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► To cite this version:

François Thomas, Simon Dittami, Maéva Brunet, Nolwen Le Duff, Gwenn Tanguy, et al.. Evaluation of a new primer combination to minimize plastid contamination in 16S rDNA metabarcoding analyses of alga-associated bacterial communities. *Environmental Microbiology Reports*, 2019, 12 (1), pp.30-37. 10.1111/1758-2229.12806 . hal-02404303

HAL Id: hal-02404303

<https://hal.sorbonne-universite.fr/hal-02404303>

Submitted on 1 Dec 2020

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Evaluation of a new primer combination to minimize plastid contamination in 16S rDNA metabarcoding analyses of alga-associated bacterial communities

Journal:	<i>Environmental Microbiology and Environmental Microbiology Reports</i>
Manuscript ID	EMIR-2019-1373.R1
Journal:	Environmental Microbiology Reports
Manuscript Type:	EMIR - Brief report
Date Submitted by the Author:	n/a
Complete List of Authors:	Thomas, François; Station Biologique de Roscoff, UMR8227 (CNRS-Sorbonne Université) Dittami, Simon; Station Biologique de Roscoff, UMR8227 (CNRS-Sorbonne Université) Brunet, Maéva; Station Biologique de Roscoff, UMR8227 (CNRS-Sorbonne Université) Le Duff, Nolwen; Station Biologique de Roscoff, UMR8227 (CNRS-Sorbonne Université) Tanguy, Gwenn; Station Biologique de Roscoff, FR2424, Genomer sequencing platform Leblanc, Catherine; Station Biologique de Roscoff, LBI2M, UMR8227, CNRS-UPMC Gobet , Angélique ; MARBEC
Keywords:	chloroplast, plastid, 16S rDNA, alga-associated bacteria, metabarcoding, V34 primer, algal holobiont

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Manuscripts

1 **Evaluation of a new primer combination to minimize plastid contamination**
2 **in 16S rDNA metabarcoding analyses of alga-associated bacterial**
3 **communities**

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32 **Keywords**

33 Chloroplast, plastid, 16S rDNA, alga-associated bacteria, metabarcoding, V34 primer, algal
34 holobiont

35

36

37 **Running title:** Avoiding plastids in metabarcoding of algal microbiota

38 **Originality-Significance statement**

- 39 • Our aim was to minimize the amplification of plastidial 16S rDNA when
40 analyzing alga-associated bacterial communities using metabarcoding.
- 41 • We compared the predicted coverage of five primer combinations *in*
42 *silico*, including a universal primer set and four new sets designed to
43 avoid amplification from plastids.
- 44 • Sequencing of a synthetic community showed similar performances for
45 the universal primer set and one new combination.
- 46 • On natural alga-associated microbiota, the relative abundance of plastid
47 sequences was reduced by 99-100% with the new primer set while the
48 recovered bacterial richness was higher than with the universal primers
49 given identical sequencing efforts and the community structure was
50 maintained.

51

52 **Summary**

53 Plant- and alga-associated bacterial communities are generally described via 16S rDNA
54 metabarcoding using universal primers. As plastid genomes encode 16S rDNA related to
55 cyanobacteria, these datasets frequently contain >90% plastidial sequences, and the bacterial
56 diversity may be under-sampled. To overcome this limitation we evaluated *in silico* the
57 taxonomic coverage for four primer combinations targeting the 16S rDNA V3-V4 region.
58 They included a forward primer universal to *Bacteria* (S-D-Bact-0341-b-S-17) and four
59 reverse primers designed to avoid plastid DNA amplification. The best primer combination

60 (NOCHL) was compared to the universal primer set in the wet lab using a synthetic
61 community and samples from 3 macroalgal species. The proportion of plastid sequences was
62 reduced by 99-100% with the NOCHL primers compared to the universal primers,
63 irrespective of algal hosts, sample collection and extraction protocols. Additionally, the
64 NOCHL primers yielded a higher richness while maintaining the community structure. As
65 *Planctomycetes*, *Verrucomicrobia*, and *Cyanobacteria* were underrepresented (70-90%)
66 compared to universal primers, combining the NOCHL set with taxon-specific primers may
67 be useful for a complete description of the alga-associated bacterial diversity. The NOCHL
68 primers represent an innovation to study algal holobionts without amplifying host plastid
69 sequences and may further be applied on other photosynthetic hosts.

70

71 **Introduction**

72 Numerous studies on algae-associated bacteria have highlighted their importance for health
73 and physiology of the algal holobiont as well as for biomass degradation (Barott *et al.*, 2011;
74 Egan *et al.*, 2013, 2014; Singh and Reddy, 2016). Bacteria can be abundant on macroalgal
75 surfaces, ranging from 10^6 to 10^7 cells per cm^2 , and encompass a large phylogenetic diversity
76 (van der Loos *et al.*, 2019). These communities are distinct from bacterial assemblages found
77 in the surrounding seawater or on inert surfaces (Burke *et al.*, 2011; Stratil *et al.*, 2014;
78 Lemay *et al.*, 2018). The composition of algae-associated bacterial communities has been
79 shown to be host-specific and to vary depending on algal tissue, seasons, sampling sites and
80 physiological status of the algae (Staufenberger *et al.*, 2008; Lachnit *et al.*, 2011; Miranda *et*
81 *al.*, 2013; Stratil *et al.*, 2013; Zozaya-Valdes *et al.*, 2015; Aires *et al.*, 2016; Paix *et al.*, 2019).
82 A common approach to characterize these algae-associated communities is metabarcoding,
83 whereby a selected variable region of the 16S rDNA is sequenced using a primer set universal
84 to *Bacteria* (e.g., forward: S-D-Bact-0341-b-S-17 / reverse: S-D-Bact-0785-a-A-21

85 (Klindworth et al, 2013)) Due to the classical sampling strategies for macroalgae-associated
86 microbiomes (e.g. algal surface scraping or tissue grinding (Bengtsson *et al.*, 2012; Aires *et*
87 *al.*, 2016)), one major issue is that bacterial DNA samples are often contaminated with algal
88 DNA. Primers with a large taxonomic coverage for *Bacteria* will tend to also amplify 16S
89 rDNA sequences from plastids. This is because plastid genomes, deriving from
90 endosymbiosis events, still encode 16S rDNA closely related to their cyanobacterial
91 ancestors. Datasets obtained from brown algal field samples using common universal primers
92 may contain over 90% of sequences affiliated to plastid DNA (Leblanc, personal data). This
93 high proportion of plastidial sequences decreases the sequencing depth for bacterial epibionts
94 and reduces the power of diversity analyses. One strategy to allow a large coverage of
95 *Bacteria* while minimizing 16S gene amplification from plastids is to design a primer in a
96 region that differs between plastid sequences and those of *Bacteria*. Chelius and Triplett have
97 identified such a region between the positions 783 and 799 of the 16S rDNA (following the *E.*
98 *coli* numbering system) and they designed the 799F primer which includes four mismatches
99 with chloroplasts to amplify *Bacteria* while avoiding chloroplast amplification from maize
100 roots (Chelius and Triplett, 2001). The resulting primer was then used in combination with
101 primer 1193R (amplification product 394 bp) in several studies on bacterial communities
102 associated with plants (Sagaram *et al.*, 2009; Bodenhausen *et al.*, 2013) or macroalgae (Vieira
103 *et al.*, 2016; Aires *et al.*, 2018; Serebryakova *et al.*, 2018). However, this primer set still
104 yielded up to 38% of sequences affiliated to chloroplasts (Sagaram *et al.*, 2009). Several sets
105 of primers including the 799F primer with or without modifications were subsequently
106 designed and tested to minimize plastid contamination on samples from plants and algae
107 (Hanshew *et al.*, 2013; Miranda *et al.*, 2013; Aires *et al.*, 2016). These primer sets target the
108 V5-V8 or V5-V9 regions, yielding products of ca. 590 bp or 750 bp, respectively. Although
109 suitable for the 454 pyrosequencing technology used in the latter studies, this fragment length

110 is not compatible with the current Illumina v3 sequencing chemistry, which produces paired-
111 end reads of 2×300 bp. Allowing for a recommended overlap of at least 50 bp, the total
112 product length for this technology should not exceed 500 bp. Our objective was to evaluate
113 primer combinations matching these criteria while minimizing plastid contamination in 16S
114 rDNA metabarcoding analyses by: (i) comparing the efficiency of a primer set universal to
115 *Bacteria* and that of four primer combinations avoiding plastid amplification *in silico*, (ii)
116 comparing the efficiency of the best primer combination from the *in silico* results with that of
117 the universal primer set in the wet lab.

118

119 **Results and Discussion**

120 *In silico* evaluation of primer combinations to minimize the amplification of plastid 121 sequences

122 We evaluated several sets of primers to amplify a 450 bp fragment spanning the V3 and V4
123 regions of the 16S rDNA while minimizing amplification of plastid sequences (Table S1).
124 These primer sets were designed by combining the universal forward primer S-D-Bact-0341-
125 b-S-17 (Klindworth *et al.*, 2013) with reverse primers corresponding to the reverse
126 complement of four V5-V8 forward primers previously used in 454 pyrosequencing to
127 minimize chloroplast contamination (Hanshew *et al.*, 2013). The four new primer
128 combinations (*E. coli* position 341 to 785) covers most of the region amplified by the original
129 V34 set (*E. coli* position 341 to 799), ensuring the comparability of data. The performance of
130 these different primer combinations was tested by running an *in silico* PCR on the SILVA
131 database ssu-132 with the RefNR sequence collection, using the online tool SILVA TestPrime
132 1.0 (Klindworth *et al.*, 2013). The universal V34 combination with the original reverse primer
133 S-D-Bact-0785-a-A-21 had an *in silico* predicted coverage for plastids (Table 1) ranging from
134 57.3% to 88.3% with zero or 2 allowed mismatches, respectively. By contrast, three of the

135 four new combinations (NOCHL, NOCHL3, and NOCHL6 but not NOCHL7, Table S1)
136 showed consistently low predicted coverage for plastids, even with 2 allowed mismatches
137 (2.7%, 1.1% and 1.1%, respectively). The overall coverage of these three primer
138 combinations for all bacterial sequences remained high (79% with no mismatch, 85-90% with
139 two mismatches). However, the NOCHL3 and NOCHL6 primer sets were predicted to
140 perform poorly on a number of phyla, including *Planctomycetes* (12% coverage with two
141 mismatches) and *Verrucomicrobia* (17% coverage), which are known to be part of alga-
142 associated microbial communities (Bengtsson and Øvreås, 2010; Lage and Bondoso, 2014;
143 Vollmers *et al.*, 2017). For all taxa, the NOCHL combination had equal or better coverage
144 than the NOCHL3 and NOCHL6 combinations, notably for *Planctomycetes* (82.3%) and
145 *Verrucomicrobia* (48.7%). The NOCHL combination was therefore considered the most
146 promising candidate to minimize plastid contamination while maintaining the overall
147 bacterial diversity, and chosen to prepare Illumina-sequencing libraries.

148

149 ***Comparison of primer performances in the wet lab***

150 Performances of the NOCHL and V34 primer combinations were compared *in vitro* using
151 different types of samples. As a positive control for the metabarcoding experiment, we
152 constructed a mock community based on the genomic DNA of pure bacterial isolates.
153 Genomic DNA was extracted from a total of 32 sequenced bacterial strains (Table S2) and
154 mixed in known proportions to assemble the mock community. Furthermore, the microbiota
155 associated with three brown algal species was sampled according to different approaches. The
156 filamentous brown alga *Ectocarpus subulatus* samples were collected 5 km North of the
157 Hopkins River Falls (Australia) and whole tissues were ground before DNA extraction. Blade
158 samples of *Laminaria hyperborea* sporophytes were collected at the coast off Roscoff
159 (Brittany, France) and soaked in a lysis buffer for DNA extraction. The blades of *L. digitata*

160 sporophytes from Roscoff were swabbed for DNA extraction. All samplings were performed
161 in triplicate. Details on sampling and DNA extraction protocols are available in
162 Supplementary Information. Amplicon and library preparation were carried out following the
163 standard Illumina protocol (Illumina web page, 2018), including several negative controls, as
164 detailed in Supplementary Information. Both the NOCHL and V34 primer pairs successfully
165 amplified a ca. 450-bp fragment covering a similar region (V3-V4) of the bacterial 16S
166 rDNA. Sequencing libraries were prepared in parallel for each sample (nine algal samples,
167 one mock community, and six negative controls) using each primer pair. Sequencing was
168 carried out using one run of an Illumina Miseq yielding a total of 5,549,008 read pairs.
169 Sequences were deposited at the ENA under project accession number PRJEB33453. After
170 quality checks and removal of exogenous contaminations, low-quality sequences, and blanks
171 (see Supplementary Information), 2,784,253 assembled reads remained for data analysis.
172 Sequences were clustered, assigned to operational taxonomic units (OTUs) at 97% identity,
173 taxonomically classified with RDP classifier (Wang *et al.*, 2007) on the Silva SSU database
174 release 132 (Quast *et al.*, 2013), and filtered to remove rare OTUs (see details in
175 Supplementary Information). The final dataset comprised 3,009 OTUs.
176 First, we compared metabarcoding results from the primer combinations V34 and NOCHL on
177 the mock community. The sequencing error rate calculated as in Kozich *et al.* (2013) from the
178 mock community datasets was 0.54% for both the V34 and NOCHL libraries. A total of 65
179 OTUs were detected in the rarefied mock community datasets. The expected relative
180 proportion p_i of 16S rDNAs from strain i was calculated as follows:

181

182

183

$$p_i = \frac{\frac{q_i}{M_i} \times c_i}{\sum_{i=1}^{32} \frac{q_i}{M_i} \times c_i}$$

184

185 where q_i is the mass of DNA from strain i added in the MOCK community, M_i is the
 186 molecular weight of the complete genome for strain i and c_i is the number of 16S rDNA gene
 187 copies in the genome of strain i . At the genus level, poor correlations (Pearson coefficient
 188 <0.4) were obtained between the expected 16S rDNA proportions in the mock community
 189 and the relative abundance of taxa found in V34 or NOCHL libraries (Table 2). From the 26
 190 genera represented in the mock community, 18 and 20 were detected in the V34 and NOCHL
 191 datasets, respectively. Both primer combinations did not detect the 6 genera *Nonlabens*,
 192 *Agrococcus*, *Arthrobacter*, *Dokdonia*, *Roseovarius*, and *Imperialibacter*. Sequences from
 193 these genera might either not be amplified by the primer sets, or more likely be merged to
 194 closely related sequences during OTU clustering. In addition, *Roseobacter* and *Hoeflea* were
 195 missed by the V34 primer combination, whereas their relative abundance was close to the
 196 expected value in the NOCHL dataset. With both primer pairs, ca. 30% of sequences could
 197 not be classified down to the genus level, leading to discrepancies between expected and
 198 measured proportions. A striking example is *Vibrio*: its expected abundance of 24.63% was
 199 largely underestimated at ca. 8% with both primer pairs, whereas 11% of sequences were
 200 assigned to unclassified *Vibrionaceae*. On the contrary, both primer combinations largely
 201 overestimated the proportion of *Pseudoalteromonas*, possibly due to the proximity with
 202 sequences from *Alteromonas* (Gauthier *et al.*, 1995). Although the NOCHL combination had
 203 a suboptimal predicted coverage for *Planctomycetes* (Table 1), it still detected the
 204 planctomycetal genus *Rhodopirellula* in the mock community, underestimating it by 56%. On
 205 the other hand, the V34 combination overestimated *Rhodopirellula* by 47%. As expected,
 206 correlations between theoretical and measured proportions were much better (Pearson
 207 coefficient > 0.9) at the family level for both primer combinations (Table S3). All 13 families

208 represented in the mock community were detected using the NOCHL combination, whereas
209 the V34 combination missed the *Phyllobacteriaceae* family. Altogether, the performance of
210 the NOCHL combination was found to be comparable to the V34 set on the mock community
211 in terms of sensitivity and accuracy.

212
213 We further compared the efficiency of the primer combinations V34 and NOCHL in field
214 samples, characterizing the bacterial community associated with *Ectocarpus subulatus*
215 (ECTO), *Laminaria digitata* (LDIG) and *Laminaria hyperborea* (LHYP). The relative
216 abundance of sequences affiliated to plastids ranged from 0.6% to 66% in the V34 dataset
217 (Figure 1). The highest relative abundances of plastid sequences were found when algal
218 tissues were ground (ECTO) or soaked in lysis buffer (LHYP) before DNA extraction,
219 compared to the swab-based technique (LDIG). It was drastically reduced by 99-100% with
220 the NOCHL combination in all three types of alga-associated samples analyzed (Figure 1),
221 with relative abundance of plastid 16S rDNA sequences ranging from 0 to 0.3%. This
222 confirms that the new primer combination succeeds at minimizing plastid contamination,
223 irrespective of the algal host, sample collection and DNA extraction protocol. The NOCHL
224 combination was more efficient at reducing plastid contamination compared to a previous
225 Illumina metabarcoding analysis of *Ectocarpus*-associated bacterial communities. In that
226 former study, a modified set of 341F and 806R primers still yielded 32% of plastid sequences
227 despite a central mismatch to avoid plastid DNA amplification (Dittami *et al.*, 2016).

228 Alpha-diversity analyses tended to show higher values of OTU richness (observed OTUs) and
229 diversity (Shannon and Simpson indices) with the NOCHL combination compared to V34
230 before removal of plastid sequences (Figure 2A). This overall effect was found significant for
231 Shannon and Simpson diversity indices and was more pronounced for samples from *E.*
232 *subulatus* and *L. hyperborea*, After removal of plastid sequences (Figure 2B), the NOCHL

233 primer combination yielded significantly higher richness than V34 (Student's t-test, $P=0.05$)
234 with an overall increase of 10%. Again, this effect was more pronounced for ECTO and
235 LHYP samples. Indeed, ECTO and LHYP samples, where plastid contamination was the
236 highest (Figure 1), showed the strongest effect of plastidial sequence removal on sequencing
237 depth (Figure S1). This exemplifies the issue faced with universal primers, where a large
238 amount of plastid-affiliated sequences can decrease the sequencing depth for target bacterial
239 sequences and reduce the evenness. After removal of plastid sequences, lower Shannon and
240 Simpson diversity indices were detected with the NOCHL combination compared with V34 (-
241 3.2% and -0.5% lower values for Shannon and Simpson, respectively). This was mostly due
242 to an increase in these indices for V34 datasets after removal of plastid sequences, while they
243 stayed stable for the NOCHL datasets. Although not the main focus of this work, we noticed
244 that bacterial communities retrieved from *Ectocarpus* were more diverse than those from the
245 two *Laminaria* species. This might be due to the nature of the samples as well as the
246 extraction protocol where entire algal specimens were ground for *Ectocarpus* (i.e. including
247 the endomicrobiota), while for *Laminaria* the extraction protocols only targeted surface-
248 attached bacteria. Joint hierarchical clustering of OTU-level datasets for both primer pairs
249 after removal of plastid sequences showed that samples grouped according to algal host rather
250 than primer pair (Figure 3A).

251 The relative abundance of phyla was generally similar between paired sets of samples (Figure
252 3B). Notable exceptions were the *Planctomycetes*, *Verrucomicrobia*, and *Cyanobacteria* that
253 had lower relative abundance in the NOCHL libraries compared to V34 (Table S4). We
254 further searched for differential OTUs between pairs of samples sequenced with the V34 or
255 NOCHL combination. This analysis was performed with the edgeR package (Robinson *et al.*,
256 2009) on the non-transformed dataset after removal of plastid sequences, and accounted for
257 the paired design of the study, *i.e.* the same samples were sequenced with the two primer

258 combinations. The significance threshold was set at $\alpha=5\%$ after Benjamini-Hochberg
259 correction for multiple testing. Out of the 2,852 OTUs remaining after discarding plastids, the
260 edgeR analysis detected 39 differential OTUs (i.e. 1.4%) between primer combinations (Table
261 S5). All differential OTUs were more abundant in the V34-amplified dataset compared to
262 NOCHL. The majority of them belonged to *Verrucomicrobiae* and *Planctomycetacia* (Figure
263 4), partly reflecting the lower predicted coverage of NOCHL for these taxa. The apparent
264 lower abundance of *Plantomycetacia* OTUs using the NOCHL primer set could also be in
265 part due to their over-estimation with the V34 combination, as shown in the mock community
266 analysis (Table 2). The highest fold-change was found for OTU00082 belonging to
267 *Cyanobacteria*. The lower abundance of *Cyanobacteria* was inevitable since plastids and
268 extant cyanobacteria share a common ancestor and therefore have homologous 16S rDNA
269 sequences (Giovannoni *et al.*, 1988; Delwiche, 1999).

270

271 **Conclusion**

272 Considering that plastid sequences can represent more than 90% of all sequences in one
273 sample when using universal primer sets, the bacterial diversity in a sample may be
274 underrepresented. The new primer set NOCHL is efficient to avoid amplifying plastid
275 sequences from an algal host while identifying a significantly higher bacterial richness than
276 with the universal primers V34, given identical sequencing efforts. This validates that fewer
277 plastid sequences in the samples lead to a larger access to bacterial sequences thus recovering
278 more of the bacterial diversity. However, as some bacterial groups may be underrepresented,
279 this primer set may be combined with taxon-specific primers (e.g. for *Planctomycetes*) for a
280 more complete coverage of *Bacteria*. Still, the data obtained with the NOCHL and universal
281 V34 primer sets are comparable since the targeted regions are the same and patterns of the
282 community structure are similar. Although validated here on macroalgal samples, this primer

283 set may be useful to characterize bacterial communities from other environments where
284 plastid contamination can be an issue such as terrestrial plants, microalgae, or gut microbiota
285 of herbivores.

286

287 **Acknowledgments and funding**

288 The authors have no conflict of interest to declare. We warmly thank Gaëlle Correc and
289 Sylvie Rousvoal for help with sampling as well as Florian de Bettignies and Dominique
290 Davoult for providing samples. We thank Frédérique Le Roux and Christian Jeanthon for
291 providing bacterial strains. This work has benefited from the facilities of the Genomer
292 platform and from the computational resources of the ABiMS bioinformatics platform (FR
293 2424, CNRS-Sorbonne Université, Roscoff), which are part of the Biogenouest core facility
294 network.

295 This work has benefited from the support of the French Government via the National
296 Research Agency investment expenditure program IDEALG (ANR-10-BTBR-04). FT, SD,
297 and CL acknowledge support by the Centre National de la Recherche Scientifique (CNRS).
298 AG acknowledges support by the Institut Français de Recherche pour l'Exploitation de la Mer
299 (IFREMER).

300

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405 known and potential bacterial pathogens. *Front Microbiol* **6**: 1–9.
- 406

408

409 **Table and Figure legends**

410

411 **Figure 1:** Relative abundance of sequences affiliated to plastid OTUs using the V34 or
412 NOCHL primer sets. Bacterial communities were analyzed in triplicate samples from
413 different algal hosts: ECTO, *Ectocarpus subulatus*; LHYP, *Laminaria hyperborea*; LDIG,
414 *Laminaria digitata*.

415

416 **Figure 2:** Richness and diversity estimates before (A) and after (B) removal of OTUs
417 affiliated to plastids. Data were subsampled at 8,358 sequences per sample prior to analysis.
418 Observed, observed OTU richness; Shannon, Shannon index; Simpson, Simpson index;
419 ECTO, *Ectocarpus subulatus*; LDIG, *Laminaria digitata*; LHYP, *Laminaria hyperborea*.
420 Results of paired Student's t-test testing the effect of the set of primers on each measured
421 estimate are reported below each panel (df=8).

422

423 **Figure 3:** Effect of the primer pair on the detected bacterial community composition, after
424 removal of OTUs affiliated to plastids.

425 (A) Hierarchical clustering calculated on Hellinger-transformed data based on Morisita-Horn
426 distance using the complete linkage algorithm

427 (B) Taxonomic composition based on the phylum level

428

429 **Figure 4:** Taxonomic affiliation of the 39 differential OTUs detected with edgeR, depending
430 on the primer pair. All of these OTUs received higher coverage with the V34 primers.

431 **Supplementary Figure 1:** Rarefaction analysis of datasets obtained with V34 and NOCHL
432 primer sets before (A) and after (B) removal of plastid-related sequences.

433

434 **Table 1:** *In silico* predicted coverage of selected bacterial taxa, relative to the sequences
435 available in the Silva SSU r132 database for different primer combinations using TestPrime
436 1.0. A length of 3 bases of 0-mismatch zone at 3' end of primers was chosen for tests with two
437 allowed mismatches in the sequence.

438

439 **Table 2:** Genus-level comparison of theoretical relative abundance in the mock community
440 with the measured abundance in libraries sequenced with V34 or NOCHL primers. unclass.,
441 unclassified.

442

1 **Evaluation of a new primer combination to minimize plastid contamination**
2 **in 16S rDNA metabarcoding analyses of alga-associated bacterial**
3 **communities**

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32 **Keywords**

33 Chloroplast, plastid, 16S rDNA, alga-associated bacteria, metabarcoding, V34 primer, algal
34 holobiont

35

36

37 **Running title:** Avoiding plastids in metabarcoding of algal microbiota

38 **Originality-Significance statement**

- 39 • Our aim was to minimize the amplification of plastidial 16S rDNA when
40 analyzing alga-associated bacterial communities using metabarcoding.
- 41 • We compared the predicted coverage of five primer combinations *in*
42 *silico*, including a universal primer set and four new sets designed to
43 avoid amplification from plastids.
- 44 • Sequencing of a synthetic community showed similar performances for
45 the universal primer set and one new combination.
- 46 • On natural alga-associated microbiota, the relative abundance of plastid
47 sequences was reduced by 99-100% with the new primer set while the
48 recovered bacterial richness was higher than with the universal primers
49 given identical sequencing efforts and the community structure was
50 maintained.

51

52 **Summary**

53 Plant- and alga-associated bacterial communities are generally described via 16S rDNA
54 metabarcoding using universal primers. As plastid genomes encode 16S rDNA related to
55 cyanobacteria, these datasets frequently contain >90% plastidial sequences, and the bacterial
56 diversity may be under-sampled. To overcome this limitation we evaluated *in silico* the
57 taxonomic coverage for four primer combinations targeting the 16S rDNA V3-V4 region.
58 They included a forward primer universal to *Bacteria* (S-D-Bact-0341-b-S-17) and four
59 reverse primers designed to avoid plastid DNA amplification. The best primer combination

60 (NOCHL) was compared to the universal primer set in the wet lab using a synthetic
61 community and samples from 3 macroalgal species. The proportion of plastid sequences was
62 reduced by 99-100% with the NOCHL primers compared to the universal primers,
63 irrespective of algal hosts, sample collection and extraction protocols. Additionally, the
64 NOCHL primers yielded a higher richness while maintaining the community structure. As
65 *Planctomycetes*, *Verrucomicrobia*, and *Cyanobacteria* were underrepresented (70-90%)
66 compared to universal primers, combining the NOCHL set with taxon-specific primers may
67 be useful for a complete description of the alga-associated bacterial diversity. The NOCHL
68 primers represent an innovation to study algal holobionts without amplifying host plastid
69 sequences and may further be applied on other photosynthetic hosts.

70

71 **Introduction**

72 Numerous studies on algae-associated bacteria have highlighted their importance for health
73 and physiology of the algal holobiont as well as for biomass degradation (Barott *et al.*, 2011;
74 Egan *et al.*, 2013, 2014; Singh and Reddy, 2016). Bacteria can be abundant on macroalgal
75 surfaces, ranging from 10^6 to 10^7 cells per cm^2 , and encompass a large phylogenetic diversity
76 (van der Loos *et al.*, 2019). These communities are distinct from bacterial assemblages found
77 in the surrounding seawater or on inert surfaces (Burke *et al.*, 2011; Stratil *et al.*, 2014;
78 Lemay *et al.*, 2018). The composition of algae-associated bacterial communities has been
79 shown to be host-specific and to vary depending on algal tissue, seasons, sampling sites and
80 physiological status of the algae (Staufenberger *et al.*, 2008; Lachnit *et al.*, 2011; Miranda *et*
81 *al.*, 2013; Stratil *et al.*, 2013; Zozaya-Valdes *et al.*, 2015; Aires *et al.*, 2016; Paix *et al.*, 2019).
82 A common approach to characterize these algae-associated communities is metabarcoding,
83 whereby a selected variable region of the 16S rDNA is sequenced using a primer set universal
84 to *Bacteria* (e.g., forward: S-D-Bact-0341-b-S-17 / reverse: S-D-Bact-0785-a-A-21

85 (Klindworth et al, 2013)) Due to the classical sampling strategies for macroalgae-associated
86 microbiomes (e.g. algal surface scraping or tissue grinding (Bengtsson *et al.*, 2012; Aires *et*
87 *al.*, 2016)), one major issue is that bacterial DNA samples are often contaminated with algal
88 DNA. Primers with a large taxonomic coverage for *Bacteria* will tend to also amplify 16S
89 rDNA sequences from plastids. This is because plastid genomes, deriving from
90 endosymbiosis events, still encode 16S rDNA closely related to their cyanobacterial
91 ancestors. Datasets obtained from brown algal field samples using common universal primers
92 may contain over 90% of sequences affiliated to plastid DNA (Leblanc, personal data). This
93 high proportion of plastidial sequences decreases the sequencing depth for bacterial epibionts
94 and reduces the power of diversity analyses. One strategy to allow a large coverage of
95 *Bacteria* while minimizing 16S gene amplification from plastids is to design a primer in a
96 region that differs between plastid sequences and those of *Bacteria*. Chelius and Triplett have
97 identified such a region between the positions 783 and 799 of the 16S rDNA (following the *E.*
98 *coli* numbering system) and they designed the 799F primer which includes four mismatches
99 with chloroplasts to amplify *Bacteria* while avoiding chloroplast amplification from maize
100 roots (Chelius and Triplett, 2001). The resulting primer was then used in combination with
101 primer 1193R (amplification product 394 bp) in several studies on bacterial communities
102 associated with plants (Sagaram *et al.*, 2009; Bodenhausen *et al.*, 2013) or macroalgae (Vieira
103 *et al.*, 2016; Aires *et al.*, 2018; Serebryakova *et al.*, 2018). However, this primer set still
104 yielded up to 38% of sequences affiliated to chloroplasts (Sagaram *et al.*, 2009). Several sets
105 of primers including the 799F primer with or without modifications were subsequently
106 designed and tested to minimize plastid contamination on samples from plants and algae
107 (Hanshew *et al.*, 2013; Miranda *et al.*, 2013; Aires *et al.*, 2016). These primer sets target the
108 V5-V8 or V5-V9 regions, yielding products of ca. 590 bp or 750 bp, respectively. Although
109 suitable for the 454 pyrosequencing technology used in the latter studies, this fragment length

110 is not compatible with the current Illumina v3 sequencing chemistry, which produces paired-
111 end reads of 2×300 bp. Allowing for a recommended overlap of at least 50 bp, the total
112 product length for this technology should not exceed 500 bp. Our objective was to evaluate
113 primer combinations matching these criteria while minimizing plastid contamination in 16S
114 rDNA metabarcoding analyses by: (i) comparing the efficiency of a primer set universal to
115 *Bacteria* and that of four primer combinations avoiding plastid amplification *in silico*, (ii)
116 comparing the efficiency of the best primer combination from the *in silico* results with that of
117 the universal primer set in the wet lab.

118

119 **Results and Discussion**

120 *In silico* evaluation of primer combinations to minimize the amplification of plastid 121 sequences

122 We evaluated several sets of primers to amplify a 450 bp fragment spanning the V3 and V4
123 regions of the 16S rDNA while minimizing amplification of plastid sequences (Table S1).
124 These primer sets were designed by combining the universal forward primer S-D-Bact-0341-
125 b-S-17 (Klindworth *et al.*, 2013) with reverse primers corresponding to the reverse
126 complement of four V5-V8 forward primers previously used in 454 pyrosequencing to
127 minimize chloroplast contamination (Hanshew *et al.*, 2013). The four new primer
128 combinations (*E. coli* position 341 to 785) covers most of the region amplified by the original
129 V34 set (*E. coli* position 341 to 799), ensuring the comparability of data. The performance of
130 these different primer combinations was tested by running an *in silico* PCR on the SILVA
131 database ssu-132 with the RefNR sequence collection, using the online tool SILVA TestPrime
132 1.0 (Klindworth *et al.*, 2013). The universal V34 combination with the original reverse primer
133 S-D-Bact-0785-a-A-21 had an *in silico* predicted coverage for plastids (Table 1) ranging from
134 57.3% to 88.3% with zero or 2 allowed mismatches, respectively. By contrast, three of the

135 four new combinations (NOCHL, NOCHL3, and NOCHL6 but not NOCHL7, Table S1)
136 showed consistently low predicted coverage for plastids, even with 2 allowed mismatches
137 (2.7%, 1.1% and 1.1%, respectively). The overall coverage of these three primer
138 combinations for all bacterial sequences remained high (79% with no mismatch, 85-90% with
139 two mismatches). However, the NOCHL3 and NOCHL6 primer sets were predicted to
140 perform poorly on a number of phyla, including *Planctomycetes* (12% coverage with two
141 mismatches) and *Verrucomicrobia* (17% coverage), which are known to be part of alga-
142 associated microbial communities (Bengtsson and Øvreås, 2010; Lage and Bondoso, 2014;
143 Vollmers *et al.*, 2017). For all taxa, the NOCHL combination had equal or better coverage
144 than the NOCHL3 and NOCHL6 combinations, notably for *Planctomycetes* (82.3%) and
145 *Verrucomicrobia* (48.7%). The NOCHL combination was therefore considered the most
146 promising candidate to minimize plastid contamination while maintaining the overall
147 bacterial diversity, and chosen to prepare Illumina-sequencing libraries.

148

149 ***Comparison of primer performances in the wet lab***

150 Performances of the NOCHL and V34 primer combinations were compared *in vitro* using
151 different types of samples. As a positive control for the metabarcoding experiment, we
152 constructed a mock community based on the genomic DNA of pure bacterial isolates.
153 Genomic DNA was extracted from a total of 32 sequenced bacterial strains (Table S2) and
154 mixed in known proportions to assemble the mock community. Furthermore, the microbiota
155 associated with three brown algal species was sampled according to different approaches. The
156 filamentous brown alga *Ectocarpus subulatus* samples were collected 5 km North of the
157 Hopkins River Falls (Australia) and whole tissues were ground before DNA extraction. Blade
158 samples of *Laminaria hyperborea* sporophytes were collected at the coast off Roscoff
159 (Brittany, France) and soaked in a lysis buffer for DNA extraction. The blades of *L. digitata*

160 sporophytes from Roscoff were swabbed for DNA extraction. All samplings were performed
161 in triplicate. Details on sampling and DNA extraction protocols are available in
162 Supplementary Information. Amplicon and library preparation were carried out following the
163 standard Illumina protocol (Illumina web page, 2018), including several negative controls, as
164 detailed in Supplementary Information. Both the NOCHL and V34 primer pairs successfully
165 amplified a ca. 450-bp fragment covering a similar region (V3-V4) of the bacterial 16S
166 rDNA. Sequencing libraries were prepared in parallel for each sample (nine algal samples,
167 one mock community, and six negative controls) using each primer pair. Sequencing was
168 carried out using one run of an Illumina Miseq yielding a total of 5,549,008 read pairs.
169 Sequences were deposited at the ENA under project accession number PRJEB33453. After
170 quality checks and removal of exogenous contaminations, low-quality sequences, and blanks
171 (see Supplementary Information), 2,784,253 assembled reads remained for data analysis.
172 Sequences were clustered, assigned to operational taxonomic units (OTUs) at 97% identity,
173 taxonomically classified with RDP classifier (Wang *et al.*, 2007) on the Silva SSU database
174 release 132 (Quast *et al.*, 2013), and filtered to remove rare OTUs (see details in
175 Supplementary Information). The final dataset comprised 3,009 OTUs.
176 First, we compared metabarcoding results from the primer combinations V34 and NOCHL on
177 the mock community. The sequencing error rate calculated as in Kozich *et al.* (2013) from the
178 mock community datasets was 0.54% for both the V34 and NOCHL libraries. A total of 65
179 OTUs were detected in the rarefied mock community datasets. The expected relative
180 proportion p_i of 16S rDNAs from strain i was calculated as follows:

181

182

183

$$p_i = \frac{\frac{q_i}{M_i} \times c_i}{\sum_{i=1}^{32} \frac{q_i}{M_i} \times c_i}$$

184

185 where q_i is the mass of DNA from strain i added in the MOCK community, M_i is the
 186 molecular weight of the complete genome for strain i and c_i is the number of 16S rDNA gene
 187 copies in the genome of strain i . At the genus level, poor correlations (Pearson coefficient
 188 <0.4) were obtained between the expected 16S rDNA proportions in the mock community
 189 and the relative abundance of taxa found in V34 or NOCHL libraries (Table 2). From the 26
 190 genera represented in the mock community, 18 and 20 were detected in the V34 and NOCHL
 191 datasets, respectively. Both primer combinations did not detect the 6 genera *Nonlabens*,
 192 *Agrococcus*, *Arthrobacter*, *Dokdonia*, *Roseovarius*, and *Imperialibacter*. Sequences from
 193 these genera might either not be amplified by the primer sets, or more likely be merged to
 194 closely related sequences during OTU clustering. In addition, *Roseobacter* and *Hoeflea* were
 195 missed by the V34 primer combination, whereas their relative abundance was close to the
 196 expected value in the NOCHL dataset. With both primer pairs, ca. 30% of sequences could
 197 not be classified down to the genus level, leading to discrepancies between expected and
 198 measured proportions. A striking example is *Vibrio*: its expected abundance of 24.63% was
 199 largely underestimated at ca. 8% with both primer pairs, whereas 11% of sequences were
 200 assigned to unclassified *Vibrionaceae*. On the contrary, both primer combinations largely
 201 overestimated the proportion of *Pseudoalteromonas*, possibly due to the proximity with
 202 sequences from *Alteromonas* (Gauthier *et al.*, 1995). Although the NOCHL combination had
 203 a suboptimal predicted coverage for *Planctomycetes* (Table 1), it still detected the
 204 planctomycetal genus *Rhodopirellula* in the mock community, underestimating it by 56%. On
 205 the other hand, the V34 combination overestimated *Rhodopirellula* by 47%. As expected,
 206 correlations between theoretical and measured proportions were much better (Pearson
 207 coefficient > 0.9) at the family level for both primer combinations (Table S3). All 13 families

208 represented in the mock community were detected using the NOCHL combination, whereas
209 the V34 combination missed the *Phyllobacteriaceae* family. Altogether, the performance of
210 the NOCHL combination was found to be comparable to the V34 set on the mock community
211 in terms of sensitivity and accuracy.

212
213 We further compared the efficiency of the primer combinations V34 and NOCHL in field
214 samples, characterizing the bacterial community associated with *Ectocarpus subulatus*
215 (ECTO), *Laminaria digitata* (LDIG) and *Laminaria hyperborea* (LHYP). The relative
216 abundance of sequences affiliated to plastids ranged from 0.6% to 66% in the V34 dataset
217 (Figure 1). The highest relative abundances of plastid sequences were found when algal
218 tissues were ground (ECTO) or soaked in lysis buffer (LHYP) before DNA extraction,
219 compared to the swab-based technique (LDIG). It was drastically reduced by 99-100% with
220 the NOCHL combination in all three types of alga-associated samples analyzed (Figure 1),
221 with relative abundance of plastid 16S rDNA sequences ranging from 0 to 0.3%. This
222 confirms that the new primer combination succeeds at minimizing plastid contamination,
223 irrespective of the algal host, sample collection and DNA extraction protocol. The NOCHL
224 combination was more efficient at reducing plastid contamination compared to a previous
225 Illumina metabarcoding analysis of *Ectocarpus*-associated bacterial communities. In that
226 former study, a modified set of 341F and 806R primers still yielded 32% of plastid sequences
227 despite a central mismatch to avoid plastid DNA amplification (Dittami *et al.*, 2016).

228 Alpha-diversity analyses tended to show higher values of OTU richness (observed OTUs ~~and~~
229 ~~Chao1 richness estimator~~) and diversity (Shannon and Simpson indices) with the NOCHL
230 combination compared to V34 before removal of plastid sequences (Figure 2A). This overall
231 effect was found significant for Shannon and Simpson diversity indices and was more
232 pronounced for samples from *E. subulatus* and *L. hyperborea*, After removal of plastid

233 sequences (Figure 2B), the NOCHL primer combination yielded significantly higher richness
234 ~~estimates~~ than V34 (Student's t-test, $P=0.05$) with an overall increase of 10%. Again, this
235 effect was more pronounced for ECTO and LHYP samples. Indeed, ECTO and LHYP
236 samples, where plastid contamination was the highest (Figure 1), showed the strongest effect
237 of plastidial sequence removal on sequencing depth (Figure S1). This exemplifies the issue
238 faced with universal primers, where a large amount of plastid-affiliated sequences can
239 decrease the sequencing depth for target bacterial sequences and reduce the evenness. After
240 removal of plastid sequences, lower Shannon and Simpson diversity indices were detected
241 with the NOCHL combination compared with V34 (-3.2% and -0.5% lower values for
242 Shannon and Simpson, respectively). This was mostly due to an increase in these indices for
243 V34 datasets after removal of plastid sequences, while they stayed stable for the NOCHL
244 datasets. Although not the main focus of this work, we noticed that bacterial communities
245 retrieved from *Ectocarpus* were more diverse than those from the two *Laminaria* species.
246 This might be due to the nature of the samples as well as the extraction protocol where entire
247 algal specimens were ground for *Ectocarpus* (i.e. including the endomicrobiota), while for
248 *Laminaria* the extraction protocols only targeted surface-attached bacteria. Joint hierarchical
249 clustering of OTU-level datasets for both primer pairs after removal of plastid sequences
250 showed that samples grouped according to algal host rather than primer pair (Figure 3A).
251 The relative abundance of phyla was generally similar between paired sets of samples (Figure
252 3B). Notable exceptions were the *Planctomycetes*, *Verrucomicrobia*, and *Cyanobacteria* that
253 had lower relative abundance in the NOCHL libraries compared to V34 (Table S4). We
254 further searched for differential OTUs between pairs of samples sequenced with the V34 or
255 NOCHL combination. This analysis was performed with the edgeR package (Robinson *et al.*,
256 2009) on the non-transformed dataset after removal of plastid sequences, and accounted for
257 the paired design of the study, *i.e.* the same samples were sequenced with the two primer

258 combinations. The significance threshold was set at $\alpha=5\%$ after Benjamini-Hochberg
259 correction for multiple testing. Out of the 2,852 OTUs remaining after discarding plastids, the
260 edgeR analysis detected 39 differential OTUs (i.e. 1.4%) between primer combinations (Table
261 S5). All differential OTUs were more abundant in the V34-amplified dataset compared to
262 NOCHL. The majority of them belonged to *Verrucomicrobiae* and *Planctomycetacia* (Figure
263 4), partly reflecting the lower predicted coverage of NOCHL for these taxa. The apparent
264 lower abundance of *Plantomycetacia* OTUs using the NOCHL primer set could also be in
265 part due to their over-estimation with the V34 combination, as shown in the mock community
266 analysis (Table 2). The highest fold-change was found for OTU00082 belonging to
267 *Cyanobacteria*. The lower abundance of *Cyanobacteria* was inevitable since plastids and
268 extant cyanobacteria share a common ancestor and therefore have homologous 16S rDNA
269 sequences (Giovannoni *et al.*, 1988; Delwiche, 1999).

270

271 **Conclusion**

272 Considering that plastid sequences can represent more than 90% of all sequences in one
273 sample when using universal primer sets, the bacterial diversity in a sample may be
274 underrepresented. The new primer set NOCHL is efficient to avoid amplifying plastid
275 sequences from an algal host while identifying a significantly higher bacterial richness than
276 with the universal primers V34, given identical sequencing efforts. This validates that fewer
277 plastid sequences in the samples lead to a larger access to bacterial sequences thus recovering
278 more of the bacterial diversity. However, as some bacterial groups may be underrepresented,
279 this primer set may be combined with taxon-specific primers (e.g. for *Planctomycetes*) for a
280 more complete coverage of *Bacteria*. Still, the data obtained with the NOCHL and universal
281 V34 primer sets are comparable since the targeted regions are the same and patterns of the
282 community structure are similar. Although validated here on macroalgal samples, this primer

283 set may be useful to characterize bacterial communities from other environments where
284 plastid contamination can be an issue such as terrestrial plants, microalgae, or gut microbiota
285 of herbivores.

286

287 **Acknowledgments and funding**

288 The authors have no conflict of interest to declare. We warmly thank Gaëlle Correc and
289 Sylvie Rousvoal for help with sampling as well as Florian de Bettignies and Dominique
290 Davoult for providing samples. We thank Frédérique Le Roux and Christian Jeanthon for
291 providing bacterial strains. This work has benefited from the facilities of the Genomer
292 platform and from the computational resources of the ABiMS bioinformatics platform (FR
293 2424, CNRS-Sorbonne Université, Roscoff), which are part of the Biogenouest core facility
294 network.

295 This work has benefited from the support of the French Government via the National
296 Research Agency investment expenditure program IDEALG (ANR-10-BTBR-04). FT, SD,
297 and CL acknowledge support by the Centre National de la Recherche Scientifique (CNRS).
298 AG acknowledges support by the Institut Français de Recherche pour l'Exploitation de la Mer
299 (IFREMER).

300

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- 406

408

409 **Table and Figure legends**

410

411 **Figure 1:** Relative abundance of sequences affiliated to plastid OTUs using the V34 or
412 NOCHL primer sets. Bacterial communities were analyzed in triplicate samples from
413 different algal hosts: ECTO, *Ectocarpus subulatus*; LHYP, *Laminaria hyperborea*; LDIG,
414 *Laminaria digitata*.

415

416 **Figure 2:** Richness and diversity estimates before (A) and after (B) removal of OTUs
417 affiliated to plastids. Data were subsampled at 8,358 sequences per sample prior to analysis.
418 Observed, observed OTU richness; ~~Chao1, Chao-1 estimated richness~~; Shannon, Shannon
419 index; Simpson, Simpson index; ECTO, *Ectocarpus subulatus*; LDIG, *Laminaria digitata*;
420 LHYP, *Laminaria hyperborea*. Results of paired Student's t-test testing the effect of the set of
421 primers on each measured estimate are reported below each panel (df=8).

422

423 **Figure 3:** Effect of the primer pair on the detected bacterial community composition, after
424 removal of OTUs affiliated to plastids.

425 (A) Hierarchical clustering calculated on Hellinger-transformed data based on Morisita-Horn
426 distance using the complete linkage algorithm

427 (B) Taxonomic composition based on the phylum level

428

429 **Figure 4:** Taxonomic affiliation of the 39 differential OTUs detected with edgeR, depending
430 on the primer pair. All of these OTUs received higher coverage with the V34 primers.

431 **Supplementary Figure 1:** Rarefaction analysis of datasets obtained with V34 and NOCHL
432 primer sets before (A) and after (B) removal of plastid-related sequences.

433

434 **Table 1:** *In silico* predicted coverage of selected bacterial taxa, relative to the sequences
435 available in the Silva SSU r132 database for different primer combinations using TestPrime
436 1.0. A length of 3 bases of 0-mismatch zone at 3' end of primers was chosen for tests with two
437 allowed mismatches in the sequence.

438

439 **Table 2:** Genus-level comparison of theoretical relative abundance in the mock community
440 with the measured abundance in libraries sequenced with V34 or NOCHL primers. unclass.,
441 unclassified.

442

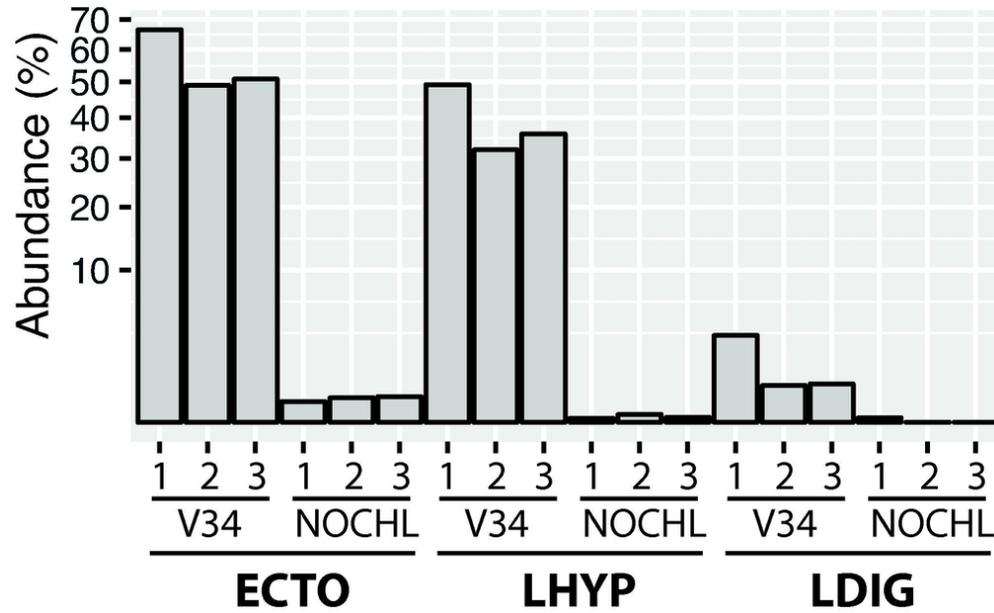


Figure 1: Relative abundance of sequences affiliated to plastid OTUs using the V34 or NOCHL primer sets. Bacterial communities were analyzed in triplicate samples from different algal hosts: ECTO, *Ectocarpus subulatus*; LHYP, *Laminaria hyperborea*; LDIG, *Laminaria digitata*.

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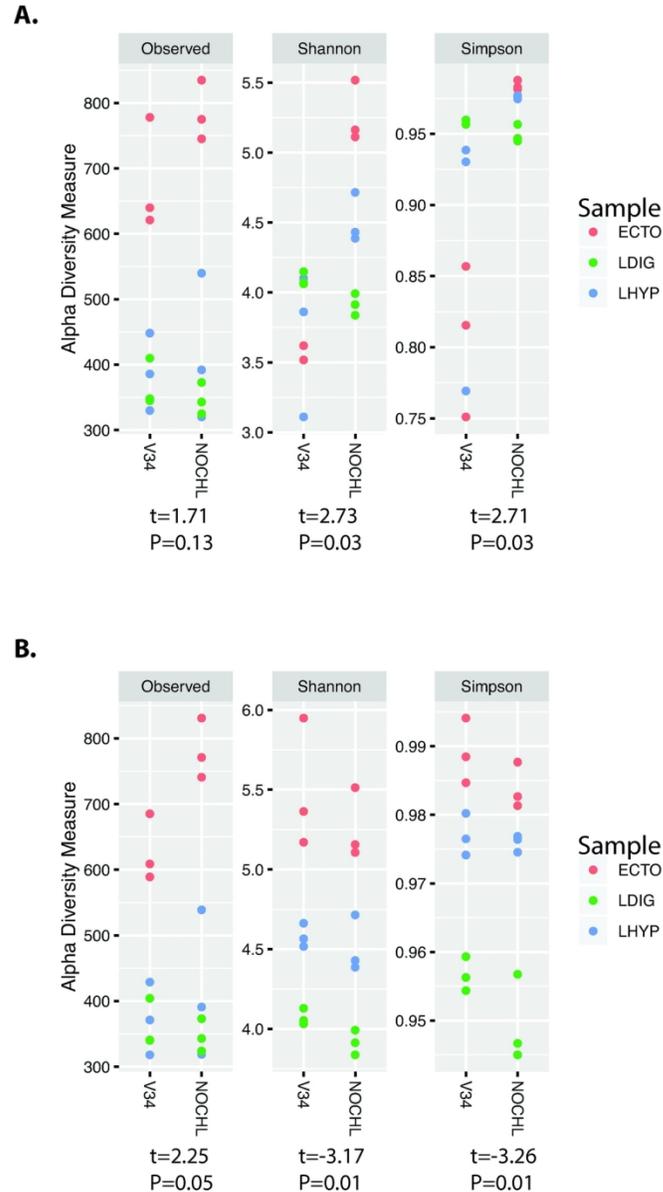


Figure 2: Richness and diversity estimates before (A) and after (B) removal of OTUs affiliated to plastids. Data were subsampled at 8,358 sequences per sample prior to analysis. Observed, observed OTU richness; Shannon, Shannon index; Simpson, Simpson index; ECTO, *Ectocarpus subulatus*; LDIG, *Laminaria digitata*; LHYP, *Laminaria hyperborea*. Results of paired Student's t-test testing the effect of the set of primers on each measured estimate are reported below each panel (df=8).

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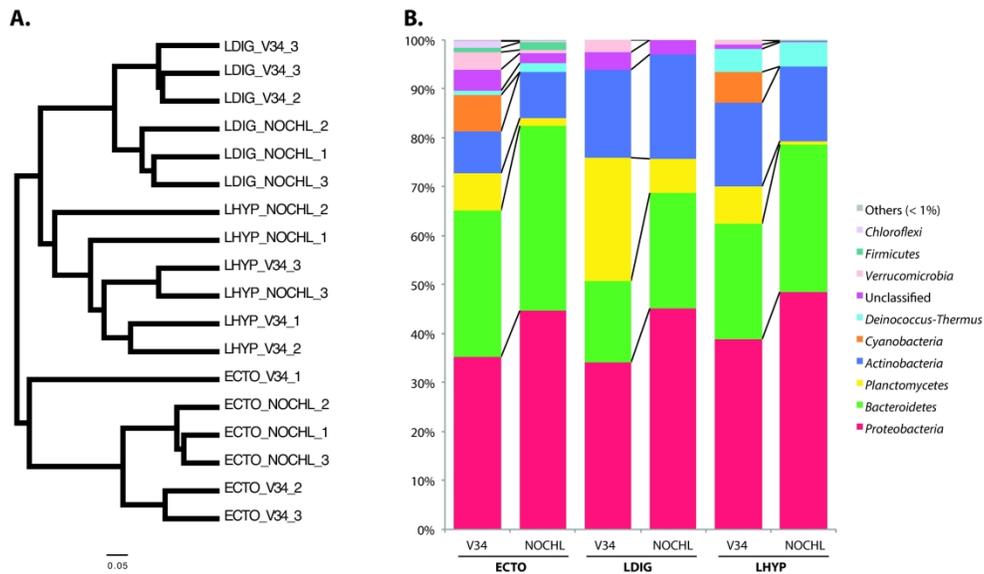


Figure 3: Effect of the primer pair on the detected bacterial community composition, after removal of OTUs affiliated to plastids.

- (A) Hierarchical clustering calculated on Hellinger-transformed data based on Morisita-Horn distance using the complete linkage algorithm
- (B) Taxonomic composition based on the phylum level

271x164mm (300 x 300 DPI)

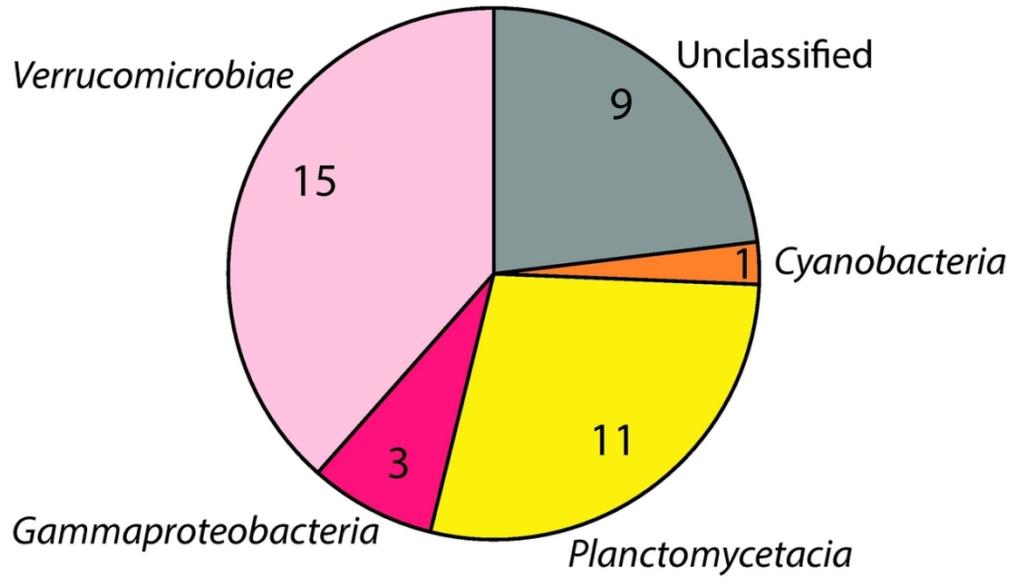


Figure 4: Taxonomic affiliation of the 39 differential OTUs detected with edgeR, depending on the primer pair

105x76mm (300 x 300 DPI)

Table 1: *In silico* predicted coverage of selected bacterial taxa, relative to the sequences available in the Silva SSU r132 database for different primer combinations using TestPrime 1.0. A length of 3 bases of 0-mismatch zone at 3' end of primers was chosen for tests with two allowed mismatches in the sequence.

	0 mismatch					2 mismatches				
	V34	NOCHL	NOCHL3	NOCHL6	NOCHL7	V34	NOCHL	NOCHL3	NOCHL6	NOCHL7
<i>Acidobacteria</i>	92.0	43.1	43.2	43.1	43.3	95.7	95.0	45.3	45.1	96.2
<i>Actinobacteria</i>	82.4	82.5	82.7	82.6	82.7	86.6	96.3	96.5	96.1	86.9
<i>Aquificae</i>	89.3	89.6	90.2	89.9	90.5	95.0	95.5	95.8	95.5	96.1
<i>Bacteroidetes</i>	89.8	88.8	89.3	89.0	89.4	95.4	95.8	95.2	94.8	95.9
<i>Chlamydiae</i>	82.8	65.9	65.9	65.9	66.4	96.7	96.9	79.7	79.7	96.7
<i>Chloroflexi</i>	39.0	19.3	20.8	20.7	21.1	88.7	48.6	30.3	30.3	88.6
<i>Cyanobacteria</i>	76.2	0.6	0.6	0.6	0.6	92.4	1.9	0.9	0.9	88.6
<i>Chloroplasts</i>	57.3	0.7	0.7	0.7	0.7	88.3	2.7	1.1	1.1	81.3
<i>Deinococcus-Thermus</i>	92.9	92.6	92.6	92.6	93.0	96.7	96.9	96.3	96.2	96.7
<i>Epsilonbacteraeota</i>	93.9	85.1	85.9	85.5	85.8	96.8	96.6	88.6	88.2	97.3
<i>Firmicutes</i>	88.2	84.0	84.9	84.7	85.0	94.2	92.8	90.5	90.1	94.7
<i>Fusobacteria</i>	87.1	86.1	87.0	86.7	87.2	94.9	94.0	93.9	93.6	95.3
<i>Gemmatimonadetes</i>	89.6	89.2	89.8	89.4	89.8	93.6	94.7	95.5	94.4	94.9
<i>Planctomycetes</i>	73.5	10.6	10.6	10.5	10.7	85.1	82.3	12.0	11.9	85.8
<i>Proteobacteria</i>	90.0	89.0	89.4	89.2	89.4	95.7	94.3	93.6	93.1	96.2
<i>Spirochaetes</i>	75.7	70.5	70.7	70.3	70.7	88.2	94.6	85.6	85.1	88.4
<i>Verrucomicrobia</i>	87.3	16.2	16.3	16.3	16.5	93.0	48.7	17.2	17.1	92.5
All Bacteria	86.6	79.1	79.6	79.3	79.6	93.6	90.1	85.3	84.9	93.9

Table 2: Comparison between the theoretical and the observed relative abundance of genera in the mock community in libraries sequenced with V34 or NOCHL primers. unclass., unclassified.

Genus	Relative abundance of sequences in the mock community (%)		
	Theory	Primers V34	Primers NOCHL
<i>Vibrio</i>	24.63	8.28	8.78
<i>Formosa</i>	9.72	3.04	2.53
<i>Maribacter</i>	9.42	5.41	5.24
<i>Zobellia</i>	7.24	5.07	3.04
<i>Alteromonas</i>	5.29	1.01	1.35
<i>Nonlabens</i>	5.02	0.00	0.00
<i>Psychrobacter</i>	2.59	6.08	4.05
<i>Microbacterium</i>	2.79	0.51	0.34
<i>Agrococcus</i>	2.74	0.00	0.00
<i>Sphingomonas</i>	2.51	1.52	2.20
<i>Arthrobacter</i>	2.42	0.00	0.00
<i>Dokdonia</i>	2.35	0.00	0.00
<i>Paracoccus</i>	2.31	2.87	4.22
<i>Polaribacter</i>	2.15	8.78	9.12
<i>Roseovarius</i>	1.94	0.00	0.00
<i>Cobetia</i>	1.94	5.24	6.25
<i>Winogradskyella</i>	1.91	1.35	2.03
<i>Roseobacter</i>	1.90	0.00	1.35
<i>Cellulophaga</i>	1.77	3.38	2.53
<i>Mariniflexile</i>	1.74	1.86	1.01
<i>Pseudoalteromonas</i>	1.71	11.82	11.99
<i>Hoeflea</i>	1.57	0.00	1.86
<i>Bosea</i>	1.30	0.68	0.84
<i>Imperialibacter</i>	1.23	0.00	0.00
<i>Rhodopirellula</i>	1.15	1.69	0.51
<i>Dinoroseobacter</i>	0.68	0.51	0.17
unclass. <i>Acidimicrobiales</i>	-	0.34	0.00
unclass. <i>Vibrionaceae</i>	-	11.49	10.81
unclass. <i>Sphingobacteriales</i>	-	5.24	6.93
unclass. <i>Micrococcaceae</i>	-	3.89	3.04
unclass. <i>Flavobacteriaceae</i>	-	5.41	4.05
unclass. <i>Rhodobacteraceae</i>	-	1.52	3.21
<i>Krokinobacter</i>	-	1.86	2.03
unclass. <i>Gammaproteobacteria</i>	-	0.34	0.34
unclass. <i>Actinobacteria</i>	-	0.34	0.00
unclass. <i>Proteobacteria</i>	-	0.17	0.00
unclass. <i>Alteromonadaceae</i>	-	0.00	0.17
unclass. <i>Hyphomonadaceae</i>	-	0.34	0.00
Correlation coefficient Theory vs. Observed for genera present in the mock community		0.39	0.38