% blocking reagent, 10% dextran
212 sulfate, 0.0015% H_2O_2 and 1 $\mu\text{g}\cdot\text{ml}^{-1}$ of Alexa₅₄₆-labeled tyramides) for amplification (45 min,
213 46°C). DAPI (1 $\mu\text{g}\cdot\text{mg}^{-1}$) counter-staining was performed and slides were mounted once samples
214 were completely dry with Citifluor:Vectashield (3:1). Membrane portions were visualized with a

215 Leica DMI8 epifluorescent microscope equipped with an oil objective 100X and a Leica
216 DFC3000 G camera (Wetzlar, Germany). Cells on algal tissues were visualized with a confocal
217 microscope Leica TCS SP8 equipped with HC PL APO 63x/1.4 oil objective and K-Cube filters
218 A UV and N2.1 using the 405 et 552 nm lasers to detect DAPI and Alexa₅₄₆ signal, respectively.
219 Z-stack images were collected due to the uneven algal surface (between 21 and 75 single layers
220 of 0.15 or 0.3 µm thickness, depending on the observed field). Total and *Zobellia* cell counts
221 were processed on maximum intensity images with Imaris v9.5.1. A total of 21 000 cells were
222 counted automatically from 7 different fields.

223 Results

224 **Optimization of qPCR parameters for specific detection of the genus *Zobellia***

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

234 We assessed the optimal annealing temperature (T_a) and primer concentration for qPCR using 10^4
235 16S rRNA gene copies from two *Zobellia* strains. Since the 16S rRNA gene sequences of all
236 valid *Zobellia* species were identical in the region targeted by primers, we focused our efforts on

237 *Z. galactanivorans* Dsjj^T and *Z. russellii* KMM 3677^T, which belong to two separate subclades
238 based on core genome phylogeny [15]. Similar results were obtained with the three qPCR primer
239 pairs and only those obtained with 142F/289R are shown in **Fig. 1**. Amplification was successful
240 for the two *Zobellia* species with primer concentration ranging between 100 - 500 nM (**Fig. 1A**)
241 and T_a ranging between 55 - 65°C (**Fig. 1B**). Agarose gel electrophoresis of qPCR products
242 showed a single band at the expected amplicon size (148 nt) with *Zobellia* gDNA (**Fig. S2A**). A
243 slight increase of C_t was observed on *Zobellia* gDNA for primer concentration below 200 nM
244 (**Fig. 1B**). Therefore, primer concentration was set at 300 nM for further experiments. C_t
245 remained stable irrespective of the temperature (around 24 and 21 for *Z. galactanivorans* and *Z.*
246 *russellii* respectively).

247 Ten-fold serial dilutions of *Z. galactanivorans* Dsjj^T gDNA were amplified (T_a = 60°C, [primers]
248 = 300 nM) to evaluate the qPCR dynamic range and efficiency of each primer pair. The highest
249 efficiency (88%) was observed for the pair 142F/289R, compared to 142F/285R and 142F/294R
250 (86% and 81%, respectively) (**Fig. 1C**). The linear dynamic range spanned 8 orders of
251 magnitude, from 10 to 10⁸ 16S rRNA gene copies. To ensure high efficiency and specificity of
252 qPCR assays on environmental samples, we further used the pair 142F/289R and a high
253 annealing temperature (T_a = 64°C). With these conditions, the strains *Cellulophaga baltica*
254 NN015840^T and *Cellulophaga* sp. Asnod2-G02 were used as negative controls to check the assay
255 specificity, since the target regions of their 16S rRNA gene sequences were the closest to that of
256 *Zobellia* based on SILVA TestPrime results (3 mismatches with 142F, 3 mismatches with 289R).
257 We also tested amplification from *Maribacter forsetii* KT02ds18-6^T (5 and 4 mismatches to 142F
258 and 289R, respectively), since *Maribacter* is the closest described genus to *Zobellia* [7]. No
259 amplification was detected with any of these negative controls (**Fig. S2A**). In addition,
260 amplification from environmental DNA yielded a single product at the expected size (**Fig. S2B**),

261 with melting curves showing a single dissociation peak (**Fig. S2D**) similar to that obtained on
262 pure *Z. galactanivorans* Dsij^T gDNA (**Fig. S2C**). qPCR products obtained with two selected
263 environmental samples (LdigO and PpalS, see below) were sequenced (**Supplementary File 3**).
264 All retrieved sequences (20/20) had their best blastn hit against 16S rRNA gene sequences from
265 *Zobellia* spp (**Supplementary Table 4**), confirming the specificity of the assay on natural
266 samples. Sensitivity and specificity were further assessed using environmental DNA extracted
267 from natural seawater devoid of *Zobellia* (no amplification detected after 45 cycles of qPCR with
268 primers 142F/289R) mixed with 10-fold serial dilutions of *Z. galactanivorans* Dsij^T gDNA. The
269 proportion of *Zobellia* was calculated by dividing *Zobellia* 16S rRNA gene copies (amplification
270 with primers 142F/289R) by total 16S rRNA gene copies (amplification with primers
271 926F/1062R) (**Fig. 1D**). Measured and theoretical proportions were highly congruent, confirming
272 the specificity of the method toward *Zobellia* spp. The assay shows a strong sensitivity, as it can
273 detect minor proportions of *Zobellia* DNA (approximately 0.01%, i.e., 5 16S rRNA genes copies)
274 when combined with environmental DNA. Detection was saturated when the proportion of
275 *Zobellia* was close to 100% (**Fig. 1D**, top-right corner), a situation unlikely to happen in natural
276 samples.

277 **Characterization of the newly-designed FISH probe ZOB137**

278 Probe design yielded one candidate, ZOB137 (**Table 1**) covering 100% of the target group.
279 ZOB137 target site was similar to that of primer 142F (**Fig. S1B**). *In silico* analysis showed
280 ZOB137 matches the 54 *Zobellia* sequences available in the SILVA SSU r138.1 database. Three
281 outgroup hits were found (uncultured members of the genera *Aquibacter* and *Euzebyella*).
282 We optimized the hybridization stringency for FISH with ZOB137, i.e. the highest formamide
283 (FA) concentration in the hybridization buffer that does not result in loss of fluorescence intensity

284 of the target cells [39]. Melting curves were obtained from hybridizations of *Z. galactanivorans*
285 *Dsij*^T and *Z. russellii* KMM 3677^T with ZOB137-Atto488 at increasing FA concentrations (**Fig.**
286 **2**). For both strains, cell fluorescence intensity was stable until 35% FA, slightly decreased using
287 40% FA and dropped using 50-70% FA. Therefore, the optimal FA concentration for ZOB137
288 was set to 35% for further experiments. ZOB137 was tested using these conditions with two
289 additional *Zobellia* strains, *Z. amurskyensis* KMM 3526^T and *Z. roscoffensis* Asnod1-F08^T, and a
290 fluorescent signal was observed (data not shown).

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

300 **Application of *Zobellia*-specific qPCR and CARD-FISH assays on natural macroalgal** 301 **microbiota**

302 These newly-designed qPCR and CARD-FISH assays were tested on environmental samples, to
303 detect and quantify the abundance of the genus *Zobellia* on the surface of macroalgae. Total and
304 *Zobellia* 16S rRNA gene copies were quantified on the surface of two fresh brown macroalgae
305 *Laminaria digitata* (base, medium and old part) and *Ascophyllum nodosum*, a fresh green

306 macroalga *Ulva lactuca* and a red macroalga *Palmaria palmata* (fresh and stranded individuals)
307 (**Fig. 4**).

308 No significant difference was observed in the average total copies.cm⁻² between the basal (0.27 ×
309 10⁶ copies.cm⁻²) and medium (1.8 × 10⁶ copies.cm⁻²) part of *L. digitata* (Tukey HSD post-hoc
310 pairwise comparisons, *P* = 0.8). However, the total number of copies was significantly higher on
311 the old blade where 14.6 × 10⁶ copies.cm⁻² were quantified (*P* = 0.003 and *P* = 0.005 in
312 comparison to LdigB and LdigM, respectively). The average total copy number on *A. nodosum*,
313 *U. lactuca* and *P. palmata* fresh and stranded was estimated at 7.2, 5.7, 5.1 and 14.6 × 10⁶
314 copies.cm⁻², respectively.

315 16S rRNA gene copies were also detected on the four macroalgae with the *Zobellia*-specific
316 primers (**Fig. 4B**). The number of *Zobellia*-16S rRNA gene copies.cm⁻² differed along the *L.*
317 *digitata* blade (ANOVA, *F*_{2,6} = 31.8, *P* = 0.001). It was higher on the old blade (10.5 × 10³
318 copies.cm⁻²) compared to the basal (1.3 × 10³ copies.cm⁻², Tukey HSD *P* = 0.001) and the
319 medium (2.5 × 10³ copies.cm⁻², *P* = 0.002) part. The proportion of *Zobellia* cells within the
320 epiphytic communities (**Fig. 4C**) was significantly higher on the basal part (0.54%) than on the
321 old blade of *L. digitata* (0.07%) (*P* = 0.01). Fresh *A. nodosum*, *U. lactuca* and *P. palmata*
322 displayed 9.7, 6.1 and 11.3 × 10³ *Zobellia* copies.cm⁻² representing 0.14, 0.12 and 0.21% of the
323 total number of copies, respectively. On the surface of the stranded *P. palmata* sample, 1.3 × 10⁵
324 *Zobellia* copies.cm⁻² were estimated, ie. 0.87% relative abundance.

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

330 on both macroalgae. *Zobellia* cells observed on algal tissues (**Fig. 5**) were shorter than those
331 observed in pure cultures in rich medium (**Fig. 3F**), indicating different morphologies depending
332 on environmental conditions. After image analysis on *P. palmata*, 1.0×10^5 *Zobellia* cells were
333 counted per cm^2 of algal surface, representing ca. 0.43% of detected bacterial cells. *Zobellia* cells
334 displayed a homogeneous distribution on stranded *P. palmata* but not on the surface of *U. lactuca*
335 where *Zobellia*-specific signal was visible only in a few areas. Hence, *Zobellia* cell count was not
336 performed on *U. lactuca* as it would not have been reliable.

337 Discussion

338 qPCR and CARD-FISH are widely used, complementary molecular methods to examine
339 microbial abundance in environmental samples. qPCR is a fast, highly sensitive and specific tool
340 allowing absolute quantification of gene copies, suitable to detect minor amounts of target DNA
341 [24]. CARD-FISH is more time-consuming but is not subject to DNA extraction or PCR biases
342 and enables direct visualization and localization of single bacterial cells associated with the host.
343 qPCR using universal bacterial primers estimated around 10^6 - 10^7 total 16S rRNA gene
344 copies. cm^{-2} on the different macroalgal species. Considering an average of 4 copies of 16S rRNA
345 gene per bacterial cell [50], these results concur with previous studies showing 10^6 - 10^9 cells per
346 cm^2 on algal surfaces [17,35]. qPCR assays on *L. digitata* samples showed that total bacterial
347 abundance increased with kelp tissue age, corroborating observations made on different kelp
348 species [36,42].

349 Newly designed qPCR and CARD-FISH assays specifically targeting the genus *Zobellia* were
350 tested and optimized on pure cultures before application on macroalgal samples. qPCR assays
351 using the novel *Zobellia*-specific primers 142F/289R were shown to be a robust, fast, high-

352 throughput and sensitive way to quantify the abundance of *Zobellia* spp. in environmental
353 samples. The detection limits were determined by pooling targeted with untargeted DNA, a
354 relevant approach as we aim to detect *Zobellia* in natural samples (as discussed in Skovhus *et al.*
355 [44]). This method allows the detection of minor proportions of *Zobellia* in environmental
356 samples (0.01%), which would be technically difficult using CARD-FISH.

357 CARD-FISH detection of non-abundant taxa directly on macroalgae is difficult due to the high
358 background autofluorescence of algal pigments. Here, algal bleaching was performed with
359 extended ethanol and methanol baths to reduce autofluorescence. Moreover, the addition of three
360 unlabeled helper oligonucleotides improved the CARD-FISH signal, facilitating cell
361 visualization. The secondary structure of the 16S rRNA target region for probe ZOB137 might
362 partly hinder hybridization [11,23]. The combined hybridization of multiple adjacent helper
363 oligonucleotides assisted to open the targeted region, facilitating probe binding. The use of
364 helpers for FISH was first described by Fuchs *et al.* [22] who recommended to test
365 experimentally their influence on the probe specificity. In our study, no hybridization signal was
366 detected using ZOB137 combined with helpers on two strains with single-mismatch, confirming
367 helpers did not impact probe specificity. A specific CARD-FISH signal was detected with
368 ZOB137 at the surface of macroalgae with a flat thin blade, such as the red seaweed *Palmaria*
369 *palmata* or the green seaweed *Ulva lactuca*. This new protocol represents a powerful tool to (i)
370 determine the spatial localization of *Zobellia* cells within macroalgal surface microbiota, (ii)
371 estimate their physical interactions with other taxa or the host and (iii) assess whether they are
372 structured into patches or evenly distributed on the surface, as it seems to be the case on *P.*
373 *palmata* thallus. Visualization was more complex with thicker algae such as *Laminaria*, since
374 multiple cell layers caused a strong autofluorescence masking the bacterial CARD-FISH signal.
375 Moreover, thick algal pieces sometimes decomposed during the CARD-FISH process as their

376 structure became weaker, more viscous and tissues tended to disintegrate during washing steps.
377 Hence, specific taxa visualization on algal surfaces remains a challenge depending on algal
378 species. A way to overcome these issues would be to embed algae in resin and cut thin thallus
379 sections using a microtome prior to CARD-FISH, as it was successfully done in Ramirez-Puebla
380 *et al.* [42] and Tourneroché *et al.* [46].

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

386 Previous studies already reported the presence of *Zobellia* spp. on *A. nodosum* (3% of the
387 cultivable microbiota) [9,34] and *L. digitata* [47] in Roscoff. Since macroalgal microbiomes are
388 diverse and can host hundreds of taxa [20], the observed frequency of 0.1-1% for *Zobellia* shows
389 the quantitative relevance of this genus in macroalgae-dominated habitats. The highest *Zobellia*
390 proportion was observed on the stranded decaying *P. palmata* individual, highlighting its putative
391 important activity in macroalgal biomass recycling. Although not dominant in the bacterial
392 communities, *Zobellia* spp. might act as primary degraders of algal polysaccharides due to their
393 outstanding CAZyme repertoire, releasing degradation products that could fuel other taxa via
394 cross-feeding interactions. In line with this, *Z. galactanivorans* Dsij^T was recently shown to
395 degrade intact kelp tissue, releasing soluble algal compounds that become accessible to
396 opportunistic taxa [52]. The absolute number of *Zobellia* 16S rRNA gene copies was positively
397 correlated with the age of the *L. digitata* tissues. Kelp decay is more pronounced at the tip of the
398 blade, which might result in more available substrates for diverse bacterial algal degraders,
399 including *Zobellia* spp. By contrast, the proportion of *Zobellia* 16S rRNA gene copies over total

400 copies was highest in the youngest region of *L. digitata* (i.e. the basal meristem). The high
401 proportion of the genus *Zobellia* on young algal tissues might thus reflect its metabolic
402 specialization towards complex polysaccharides found in the meristem, while it could be
403 outcompeted by opportunistic taxa on the decaying old blade. *Zobellia* spp. might also be early
404 colonizers of newly-produced meristematic tissues, reflecting their adaptation to attach to and
405 live on algal surfaces. Moreover, intra-thallus variability of algal defense metabolites, such as
406 iodine, reactive oxygen and nitrogen species and phlorotannins, might create different ecological
407 micro-niches [4,27,30] selecting for specific taxa.

408
409 Using *P. palmata* surface microbiota as a test case, we showed that both the absolute number and
410 proportion of ZOB137-hybridized cells concur with the qPCR-estimated *Zobellia* 16S rRNA
411 gene copy number. However, it should be interpreted carefully as available *Zobellia* genomes
412 feature between 1 to 4 copies of 16S rRNA genes (**Supplementary Table 3**), so qPCR results
413 cannot be directly converted into a number of cells. Another bias might lie in the size of the
414 sampled algal area, as bacteria were quantified from a 50-cm² surface with qPCR but CARD-
415 FISH was performed only on 30-mm² algal pieces. Tourneroche and co-workers recently drew
416 attention to the potential within-tissue spatial variability of kelp-associated bacterial communities
417 [46]. Finally, CARD-FISH analyses detect metabolically active cells as the probe targets 16S
418 rRNA, while here qPCR was applied on DNA samples and therefore could detect resting or dead
419 cells. The close proximity of values obtained on stranded *P. palmata* with qPCR (1.3×10^5
420 copies.cm⁻² for *Zobellia* 16S rRNA gene) and CARD-FISH (1.0×10^5 *Zobellia* cells.cm⁻²)
421 suggests that most *Zobellia* cells in this sample were active. In the future, the newly-designed
422 primer pair 142F/289R could be used for reverse-transcription qPCR on RNA extracts to better
423 estimate *Zobellia* transcriptional activity.

424
425 To conclude, we validated novel qPCR and CARD-FISH protocols to specifically target the
426 marine flavobacterial genus *Zobellia*. We provide the first quantitative estimates of *Zobellia*
427 absolute and relative abundance on different macroalgae, suggesting a widespread distribution.
428 This work paves the way for further studies of the spatiotemporal dynamics of *Zobellia* in marine
429 environments.

430

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440 Conceptualization: FT; Formal analysis: MB, NLD, FT; Funding acquisition: FT; Investigation:
441 MB, NLD, FT; Project administration: FT; Supervision: FT, TB, BF, RA; Visualization: MB,
442 NLD, FT; Writing – original draft: MB, FT; Writing – review & editing: MB, BF, RA, TB, FT.

443

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624 **Table**625 **Table 1. Oligonucleotides used in this study.** Specificity was tested *in silico* using the SILVA tools TestPrime and TestProbe for qPCR primers

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

627

628 **Figure legends**

629

630 **Figure 1. Optimization of qPCR parameters.** A-B: Effect of primer concentration (A) and annealing
631 temperature (T_a) (B) on the cycle threshold (C_t) was determined using the primer pair 142F/289R and 10^4
632 16S rRNA gene copies. T_a was set to 60°C in A and primer concentration to 300 nM in B. C: Standard
633 curves obtained in qPCR with the three sets of primers using *Z. galactanivorans* Dsij^T gDNA diluted from
634 10^8 to 10^1 16S rRNA gene copies. D: Comparison of measured and theoretical proportion of *Zobellia* 16S
635 rRNA gene copies in environmental DNA with increasing load of *Zobellia* gDNA. The 1:1 line was drawn
636 in light grey as a comparison. Values are mean \pm s.d (n = 3). In C and D, $T_a = 64^\circ\text{C}$ and [primer] = 300 nM.
637 In all panels (A-D), measurements were performed in technical triplicates.

638

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

644

645 **Figure 3. Epifluorescence microscopy images of bacterial cells hybridized with the CARD-FISH**
646 **probe ZOB137 (E-H) and counterstained with DAPI (A-D).** *Lewinella marina* MKG-38T and
647 *Marinirhabdus citrea* MEBiC09412T were used as negative controls to assess the specificity of the newly-
648 designed probe ZOB137. Addition of the three helpers in the hybridization mix increased the fluorescence
649 of the *Zobellia galactanivorans* cells (F) without affecting the probe specificity (G-H). Upper (DAPI-
650 stained cells) and lower (CARD-FISH signal) epifluorescent micrographs show identical fields. Exposure
651 time was 100 ms for CARD-FISH. FA = 35%.

652

653 **Figure 4. Number of 16S rRNA gene copies per cm² of algal surface detected either with the universal**
654 **bacterial primers (A) or with the *Zobellia*-specific primers 142F/289R (B).** Proportion of *Zobellia* (C)
655 was obtained by dividing the number of *Zobellia* 16S copies by the number of 16S copies detected with the
656 universal primers for each sample. Measurements were performed in technical triplicates. LdigB, LdigM
657 and LdigO are basal, median and apical parts of *Laminaria digitata*, respectively. Anod, *Ascophyllum*
658 *nodosum*; Ulac, *Ulva lactuca*; Ppal, healthy *Palmaria palmata*; PpalS, stranded *P. palmata*. For *L. digitata*
659 samples, within-blade variations were tested using one-way ANOVA analyses followed by pairwise post-
660 hoc Tukey HSD. Different letters denote significant difference ($P < 0.05$).

661

662 **Figure 5. *Zobellia* cells visualization in the stained biofilm of *Palmaria palmata* (A) and *Ulva lactuca***
663 **(B).** Micrographs are overlay of the CARD–FISH signal (green cells, ZOB137-HRP probe with Alexa₅₄₆ as
664 the reporter signal) and the DAPI signal (magenta cells) and represent the maximum intensity projection of
665 Z-stack. Transversal view is shown at the bottom. Bars: 10 μm (A) and 15 μm (B).