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1 For submission to:

2 Development of a real-time quantitative PCR assay for detection and quantification of

3 the marine bacterium *Alteromonas macleodii* from coastal environments

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Abstract: *Alteromonas macleodii* is a ubiquitous marine bacterial species found in a variety of habitats that displays both planktonic and particle-associated lifestyles. Transcriptomic studies demonstrate that, even when present at low abundance, it can make significant contributions to biogeochemical cycles, and its specific association with key marine phytoplankton species indicates other ecological roles as well. It has also been shown to be one of the early colonizers of copper-treated marine vessels. There currently exist no rapid, reliable molecular assays for the detection and quantification of *A. macleodii* from its different environments. We developed a real-time PCR assay, specific to *A. macleodii*. This assay targets the DNA gyrase B subunit (*gyrB*) gene, which occurs as a single copy in the genome. The assay possesses an amplification efficiency of 94.3 %, with a limit of detection of 2.5 *gyrB* copies per μ l. Assay specificity was validated by melt curve analysis, followed by sequencing of the amplified product. The assay was specific to thirteen *A. macleodii* strains and did not amplify other marine bacteria, including *Roseobacter denitrificans*, *Silicibacter* sp. TM1040, *Vibrio coralliilyticus*, *Vibrio harveyi*, and *Vibrio alginolyticus*. It also did not amplify *Alteromonas mediterranea*, a close relative that can occur in the same environment as *A. macleodii*. This assay was used to determine the presence and abundance of *A. macleodii* from a range of coastal habitats. The assay was also used to monitor the *A. macleodii* growth in biofilm and planktonic cultures over time in the presence of elevated copper. This assay provides a rapid and reliable means to assess the presence and abundance of a ubiquitous marine bacterium that, even at low abundance, has been shown to make significant contributions to key marine processes.

Keywords

32 *Alteromonas macleodii*, *gyrB*, qPCR, Marine, Biofilm, Planktonic

33 **Introduction**

34 *Alteromonas macleodii* is a common marine bacterium representing approximately 3 –
35 5% of the bacterial population in ocean surface water (Fadeev et al., 2016; García-
36 Martínez et al., 2002). *A. macleodii* isolates have been obtained from different
37 geographical regions such as the Pacific Ocean, Mediterranean Sea, English Channel,
38 Black Sea, and Andaman Sea (Ivars-Martinez et al., 2008). They are found ubiquitously
39 in marine environments from the surface level to a depth of ca. 3500 meters (Ivars-
40 Martinez et al., 2008). *A. macleodii* was initially classified into two ecotypes: surface and
41 deep-sea. The deep-sea ecotype was later reclassified as *A. mediterranea* based on
42 differences in physiological, biochemical, phylogenetic, and genomic (DNA-DNA
43 hybridization) features (Ivanova et al., 2015). The deep-sea ecotype shares less than
44 85% average nucleotide identity over the core genome with the surface ecotype (Ivars-
45 Martinez et al., 2008). Previous studies have shown that *A. macleodii* maintains both a
46 free-living and particle-associated lifestyle, and readily forms biofilms (Acinas et al.,
47 1999; García-Martínez et al., 2002).

48 *A. macleodii* is an important contributor to biogeochemical cycling. Previous
49 metatranscriptomic studies revealed its role in the marine carbon and nitrogen recycling
50 (Baker et al., 2013). *A. macleodii* species also indirectly affect marine carbon and
51 nitrogen cycles because of their association with marine cyanobacteria such as
52 *Prochlorococcus* and *Trichodesmium* (Hou et al., 2018; Morris et al., 2008). For
53 example, studies have suggested that *Alteromonas* strain EZ55 acts as a heterotrophic
54 helper bacterium promoting the growth of a *Prochlorococcus* by helping reduce oxidative

stress (Morris et al., 2008). *Prochlorococcus* might provide *Alteromonas* organic carbon and other nutrients (Biller et al., 2015).

In the marine environment, copper is commonly used as an anti-fouling agent on ship hulls to inhibit bacterial growth and subsequent biofilm formation. Previous studies have indicated *Alteromonas* species as early colonizers of copper-based anti-fouling surfaces (Chen et al., 2013). More recently, an *A. macleodii* strain CUKW isolated from copper test coupons exhibited a high copper tolerance (Cusick et al., 2017). Genomic and transcript analyses revealed that CUKW possesses an enhanced genetic repertoire of homologs of copper resistance systems, most of which were induced when grown in the presence of copper in the laboratory (Cusick et al., 2021).

Previous metatranscriptomic studies showed an abundance of *A. macleodii* transcripts in the marine environment, but *A. macleodii* was not well represented in a corresponding metagenome from the same environment (Baker et al., 2013; Lesniewski et al., 2012). This emphasizes the functional importance of the low abundance, yet transcriptionally active population of *A. macleodii* in marine habitats (Baker et al., 2013). Thus, accurate detection and quantification of *A. macleodii* is essential to better understand the ecology of the species and its contributions within various marine ecosystems. Currently, there are limitations to available culture-based techniques for the enumeration of total bacteria from an environment. Previously, *A. macleodii* has been detected and enumerated using the plate count method (Kimes et al., 2014). Regarding culture-independent methods, flow cytometry was previously used to quantify *A. macleodii* cell abundance as a co-occurring heterotroph (Coe et al., 2016). *A. macleodii* was also studied from a natural population by performing metagenomic

sequencing (Gonzaga et al., 2012). However, these techniques are laborious and expensive. Culture-independent techniques are important in identifying marine microbes as they cannot be readily isolated and cultured (Giovannoni & Stingl, 2005). The molecular methods of microbial identification and enumeration, such as the real-time quantitative PCR (qPCR) assay, are extensively used today in microbial ecology. For absolute quantification, the qPCR method compares a sample with the standard curve of known concentrations of the gene of interest and its corresponding cycle threshold.

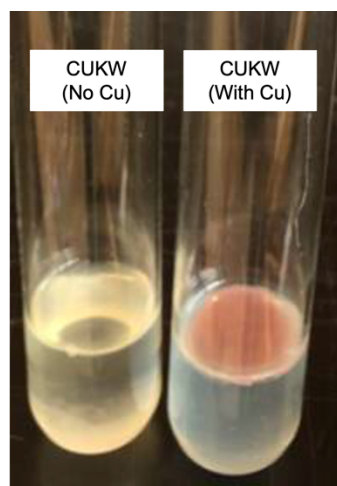
The objective of this study was to develop a qPCR assay for the detection and quantification of *A. macleodii* from different environments. A SYBR Green-based qPCR assay was developed that targeted the *gyrB* (DNA gyrase subunit B) gene. DNA gyrase is an enzyme that maintains the supercoiling of DNA and plays an important role in replication, transcription, and translation (Gellert et al., 1976). The *gyrB* gene sequence was used for this assay design because the protein-coding genes are known to evolve much faster than the rRNAs (Yamamoto & Harayama, 1995). Thus, *gyrB* is more reliable in differentiating *A. macleodii* from closely related species. The *gyrB* gene is present as a single copy in the *A. macleodii* genome and thus can be used to estimate the total cell numbers. Following development and optimization, the assay was used to detect and quantify *A. macleodii* from a range of environmental samples and track biofilm development over time under elevated copper levels.

Materials and Methods

1. *Bacterial strains and maintenance.* The following *A. macleodii* strains were used in the study: *A. macleodii* CUKW, *A. macleodii* MIT1002, *A. macleodii* KCC02, *A. macleodii* J912, *A. macleodii* EZ55, *Alteromonas* species W12, *A. macleodii* J589, *Alteromonas*

species OCN 004, *A. macleodii* V450, *A. macleodii* 5003, *Alteromonas* MR32A, *Alteromonas* species PC21ay, and *Alteromonas* species BT3 (Table 1). Isolation and maintenance of CUKW was described previously (Cusick et al., 2017; Cusick et al., 2020). The following marine bacterial species were also used in this study: *Roseobacter denitrificans*, *Silicibacter* sp. TM1040, *Vibrio coralliilyticus*, *Vibrio harveyi*, *Vibrio alginolyticus*, and *Alteromonas mediterranea*. All bacterial isolates were routinely grown in Burkholder's Formulation B medium in Artificial Sea Water (Burkholder's B) broth (Bidwell & Spotte., 1985) at 28 °C for 18 to 24 hours. Identification of individual bacterial isolates acquired from multiple laboratories was confirmed via PCR and the sequencing of the 16S rRNA gene. A single colony of bacteria was suspended in 3 ml of Burkholder's B liquid medium and incubated on a shaking platform (120 rpm) at 28 °C for 24 hours for routine DNA extraction. The cell pellet was collected after centrifugation of samples for 10 min at 10,000 rpm and stored at -80 °C until extracted. PCR was conducted using universal primers (8F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1541R 5'-AAGGAGGTGATCCAACC-3') targeting the small subunit ribosomal RNA gene (Zhou et al., 1995). A single band of the expected size (1527 bp) was obtained for all isolates. The PCR products were purified using the QIAGEN QIAquick PCR purification kit (Valencia, CA, USA), following the manufacturer's protocol. The DNA concentration was measured using the NanoDrop One Microvolume UV-Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The purified PCR products were sequenced (Genewiz, Germantown, MD, USA) using universal 16S primers (8F and 1541R), and the organisms were identified by NCBI standard nucleotide BLAST.

124 2. *Biofilm growth and culture condition for CUKW*. Single colonies of CUKW were
125 inoculated into 3 ml of Burkholder's B medium and grown overnight with agitation (120
126 rpm) in a sterile 50-ml tube. Upon reaching an OD₆₀₀ of 1.8 – 2.0, they were diluted
127 1:100 into 5 ml of Burkholder's B and grown statically for 6 hours in glass culture tubes
128 (30-ml). Copper (CuSO₄ · 5H₂O) (Sigma Aldrich, St. Louis, MO, USA) was added to the
129 culture to a final concentration of 2.7 mM. Controls consisted of nuclease-free water
130 added at the same volume as the copper solution. Abiotic controls consisted of a cell-
131 free medium with copper. Cultures were incubated statically at 28 °C. A pellicle biofilm
132 was generally seen upon visual inspection 15 -16 hours after addition of copper (Figure
133 1).



134
135 Figure 1: Pellicle biofilm of *A. macleodii* CUKW in static culture 27 hours after the
136 addition of copper. There was no observable biofilm when *A. macleodii* CUKW was
137 grown with no additional copper under the same conditions.

138 3. *Environmental samples*. Environmental samples used in this study were collected as
139 substratum-associated samples and water samples from temperate and tropical marine
140 sites (Table 3). The sampling site and the amount of biofilm collected for DNA

extractions are shown in Supplemental Table 1. In most cases, samples were collected as a biofilm from various substrates, including ship hulls, different composites being tested for anti-fouling properties, piping, ladders, and vegetation, including mangroves and marine invertebrates or water samples from the vicinity of the biofilm-containing area. Biofilms were collected using cell lifters (Celltreat Scientific Products, Pepperell, MA, USA). Biofilms and water samples were placed in sterile 1.5-ml or 50-ml tubes for transport back to the laboratory, where they were stored at -20 °C until DNA extraction.

4. *DNA extraction.* Genomic DNA was extracted from all laboratory cultures (CUKW, other *Alteromonas* strains, and other bacterial genera) using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's instructions for Gram-positive and Gram-negative bacteria. To extract the DNA from water samples, 50 ml of water sample was centrifuged at 13,000 rpm for 5 min. Genomic DNA from environmental and laboratory-generated biofilm samples was extracted using the DNeasy PowerBiofilm kit (Qiagen). DNA from all samples was eluted in a final volume of 100 µL of nuclease-free water. DNA concentration and purity were assessed using the NanoDrop and stored at -20 °C until use.

5. *gyrB primer design and validation.* A quantitative PCR assay was developed based on SYBR Green chemistry that targeted the *gyrB* gene. The *gyrB* gene sequences of all available *A. macleodii* strains were downloaded from GenBank and aligned using MEGA 7 (Kumar et al., 2016). Primers were designed to target a conserved 140-bp region of the *A. macleodii gyrB* gene. Primers were designed using the Primer3 software (Untergasser et al., 2007) with an optimal annealing temperature of 60 °C and examined for potential secondary structures using Mfold (Zuker, 2003). Self and cross-

dimer formation were tested using the Operon Oligo analysis tool (<https://www.eurofinsgenomics.com/en/resources/design-tools/oligo-analysis-tool/>). Self-complementarity was examined using an online oligonucleotide properties calculator (<http://www.basic.northwestern.edu/biotools/oligocalc.html>). Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). The assay condition was optimized by testing different parameters. These included: two different commercially available SYBR Green-based master mixes (PowerUp and Fast SYBR Green, Applied Biosystems, Waltham, MA, USA), four different primer concentrations (200 nM, 300 nM, 400 nM, and 600 nM), and two different annealing temperatures (58 °C and 60 °C). Product specificity was determined via melt curve analysis. The dissociation curve showing a single discrete peak of the amplified product from environmental samples and pure cultures, was used to validate a single product of *gyrB* gene amplification. The PCR product specificity of the *gyrB* assay was confirmed by sequencing the amplicons produced from the PCR reactions with genomic CUKW DNA and the environmental samples. The product was identified as the *A. macleodii gyrB* gene by standard nucleotide BLAST function on the NCBI website. Assay specificity was evaluated with pure cultures of twelve strains of *A. macleodii* (Table 1). The assay selectivity was validated by testing the amplification of the common marine bacteria *Roseobacter denitrificans*, *Silicibacter* sp. TM1040, *Vibrio coralliilyticus*, *Vibrio harveyi*, *Vibrio alginolyticus*, and *Alteromonas mediterranea* C34D.

6. Standard curve construction.

6.1 *A. macleodii gyrB* standard curve: An external calibration curve was constructed for absolute quantification of the *gyrB* copy number. A PCR-based standard was created by

amplifying genomic DNA using *A. macleodii gyrB*-specific primers that yielded a 1194-bp *gyrB* gene product (Table 2). The PCR was performed in a 25- μ l reaction volume with the Applied Biosystems ProFlex PCR system and contained (as final concentration): 1x PCR KCl buffer (without Mg), 1.5 mM MgCl₂, 0.5 mM each dNTP, 400 nM each forward and reverse primer, 0.625 units *Taq* DNA polymerase and brought to a final volume of 25- μ l with nuclease-free water. Thermocycling conditions were as follows: denaturation for 2 min at 95 °C; 35 cycles of 95 °C, 15 sec, 53 °C, 15 sec, 72 °C, 1 min 10 sec, and a final extension at 72 °C for 7 min. The PCR product was visualized via gel electrophoresis to confirm product amplification of the correct size. The PCR amplicon was purified using the QIAquick PCR purification kit (Qiagen) and sequenced in both directions using the gene-specific primers. Sequence identity was confirmed using the standard nucleotide BLAST function on the NCBI website. The DNA concentration of the purified PCR product was quantified using the NanoDrop. The purified PCR product served as the template for preparing the standard curve. The number of copies per μ l was calculated using the following equation (Brankatschk et al., 2012).

$$\text{Copy number} = \text{Number of targets per DNA fragment} \times \frac{\text{DNA concentration [ng } \mu\text{l}^{-1}] \times \text{Avogadro Constant [bp mol}^{-1}]}{\text{Length of DNA fragment [bp]} \times \text{Average weight of double stranded base pair [Mbp]}}$$

Ten-fold serial dilutions of the PCR amplicon were used to create an external calibration curve that spanned eight orders of magnitude ranging from 2.5E+01 copies/ μ l to 2.5E+08 copies/ μ l. Two microliters of each dilution were used per reaction, so the standard curve ranged from 5E+01 copies per reaction to 5E+08 copies per reaction. Each dilution was tested in duplicate. The linear regression of the dilution series of known DNA concentration (copies per reaction) versus its corresponding cycle

threshold (Ct) value was used to make a standard curve. The PCR amplification efficiency was calculated using the slope of the standard curve.

6.2 Universal 16S rRNA gene standard curve: A previously developed TaqMan-based universal 16S rRNA gene assay was used to normalize the abundance of *A. macleodii* relative to the total 16S rRNA copies (Harms et al., 2003; Ritalahti et al., 2006). For this assay, the standard curve was constructed for the bacterial 16S rRNA gene by amplifying genomic *A. macleodii* CUKW DNA using universal 16S rRNA PCR primers (Table 2) that yielded a 1527-bp 16S rRNA gene product. The purified PCR product served as a template to prepare the 16S rRNA standard curve, constructed as described for the *gyrB* assay. The standard curve spanned from 1.0E+01 to 1.0E+09 copies. The primers and TaqMan probe used in the qPCR TaqMan assay are shown in Table 2 (LGC Bioresearch Technology, Petaluma, CA, USA). The assay conditions were optimized by testing different primer concentrations (forward and reverse primers at 300 nM and probe at 300 nM; forward and reverse primers at 400 nM and probe at 100 nM). The qPCR assay was performed in a 20- μ l reaction with 2X TaqMan Fast Advanced master mix (ThermoFisher Scientific, Waltham, MA, CA, USA), optimized primer concentration (forward and reverse primers and probes at a final concentration of 300 nM), and 2 μ l of template DNA. The TaqMan assay was performed under the standard cycling condition for the Fast Advance master mix: 2 min at 95 °C; 40 cycles of 1 sec at 95 °C, 20 sec, at 60 °C. Product specificity was verified by amplification of CUKW DNA as a template and by amplification of DNA from low concentration environmental samples. The PCR reaction was performed in triplicates to generate a 16S rRNA gene-specific standard curve.

233 7. *Application of the qPCR gyrB assay*: Each reaction contained 2X PowerUp master mix
234 buffer, 300 nM each primer, 2 µl of the template DNA, and brought to a final volume of
235 20 µl with nuclease-free water. The PCR protocol consisted of an initial 20 sec
236 incubation at 95 °C, followed by 40 cycles of 95 °C for 1 sec, and 60 °C for 20 sec,
237 followed by a melt curve analysis of 95 °C for 15 sec, 60 °C for 1 min, and 95 °C for 15
238 sec to confirm product specificity. All qPCR assays were carried out in Microamp Fast
239 96-well reaction plates on the Quant Studio 6 Flex Real-Time PCR systems (Applied
240 Biosystems). Genomic *A. macleodii* CUKW DNA samples and no-template controls in
241 which nuclease-free water was added instead of template were included as positive
242 and negative controls on each plate.

243 7.1 *Quantification of A. macleodii from environmental samples*: The biofilm and water
244 samples collected from various locations were screened for *A. macleodii* using the
245 qPCR assay. The DNA extracted from the environmental samples was diluted in
246 nuclease-free water (1:1 dilution). The cycle threshold (Ct) values obtained were
247 compared with the standard curve to determine the total copies of the *gyrB* gene in
248 environmental samples. The absolute abundance of *A. macleodii gyrB* gene copies
249 detected in the environmental samples was normalized by the total 16S rRNA gene
250 copy number (representing the total bacterial abundance) to obtain a relative
251 abundance of *gyrB* gene per 16S rRNA gene. The melt curve obtained from the
252 environmental samples verified the product of a single specific size. Environmental
253 samples with high Ct values (indicative of low abundance) were purified and sequenced
254 to verify amplification of the *A. macleodii gyrB* target.

7.2 Quantification of *A. macleodii* from laboratory culture of biofilm and planktonic cells:

The *gyrB* gene-specific qPCR assay was used to enumerate the total *A. macleodii* CUKW cells in the planktonic and biofilm counterparts of the culture in the presence of elevated copper. The biofilm and planktonic cells were collected separately from within the same tubes. They were collected at different time points spanning the exponential to stationary phase of growth (Cusick et al., 2021): 19 hours, 27 hours, and 42 hours. The assay was carried out in biological triplicates to verify its reproducibility. To collect the biofilm, pellicles on the surface of the air-water interface were carefully collected using a sterile disposable inoculating loop and placed in a 1.5-ml centrifuge tube. Biofilm attached to the glass surface, if any, was also carefully removed from the side by tilting the tubes and collecting with the same inoculating loop. Biofilm samples were pelleted by centrifugation for 1 min at 10,000 rpm, and the excess supernatant was removed. Tubes were stored at -80 °C until DNA extraction.

To collect planktonic cells, the remaining culture was harvested by centrifugation at room temperature for 2 min at 10,000 rpm, and the resulting cell pellet was stored at -80 °C until DNA extraction. DNA extraction was performed as described earlier. DNA from biofilm and planktonic cells were diluted to 1:10 with nuclease-free water prior to their use in the qPCR assay. Reactions were performed in duplicate for each sample. An external calibration curve in the range of 2.5E+01 copies/μl to 2.5E+08 copies/μl was used to quantify total *gyrB* copies from each sample. DNA extracted from biofilm was diluted 10 and 100-fold to look for the presence of inhibitors in the DNA extract. No inhibitors were present, as indicated by a nearly 3.3 Ct difference between ten-fold

dilutions (data not shown). The *gyrB* copy number was then normalized to colony forming units (CFUs).

Both biofilm and planktonic phases of individual cultures were enumerated by the plate count (cfu/ml) method (Wilson et al., 2017). The biofilm and planktonic cultures were collected as described for the *gyrB* assay. Biofilm was homogenized in Burkholder's B liquid medium by vigorously pipetting the culture up and down until dispersed. Dilutions spanning from 1.0E-01 to 1.0E-06 of biofilm and planktonic cultures were plated on Burkholder's B plates. The plates were incubated at 28 °C for 48 hours before colony enumeration.

Statistical Analysis: Pearson's correlation coefficient was used to examine the relationship between the quantification from the gene-based qPCR assay and the plate count technique. Total *A. macleodii* cells quantified from the biofilm and planktonic cultures by *gyrB* assay and CFU counts were used to measure the linear relationship between the results of the two methods. The correlations were considered statistically significant if the p-value ≤ 0.05 .

The data from the qPCR assay and plate count technique from biofilm and planktonic cells were assessed for normality using the Shapiro-Wilk test. Normally distributed samples were tested for correlation using the independent samples t-test, and non-normal samples were tested for correlation with the non-parametric Mann-Whitney U test. The independent samples t-test and Mann-Whitney U test (p-value ≤ 0.05) were used to compare differences between the means of the two quantification methods. The independent samples t-test was used to compare differences between the means of both biofilm cells and planktonic cells at different time points. Statistical

analysis was performed with SPSS Version 29.0 statistical software package (IBM Corp., Armonk, NY, USA).

Results

1. *A. macleodii gyrB*-specific qPCR assay development and optimization: The assay was evaluated under a range of conditions, including two commercially available SYBR Green master mixes (PowerUp and Fast SYBR), a range of primer concentrations (200 nM, 300 nM, 400 nM, and 600 nM), and two different annealing temperatures (58 °C and 60 °C). DNA from environmental samples and pure culture of *A. macleodii* CUKW was used to confirm specificity. The optimal conditions consisted of a PowerUp master mix, with both forward and reverse primers at a final concentration of 300 nM and an annealing temperature of 60 °C. Melt curve analysis showed a single discrete peak, indicative of a single product, with a T_m of ca. 79 °C (Figure 2).

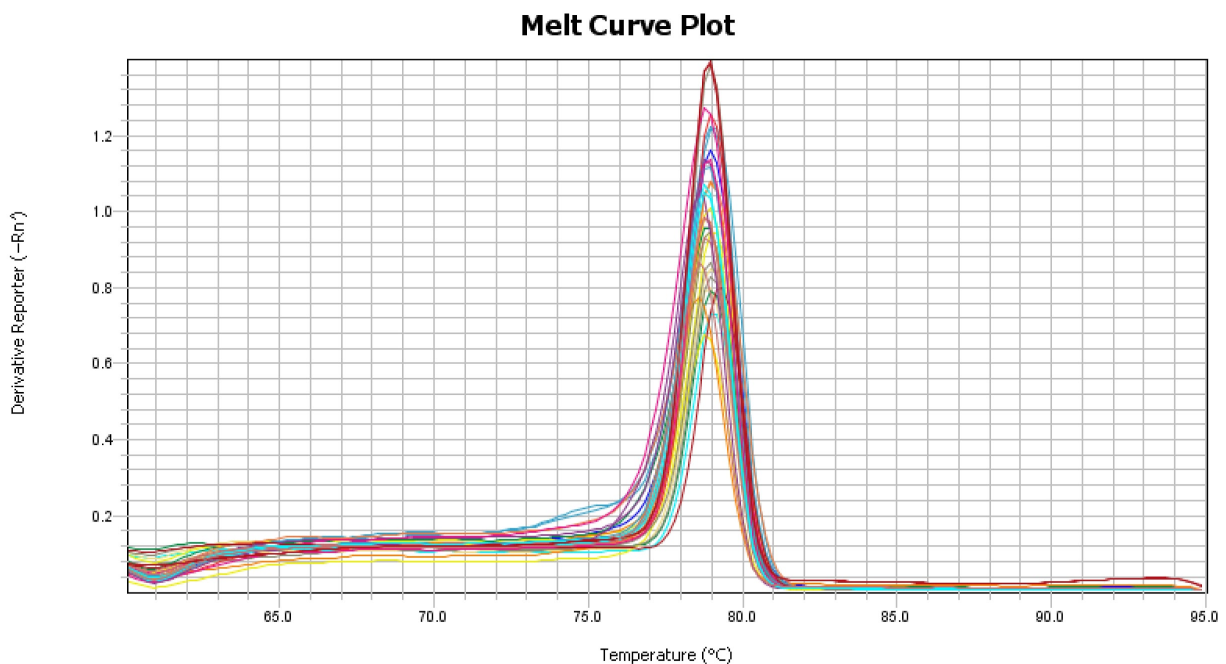


Figure 2: A single melt peak (79 °C) was obtained for genomic DNA of *A. macleodii* CUKW and all environmental samples, indicative of *A. macleodii* presence. Melt curve specificity was confirmed by sequencing a subset of samples.

2. Evaluation of assay specificity and sensitivity:

2.1 Assay sensitivity. Performance of the standard curve and detection limit: The standard curve of the known *gyrB* copies and its corresponding cycle threshold values from the qPCR assay were used for the quantification of *A. macleodii*. The standard curve was generated by preparing ten-fold serial dilutions of the known concentration of a PCR purified, *gyrB* gene of *A. macleodii* (Figure 3A). The slope of the standard curve was -3.466, which corresponds to an amplification efficiency of 94.3 % (Figure 3A), and an R^2 of 0.998 (Figure 3A). The assay can reliably and consistently detect down to 2.5 *gyrB* copies per μ l (i.e., 50 copies per 20 μ l reaction).

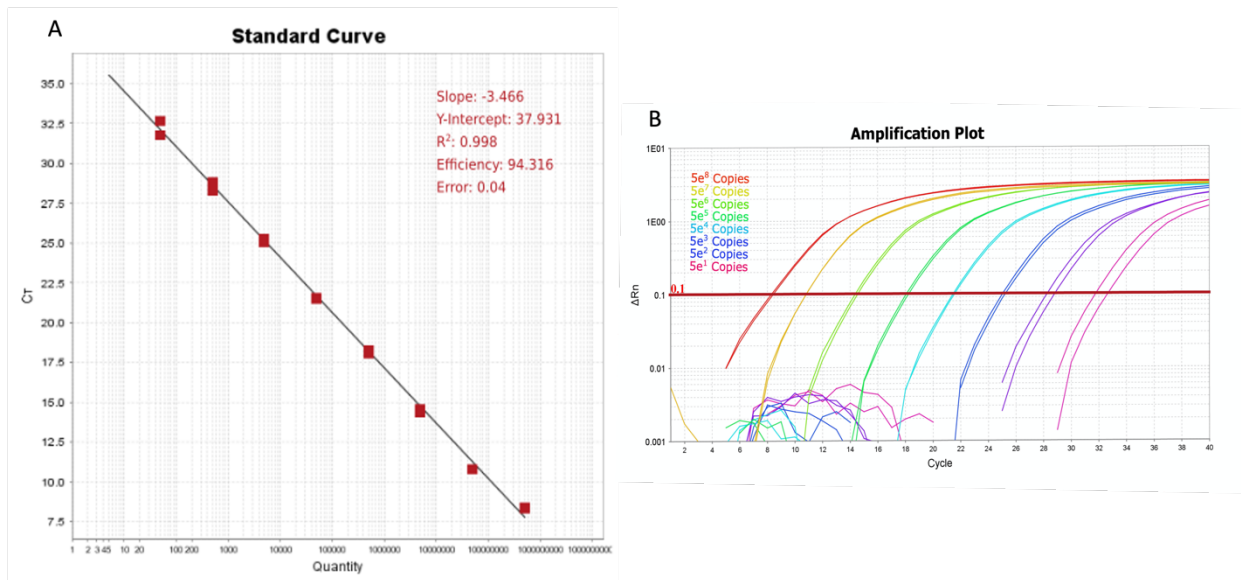


Figure 3: Standard curve of qPCR assay with *A. macleodii*. A) Standard curve with serial decimal dilutions of *A. macleodii gyrB* gene equivalent to 5E+08 copies to 5E+01 copies of 1194-bp *gyrB* gene fragment. The curve was generated using the

329 QuantStudio 6 Real time PCR system software. The software automatically calculated
330 the slope, Y-intercept, R^2 , efficiency, and error. B) Amplification plot of 10-fold serial
331 dilutions containing $5E+08$ to $5E+01$ copies *gyrB* gene copies.

332 *2.2 Assay specificity.* Primer specificity was evaluated using 13 *A. macleodii* strains.
333 The primer pair showed amplification with all the tested *A. macleodii* strains (Table 1).
334 The qPCR assay yielded no amplicon when applied to other marine bacterial species,
335 *R. denitrificans*, *Silicibacter* sp. TM1040, *V. coralliilyticus*, *V. harveyi*, *V. alginolyticus*,
336 and the closely related *A. mediterranea* C34D. These species are frequently found in
337 marine habitats and are known to co-occur with *A. macleodii* in coastal marine systems
338 (Floyd et al., 2005). This shows the PCR assay to be selective for *A. macleodii* species
339 (Supplemental Figure 1). Collectively, these results demonstrate that the qPCR assay is
340 specific and selective to *A. macleodii*.

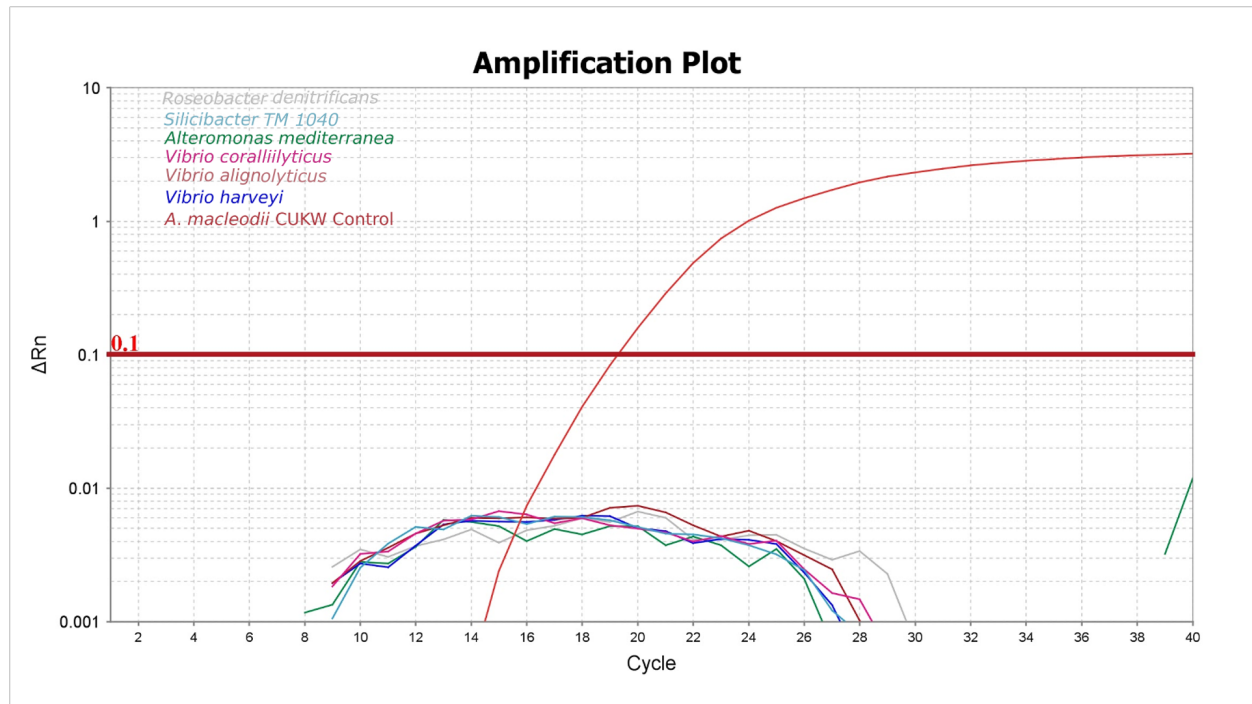
341 Table 1: List of *Alteromonas* strains and species used in this study.

Name	<i>gyrB</i> gene amplification	Isolation Source	Source
<i>A. macleodii</i> CUKW	+	Key West, Florida	(Cusick et al., 2017)
<i>A. macleodii</i> KCC02	+	Key West, Florida	(Cusick et al., 2017)
<i>A. macleodii</i> MIT1002	+	<i>Prochlorococcus</i> NATL2A, North Atlantic	(Biller et al., 2015)
<i>A. macleodii</i> J912	+	Harbor Branch Oceanographic Institute, Florida Atlantic University	(Peter McCarthy, HBOI, personal collection)
<i>A. macleodii</i> EZ 55	+	<i>Prochlorococcus</i> strain MIT 9215	(Morris et al., 2008)
<i>Alteromonas</i> sp. W12	+	<i>Thalassiosira rotula</i> strain CCMP3362	(Garcia et al., 2017)
<i>A. macleodii</i> J589	+	Harbor Branch Oceanographic Institute, Florida Atlantic University	(Peter McCarthy, HBOI, personal collection)
<i>Alteromonas</i> sp. OCN004	+	<i>Montipora capitata</i> in Hawaii	(Ushijima et al., 2012)
<i>Alteromonas macleodii</i> V450	+	Marine Sponge <i>Leiodermatium</i> sp.	(Wang et al., 2017)
<i>A. macleodii</i> HBOI 5003	+	Harbor Branch Oceanographic Institute, Florida Atlantic University	(Peter McCarthy, HBOI personal collection)
<i>Alteromonas</i> sp. MR32A	+	Coral Reef in Kaneohe, Hawaii	(Tran & Hadfield, 2011)
<i>Alteromonas</i> PC21ay	+	Coral Reef in Kaneohe, Hawaii	(Tran & Hadfield, 2011)
<i>Alteromonas</i> B3T	+	Coral Reef in Kaneohe, Hawaii	(Tran & Hadfield, 2011)

342

Table 2: List of primers and probes used in this study.
T_m = Melting temperature of the primers

Primer name	Description	Primer sequence (5'–3')	<i>T_m</i> (°C)	Source
480Fq2	<i>gyrB</i> Forward	CGATACTGACAAAACGGGAATA	62.9	This Study
619Rq2	<i>gyrB</i> Reverse	TCAAGCGAATAGACACACCAGAG	62.9	This Study
gyrBstdF	<i>gyrB</i> standard Forward	AGCTTCGTATTCGTCGCG	56.3	This Study
gyrB1650R	<i>gyrB</i> standard Reverse	ATACATATCCACGCTCAATGAT	56.4	This Study
8F	Universal 16S rRNA Forward	AGAGTTTGATCCTGGCTCAG		Zhou <i>et al</i> , 1995
1541R	Universal 16S rRNA Reverse	AAGGAGGTGATCCAACC		Zhou <i>et al</i> , 1995
Bac1055YF	Universal 16S rRNA qPCR Forward	ATGGYTGTCTCAGCT		Ritalahti, <i>et al.</i> , 2006
Bac1392R	Universal 16S rRNA qPCR Reverse	ACGGGCGGTGTGTAC		Lane, 1991
Bac1115Probe	Universal 16S rRNA qPCR Probe	FAM-CAACGAGCGCAACCC-BHQ		Harms, <i>et al.</i> , 2003

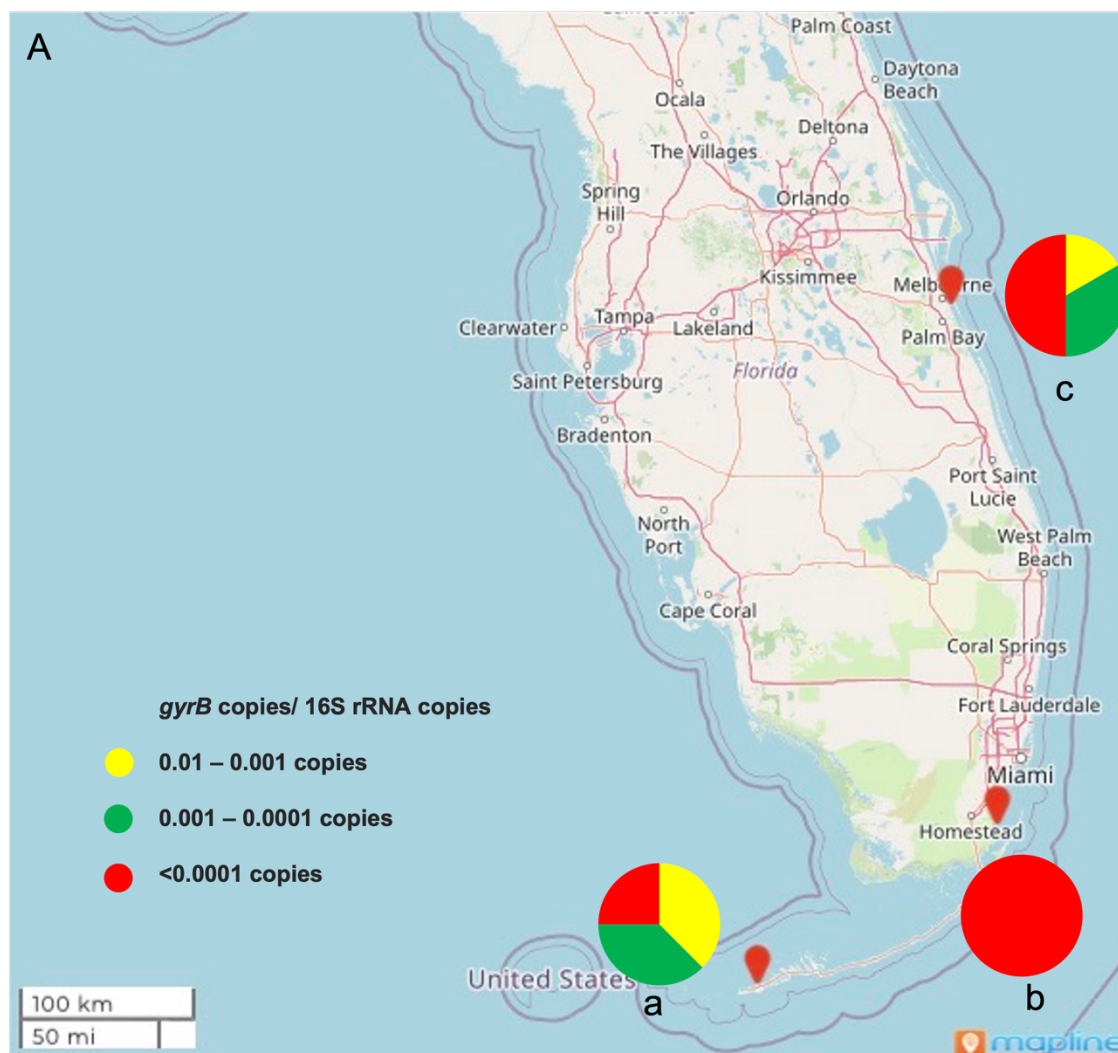


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350 Supplemental Figure 1: Selectivity of the qPCR assay. The real time PCR assay
 351 showed no amplification of the *gyrB* gene in six common marine bacterial species. The
 352 red line shows the amplification of the *gyrB* gene using genomic DNA of *A. macleodii*
 353 CUKW as a template.

354 3. *Quantification of environmental samples:* The assay was applied to samples from
 355 various marine habitats to screen for the presence of *A. macleodii*. DNA extracted from
 356 54 environmental samples was quantified for the abundance of *A. macleodii*.
 357 Environmental samples were collected from coastal areas, including temperate and
 358 tropical latitudes, primarily in the western Atlantic. *A. macleodii* was detected in nearly
 359 all samples (50 of 54) (Table 3A and 3B). The relative abundance of *A. macleodii* was
 360 calculated as a ratio of *A. macleodii gyrB* gene copies to the bacterial 16S rRNA gene
 361 copies. The standard curve for the 16S rRNA gene is shown in Supplemental Figure 2.

The relative abundance varied from 2.8E-06 to 1.17E-01 copies per 16S rRNA gene copy. The distribution of the *gyrB* gene in the environmental samples collected from various places is shown in Figure 4. *A. macleodii* was also detected in the dinoflagellates *Karlodinium* and *Pyrodinium* cultures collected from seawater (Table 3B). Previous studies have shown that *Alteromonas* is found in abundance with phytoplankton blooms in the presence of organic matter (Tada et al., 2011). The environmental samples that were quantified as less than 100 *gyrB* gene copies were further verified to be *A. macleodii* by sequencing the resulting qPCR product.



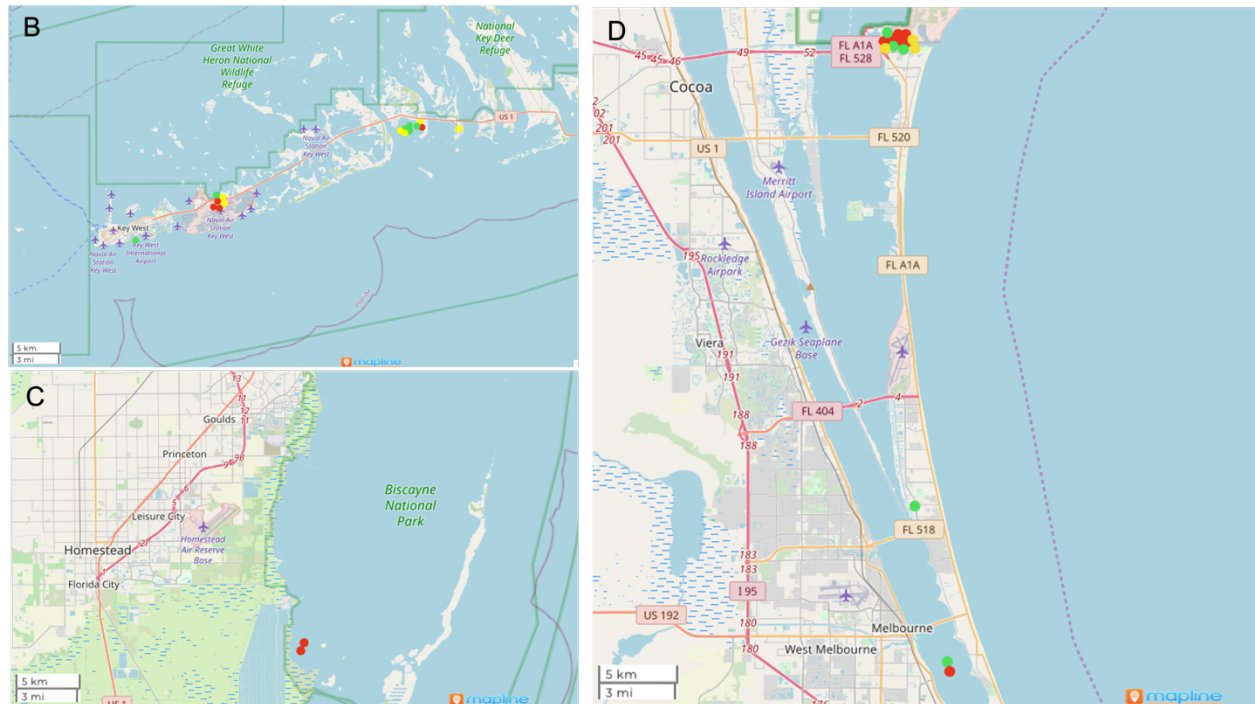


Figure 4: Overview of geographic location. A. The map shows profiling of the distribution of *A. macleodii gyrB* gene copies as pie charts in tropical marine sites: (a) Key West, (b) Miami, (c) Palm Bay. B, C, and D show the detailed locations from respective sites zoomed in respective panels to show the total *gyrB* gene count per 16S rRNA gene copy. Of 54 marine habitats tested, *A. macleodii* was identified in 50 habitats. The colored circles represent a ubiquitous distribution of *A. macleodii*. The map was generated in mapline (<https://mapline.com/>) using GPS coordinates of the sampling location and annotated with Inkscape software (<https://inkscape.org/>).

382 Table 3A: Quantification of *A. macleodii gyrB* gene from environmental biomass
 383 samples (ND: Not Detected) n/a (not applicable)

Sample Name	GPS coordinates	<i>gyrB</i> copies/16S copies
Telemar Bay Ship Surface	n/a	ND
Orange Sponge Cudjoe Bay	24.65, -81.49	8.32E-04
Orange Sponge Cudjoe Bay	24.65, -81.49	5.24E-04
Anne Bonny Canal Boat	24.66, -81.48	2.25E-03
KW Marina A-LI Wood Pileag	24.58, -81.69	2.20E-05
Anne Bonny Canal Metal Ladder	24.66, -81.48	1.07E-04
Mangrove	25.40, -80.32	7.21E-05
KW Marina Boat Surface A-LI Fiberglass	24.58, -81.69	6.87E-05
Lawrence A-LI Sailboat	24.65, -81.44	4.36E-03
Keywest biomass	24.58, -81.69	5.61E-06
Cudjoe Bay Personal vessel	24.65, -81.49	1.18E-04
Anne Bonny Canal PVC Pipe	24.66, -81.48	2.64E-05
Lawrence A-LI Sailboat	24.65, -81.44	3.71E-03
Tarpon Marina KW Boat	24.58, -81.69	2.56E-03
Tarpon Marina KW Boat	24.58, -81.69	5.06E-03
Mangrove	25.40, -80.32	7.85E-05
Tarpon Marina A-LI Boat	Not available	2.27E-04
Tarpon Marina A-LI Boat	Not available	3.26E-04
Cape Canaveral FIT Biocorrosion Test Site Panel (proprietary)	28.41, -80.61	1.58E-05
Cape Canaveral FIT Biocorrosion Test Site Panel (proprietary)	28.41, -80.61	4.66E-06
Cape Canaveral FIT Biocorrosion Test Site Panel (proprietary)	28.41, -80.61	2.86E-06
Cape Canaveral FIT Biocorrosion Test Site Panel 1: BRA 640 I	28.41, -80.61	5.14E-06
Cape Canaveral FIT Biocorrosion Test Site Panel 1: BRA 640 II	28.41, -80.61	2.90E-06
Cape Canaveral FIT Biocorrosion Test Site Panel 1: BRA 640 III	28.41, -80.61	7.75E-06
Cape Canaveral FIT Biocorrosion Test Site Panel 1: Inter900 (Cu-free)	28.41, -80.61	3.48E-05
Cape Canaveral FIT Biocorrosion Test Site Panel 2: BRA 1 I	28.41, -80.61	2.02E-03
Cape Canaveral FIT Biocorrosion Test Site Panel 2: BRA 1II	28.41, -80.61	9.93E-04
Cape Canaveral FIT Biocorrosion Test Site Panel 2: Nature Coat white panel	28.41, -80.61	4.32E-04
Cape Canaveral FIT Biocorrosion Test Site Panel 2: green panel	28.41, -80.61	5.58E-04
Cape Canaveral FIT Biocorrosion Test Site Panel 2: Epoxy panel	28.41, -80.61	1.30E-03
Cape Canaveral FIT Biocorrosion Test Site Panel 2: unknown	28.41, -80.61	1.12E-03
Harbortown Marine, recreational vessel	30.49, -80.93	6.01E-06
Indian River sediment	28.06, -80.58	1.21E-04
Indian River algae from mangrove	28.06, -80.58	1.64E-05
Indian River mangrove	n/a	ND

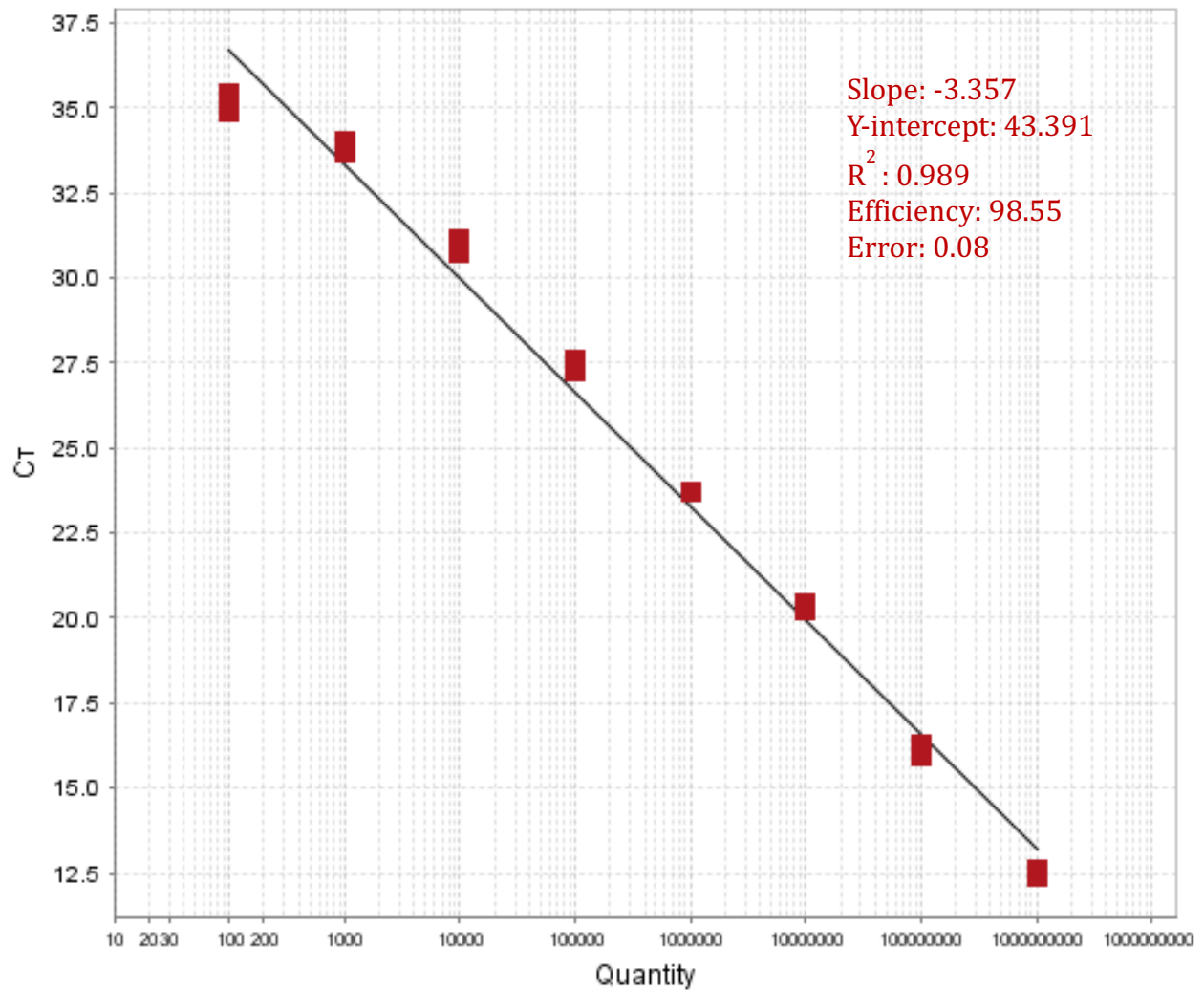
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385 Table 3B: Quantification of *A. macleodii gyrB* gene from environmental water samples
 386 and laboratory cultures (ND: Not Detected) n/a (not applicable)

Sample Name	GPS coordinates	<i>gyrB</i> copies/16S copies
Telemar Bay Ship surface water	28.15, -80.60	3.05E-04
Smathers Beach	24.55, -81.77	4.21E-04
Cudjoe Bay water	n/a	ND
Orange Sponge Cudjoe Bay water	24.65, -81.49	1.17E-01
KW Marina Boat Surface A-LI Fiberglass water	24.58, -81.69	8.56E-04
Telemar Bay surface water	28.15, -80.60	1.22E-04
Baltimore Harbor Surface water	n/a	ND
Cape Canaveral FIT Biocorrosion Test Site Panel (proprietary) coastal water	28.41, -80.61	3.98E-05
<i>Karlodinium dinoflagellate</i> cultures 1	n/a	7.32E-04
<i>Karlodinium dinoflagellate</i> cultures 2	n/a	1.80E-04
<i>Karlodinium dinoflagellate</i> cultures 3	n/a	5.53E-05
<i>Karlodinium dinoflagellate</i> cultures 4	n/a	1.19E-04
<i>Karlodinium dinoflagellate</i> cultures 5	n/a	5.32E-04
<i>Karlodinium dinoflagellate</i> cultures 6	n/a	3.13E-05
<i>Karlodinium dinoflagellate</i> cultures 7	n/a	1.91E-05
<i>Pyrodinium bahamense</i> grown with 0.5 mM Cu (culture 1)	n/a	1.24E-04
<i>Pyrodinium</i> laboratory culture	n/a	1.33E-04
<i>Pyrodinium bahamense</i> grown with 0.5 mM Cu (culture 1)	n/a	3.68E-04
<i>Pyrodinium</i> laboratory culture	n/a	2.69E-04

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390 Supplemental Figure 2: Standard curve with serial dilutions of *A. macleodii* 16S rRNA

391 gene equivalent to 1.0E+09 to 1.0E+01 full-length 16S rRNA gene fragment. The curve

392 is made from the cycle threshold of triplicates corresponding to the 16S rRNA copies.

393 The slope, Y-intercept, R^2 , Efficiency, and error is shown in the figure.

394 4. Quantification of *A. macleodii* in the biofilm and planktonic laboratory cultures. The

395 assay was applied to laboratory cultures of *A. macleodii* CUKW grown under elevated

396 copper conditions to enumerate total *A. macleodii* in the biofilm and planktonic cells

397 over time. A pellicle biofilm was observed in the tube after 16 hours of exposure to

copper under static growth. Biofilm and planktonic phases from individual tubes were collected in triplicates at 19 hours, 27 hours, and 42 hours. The *gyrB* copies in the biofilm and planktonic cells were normalized to the total volume of the culture. To evaluate the qPCR assay performance, biofilm and planktonic cultures collected from three biological replicates at the same time points were also quantified with the plate count method. Table 4 shows the total cell abundance in the biofilm and planktonic cells, quantified using the two methods (qPCR assay and CFU counts). A linear correlation was observed between quantifications done by gene-based assay and CFU counts (in both biofilm and planktonic cells). Pearson's correlation performed between quantification methods (culture-based and qPCR assays) showed a strong correlation with Pearson's correlation coefficient $r = 0.89$ and $p\text{-value} = 0.008$ (Figure 5).

The change in the total *gyrB* gene copies over time in the biofilm and the planktonic culture was observed. Similar changes in cfu/ml were observed over time in the biofilm and the planktonic culture. The statistical similarity between the means of the two quantification methods could not be validated for some groups (Figure 6). This could be due to the limitation of accuracy of the plate count method (refer to the discussion). Moreover, a Pearson's correlation coefficient of 0.89 ($p\text{-value} = 0.008$) indicates that the qPCR quantification results positively correlate to the CFU quantification results (Figure 5). Similarly, the differences between the mean *gyrB* gene copies obtained at three-time points were statistically validated using the independent sample t-test (Figure 6).

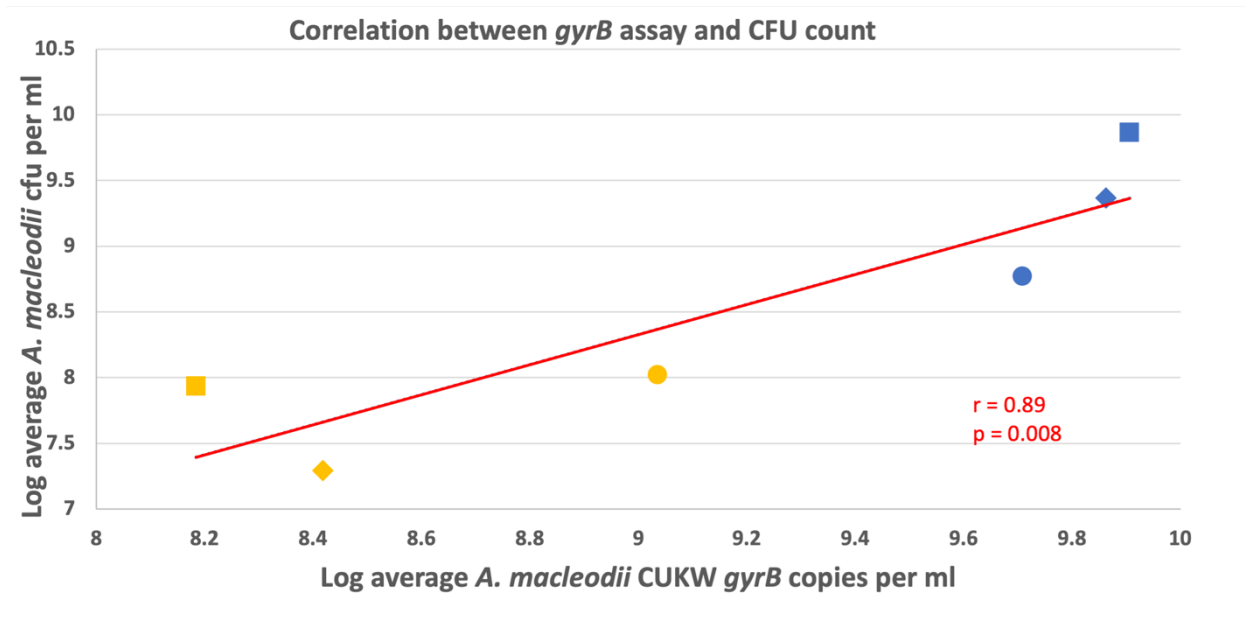


Figure 5: Correlation of quantification methods: The scatter plot of total *A. macleodii* quantified by gene-based qPCR and culture-based plate count in the biofilm and planktonic cells. X-axis shows *A. macleodii gyrB* copies per ml, and y-axis shows *A. macleodii* cfu per ml. The correlation between the two quantification methods was assessed using Pearson's correlation. Quantification data from biofilm is shown in blue, and that from planktonic cells is shown in orange. Circles show data from 19 hours, diamonds show data from 27 hours, and squares data from 42 hours. The linear fittings of data from two quantification techniques show Pearson's correlation coefficient of 0.89 and a p-value of 0.008.

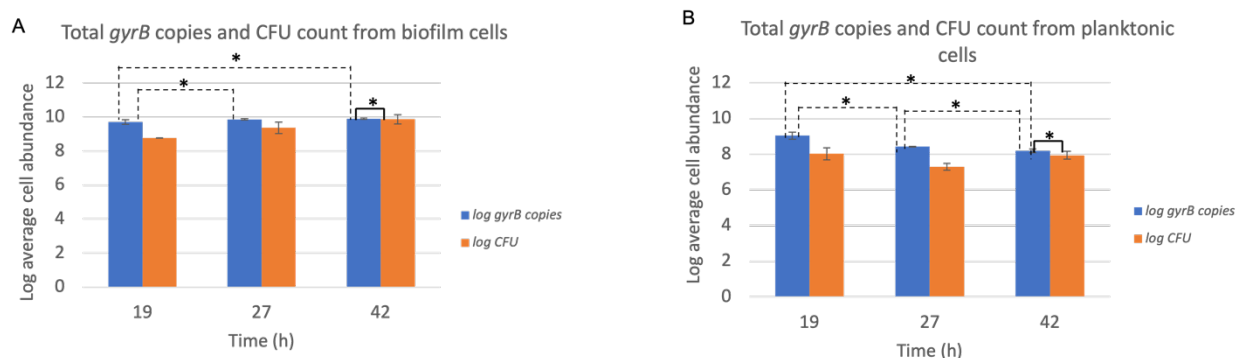


Figure 6: Comparison between *A. macleodii gyrB* gene copies and CFU counts from biofilm and planktonic cells. Bar graph comparing the total *gyrB* gene/ml and total cfu/ml of *A. macleodii* over time from the biofilm (A) and planktonic cells (B). The results are shown as log-transformed mean values of the replicates collected at 19 hours, 27 hours, and 42 hours. The statistical significance of the similarity between qPCR and plate count measurements is indicated by * and a straight-line ($p > 0.05$). The statistical significance of the differences in *gyrB* quantities among three-time points is indicated by * and a dotted line ($p < 0.05$).

Table 4: Quantification of *gyrB* gene from the biofilm and planktonic cells

Sample Name	<i>gyrB</i> copies/ml	cfu/ml
Biofilm -19 hours	9.73	8.77
Biofilm -27 hours	9.86	9.37
Biofilm -42 hours	9.91	9.87
Planktonic -19 hours	9.04	8.02
Planktonic -27 hours	8.42	7.29
Planktonic -42 hours	8.18	7.93

Discussion

A. macleodii is typically found in surface seawater where it contributes to marine biogeochemical cycling. Its accurate quantification aids in understanding the ecology of the species and its contributions within various marine ecosystems. This study developed an assay to detect and quantify *A. macleodii* in marine environments. As a quantitative measure, the popular cell culture technique has its limitations, as some marine bacteria are difficult to grow (Giovannoni & Stingl, 2005). Similarly, the “gold standard” plate count method is influenced by factors such as dispersal of cells in a liquid medium, clumping of the bacterial cells, and analyst error for the result

interpretation (Silvestri et al., 2017). Other methods of bacterial quantifications, like microscopic count have limitations of the large limit of detection and require a dense population to be accurately measured (Bedrossian et al., 2017). The qPCR method accurately quantifies *A. macleodii* from biofilm (multispecies environmental biofilm and single-species biofilm from the laboratory). Other quantification methods may generate an inaccurate result as the biofilm is difficult to disperse evenly in solution, influencing quantification methods like cell culture and flow cytometry.

This assay detected *A. macleodii* from both open water and different substrate-associated environments, including marine ship surfaces, marine vegetation and organisms, and wood pilings, as shown in Table 3A, 3B. *A. macleodii* is ubiquitous in temperate marine surface waters (Ivars-Martinez et al., 2008) and occurs in different marine environments. However, they are typically found in low abundance in most of these environments. *Alteromonas* are known to be r-specialist which include organisms that can reproduce very fast when found in nutrient-rich micro niches (Lopez-Perez et al., 2012). Its lower abundance in water samples might be due to its inability to compete efficiently with other microbes in the low-nutrient water environment. However, when organic matter was available in abundance, they were found in higher density, as in the case of biomass from the Tarpon marina boat sample (Table 3A). It should also be considered that the relative abundance of the *gyrB* gene from environmental samples presented here is lower than the actual abundance as they are normalized by the 16S rRNA gene, which is found at a different copy number than a single copy *gyrB* gene per bacterial genome.

In this study, we also monitored the biofilm formation by *A. macleodii* in the laboratory and quantified its *gyrB* gene number over time in the biofilm and planktonic cultures using our newly developed assay. The observed differences in the measures between the gene-based assay and CFU technique are due to the limitations of the methods, such as the inability to disperse the biofilm efficiently for plating, technical error, and DNA extraction bias between different extractions kits.

Conclusion

Overall, the qPCR assay developed in this study provides a robust and sensitive method of detecting and quantifying *A. macleodii* from planktonic and substratum-associated marine environments. It circumvents the need for culture-based methods of enumeration as well as expensive equipment such as flow cytometers. This assay should provide insight into the ecological role(s) of *A. macleodii* within varied marine ecosystems and aid in studies related to surface colonization and biofilm formation in high copper environments.

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494 Supplemental Table1: Amount of the biofilm collected from various locations

Source	Weight (g)
Telemark Bay Ship Surface	0.2048
Orange Sponge Cudjoe Bay	0.45
KW Marina A-LI Wood Pileag	0.2004
Anne Bonny Canal Metal Ladder	0.2006
Mangrove	0.2023
KW Marina Boat Surface A-LI Fiberglass	0.1695
Lawrence A-LI Sailboat	0.1589
Key West Biomass	0.2194
Cudjoe Bay Personal vessel	0.2034
Anne Bonny Canal PVC Pipe	0.2077
Lawrence A-LI Sailboat	0.1948
Tarpon Marina KW Boat	0.2055
Mangrove	0.2346
Tarpon Marina A-LI Boat	0.2012
Cape Canaveral FIT Biocorrosion Test Site Panel (proprietary)	0.2
Cape Canaveral FIT Biocorrosion Test Site Panel (proprietary)	0.037
Cape Canaveral FIT Biocorrosion Test Site Panel (proprietary)	0.109
Cape Canaveral FIT Biocorrosion Test Site Panel 1: BRA 640 I	0.176
Cape Canaveral FIT Biocorrosion Test Site Panel 1: BRA 640 II	0.2099
Cape Canaveral FIT Biocorrosion Test Site Panel 1: BRA 640 III	0.1661
Cape Canaveral FIT Biocorrosion Test Site Panel 1: Inter900 (Cu-free)	0.1077
Cape Canaveral FIT Biocorrosion Test Site Panel 2: BRA 1 I	0.0886
Cape Canaveral FIT Biocorrosion Test Site Panel 2: BRA 1 II	0.1348
Cape Canaveral FIT Biocorrosion Test Site Panel 2: Nature Coat white panel	0.003
Cape Canaveral FIT Biocorrosion Test Site Panel 2: green panel	0.0908
Cape Canaveral FIT Biocorrosion Test Site Panel 2: Epoxy panel	0.0499
Cape Canaveral FIT Biocorrosion Test Site Panel 2: unknown	0.0026
Harbortown Marine, recreational vessel	0.2091
Indian River sediment	0.2023
Indian River algae from mangrove	0.2081
Indian River mangrove	0.2009

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