bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1 **Isolation of SAR11 marine bacteria from cryopreserved seawater**

2

3 Elizabeth A. Monaghan[a,b], Kelle C. Freel[a], and Michael S. Rappé[a]#

4

5 [a]Hawai‘i Institute of Marine Biology, School of Ocean and Earth Science and Technology,

6 University of Hawai‘i at Mānoa, Kāne‘ohe, Hawai‘i, USA

7 [b]Marine Biology Graduate Program, University of Hawai‘i at Mānoa, Honolulu, Hawai‘i, USA

8

9 Running Title: Isolation of SAR11 from cryopreserved seawater

10

11 #Address correspondence to Michael S. Rappé, rappe@hawaii.edu

12

13

14

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

15 **Abstract**

16 In this study, we sought a means to increase current culture collections of SAR11 marine bacteria
17 by testing the use of seawater cryopreserved with glycerol as an inoculum. In July 2017, raw
18 seawater was collected outside of Kāneʻohe Bay, Hawaiʻi, in the tropical Pacific Ocean. A
19 portion of this sample was diluted in seawater-based growth medium to create 576 × 2 mL
20 dilution cultures containing 5 cells each and incubated for a high-throughput cultivation
21 experiment, while another portion was cryopreserved in 10% glycerol. After ten months, a
22 cryopreserved aliquot of seawater was thawed, diluted in seawater-based growth medium, and
23 distributed to create a second high-throughput cultivation experiment of 480 × 2 mL dilution
24 cultures containing 5 cells each and 94 cultures containing 105 cells each. The raw seawater
25 cultivation experiment resulted in the successful isolation of 54 monocultures and 29 mixed-
26 cultures, while cryopreserved seawater resulted in 59 monocultures and 29 mixed cultures.
27 Combined, the cultures included 51 SAR11 isolates spanning 11 unique 16S rRNA gene
28 amplicon sequence variants (ASVs) from raw seawater inoculum and 74 SAR11 isolates
29 spanning 13 unique ASVs from cryopreserved seawater. A vast majority (115 of 125) of SAR11
30 isolates from the two HTC experiments were members of SAR11 subclade Ia, though isolates of
31 subclades IIIa and Va were also recovered from cryopreserved seawater and subclade Ib was
32 recovered from both. The four most abundant SAR11 subclade Ia ASVs found in the initial
33 seawater sample used to create both culture experiments were isolated by both approaches.
34

35 **Importance**

36 High-throughput dilution culture has proved to be a successful approach to bring some difficult-
37 to-isolate planktonic microorganisms into culture, including the highly abundant SAR11 lineage

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

38 of marine bacteria. While the long-term preservation of bacterial isolates by freezing in the
39 presence of cryoprotectants such as glycerol has been shown to be an effective method of storing
40 viable cells over long time periods (i.e. years), to our knowledge it had not previously been
41 tested for its efficacy in preserving raw seawater for later use as inoculum for high-throughput
42 cultivation experiments. We found that SAR11 and other abundant marine bacteria could be
43 isolated from seawater that was previously cryopreserved for nearly 10 months, at a rate of
44 culturability similar to that of the same seawater used fresh, immediately after collection. Our
45 findings expand the potential of high-throughput cultivation experiments to include opportunities
46 where immediate isolation experiments are impractical, allow for targeted isolation experiments
47 from specific samples based on analyses such as microbial community structure, and enable
48 cultivation experiments across a wide range of other conditions that would benefit from having
49 source inoculum available over extended periods of time.
50

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

51 **Introduction**

52 The rapid advancement of molecular tools to investigate marine microorganisms in their
53 natural environment has led to unprecedented access to the genomic repertoire and transcription-
54 and protein-based assessments of activity within natural microbial cells, populations, and
55 communities (1, 2). It is currently feasible for a few liters of seawater to provide sequence data
56 that reveals microbial population structure, the identities of microbial community members, and
57 the presence and activity of potential metabolic functions they harbor (e.g. 3, 4). In recent years,
58 however, the value of having cultivated representatives of numerically abundant and
59 environmentally relevant microbial lineages has received renewed recognition (5–8). Access to
60 isolated strains or low-diversity enrichments of marine microorganisms that are commonly found
61 in the natural environment has provided a means to definitively test many hypotheses generated
62 from environmental observations and experiments, as well as whole genome sequences useful
63 for informing and guiding environmental genomics, transcriptomics, and proteomics research
64 (e.g. 5, 6). The importance of cultivating environmentally-relevant microorganisms from pelagic
65 marine ecosystems for laboratory-based experimentation is now generally appreciated. However,
66 evidence provided by the sequencing of environmental DNA continues to support the conclusion
67 that most of the microorganisms that appear to dominate pelagic marine ecosystems have not yet
68 been cultivated from seawater (11).

69 Several different isolation methods and strategies have been developed in order to coax
70 recalcitrant environmental microorganisms into laboratory culture (e.g 12–15). Among these
71 novel approaches is an isolation technique based on dilution-to-extinction culturing methodology
72 first developed by Button and colleagues (16). Although early dilution-to-extinction culturing
73 studies resulted in cultures of novel oligotrophs (16–18), the dilution culture strategy was not

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

74 without limitations. For instance, the technique yielded only a small number of isolates, while
75 requiring a significant amount of time and effort per experiment. The high-throughput culturing
76 (HTC) approach is a variation of dilution-to-extinction culturing methodology tailored to
77 facilitate rapid, high-throughput experiments with high rates of replication and greater
78 opportunities to investigate physical, chemical, and biological variables (19, 20).

79 For over a decade since its initial discovery in 1990, the marine planktonic bacterial
80 lineage known as SAR11 served as a notorious example of an abundant and widespread
81 microorganism in nature that was recalcitrant to cultivation as an isolated strain in a controlled
82 laboratory setting (21, 22). The value of the HTC strategy was solidified when early trials
83 yielded the first cultured representatives of many marine microbial groups that were previously
84 known only from environmental SSU rRNA genes (19), including the first cultivated strains of
85 SAR11 (20). In general, the HTC approach employs growth media created from natural or
86 artificial seawater in order to dilute the cells within a fluid sample, which is then arrayed in high
87 density replicate cultures, propagated, and monitored under controlled conditions. In addition to
88 diverse SAR11 strains (20, 23–28), the application of this method has resulted in the isolation of
89 numerous other important lineages of marine bacteria including OM43 (19, 29), SAR116 (23,
90 30), SAR92 (23, 31), and SUP05 (32), among others. While efforts have succeeded in isolating
91 many abundant planktonic marine bacteria, it remains that the genetic diversity harbored by these
92 lineages in nature greatly surpasses what has been isolated in the laboratory.

93 A current limitation of the HTC approach is that, thus far, it has only been used with
94 freshly collected inoculum. This presents a potential constraint on HTC experiments using fluid
95 samples collected in the field as it requires all of the resources necessary for setting up an HTC
96 experiment (appropriate laboratory space, biosafety cabinet, etc.) be available at or near the time

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

97 and location of sampling. The preservation of cultivated bacterial strains by freezing in the
98 presence of cryoprotectants such as glycerol or dimethyl sulfoxide (DMSO) has proved an
99 effective method for preserving viable cells over long time periods (i.e. years), including
100 cultivated strains of SAR11 (20, 33, 34). However, to our knowledge it has not previously been
101 tested for its efficacy in preserving raw seawater for subsequent use as inoculum for high-
102 throughput cultivation. In this study, we conducted HTC experiments to compare the use of a
103 raw seawater sample collected from the coast of O'ahu, Hawai'i, in the tropical Pacific Ocean,
104 with a subsample of the same seawater that was cryogenically preserved for nearly ten months.
105 In particular, we sought to determine if members of the SAR11 lineage of marine bacteria could
106 be isolated from cryopreserved seawater and thus open the possibility to expand existing culture
107 collections of SAR11 to potentially include any locations where seawater samples could be
108 collected and preserved.
109

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

110 **Results**

111 **Overview of HTC cultivation experiments**

112 Using seawater sampled outside of Kāneʻohe Bay on the island of Oʻahu, Hawaiʻi (Fig.
113 1), two high-throughput cultivation experiments were conducted: one that used fresh seawater as
114 an inoculum, labeled HTC2017, and one that used a cryopreserved sample of the same seawater
115 ~10 months later (HTC2018) (Fig. 2). Of 576 initial 2 mL cultures inoculated with raw seawater
116 for the HTC2017 experiment, 150 exhibited positive growth after 56 days of incubation. Of
117 these, 123 contained sufficient volume of culture to be sub-cultured into 20 mL of fresh medium.
118 Following DNA extraction, sequencing, and the assignment of ASVs, 54 monocultures and 29
119 mixed cultures were recovered (Table 1). The remainder either did not yield an amplification
120 product or did not contain an ASV ≥50% of the culture and thus were not considered further.
121 Fifty-four isolates were identified in the 29 mixed cultures (Table S1); the 108 unique isolates
122 identified in the HTC2017 experiment (monocultures plus isolates contained in mixed cultures)
123 were distributed amongst 28 ASVs in total (Table 2, Table S1). The HTC2017 experiment
124 yielded a culturability of 3.1% (2.5% – 3.9%) when both monocultures and mixed cultures were
125 considered, and 2.0% (1.5% – 2.6%) when considering only monocultures (Table 1).

126 For the cryopreserved seawater experiment (HTC2018), wells were inoculated with either
127 5 or 105 cells. Of the 480 initial 2 mL cultures inoculated with 5 cells well^{-1} of cryopreserved
128 seawater (HTC2018), 142 exhibited positive growth after 30 days of incubation. The 142
129 positive wells were subcultured into 20 mL seawater media and, after 72 days, 95 subcultures
130 ultimately exhibited growth. Following DNA extraction, sequencing, and the assignment of
131 ASVs, 39 monocultures and 11 mixed cultures were identified (Table 1). The remainder either
132 did not yield an amplification product or did not contain an ASV ≥ 50% of the culture and thus

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

133 were not considered further. Sixteen isolates were identified in the 11 mixed cultures (Table S1).
134 Combined, the 55 unique isolates identified in the HTC2018 5 cells well^{-1} experiment were
135 distributed amongst 17 ASVs (Table 2, Table S1). The HTC2018 5 cells well^{-1} experiment
136 yielded a culturability of 2.2% (1.7% – 2.9%) when both monocultures and mixed cultures were
137 considered, and 1.7% (1.2% – 2.3%) when considering only monocultures (Table 1).
138 Of the 470 initial 2 mL cultures inoculated with 105 cells well^{-1} of cryopreserved
139 seawater (HTC2018), 343 exhibited positive growth after 31 days of incubation. A single 96-
140 well cultivation plate containing 64 positive wells and two uninoculated control wells were
141 selected for further processing. Following DNA extraction, sequencing, and the assignment of
142 ASVs, 20 monocultures and 18 mixed cultures were identified (Table 1). The remainder either
143 did not yield an amplification product or did not contain an ASV ≥ 50% of the culture and thus
144 were not considered further. Thirty-five isolates were identified in the 18 mixed cultures (Table
145 S1); combined, the 55 unique isolates identified in the 105 cells well^{-1} HTC2018 experiment
146 were distributed amongst 21 ASVs (Table 2, Table S1). The HTC2018 105 cells well^{-1}
147 experiment yielded a culturability of 0.5% (0.3% – 0.7%) when both monocultures and mixed
148 cultures were considered, and 0.2% (0.1% – 0.4%) when considering only monocultures (Table
149 1).
150
151 **Identity of isolates**
152 After quality control, each culture was sequenced to an average depth of 14,047 ± 8,014
153 (s.d.; range of 679 – 57,557) reads. Regardless of whether they originated from raw or
154 cryopreserved seawater, the broad, bacterial family-level taxonomic identity of isolates revealed
155 substantial overlap between culture experiments (Table 2, Table S1). Members of the

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

156 alphaproteobacterial SAR11 subclade I, the marine gammaproteobacterial family *Halieaceae*,
157 and the alphaproteobacterial family *Rhodobacteraceae* were the first-, second-, and third-most
158 abundant families isolated in both the fresh seawater (HTC2017) and cryopreserved seawater
159 (HTC2018) cultivation experiments (Table 2, Table S1). Combined, these three groups made up
160 82% (89 of 108) and 88% (97 of 110) of isolates recovered from HTC2017 and HTC2018,
161 respectively. Other bacterial families with isolates shared between HTC2017 and HTC2018
162 include the PS1 clade of *Alphaproteobacteria* and *Burkholderiaceae* within the
163 *Betaproteobacteria* (Table 2, Table S1). When the five bacterial families shared between
164 HTC2017 and HTC2018 are considered, the fresh seawater and cryopreserved seawater
165 cultivation experiments shared 89% (96 of 108) and 93% (102 of 110) of isolated strains,
166 respectively.

167 **SAR11.** A total of 51 strains representing 11 unique ASVs of SAR11 marine bacteria
168 (alphaproteobacterial order *Pelagibacterales*) were cultivated in HTC2017, while 74 strains
169 representing 13 ASVs were cultivated in HTC2018 (Fig. 3, Table 2, Table S1). They make up
170 47% and 67% of the isolates recovered in the two experiments, respectively. The vast majority of
171 these isolates were members of SAR11 subclade Ia, including 47 strains from HTC2017 and 68
172 strains from HTC2018. Each experiment resulted in the isolation of nine subclade Ia ASVs,
173 including five that were common between the two experiments (Figs. 3 & 4, Table S1). The two
174 most often isolated SAR11 subclade Ia ASVs were shared between the two experiments:
175 ASV003 (25 and 37 isolates) and ASV002 (11 and 13 isolates) from HTC2017 and HTC2018,
176 respectively (Fig. 3, Table S1). Two other subclade Ia ASVs (ASV034 and ASV046) consisted
177 of multiple strains from both experiments, while 7 subclade Ia ASVs consisted of a single isolate
178 from one experiment (Fig. 3, Table S1).

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

179 Strains affiliated with SAR11 subclade Ib were also isolated from both fresh and
180 cryopreserved seawater, including 4 isolates across 2 ASVs from HTC2017 and 3 isolates across
181 2 ASVs from HTC2018 (Fig. 4, Table S1). SAR11 subclade Ib ASV060 consisted of 3 and 2
182 isolates from HTC2017 and HTC2018, respectively, while each experiment also yielded an
183 isolate with a unique subclade Ib ASV (Fig. 3, Table S1). Two SAR11 subclades were only
184 isolated from the cryopreserved seawater sample, including two isolates from subclade IIIa
185 (ASV188) and one isolated from subclade Va (ASV200) (Fig. 3, Table 2, Table S1).

186 **OM60(NOR5).** Within the gammaproteobacterial family *Halieaceae*, the marine
187 OM60(NOR5) clade made up 24 and 21 isolates, or 22% and 19% of HTC2017 and HTC2018,
188 respectively (Table 2). The 4 ASVs that accounted for the 24 isolates from HTC2017 were
189 shared with HTC2018, where they accounted for 19 of the 21 isolates recovered from that
190 experiment (Fig. 5, Table S1). One additional OM60(NOR5) ASV (ASV201) consisting of 2
191 isolates was recovered from cryopreserved seawater (Fig. 5, Table S1). Two closely related
192 ASVs (ASV032 and ASV018) accounted for most of the OM60(NOR5) strains isolated from
193 both experiments of this study (Fig. 5, Table S1). ASV32 was identical to strain HIMB55, a
194 genome-sequenced member of the OM60(NOR5) clade previously isolated from Kāneʻohe Bay,
195 Hawaiʻi (35).

196 ***Rhodobacteraceae*.** The marine alphaproteobacterial family *Rhodobacteraceae* made up
197 14 and 5 isolates, or 13% and 5% of HTC2017 and HTC2018, respectively (Table 2). The strains
198 were distributed amongst 4 (HTC2017) and 5 (HTC2018) ASVs, including three (ASV12,
199 ASV71, ASV124) that were shared between the two experiments (Fig. 5, Table S1). Eight of 14
200 *Rhodobacteraceae* isolates recovered from HTC2107 belonged to a single ASV (ASV012).

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

201 ASV71 was identical to strain HIMB11, a genome-sequenced member of the *Rhodobacteraceae*
202 previously isolated from Kāneʻohe Bay, Hawaiʻi (Fig. 5) (36).
203 **Other isolates.** In addition to the SAR11, OM60(NOR5), and *Rhodobacteraceae* ASVs
204 described above, one additional ASV was isolated in both experiments: ASV190 within the
205 betaproteobacterial family *Burkholderiaceae* was represented by 4 strains in HTC2017 and 1 in
206 HTC2018 (Table S1). While identical ASVs were not isolated, members of the marine
207 alphaproteobacterial PS1 clade within the order *Parvibaculales* were recovered from both
208 cultivation experiments, including 3 isolates from a single ASV (ASV137) in HTC2017 and 4
209 isolates from 3 ASVs in HTC2018 (Fig. 5, Table S1). The remaining ASVs were recovered as
210 either singletons or pairs of strains except for the OM43 clade of the *Betaproteobacteriales*,
211 which constituted five strains across 2 ASVs from the fresh seawater inoculum (HTC2017) only
212 (Table S1).
213
214 **Comparisons with inoculum microbial community**
215 After quality control, the 16S rRNA gene amplicon from the inoculum seawater sample
216 was sequenced to a depth of 65,924 reads. This sample harbored a microbial community
217 dominated by typical marine bacteria, including the marine picocyanobacteria *Prochlorococcus*
218 and *Synechococcus*, multiple subclades of the SAR11 lineage, the family *Flavobacteriaceae* of
219 the bacterial phylum *Bacteroidetes*, diverse members of the gammaproteobacterial SAR86 and
220 OM60(NOR5) lineages and alphaproteobacterial SAR116 and *Rhodobacteraceae* lineages, and
221 *Actinomarinaceae* of the bacterial phylum *Actinobacteria*, among others (Table S1).
222 Within the inoculum sample, 53 SAR11 ASVs totaling 26% of the microbial community
223 were identified. These spanned a diverse array of subclades that included Ia, Ib, IIa, IIIa, IV, Va,

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

224 and Vb (Fig. 3, Table S1). Eight of the 53 SAR11 ASVs were isolated from at least one
225 cultivation experiment, including ASVs within subclades Ia, Ib, IIIa, and Va, while 5 of the 8
226 were isolated from both HTC2017 and HTC2018 (Figs. 3 & 4, Table S1). Of 10 SAR11 subclade
227 Ia ASVs present in the inoculum, the four most abundant were cultivated from both experiments
228 (ASV002, ASV003, ASV034, and ASV046), including the second- and third- most abundant
229 individual ASVs in the inoculum seawater community (Table S1). A fifth was cultivated in
230 HTC2017 only (Fig. 3, Table S1). Eight SAR11 subclade Ia ASVs were isolated that did not
231 appear in the inoculum seawater community (Figs. 3 & 4, Table S1).
232 In contrast to SAR11 subclade Ia, other SAR11 subclades present in the inoculum
233 seawater microbial community were cultivated rarely or not at all. For example, only 1 of 14
234 subclade Ib ASVs that appeared in the inoculum was isolated (ASV060; Fig. 3, Table S1),
235 although it was cultivated in both HTC2017 and HTC2018 experiments. From the HTC2018
236 experiment, one of four subclade IIIa ASVs (ASV188) was isolated, as well as one of two
237 subclade Va ASVs (ASV200; Fig. 3, Table S1). Thirteen *Rhodobacteraceae* ASVs were
238 identified in the environmental sample, of which the same three (ASV012, ASV071, ASV124)
239 were cultivated from both fresh and cryopreserved seawater (Fig. 5, Table S1). Four of the five
240 total most environmentally abundant OM60(NOR5) clade ASVs were also cultivated in both
241 experiments (Table S1). Despite 19 ASVs appearing in the inoculum, only one SAR116 ASV
242 (ASV142; Fig. 5, Table S1) was cultivated in the HTC2017 experiment.
243
244 **Mixed cultures**
245 Twenty-nine mixed cultures were identified within each of the HTC2017 and HTC2018
246 experiments, yielding a total of 58 mixed cultures (Table S1). A majority of the mixed cultures

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

247 contained ASVs from either SAR11 subclade Ia or the OM60(NOR5) clade, which is logical
248 given the high recovery of monocultures from these two groups in both cultivation experiments
249 (Fig. S1). Of nine mixed cultures containing OM60(NOR5) ASV018, eight also contained
250 SAR11 subclade Ia ASV002, ASV003, or ASV034 (Fig. S1, Table S1). Eight of the 11 mixed
251 cultures containing OM60(NOR5) ASV032 also contained a SAR11 subclade Ia ASV as well
252 (Fig. S1, Table S1). All cultivated OM43 clade ASVs were in mixed cultures; both of the OM43
253 clade ASV202 isolates appeared in co-culture with the OM43 clade ASV195 (Fig. S1, Table S1).
254

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

255 **Discussion**

256 For a variety of reasons, SAR11 marine bacteria remain a target for culturing
257 experiments, despite being first isolated nearly 20 years ago (20). In large part, this is driven by
258 the enormous genomic diversity harbored by this lineage and the probability for ecotypic
259 differentiation across the SAR11 phylogenetic tree (3, 26, 37, 38). Living cultures offer a direct
260 means to characterize and quantify the cellular and physiological features that underly
261 differences in abundance or activity observed via direct environmental sampling (4, 39, 40). The
262 primary goal of this study was to test the hypothesis that SAR11 marine bacteria can be isolated
263 from cryogenically preserved seawater. We reasoned that, since existing SAR11 isolates could
264 be cryopreserved in the presence of 10% glycerol (e.g. 20, 33, 34), then there was no *a priori*
265 reason to believe that natural populations of SAR11 cells could not similarly be preserved. One
266 of many unknown variables, however, was whether or not the process of cryopreservation would
267 result in significant cell loss and thus affect cultivation efficiency. At an equivalent-sized
268 inoculum of five cells, we found that not only were SAR11 strains able to be cultivated from
269 cryopreserved seawater, but the overall culturability was similar between the fresh and
270 cryopreserved seawater samples. Both experiments resulted in the isolation of representatives
271 from the four most abundant SAR11 subclade Ia ASVs in the original inoculum seawater
272 sample, as well as strains from subclade Ib. The cryopreserved seawater sample also proved
273 capable of serving as an inoculum to isolate other SAR11 subclades, as evidenced by the
274 recovery of isolates from within subclade IIIa (two strains) and Va (one strain) from the
275 cryopreserved sample only.

276 In addition to numerous isolates from SAR11 subclade Ia that appear to represent
277 abundant ASVs in the seawater sample used as inoculum for these experiments, this study

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

278 yielded seven strains of SAR11 subclade Ib in either mono- or mixed-culture. Despite being a
279 widespread and frequently abundant lineage of SAR11 in the global surface ocean (41–43), only
280 one cultivated representative of subclade Ib had been previously reported, from the Red Sea (28).
281 In addition to the novel isolates of subclade Ib, two strains of subclade IIIa and one of Va were
282 isolated from cryopreserved seawater indicating that a broad range of SAR11 diversity covering
283 at least four major sublineages can be cultivated by this approach, with no apparent negative
284 affect from the cryopreservation treatment itself.

285 As demonstrated by their recovery here, a range of other oligotrophic marine bacteria can
286 be isolated from cryopreserved seawater coupled with an HTC approach. This includes
287 representatives from the OM60(NOR5) clade, a ubiquitous lineage of oligotrophic marine
288 *Gammaproteobacteria* (OMG) that has been consistently isolated via HTC approaches (e.g. 19,
289 27, 31), including from coastal Hawai‘i (35). The OM60(NOR5) lineage was the second most-
290 commonly isolated group of marine bacteria, behind only SAR11 subclade Ia, whether using
291 fresh or cryopreserved seawater as inoculum. Of five OM60(NOR5) ASVs present in the
292 seawater used as inoculum, the four most abundant were isolated in both cultivation experiments,
293 indicating no apparent effect of using cryopreserved seawater as an inoculum for isolating
294 members of the OM60(NOR5) lineage. A similar pattern emerged for the *Rhodobacteraceae*
295 lineage of marine *Alphaproteobacteria*, where isolates from the same three ASVs were
296 recovered in each of the two cultivation experiments, out of 13 total *Rhodobacteraceae* ASVs
297 identified in the environmental sample. This included the most abundant *Rhodobacteraceae* ASV
298 from the seawater inoculum, as well as an ASV identical to the previously isolated and genome-
299 sequenced strain HIMB11 from the same sampling location (36). We found only one abundant
300 (> 3) set of strains that was isolated by using raw seawater as inoculum without corresponding

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

301 strains also isolated using cryopreserved seawater: five strains belonging to two ASVs within the
302 OM43 clade of *Betaproteobacteria* were isolated in mixed- and mono-cultures. While this may
303 indicate that the cryopreservation process had a negative impact on the viability of OM43 clade
304 cells, we note that previously isolated members of this lineage have been successfully
305 cryopreserved in an identical fashion to the method employed in the current study (29, 44). Thus,
306 there is also the potential that this difference stems from stochasticity related to diluting the two
307 inocula nearly one million-fold.

308 Combining a barcoded next-generation 16S rRNA gene amplicon sequencing approach
309 with a high-throughput dilution culture strategy proved to be a rapid and sensitive means with
310 which to identify strains and assess the constituent taxa within mixed cultures. By barcoding and
311 sequencing each individual culture in the same manner as if it were a mixed microbial
312 community, we obtained taxonomic and proportional data on the microorganisms growing within
313 58 mixed cultures of up to four constituent ASVs. Recent studies have highlighted the intricacies
314 that interweave the metabolisms of microorganisms inhabiting seawater (e.g. 4, 45); in natural
315 systems, it is probable that a portion of marine microorganisms require as-yet-unidentified
316 growth factors from co-existing cells (46, 47). These dependencies can be identified and
317 investigated by combining a miniaturized, high-throughput approach to cultivate and screen 100s
318 to 1000s of dilution cultures with an inoculum size aimed at growing mixed consortia and a rapid
319 sequence-based screening method that is appropriate for mixed communities, like the one used
320 here.

321 Consistent with recent observations (27), this set of experiments resulted in the isolation
322 of several bacterioplankton lineages that have been isolated numerous times via HTC and thus
323 appear readily amenable to cultivation via this approach, including members of SAR11 subclade

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

324 Ia, the OM60(NOR5) lineage, and the *Rhodobacteraceae*. However, it remains that a large
325 portion of the diversity of marine microbes is still being missed in contemporary cultivation
326 efforts. For example, when considering only the putatively heterotrophic, non-cyanobacterial
327 fraction of the microbial community targeted in this study, major lineages including the
328 *Flavobacteriaceae*, SAR86 clade, *Marinimicrobia* (SAR406 clade), and marine Actinobacteria
329 (*Candidatus* Actinomarina) were missed completely. At the single-nucleotide resolution of
330 ASVs, abundant lineages of SAR116 and SAR11 subclades Ib, IIa, and Vb were also
331 conspicuously missed. While this study does not offer a panacea for isolating any of these well-
332 known but as-yet-uncultivated (or undercultivated) lineages in laboratory culture, it presents a
333 method by which high-throughput isolation experiments can be repeatedly performed on an
334 identical set of cryopreserved seawater samples such that requirements for growth can be
335 systematically tested in a cumulative fashion.

336 In summary, we have demonstrated that a broad range of marine bacterioplankton taxa
337 can be isolated from glycerol-cryopreserved seawater via an HTC approach, and that the
338 cryopreservation process itself did not negatively affect culturability or influence the taxonomic
339 identify of the resulting isolates. Strains of SAR11 subclades Ia, Ib, IIIa, and Va are amenable to
340 isolation from cryopreserved seawater, as well as other abundant lineages of marine bacteria
341 such as OM60(NOR5), oligotrophic *Rhodobacteraceae*, and the PS1 clade. This study
342 demonstrates that cryopreserved seawater can be used as a means to expand the breadth of HTC
343 studies to anywhere cryopreserved stocks can be made, and opens new opportunities to
344 repeatedly interrogate individual water samples or selectively target specific samples for
345 cultivation once ancillary data is in hand.

346

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

347 **Materials and Methods**

348 **Processing of seawater for growth experiments**

349 On 26 July 2017, a 4 L seawater sample was collected in an acid washed polycarbonate
350 (PC) bottle from a depth of 2 m at station STO1 (N 21° 28.974', W 157° 45.978') outside of
351 Kāneʻohe Bay, Oʻahu, Hawaiʻi (Fig. 1). Within 1 hr of collection, subsamples of the raw
352 seawater were used to enumerate planktonic microorganisms, cryopreserve subsamples, collect
353 microbial biomass for environmental DNA, and serve as inoculum for a high-throughput
354 cultivation experiment (Fig. 2). Microbial cells were enumerated by staining with SYBR Green I
355 nucleic acid stain (Invitrogen, Carlsbad, CA, USA) and counted on a Guava easyCyte 5HT flow
356 cytometer (Millipore, Burlington, MA, USA) following a previously published protocol (48). To
357 cryopreserve the raw seawater, individual 1.5 mL subsamples were added to 375 µL of 50%
358 glycerol solution (v/v in sterile Kāneʻohe Bay seawater; 10% final concentration) in 2 mL
359 cryovials (Nalgene, Rochester, NY, USA) at room temperature (24ºC), mixed by inverting, and
360 cooled at a rate of -1ºC min^{-1} with a Cryo 1ºC Freezing Container (Nalgene) inside a -80ºC
361 ultracold freezer. Approximately 1.3 L of the raw seawater sample was collected on a 25 mm
362 diameter, 0.1 µm pore-sized polyethersulfone membrane (Supor-100; Pall Gelman Inc., Ann
363 Arbor, MI). The filter was submerged in 500 µL DNA lysis buffer (49, 50) and stored at -80ºC
364 until DNA extraction.

365

366 **High-throughput cultivation experiment with raw seawater**

367 Growth medium was made following previously published methods (51). Briefly, 20 L
368 seawater samples were collected on 8 July 2017 and subsequently again on 20 September 2017
369 from a depth of 2 m at station SR4 (N 21° 46.165', W 157° 78.350') near Kāneʻohe Bay, Oʻahu,

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

370 Hawaiʻi (Fig. 1), in acid-washed 4-L PC bottles. Within 1 hr of collection, the seawater was
371 sequentially filtered through pre-rinsed (10 L sterile water followed by 10 L seawater) 0.8-, 0.2-,
372 and 0.1-µm pore-sized polyethersulfone (PES) membranes (AcroPak 20 and Supor 100; Pall
373 Corp., Port Washington, NY, USA) into clean 4-L PC bottles. Bottles were then autoclaved for 3
374 hours (h) at 121ºC and allowed to cool. The sterile seawater was sparged with CO_2, followed by
375 air, through three in-line HEPA vent filters (0.3-µm glass-fiber to 0.2-µm PTFE to 0.1-µm
376 PTFE; Whatman, GE Healthcare Life Sciences, Chicago, IL, USA) and stored at 4ºC until use.
377 The pH of the seawater was checked prior to autoclaving and after sparging to ensure continuity
378 of the inorganic carbon chemistry.

379 Subsamples of raw seawater were diluted in the sterile seawater to 2.5 cells mL^{-1} and
380 arrayed in 2 mL volumes (5-cell inoculum) into 576 wells of custom fabricated 96-well Teflon
381 microtiter plates. Plates were sealed with breathable polypropylene microplate adhesive film
382 (VWR, Radnor, PA, USA) and incubated at 27ºC in the dark. The presence of cellular growth
383 was monitored at 3.5 and 8 weeks via flow cytometry (Tripp et al. 2008). This experiment is
384 hereafter referred to as HTC2017.

385 Wells that exhibited positive growth of $>10^4$ cells mL^{-1} were sub-cultured by transferring
386 1 mL into 20 mL of sterile seawater media amended with 400 µM $(NH_4)_2SO_4$, 400 µM NH_4Cl,
387 50 µM NaH_2PO_4, 1 µM glycine, 1 µM methionine, 50 µM pyruvate, 800 nM niacin (B3), 425
388 nM pantothenic acid (B5), 500 nM pyridoxine (B6), 4 nM biotin (B7), 4 nM folic acid (B9), 6
389 µM myo-inositol, 60 nM 4-aminobenzoic acid, and 6 µM thiamine hydrochloride (B1).
390 Subcultures were subsequently incubated at 27ºC in the dark and monitored for growth after 4.5
391 weeks. Those that again reached $>10^{-4}$ cells mL^{-1} were cryopreserved (500 µL of culture with
392 10% v/v glycerol, final concentration) in the same manner as described for raw seawater. Cells in

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

393 the remaining volume of culture (~18 mL) were collected by filtration through 13 mm diameter,
394 0.03 µm pore-sized PES membrane filters (Sterlitech, Kent, WA, USA), submerged in 250 µL
395 DNA lysis buffer, and stored at -80°C until DNA extraction.
396

397 **High-throughput cultivation experiment with cryopreserved seawater**

398 After 42 weeks of storage at -80ºC, one cryopreserved stock of raw seawater from station
399 STO1 was thawed to room temperature (~24ºC), diluted ten-fold in sterile seawater growth
400 medium, and enumerated via staining with SYBR Green I and flow cytometry. The
401 cryopreserved sample was subsequently diluted with nutrient-amended sterile seawater growth
402 medium to two different concentrations: 2.5 and 52.5 cells mL^{-1}. The 2.5 cells mL^{-1} dilution was
403 used to create 480 2-mL dilution cultures (5-cell inoculum) in custom fabricated 96-well Teflon
404 microtiter plates, while the 52.5 cells mL^{-1} dilution was used to create 470 2-mL dilution cultures
405 (105-cell inoculum). Ten control wells containing uninoculated sterile seawater growth medium
406 were also included. Teflon plates were sealed with breathable polypropylene microplate adhesive
407 film (VWR) and incubated at 27ºC in the dark. Growth was monitored at 2, 3, and 5 weeks post-
408 inoculation as described above. Dilution cultures from the 5-cell inoculum showing positive
409 growth (>10^4 cells mL^{-1}) after 5 weeks of incubation were subcultured by distributing 1 mL of
410 initial culture into 20 mL of sterile seawater growth medium, and monitored for growth during
411 incubation for up to 10 weeks at 27ºC in the dark. This experiment is hereafter referred to as
412 HTC2018.

413 Subcultures from the 5-cell cryopreserved seawater inoculum that reached >10^{-4} cells
414 mL^{-1} were cryopreserved (500 µL of culture with 10% v/v glycerol, final concentration) as
415 described above. Cells in the remaining volume (~18 mL) were collected by filtration through

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

416 0.03 μm pore-sized PES membrane filters (Sterlitech), submerged in 250 μL DNA lysis buffer,
417 and subsequently stored at -80ºC until DNA extraction.
418 The 105-cell inoculum was not subcultured. Instead, wells from one 96-well microtiter
419 plate that exhibited growth (>10^{-4} cells mL^{-1}) were cryopreserved by combining glycerol solution
420 to a final concentration of 10% v/v in 250 μL of subculture and frozen as above. Cells in the
421 remaining volume of subculture (~1 mL) were collected by filtration through 0.03 μm pore-sized
422 PES membrane filters (Sterlitech), submerged in 250 μL DNA lysis buffer, and subsequently
423 stored at -80°C until DNA extraction.
424

425 **DNA extraction and sequencing**

426 Genomic DNA was extracted from the environmental sample and all 5-cell subcultures
427 that exhibited growth using the Qiagen DNeasy Blood & Tissue Kit following the
428 manufacturer's instructions for bacterial cells (Qiagen, Germantown, Maryland, USA). Genomic
429 DNA was extracted from one 96-well microtiter plate of the 105-cell cryopreserved seawater
430 inoculum cultures that exhibited growth using the DNEasy 96 Blood & Tissue Kit (Qiagen) in
431 accordance with the manufacturer's protocol. Genomic DNA from the environmental sample and
432 all 5-cell subcultures was used as template for polymerase chain reaction (PCR) amplification
433 (Bio Rad C1000 Touch, Bio Rad, Hercules, CA, USA) using barcoded 515F and 926R primers
434 targeting the V4 region of the SSU rRNA gene (52) in a total reaction volume of 25 μL
435 containing 2 μL of genomic DNA template, 0.5 μL each forward and reverse primer, 10 μL
436 5PRIME HotMasterMix (Quantabio, Beverly, MA, USA), and 12 μL of H_2O. The reaction
437 included an initial denaturing step of 3 min at 94ºC followed by 40 cycles of 45 sec at 94ºC, 1
438 min at 50ºC and 1.5 min at 72ºC, and a final extension of 10 min at 72ºC.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

439 A nested-PCR approach was used to amplify SSU rRNA gene fragments from genomic
440 DNA recovered from the 105-cell inoculum cryopreserved seawater cultures. The first reaction
441 employed bacterial 27FB (53) and 1492R (54) primers in a 25 µL total reaction volume as
442 described above. The reaction included an initial denaturing step of 3 min at 94ºC followed by 35
443 cycles of 30 sec at 94ºC, 1 min at 50ºC and 45 sec at 72ºC, and a final extension of 18 min at
444 72ºC. PCR products from the first amplification were then used as template for a second
445 amplification reaction using the barcoded 515F and 926R primers (52), using the same reaction
446 conditions as described for the 5-cell inoculum samples.
447 All PCR products were quantified (Qubit 2.0, Invitrogen), pooled at a concentration of
448 240 ng sample^{-1}, and cleaned (QIAquick PCR Purification Kit, Qiagen). Pooled products were
449 sequenced via three Illumina MiSeq 250 bp paired-end runs using v.2 reagent kits.
450

451 **Sequence analysis**

452 The three Illumina MiSeq runs were each separately imported into QIIME2 v2019.4.0,
453 demultiplexed, and paired ends were analyzed for sequence quality and merged (55). The
454 DADA2 software package (56) was then used to denoise sequences, including removal of
455 chimeras. Due to the low quality at the end of the sequences, 10 bases were truncated from the 3'
456 end of the reverse reads. Sequence reads from the three runs were then merged post-denoising.
457 Amplicon sequence variant (ASV) identities were defined by DADA2 for all reads that varied by
458 at least one base pair. Taxonomy was assigned to each ASV using a Naïve Bayes classifier
459 trained on the Silva rRNA v132 database (57) clustered at 99% similarity and subsequently
460 modified manually based on phylogenetic analyses and the results of previous work. Denoised
461 sequences, ASVs, and taxonomy classifications were imported into R v3.5 (58) using the

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

462 phyloseq v1.26.1 package (59) for additional manual curation as outlined below. Visualizations
463 were created in R using ggplot2 (60) and in BioVenn (61).

464 The identities of ASVs found within the cultures and the environmental sample were
465 assigned by QIIME2. For each culture, ASVs represented by fewer than 20 reads were discarded
466 from the data set in order to account for potential sequencing error. Subsequently, the proportion
467 of each ASV in an individual culture was calculated using the read count for that ASV divided
468 by the total reads from the culture, post-curation. Cultures were functionally divided into three
469 separate categories: "monocultures", "mixed cultures", and cultures with no discernable,
470 dominant member. All cultures that consisted of ≥90% of reads from a single ASV and contained
471 no other ASVs that were ≥5% of reads were categorized as "monocultures", and that ASV was
472 assigned a unique isolate identifier in the Hawai'i Institute of Marine Biology Culture Collection
473 (prefix "HIMB", followed by unique number). Cultures were defined as "mixed" if they (i)
474 contained an ASV that accounted for <90% but ≥50% of the total reads for that particular
475 culture. This ASV, as well as any other ASV within the mixed culture that contained >5% of the
476 total reads, were assigned unique HIMB identification numbers. (ii) The culture consisted of
477 ≥90% of reads from a single ASV and an additional ASV that was ≥5% of reads. Each of these
478 were also assigned unique HIMB identification numbers. The final category consisted of culture
479 wells that contained no ASVs accounting for ≥50% of the total reads; these were not considered
480 further in the context of this study.

481

482 **Analysis of environmental sample**

483 All ASVs represented by <20 reads in the environmental sample were removed in order
484 to account for sequencing error and artifacts. All ASVs that were taxonomically identified as

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

485 "chloroplast" at the bacterial order-level in the Silva taxonomy were also removed from the data
486 set. The relative abundance of each remaining ASV was calculated as the read count of the
487 individual ASV divided by the total number of reads in the environmental sample, post-curation.
488 Unique identifiers were assigned to all ASVs that remained in the dataset post-curation.
489

490 **Culturability statistics**

491 Fundamental culturability statistics were derived as outlined previously (16). Briefly,
492 percent viability (culturability), V, is defined as the ratio of the number of viable cells to the total
493 number of cells initially present. It was calculated using the formula:

494 $$V = -\ln(1-p)/X$$

495 Where p is the proportion of wells that scored positive for growth and X is the number of cells
496 used for the initial inoculation. To obtain 95% confidence intervals, the exact upper and lower
497 95% confidence limits for p were calculated and inserted back into the original viability equation
498 in place of p. The result is the exact upper and lower 95% confidence limits for percent
499 culturability. For this experiment, p is defined as the number of cultures that were determined to
500 be either monocultures or mixed cultures as described above. Two separate culturability statistics
501 were calculated: one including mono- and mixed cultures, and one only including monocultures.
502

503 **Phylogenetic analyses**

504 Amplicon sequences corresponding to all ASVs were imported into the ARB software
505 package (62) and aligned to a curated database of marine bacterial 16S rRNA gene sequences.
506 Phylogenetic analyses were performed using the RAxML maximum likelihood method with the
507 GTR model of nucleotide substitution under the gamma and invariable- models of rate

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

508 heterogeneity (63). The heat map of ASV relative abundance was constructed in R v.3.5 (58)
509 using the ggplot2 package (60).
510
511 **Data availability**
512 Amplicon sequencing data are available in the Sequencing Read Archive (SRA) under bioproject
513 number xxxxxxxx.
514
515

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

516 **Acknowledgements**

517 We thank the Center for Genomic Research and Biocomputing at Oregon State University for
518 DNA sequencing, and Dr. Catherine M. Foley for her generous help with the creation of the
519 maps used in Figure 1. This research was supported by funding from the National Science
520 Foundation (grant OCE-1538628 to MSR), the Hawai‘i Institute of Marine Biology (Lord
521 Scholarship fund and graduate research assistantship to EM), and the University of Hawai‘i
522 Marine Biology Graduate Program (to EM). This is SOEST contribution xxx and HIMB
523 contribution xxx.

524

525

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

526 **References**

527 1. Baker BJ, Dick GJ. 2013. Omic approaches in microbial ecology: charting the unknown.
528 Microbe 8:353–360.
529 2. Solden L, Lloyd KG, Wrighton K. 2016. The bright side of microbial dark matter: Lessons
530 learned from the uncultivated majority. Curr Opin Microbiol 31:217-226.
531 3. Tsementzi D, Wu J, Deutsch S, Nath S, Rodriguez-R LM, Burns AS, Ranjan P, Sarode N,
532 Malmstrom RR, Padilla CC, Stone BK, Bristow LA, Larsen M, Glass JB, Thamdrup B,
533 Woyke T, Konstantinidis KT, Stewart FJ. 2016. SAR11 bacteria linked to ocean anoxia
534 and nitrogen loss. Nature 536:179–183.
535 4. Durham BP, Boysen AK, Carlson LT, Groussman RD, Heal KR, Cain KR, Morales RL,
536 Coesel SN, Morris RM, Ingalls AE, Armbrust EV. 2019. Sulfonate-based networks
537 between eukaryotic phytoplankton and heterotrophic bacteria in the surface ocean. Nat
538 Microbiol 4:1706–1715.
539 5. Giovannoni SJ, Stingl U. 2007. The importance of culturing bacterioplankton in the
540 "omics" age. Nat Rev Microbiol 5:820-826.
541 6. Rappé MS. 2013. Stabilizing the foundation of the house that 'omics builds: The evolving
542 value of cultured isolates to marine microbiology. Curr Opin Microbiol 16:618-624.
543 7. Carini P. 2019. A "cultural" renaissance: genomics breathes new life into an old craft.
544 mSystems 4:e00092-19.
545 8. Thrash JC. 2019. Culturing the uncultured: risk versus reward. mSystems 4:e00130-19.
546 9. Gifford SM, Sharma S, Booth M, Moran MA. 2013. Expression patterns reveal niche
547 diversification in a marine microbial assemblage. ISME J 7:281–298.
548 10. Yooseph S, Nealson KH, Rusch DB, McCrow JP, Dupont CL, Kim M, Johnson J,

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

549 Montgomery R, Ferriera S, Beeson K, Williamson SJ, Tovchigrechko A, Allen AE,
550 Zeigler LA, Sutton G, Eisenstadt E, Rogers YH, Friedman R, Frazier M, Venter JC. 2010.
551 Genomic and functional adaptation in surface ocean planktonic prokaryotes. Nature
552 468:60–66.
553 11. Lloyd KG, Steen AD, Ladau J, Yin J, Crosby L. 2018. Phylogenetically novel uncultured
554 microbial cells dominate Earth microbiomes. mSystems 3:e00055-18.
555 12. Zengler K, Toledo G, Rappé M, Elkins J, Mathur EJ, Short JM, Keller M. 2002.
556 Cultivating the uncultured. Proc Natl Acad Sci U S A 99:15681–15686.
557 13. Kaeberlein T, Lewis K, Epstein SS. 2002. Isolating “uncultivable” microorganisms in
558 pure culture in a simulated natural environment. Science 296:1127–1129.
559 14. Aoi Y, Kinoshita T, Hata T, Ohta H, Obokata H, Tsuneda S. 2009. Hollow-fiber
560 membrane chamber as a device for in situ environmental cultivation. Appl Environ
561 Microbiol 75:3826–3833.
562 15. Vartoukian SR, Palmer RM, Wade WG. 2010. Strategies for culture of ‘unculturable’
563 bacteria. FEMS Microbiol Lett 309:1–7.
564 16. Button DK, Schut F, Quang P, Martin R, Robertson BR. 1993. Viability and isolation of
565 marine bacteria by dilution culture: theory, procedures, and initial results. Appl Environ
566 Microbiol 59:881–891.
567 17. Schut F, de Vries EJ, Gottschal JC, Robertson BR, Harder W, Prins RA, Button DK.
568 1993. Isolation of typical marine bacteria by dilution culture: growth, maintenance, and
569 characteristics of isolates under laboratory conditions. Appl Environ Microbiol 59:2150–
570 2160.
571 18. Schut F, Gottschal JC, Prins RA. 1997. Isolation and characterisation of the marine

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

572 ultramicrobacterium *Sphingomonas* sp. strain RB2256. FEMS Microbiol Rev 20:363–369.
573 19. Connon SA, Giovannoni SJ. 2002. High-throughput methods for culturing
574 microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl
575 Environ Microbiol 68:3878–3885.
576 20. Rappé MS, Connon SA, Vergin KL, Giovannoni SJ. 2002. Cultivation of the ubiquitous
577 SAR11 marine bacterioplankton clade. Nature 418:630–633.
578 21. Morris RM, Rappé MS, Connon SA, Vergin KL, Siebold WA, Carlson CA, Giovannoni
579 SJ. 2002. SAR11 clade dominates ocean surface bacterioplankton communities. Nature
580 420:806–810.
581 22. Giovannoni SJ, Britschgi TB, Moyer CL, Field KG. 1990. Genetic diversity in Sargasso
582 Sea bacterioplankton. Nature 345:60–63.
583 23. Stingl U, Tripp HJ, Giovannoni SJ. 2007. Improvements of high-throughput culturing
584 yielded novel SAR11 strains and other abundant marine bacteria from the Oregon coast
585 and the Bermuda Atlantic Time Series study site. ISME J 1:361–371.
586 24. Song J, Oh HM, Cho JC. 2009. Improved culturability of SAR11 strains in dilution-to-
587 extinction culturing from the East Sea, West Pacific Ocean. FEMS Microbiol Lett
588 295:141–147.
589 25. Oh HM, Kang I, Lee K, Jang Y, Lim S Il, Cho JC. 2011. Complete genome sequence of
590 strain IMCC9063, belonging to SAR11 subgroup 3, isolated from the Arctic Ocean. J
591 Bacteriol 193:3379–3380.
592 26. Grote J, Cameron Thrash J, Huggett MJ, Landry ZC, Carini P, Giovannoni SJ, Rappé MS.
593 2012. Streamlining and core genome conservation among highly divergent members of
594 the SAR11 clade. MBio 3: e00252-12.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

595 27. Henson MW, Celeste Lanclos V, Pitre DM, Lee Weckhorst J, Cheng C, Temperton B,
596 Cameron Thrash J. 2020. Expanding the diversity of bacterioplankton isolates and
597 modeling isolation efficacy with large scale dilution-to-extinction cultivation. Appl
598 Environ Microbiol 86:1104-1123.
599 28. Jimenez-Infante F, Ngugi DK, Vinu M, Blom J, Alam I, Bajic VB, Stingl U. 2017.
600 Genomic characterization of two novel SAR11 isolates from the Red Sea, including the
601 first strain of the SAR11 Ib clade. FEMS Microbiol Ecol 93.
602 29. Huggett MJ, Hayakawa DH, Rappé MS. 2012. Genome sequence of strain HIMB624, a
603 cultured representative from the OM43 clade of marine Betaproteobacteria. Stand
604 Genomic Sci 6:11-20.
605 30. Grote J, Bayindirli C, Bergauer K, de Moraes PC, Chen H, D'Ambrosio L, Edwards B,
606 Fernández-Gómez B, Hamisi M, Logares R, Nguyen D, Rii YM, Saeck E, Schutte C,
607 Widner B, Church MJ, Steward GF, Karl DM, Delong EF, Eppley JM, Schuster SC,
608 Kyrpides NC, Rappé MS. 2011. Draft genome sequence of strain HIMB100, a cultured
609 representative of the SAR116 clade of marine Alphaproteobacteria. Stand Genomic Sci
610 5:269–278.
611 31. Cho JC, Giovannoni SJ. 2004. Cultivation and growth characteristics of a diverse group of
612 oligotrophic marine Gammaproteobacteria. Appl Environ Microbiol 70:432–440.
613 32. Marshall KT, Morris RM. 2013. Isolation of an aerobic sulfur oxidizer from the
614 SUP05/Arctic96BD-19 clade. ISME J 7:452–455.
615 33. Carini P, Steindler L, Beszteri S, Giovannoni SJ. 2013. Nutrient requirements for growth
616 of the extreme oligotroph "Candidatus Pelagibacter ubique" HTCC1062 on a defined
617 medium. ISME J 7:592–602.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

618 34. White AE, Giovannoni SJ, Zhao Y, Vergin K, Carlson CA. 2019. Elemental content and
619 stoichiometry of SAR11 chemoheterotrophic marine bacteria. Limnol Oceanogr Lett
620 4:44–51.
621 35. Huggett MJ, Rappé MS. 2012. Genome sequence of strain HIMB55, a novel marine
622 gammaproteobacterium of the OM60/NOR5 clade. J Bacteriol 194:2393–2394.
623 36. Durham BP, Grote J, Whittaker KA, Bender SJ, Luo H, Grim SL, Brown JM, Casey JR,
624 Dron A, Florez-Leiva L, Krupke A, Luria CM, Mine AH, Nigro OD, Pather S, Talarmin
625 A, Wear EK, Weber TS, Wilson JM, Church MJ, DeLong EF, Karl DM, Steward GF,
626 Eppley JM, Kyrpides NC, Schuster S, Rappé MS. 2014. Draft genome sequence of marine
627 alphaproteobacterial strain HIMB11, the first cultivated representative of a unique lineage
628 within the Roseobacter clade possessing an unusually small genome. Stand Genomic Sci
629 9:632–645.
630 37. Haro-Moreno JM, Rodriguez-Valera F, Rosselli R, Martinez-Hernandez F, Roda-Garcia
631 JJ, Gomez ML, Fornas O, Martinez-Garcia M, López-Pérez M. 2020. Ecogenomics of the
632 SAR11 clade. Environ Microbiol 22:1748–1763.
633 38. Delmont TO, Kiefl E, Kilinc O, Esen OC, Uysal I, Rappé MS, Giovannoni S, Eren AM.
634 2019. Single-amino acid variants reveal evolutionary processes that shape the
635 biogeography of a global SAR11 subclade. Elife 8:e46497.
636 39. Steindler L, Schwalbach MS, Smith DP, Chan F, Giovannoni SJ. 2011. Energy starved
637 candidatus *Pelagibacter ubique* substitutes light-mediated ATP production for
638 endogenous carbon respiration. PLoS One 6:e19725.
639 40. Carini P, Van Mooy BAS, Thrash JC, White A, Zhao Y, Campbell EO, Fredricks HF,
640 Giovannoni SJ. 2015. SAR11 lipid renovation in response to phosphate starvation. Proc

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

641 Natl Acad Sci U S A 112:7767–7772.

642 41. Carlson CA, Morris R, Parsons R, Treusch AH, Giovannoni SJ, Vergin K. 2009. Seasonal
643 dynamics of SAR11 populations in the euphotic and mesopelagic zones of the
644 northwestern Sargasso Sea. ISME J 3:283–295.

645 42. Morris RM, Frazar CD, Carlson CA. 2012. Basin-scale patterns in the abundance of
646 SAR11 subclades, marine Actinobacteria (OM1), members of the Roseobacter clade and
647 OCS116 in the South Atlantic. Environ Microbiol 14:1133–1144.

648 43. Ngugi DK, Antunes A, Brune A, Stingl U. 2012. Biogeography of pelagic
649 bacterioplankton across an antagonistic temperature-salinity gradient in the Red Sea. Mol
650 Ecol 21:388–405.

651 44. Giovannoni SJ, Hayakawa DH, Tripp HJ, Stingl U, Givan SA, Cho JC, Oh HM, Kitner
652 JB, Vergin KL, Rappé MS. 2008. The small genome of an abundant coastal ocean
653 methylotroph. Environ Microbiol 10:1771–1782.

654 45. Paerl RW, Sundh J, Tan D, Svenningsen SL, Hylander S, Pinhassi J, Andersson AF,
655 Riemann L. 2018. Prevalent reliance of bacterioplankton on exogenous vitamin B1 and
656 precursor availability. Proc Natl Acad Sci U S A 115:E10447–E10456.

657 46. Garcia SL. 2016. Mixed cultures as model communities: hunting for ubiquitous
658 microorganisms, their partners, and interactions. Aquat Microb Ecol 77:79–85.

659 47. Garcia SL, Buck M, Hamilton JJ, Wurzbacher C, Grossart H-P, McMahon KD, Eiler A.
660 2018. Model communities hint at promiscuous metabolic linkages between ubiquitous
661 free-living freshwater bacteria. mSphere 3:e00202-18.

662 48. Tripp HJ, Kitner JB, Schwalbach MS, Dacey JWH, Wilhelm LJ, Giovannoni SJ. 2008.
663 SAR11 marine bacteria require exogenous reduced sulphur for growth. Nature 452:741–

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

664 744.

665 49. Suzuki MT, Béjà O, Taylor LT, DeLong EF. 2001. Phylogenetic analysis of ribosomal
666 RNA operons from uncultivated coastal marine bacterioplankton. Environ Microbiol
667 3:323–331.

668 50. Yeo SK, Huggett MJ, Eiler A, Rappé MS. 2013. Coastal bacterioplankton community
669 dynamics in response to a natural disturbance. PLoS One 8:e56207.

670 51. Grant SR, Church MJ, Ferrón S, Laws EA, Rappé MS. 2019. Elemental composition,
671 phosphorous uptake, and characteristics of growth of a SAR11 strain in batch and
672 continuous culture. mSystems 4:e00218-18.

673 52. Parada AE, Needham DM, Fuhrman JA. 2016. Every base matters: Assessing small
674 subunit rRNA primers for marine microbiomes with mock communities, time series and
675 global field samples. Environ Microbiol 18:1403–1414.

676 53. Vergin KL, Urbach E, Stein JL, DeLong EF, Lanoil BD, Giovannoni SJ. 1998. Screening
677 of a fosmid library of marine environmental genomic DNA fragments reveals four clones
678 related to members of the order Planctomycetales. Appl Environ Microbiol 64:3075–3078.

679 54. Lane DJ. 1991. 16S/23S rRNA sequencing, p. 115–175. *In* Stackebrandt, E, Goodfellow,
680 M (eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons, New
681 York.

682 55. Bolyen E, Rideout JR, Dillon MR, *et al*. 2019. Reproducible, interactive, scalable and
683 extensible microbiome data science using QIIME 2. Nat Biotechnol 37:852-857.

684 56. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016.
685 DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods
686 13:581–583.

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

687 57. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO.
688 2013. The SILVA ribosomal RNA gene database project: improved data processing and
689 web-based tools. Nucleic Acids Res 41:D590–D596.
690 58. R Core Team. 2013. R: A language and environment for statistical computing. R
691 Foundation for Statistical Computing, Vienna, Austria.
692 59. McMurdie PJ, Holmes SP. 2013. phyloseq: An R package for reproducible interactive
693 analysis and graphics of microbiome census data. PLoS One 8:e61217.
694 60. Wickham H. 2009. ggplot2: elegant graphics for data analysis. Springer-Verlag, New
695 York.
696 61. Hulsen T, de Vlieg J, Alkema W. 2008. BioVenn - A web application for the comparison
697 and visualization of biological lists using area-proportional Venn diagrams. BMC
698 Genomics 9:488.
699 62. Ludwig W, Strunk O, Westram R, Richter L, Meier H, Buchner A, Lai T, Steppi S, Jobb
700 G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost
701 R, König A, Liss T, Lüßmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis
702 A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer K-H. 2004. ARB: a
703 software environment for sequence data. Nucleic Acids Res 32:1363–1371.
704 63. Stamatakis A. 2006. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses
705 with thousands of taxa and mixed models. Bioinformatics 22:2688–2690.
706

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

707 **Figure Legends**

708 **Figure 1.** Map indicating the location of stations STO1 (red triangle) and SR4 (black circle) in
709 the vicinity of Kāneʻohe Bay on the island of Oʻahu, Hawaiʽi, where seawater used as inoculum
710 (STO1) and media preparation (SR4) were collected.
711
712 **Figure 2.** Outline of experiments performed in this study.
713
714 **Figure 3.** Phylogenetic analysis of the SAR11 clade illustrating relationships among 16S rRNA
715 gene ASVs recovered from isolates and the source seawater used as inoculum in this study. The
716 scale bar corresponds to 0.1 substitutions per nucleotide position. A variety of
717 *Alphaproteobacteria* were used as an outgroup. Previously cultured isolates ("str.") and select
718 environmental gene clones were included as references. Boxes labeled "Env" indicate the
719 relative environmental abundance of each ASV (blue gradient), while orange ("2017") and green
720 ("2018") boxes indicate the presence of an ASV in the fresh (HTC2017) and cryopreserved
721 (HTC2018) seawater cultivation experiments respectively. Boxes containing a slash in the "Env"
722 column indicate ASVs was found in a culture but were not detected in the environmental sample.
723
724 **Figure 4.** Venn diagrams comparing (A) SAR11 ASVs identified within the environmental
725 seawater sample used as inoculum, isolates from the fresh seawater cultivation experiment
726 (HTC2017), and isolates from the cryopreserved seawater cultivation experiment (HTC2018).
727 (B) Same as (A), except limited to SAR11 subclade Ia ASVs.
728

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

729 **Figure 5.** Phylogenetic analysis of select lineages of *Alpha*- and *Gammaproteobacteria*
730 illustrating relationships among 16S rRNA gene ASVs recovered from isolates and the source
731 seawater used as inoculum in this study. The scale bar corresponds to 0.1 substitutions per
732 nucleotide position. A variety of *Betaproteobacteria* were used as an outgroup. Previously
733 cultured isolates ("str.") and select environmental gene clones were included as references.
734 Boxes labeled "Env" indicate the relative environmental abundance of each ASV (blue gradient),
735 while orange ("2017") and green ("2018") boxes indicate the presence of an ASV in the fresh
736 (HTC2017) and cryopreserved (HTC2018) seawater cultivation experiments respectively. Boxes
737 containing a slash in the "Env" column indicate ASVs was found in a culture but were not
738 detected in the environmental sample.
739
740 **Figure S1.** Relative abundance (bubble size) of ASVs identified in mixed cultures from
741 cultivation experiments using fresh seawater (HTC2017, 5-cell inoculum) and cryopreserved
742 seawater (HTC2018 5-cell and 105-cell inocula). Bar charts represent the number of
743 monocultures matching these ASVs cultivated in the two experiments.
744

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

745 **TABLES**

746 **Table 1.** Culturability statistics from fresh (HTC2017) and cryopreserved (HTC2018) seawater
747 cultivation experiments.

748

749 **Table 2.** Summary of isolates from fresh (HTC2017) and cryopreserved (HTC2018) seawater
750 cultivation experiments, including the relative abundance of each taxonomic group in the
751 environmental sample based on SSU rRNA gene sequencing.

752

753 **Table S1.** Summary of ASVs, mixed cultures, and isolates recovered in this study.

754

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Table 1. Culturability statistics from fresh (HTC2017) and cryopreserved (HTC2018) seawater cultivation experiments.

Inoculum source	Inoculum size (cells)[a]	Inoculated cultures	Mono- & mixed cultures		Monocultures only	
			Positive cultures[b]	Culturability (%)[c]	Positive cultures[b]	Culturability (%)[c]
HTC2017 fresh seawater	5	576	83	3.1 (2.5, 3.9)	54	2.0 (1.5, 2.6)
HTC2018 cryopreserved seawater	5	480	50	2.2 (1.6, 2.9)	39	1.7 (1.2, 2.3)
	105	94	38	0.5 (0.3, 0.7)	20	0.2 (0.1, 0.4)

[a]In a culture volume of 2 mL

[b]Calculated as the number of inoculated cultures that resulted in growth of either a monoculture or mixed culture (see Methods for definition). The constituent members of all mixed cultures are listed in Table S1.

[c]Ninety-five percent confidence intervals are shown in parentheses

bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

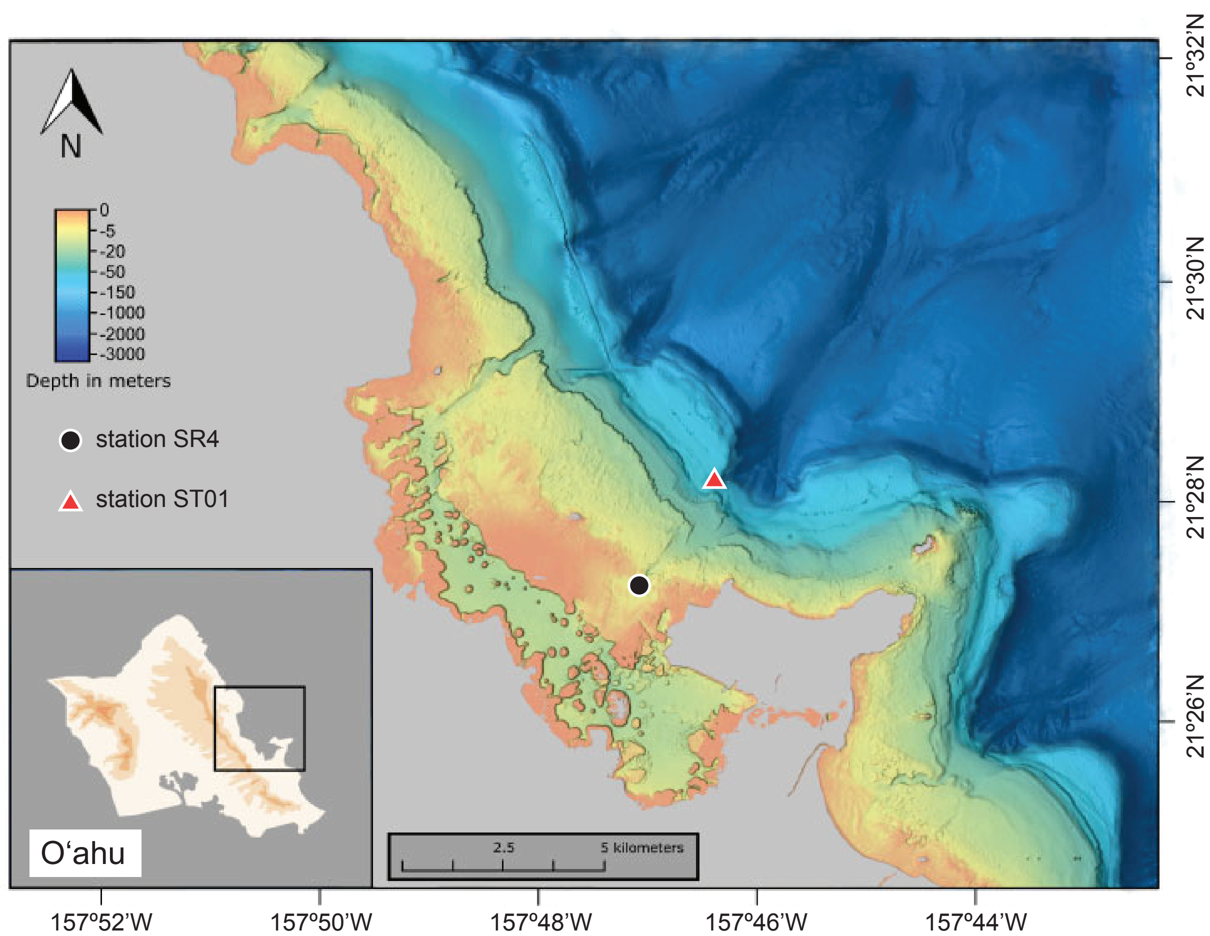
Table 2. Summary of isolates from fresh (HTC2017) and cryopreserved (HTC2018) seawater cultivation experiments, including the relative abundance of each taxonomic group in the environmental sample based on SSU rRNA gene sequencing.

	HTC2017		HTC2018		Seawater[b]	
Taxonomy[a]	ASVs	Strains	ASVs	Strains	ASVs	Abundance (rel. %)
Alphaproteobacteria, SAR11 subclade Ia	9	47	9	68	10	10.23
Alphaproteobacteria, SAR11 subclade Ib	2	4	2	3	14	7.74
Alphaproteobacteria, SAR11 subclade IIIa	0	0	1	2	4	0.42
Alphaproteobacteria, SAR11 subclade Va	0	0	1	1	2	2.13
Alphaproteobacteria, Rhodobacteraceae	4	14	5	5	12	5.81
Alphaproteobacteria, SAR116 clade	1	1	0	0	23	5.41
Alphaproteobacteria, PS1 clade	1	3	3	4	3	0.42
Gammaproteobacteria, Halieaceae, OM60(NOR5) clade	4	24	5	21	5	1.91
Gammaproteobacteria, KI89A clade	1	1	0	0	4	0.60
Betaproteobacteriales, Burkholderia-Caballeronia-Paraburkholderia	1	4	1	1	2	0.11
Betaproteobacteriales, Methylophilaceae, OM43 clade	2	5	0	0	1	0.04
Gammaproteobacteria, Pseudomonadaceae, Pseudomonas	1	2	0	0	0	0
Gammaproteobacteria, Rhodanobacteraceae	1	2	0	0	0	0
Actinobacteria, Corynebacteriaceae	0	0	1	1	0	0
Actinobacteria, Geodermatophilaceae	0	0	1	1	0	0
Bacteroidetes, Chitinophagaceae, Sediminibacterium	0	0	1	1	0	0
Fungi	1	1	1	2	0	0

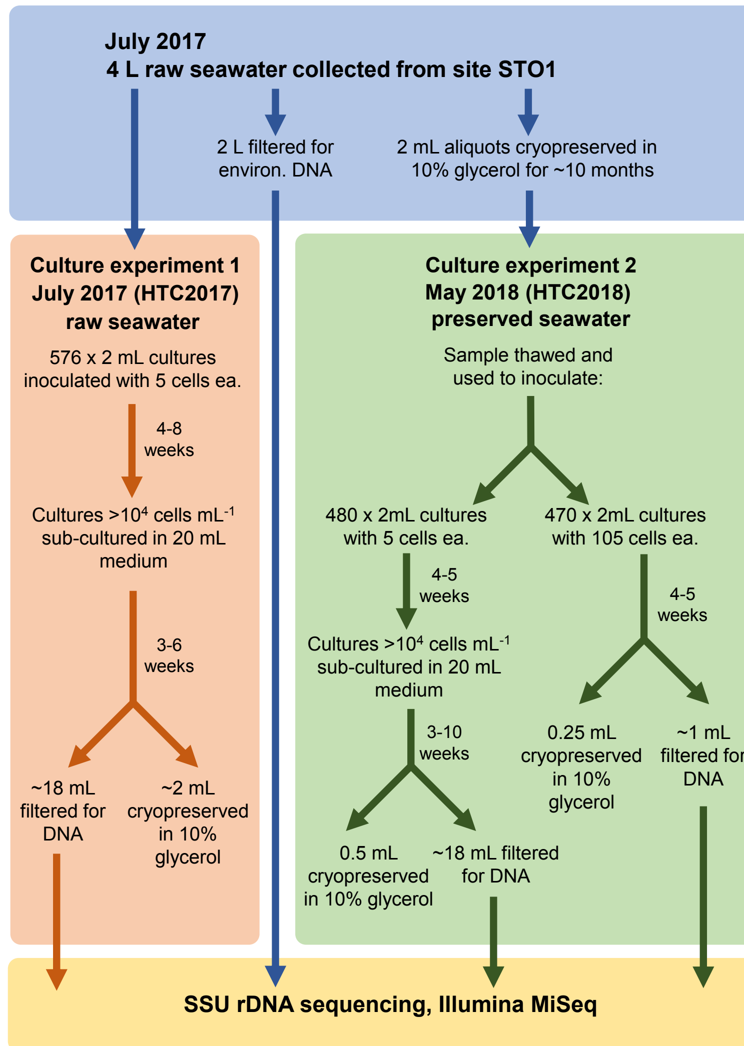
[a]Abbreviated taxonomy adapted from Silva release 132; the full taxonomy of each ASV is available in Table S1.

[b]Environmental relative abundance was calculated using read counts of all environmental ASVs detected in the seawater sample from within each taxonomic grouping, after curation to remove ASVs originating from chloroplasts.

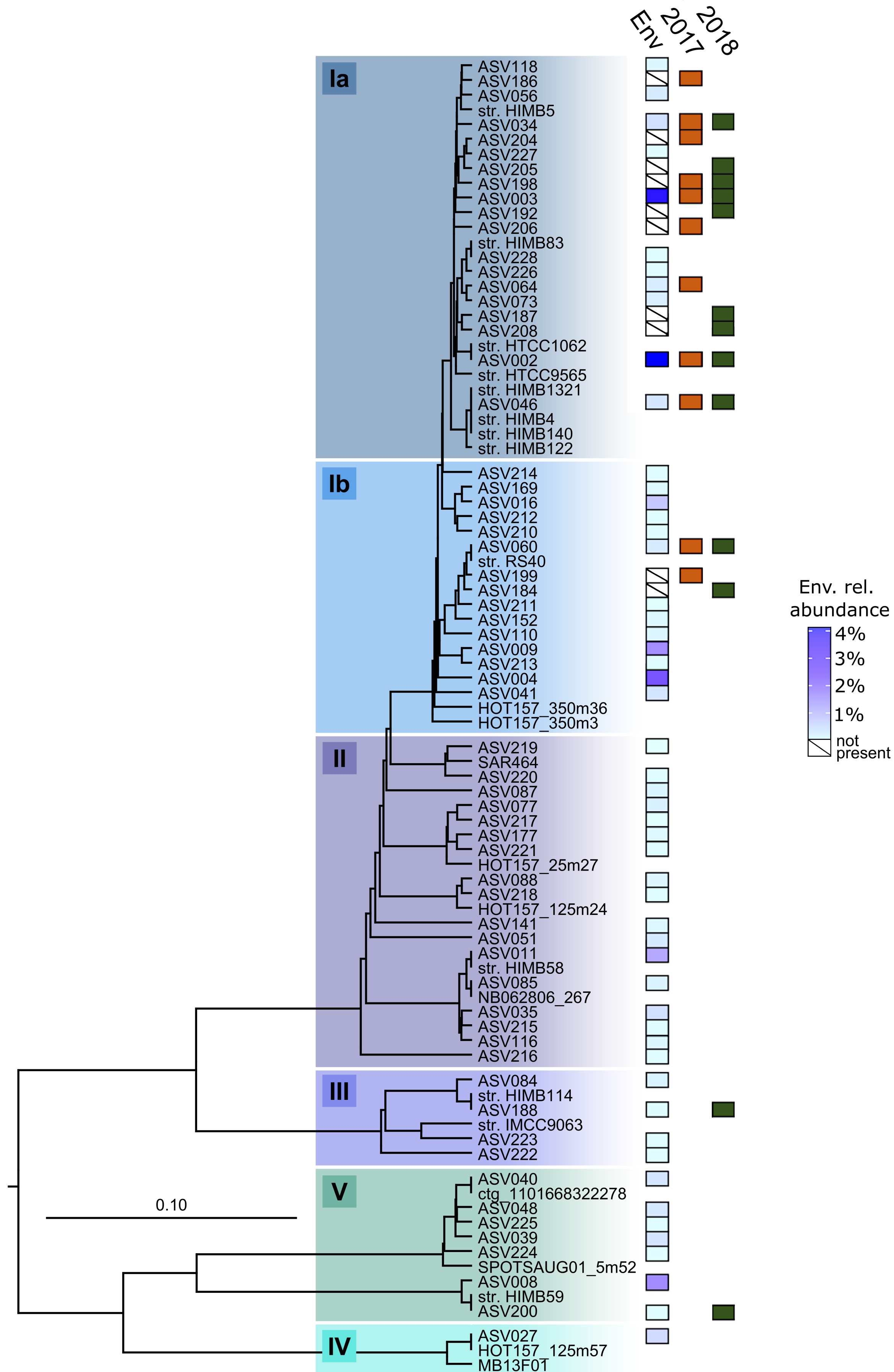
bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



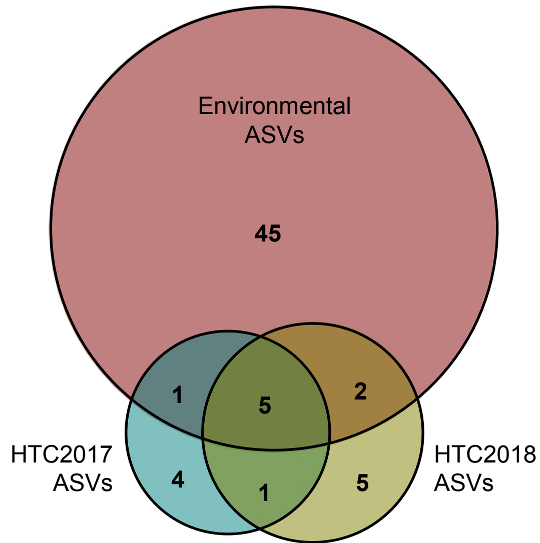
bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

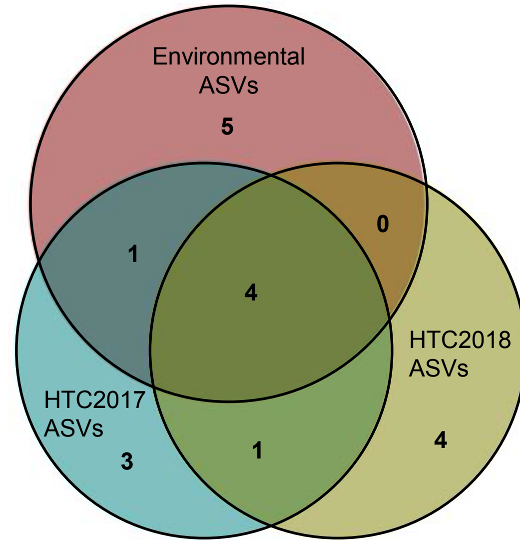


bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

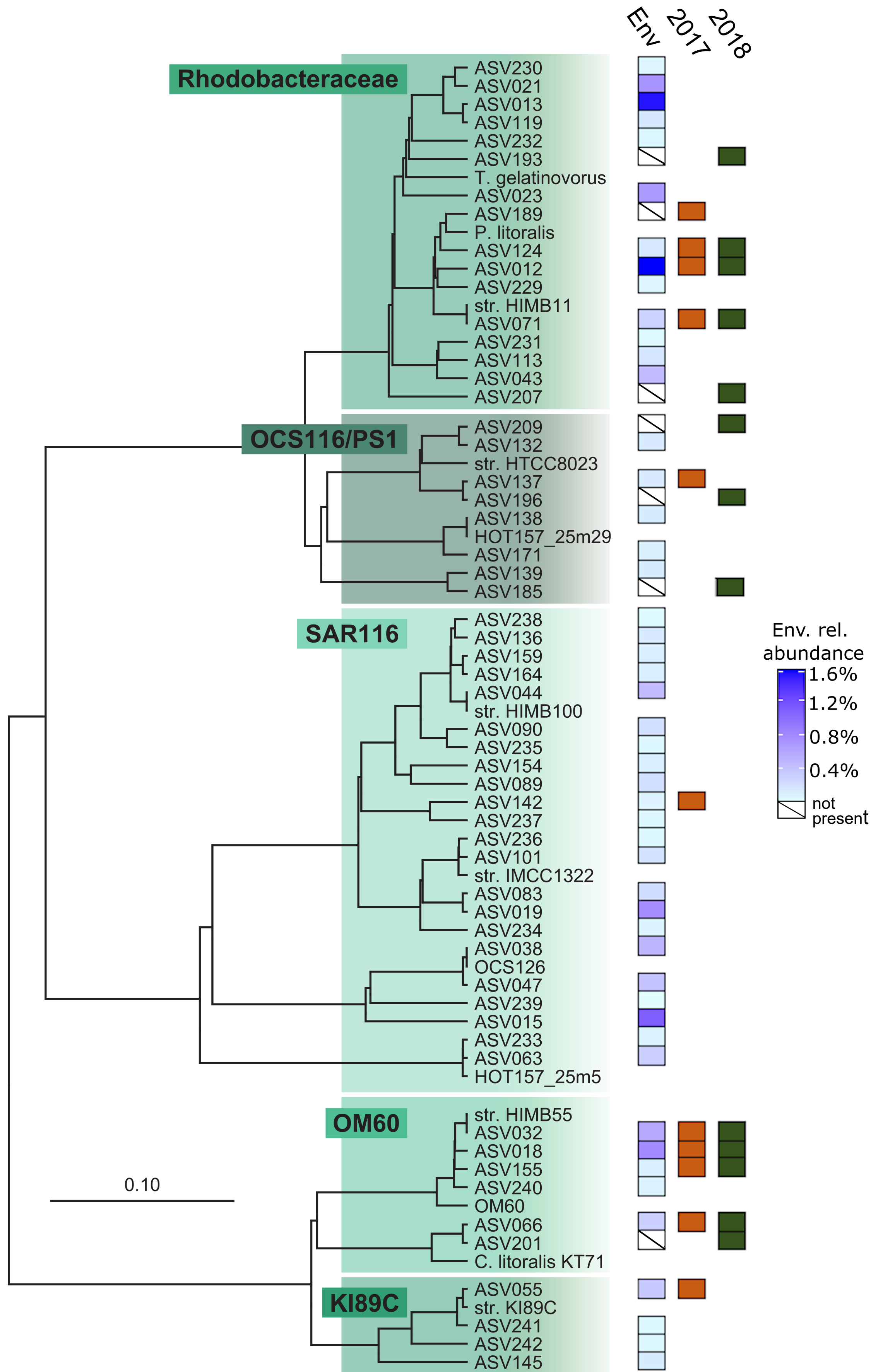


bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.





bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



bioRxiv preprint doi: https://doi.org/10.1101/2020.09.22.309336; this version posted September 23, 2020. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.