Potential probiotic aquaculture microbiomes: detecting inhibition of the fish pathogen *Vibrio anguillarum* by a complex algal community

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ABSTRACT

Intensive fish rearing in aquaculture is challenged by infectious diseases. Although vaccines have been successfully developed for mature fish, alternative disease control measures are needed for fish larvae and juveniles, whose immune systems are not yet fully developed. Probiotics, typically isolated as pure cultures from aquaculture microbiomes, offer a promising alternative to antibiotics, with the potential to reduce antibiotic use and, consequently, the risk of antibiotic resistance. In this study, we developed an *in vitro* assay to evaluate the anti-pathogen efficacy of not only individual bacterial cultures but also of mixed algal microbiomes derived from the live-feed microalgae *Tetraselmis suecica* and *Isochrysis galbana*, based on the premise that natural microbiomes have inherently evolved to combat pathogens. This assay was used to compete a GFP-tagged *Vibrio anguillarum*, a key fish pathogen, against different microbial communities, allowing us to quantify the inhibitory activity through fluorescence. We found that the *Isochrysis galbana* microbiome was more inhibitory to *V. anguillarum* than the *Tetraselmis suecica* microbiome. During co-culture with the pathogen, the *Isochrysis* microbiomes increased in bacterial density and reduced in diversity as determined by metataxonomic analyses. The enriched inhibitory microbiomes could fully inhibit the pathogen. Bacteria isolated from these microbiomes were members of *Alteromonadaceae, Halomonadaceae, Rhodobacteraceae, Vibrionaceae, Flavobacteriaceae,* and *Erythrobacteraceae*. Although some strains individually inhibited the pathogen, enhanced inhibition was observed when *Sulfitobacter pontiacus* D3 and *Halomonas campaniensis* D2 were co-cultured, even though neither were inhibitory as monocultures. Thus, this study demonstrates that microbial communities derived from natural algal microbiomes can have anti-pathogen effects, suggesting their potential as aquaculture probiotics. Furthermore, the results indicate that bacterial co-cultures may offer synergistic advantages over monocultures, highlighting their promise for aquaculture health strategies.

IMPORTANCE

Aquaculture is the fastest growing food protein producing sector and sustainable disease control measures are required. Here we demonstrate that bacteria, single or mixed cultures, from algal microbiomes can inhibit the potent fish pathogen *Vibrio anguillarum*. We developed an assay, by GFP-tagging of a potent fish pathogen, allowing the anti-pathogen effect of a complex microbiome to be quantitatively determined. The microbiomes were derived from algae normally used in marine larviculture and directing these microbiomes in a disease suppressive direction can limit the use of antibiotics for disease control and reduce the risk of spread of antibiotic resistance.

58 **INTRODUCTION**

59

60 As the human population continues to rise, sustainable production of dietary protein is becoming
61 increasingly important, and aquaculture is a key sector in supplying such high quality protein and has
62 for decades been the fastest growing protein producing food sector (1). However, intensive rearing
63 of fish carries with it the risk of disease outbreaks, which are especially damaging during larval
64 development (1, 2) where disease outbreaks can result in substantial economic losses, as seen in a
65 1988 incident where vibriosis led to a 40% loss of juvenile turbot in a Norwegian hatchery (3, 4). Fish
66 larvae lack a mature immune system, rendering vaccination ineffective as a control measure against
67 disease (1, 2). *Vibrionaceae* infections pose a considerable challenge to marine larviculture and can
68 be caused by a number of *Vibrio* species, including *V. anguillarum* that can infect up to 80 different
69 fish species (5, 6). Despite advances in vaccine development, vibriosis remains a significant problem
70 in aquaculture, primarily affecting marine fish and crustaceans (6, 7).

71

72 Many commercially valuable marine fish species require live feed, such as microalgae, at the larval
73 stages since suitable artificial feed formulations are not available (8, 9), however, live feed can serve
74 as the infection vector for pathogenic bacteria in larval rearing, leading to swift and widespread
75 infections and complicating disease management (9, 10). Historically, antibiotics have been used to
76 control these infections among larvae and juveniles, but since this may lead to spread of
77 antimicrobial resistance (AMR) of both clinical and agri- and aquacultural relevance, alternative
78 approaches are urgently needed (11). A promising strategy to mitigate bacterial pathogen
79 proliferation in live feed and fish larvae is the use of probiotic bacteria which can inhibit pathogenic
80 bacteria (12–14). Probiotic microorganisms, as defined by FAO and WHO, are "live microorganisms
81 that, when administered in adequate amounts, confer a health benefit on the host" (15). As
82 reviewed in Sonnenschein *et al.* (16), probiotics can be an environmentally sustainable and
83 economically viable approach to counteract economic losses due to bacterial pathogens in
84 aquaculture, and have been intensively studied in the past decades (2, 17). Of special note are
85 members of the *Phaeobacter* and *Ruegeria* genera which are particularly promising as probiotics in
86 marine larviculture. The genera are part of the widely occurring marine *Roseobacter* group and they
87 occur naturally in the aquaculture environment and produce a vibrio-antagonizing compound
88 tropodithietic acid (TDA) (18). The TDA-producing roseobacters can protect fish larvae against
89 vibriosis (12, 19, 20), and several studies have demonstrated that resistance does not develop (21,
90 22) likely due to the molecular effect of TDA as an antiporter (23).

91

92 As exemplified by the TDA-producing roseobacters, harnessing the potential of natural microbiomes
93 for the use of probiotics is a promising approach for pathogen control in aquaculture. This method
94 leverages the microbial communities found in aquatic ecosystems to protect and enhance the health
95 of aquaculture organisms (16). Studies on probiotics have typically relied on the isolation and testing
96 of pure bacterial cultures, however, in contrast to traditional single-isolate probiotics, complex
97 microbiomes may offer a more holistic approach by capitalizing on the synergistic interactions
98 among multiple microorganisms, including bacteria, to effectively combat pathogens (24). Several
99 studies have focused on use of probiotics in larviculture and have suggested that deriving potential
100 probiotic cultures from the aquaculture system offers an ecological advantage when they are re-
101 introduced (12, 16, 25, 26).
102
103 Microalgae, such as *Tetraselmis suecica* and *Isochrysis galbana*, that are used as live feed in many
104 larviculture productions are colonized by a microbiome, called the phycosphere, that can influence
105 the growth and metabolism of the algae (27–29). The algal microbiome depends on the host species
106 (30), is taxonomically diverse and harbours a large bioactive potential (31, 32). Roseobacters,
107 including the TDA-producers, have repeatedly been isolated from microalgae (18, 26, 33, 34),
108 however, they represent only a part of the algal microbiome where they co-exist with several other
109 taxa. The microbial world is characterized by an intricate web of interactions, some of which are
110 mediated by small molecules through which microorganisms exchange information with their
111 neighbours, competitors, and hosts. These interactions shape the microbial community and its
112 functionality, however, monocultures lack these interactions that are important for e.g. inducing
113 expression of biosynthetic gene clusters producing bioactive compounds (35–37). We therefore
114 hypothesize that the collective microalgal microbiome and not only pure cultures of roseobacters
115 could serve to counteract invasion of aquaculture pathogens. Using a complex microbiome to inhibit
116 a target bacterium, we hope to harvest the enhanced inhibitory properties promoted by the
117 microbiome interactions.
118
119 Whilst the study of single culture inhibition is straight forward, the high throughput screening of
120 pathogen inhibition by a complex microbiome is more challenging, and a prime purpose of the
121 present study was to develop a simple screening assay that would allow us to determine the
122 pathogen inhibition by a complex microbiome. Therefore, we developed an assay that allows for the
123 measurement of the antibacterial effect of complex microbiomes and subsequently determined the
124 composition of an inhibitory microalgal microbiome based on 16S rRNA gene amplicon sequencing.
125 Additionally, we isolated bacterial strains for future use as mixed probiotic cultures.

126 **MATERIALS AND METHODS**

127 **Bacterial and algal strains and culture conditions**. The fish pathogen *Vibrio anguillarum* NB10 with a
128 fluorescent tag (NB10_pNQFlaC4-gfp27) (38) (later referred to as *Vibrio anguillarum* NB10_gfp) was
129 used as target bacterium in the inhibition assays. *Phaeobacter piscinae* S26 (26) was used as a known
130 antagonist of *Vibrio* as a positive control for establishing the fluorescence-based screening assay. *P.*
131 *piscinae* and *V. anguillarum* were stored at –80°C and routinely cultivated on Marine Agar (MA;
132 Difco2216 BD) at 25°C or in Marine Broth (MB; Difco2216 BD) at 25°C and 200 rpm, supplemented
133 with 4 µg/mL chloramphenicol in case of *V. anguillarum* NB10_gfp. All bacterial cultures were stored
134 and cultivated similarly, including *V. anguillarum* NB10 wild type (WT), and bacterial isolates
135 obtained from algal cultures and enriched inhibitory microbiomes (see below).

136 Xenic microalgal cultures of *Tetraselmis suecica* and *Isochrysis galbana* were provided by
137 aquaculture industry partners. Axenic algae *Isochrysis galbana* CCMP 1323 and *Tetraselmis suecica*
138 CCAP 66/4 were purchased from the Bigelow National Center for Marine Algae and Microbiota
139 (NCMA) and the Culture Collection of Algae and Protozoa (CCAP), respectively, and their axenic
140 status determined by plating on MA. The algae were cultured in f/2 medium without silicate (f/2 –
141 Si; NCMA; (39, 40)) made with 3% Instant Ocean (IO; Aquarium Systems Inc., Sarrebourg, France)
142 and cultivated at 18°C and constant illumination at ~50 µE m^{-2} s^{-1}. Cultures were maintained by
143 continuous subculturing every 4 weeks, transferring 1% of the culture to 25 mL fresh medium.

144

145 **Fluorescence-based screening assay for GFP-labelled target organism**. *V. anguillarum* NB10_gfp
146 was grown overnight as described above and a 10-fold serial dilution was prepared using MB as a
147 diluent. 180 µL of the dilutions were distributed to the wells of a sterile, black 96-well microplate
148 with flat, clear bottom (Perkin Elmer, 6005430) in technical triplicates. *V. anguillarum* NB10 WT was
149 included as a negative control for the fluorescence measurement, and sterile medium as a no-
150 growth negative control. Fluorescence and absorbance were measured on a BioTek Cytation™ 5
151 multi-mode microplate reader at 25°C with shaking (linear, continuous, 567 cpm). Measurements
152 were taken every 20 minutes over 40 hours, measuring fluorescence (excitation: 485/15 nm,
153 emission: 513/15 nm) and OD_{600}.

154

155 **Fluorescence-based inhibition assay by *Phaeobacter piscinae* S26**. *Phaeobacter piscinae* S26 was
156 co-cultured with *V. anguillarum* NB10_gfp in the fluorescence-based inhibition assay. Overnight
157 cultures of both bacteria were prepared, 10-fold serially diluted in sterile medium (MB) and 90 µL of
158 each dilution of both bacterial cultures was loaded into sterile, black 96-well microplates allowing
159 crossed combinations of the two strains at the different dilutions (total well-content of 180 µL).

160 Dilutions of *P. piscinae* S26 were included as a negative control for the fluorescence measurement,
161 and sterile medium as a no-growth negative control. Dilutions of *V. anguillarum* were included as a
162 positive control. Absorbance and fluorescence of the plates were measured as described above.
163 Before the experiment, bacterial counts were determined by 10-fold serial dilution and plating on
164 MA.
165
166 **Inhibition of *V. anguillarum* by complex algal microbiomes**. Microbiomes from two xenic
167 microalgae, *Tetraselmis suecica* and *Isochrysis galbana,* were co-cultured with *V. anguillarum*
168 NB10_gfp using the fluorescence-based screening assay (Figure 1). Samples from axenic algal
169 cultures were included as controls without microbiomes. Five algal cultures were tested in the assay
170 described above. This included the two axenic cultures (AXT: axenic *Tetraselmis*; AXI: axenic
171 *Isochrysis*), two xenic cultures of *Tetraselmis suecica* (NT) and *Isochrysis galbana* (NI) that had been
172 re-cultivated in the lab since 2019 and 2020, respectively, and a newly acquired (2023) *Isochrysis*
173 *galbana* culture (NNI) from an aquaculture collaborator. Cultures (200 mL) of the two microalgae,
174 both axenic and xenic, were prepared by inoculating 10% stock culture to fresh media and
175 incubating them at 18°C and constant illumination for five days reaching approx. 6 log algal cells mL^{-}
176 ^{1}. The *V. anguillarum* NB10_gfp strain was grown overnight, and algal and bacterial counts were
177 determined at the beginning of the experiment. Bacterial counts were determined by 10-fold serial
178 dilution and plating on MA. Plates were incubated for two days for *V. anguillarum* and for four days
179 for the bacterial counts from algal cultures. All CFU counts were log-transformed before further
180 statistical analyses. Algal cell counts were determined using microscopy and a Neubauer-improved
181 chamber. To determine if any pathogen inhibition was caused by the algae or by their microbiomes,
182 the xenic algal cultures were divided into two 100 mL fractions: the full culture (FC) with algal and
183 bacterial cells, and a filtered microbiome (FM) fraction, where algal cells were removed by filtering
184 (47-mm Ø, pore size 3-µm, mixed cellulose ester, Advantec A300A047A). Before filtering, the
185 cultures were vortexed vigorously to detach bacteria from the algal cells.

186 To increase the bacterial density (target ~ 7 – 8 log CFU mL^{-1}), 100 ml of algal cultures were
187 harvested (2,445 x g, 10 min, 20°C) and resuspended in 1 mL sterile 3% IO. 10-fold serial dilutions of
188 both up-concentrated algal fractions (FC and FM; 3% IO as diluent) and *Vibrio* cultures (MB as
189 diluent) were prepared, and loaded into sterile, black 96-well microplates. Combinations of dilutions
190 of the pathogen (target: 3 log CFU mL^{-1} and 5 log CFU mL^{-1}) and algal samples (10^{-1} to 10^{-5} dilutions)
191 were loaded. Algal cultures with no addition of the GFP tagged pathogen were included as negative
192 control for the fluorescence measurement, and sterile medium as a no-growth negative control.
193 Different dilutions of the pure culture of *V. anguillarum* NB10_gfp were included as a positive

194 control. Fluorescence and OD_{600} were measured as described above. FM samples were measured on
195 a different instrument (BioTek Synergy HTX) than all other samples, resulting in a different scale of
196 the fluorescence measurements. The overview of sample preparation and experimental setup are
197 summarized in Figure 1.
198
199 **Characterizing the enriched inhibitory microbiomes**. During the co-culture inhibition assay, the
200 bacterial density of the algal microbiomes was increased. Several dilutions of the algal microbiomes
201 inhibited growth of *V. anguillarum* in a "dose dependent" manner and in all co-cultures, the
202 bacterial density of the algal microbiomes increased. These microbiomes which caused suppression
203 of the pathogen will be referred to as enriched inhibitory microbiomes. DNA was extracted from a
204 selection of the wells taking samples from wells exhibiting a complete inhibition and wells allowing
205 only marginal growth of *V. anguillarum*. Also, samples were taken from no-growth negative controls
206 of the experiment, resulting in a total of 41 samples. Upon completion of the assay, the plates and
207 samples were stored at -80°C until further processing, and then thawed at 4°C and samples kept on
208 ice. 2 x 200 µL lysis buffer (400 mM NaCl, 750 sucrose, 20 mM EDTA, 50 mM Tris-HCl, 1 mg/mL
209 lysozyme [pH 8.5]) was added to the wells. The contents of the wells were then resuspended and
210 transferred to Eppendorf tubes and incubated at 37°C for 30 min. Proteinase K was added to a final
211 concentration of 100 mg/mL and sodium dodecyl sulphate (SDS) to a final concentration of 1%
212 vol/vol, and samples were then incubated overnight at 55°C, 300 rpm (41). The order of handling of
213 samples was then randomized before extraction. DNA extraction was done by the Promega Maxwell
214 16 using the Maxwell LEV Blood DNA Kit, and the quality and purity of the DNA were measured using
215 a DeNovix 439 DS-11+ spectrophotometer (DeNovix Inc., Wilmington, DE, USA) and confirmed by gel
216 electrophoresis.
217 The V3-V4 region of the 16S rRNA gene of all samples was amplified by PCR as described in
218 Roager *et al*. (2023), using the primer set 341f (59-CCTACGGGNGGCWGCAG-39) and 805r (59-
219 GACTACHVGGGTATCTAATCC-39) tagged with 30 unique octametric barcodes (Table S1). PCR
220 products were purified using Agencourt AMPure XP magnetic beads (0.8:1 bead volume to DNA
221 solution; Agencourt Bioscience Corporation, Beverly, MA, USA). Concentrations of the amplicons
222 were determined using a Qubit 2.0 Fluorometer with the high sensitivity (HS) assay kit (Qubit™
223 dsDNA HS assay; Invitrogen by Thermo Fisher Scientific Inc., Eugene, OR, USA). Samples were pooled
224 in equimolar ratios before library preparation and sequencing on an Illumina NovaSeq 6000 platform
225 with paired-end 250-bp reads (Novogene; Cambridge, United Kingdom).
226

227 **Analyses of sequencing data**. Sequencing data analyses were performed using QIIME 2 version
228 2023.5 (42) and R version 4.2.1 (43). The raw reads were imported to QIIME2, demultiplexed, and
229 primers and barcodes were removed using the cutadapt demux-paired plugin (44). Reads were then
230 denoised and dereplicated using the DADA2 package (45) and an amplicon sequence variant (ASV)
231 table was created leaving out sequences shorter than 400bp. A phylogenetic tree was constructed
232 using MAFFT (46) and FastTree (47), via the phylogeny plugin. Alpha- and beta-diversity metrics were
233 computed using the diversity plugin (48) at an even sampling depth of 26,226. A classifier trained on
234 the Silva database 138 (49) and the specific primer sequences employed as mentioned above was
235 used for taxonomic classification of the reads via the feature-classifier plugin (50, 51). ASVs which
236 were either not assigned to a phylum, classified as chloroplasts/mitochondria or had a frequency <
237 10 were filtered out before further analysis in R. Two sterility controls were excluded from the
238 dataset before further analysis. QIIME 2 artifacts were imported to R v4.2.1 using the qiime2R
239 package (52) and analysis was continued in RStudio (53) with the tidyverse (54) package. For
240 multivariate analyses using the vegan package, the ASV table was normalized by square-root
241 transformation and Wisconsin standardization (55). PERMANOVA analysis was then performed using
242 the beta-group-significance function of Qiime2 and the adonis plugin (56) in R with 999
243 permutations to test significant differences in beta diversity as a function of source culture (NI and
244 NNI), degree of pathogen inhibition, microbiome dilution and sample fraction (FC and FM), based on
245 Bray-Curtis dissimilarities calculated using the normalized ASV table. The analysis was conducted
246 both on the complete dataset and on subsets divided by samples before and after the enrichment
247 assay. Interactive effects were included in the models but were removed due to lack of significance.
248
249 **Isolation and identification of bacterial strains from *Isochrysis galbana* microbiomes***.* Culturable
250 bacterial isolates were obtained from a subset of the enriched inhibitory *I. galbana* microbiomes (NI
251 and NNI) analysed for their microbiome composition. Culture from wells where *V. anguillarum* was
252 inhibited by the microbiomes was serially diluted and plated on MA. Isolates were picked
253 representing colonies with different morphology. Also, strains were picked from the plating of the
254 algal culture before testing inhibition (native microbiome), resulting in a total of 64 bacterial isolates.
255 The strains were re-streaked for purity and glycerol stocks (20% v/v) were made and stored at -80°C.
256 The isolates were later identified by 16S rRNA gene amplification by colony PCR using universal
257 primers 27F and 1492R and subsequent Sanger sequencing by Macrogen (Amsterdam, Netherlands).
258 Sequencing results were compared against the NCBI nucleotide database using the BLASTn function
259 (57) to determine the putative bacterial taxa.
260

261 **Inhibition of *V. anguillarum* by pure cultures and co-cultures of bacterial isolates**. Antagonism of
262 pure cultures of the 64 bacterial isolates against *V. anguillarum* NB10_gfp was tested using an agar-
263 based assay (58) using 50 µL of culture to 50 ml of agar and spotting 5 µL of an overnight culture of
264 the isolates on the pathogen-embedded plates. Plates were incubated at 25°C and visually inspected
265 for clearing zones after 24 and 48 hours. In a similar manner, dual cultures of isolates from the
266 enriched inhibitory microbiome were spotted on embedded *Vibrio* and inspected for inhibition. Five
267 isolates were chosen for investigating co-culture effects as representatives of the five non-*Vibrio*
268 genera isolated from the enriched inhibitory *I. galbana* microbiomes (Table 2) with complete
269 inhibition of 5.3 log CFU mL^{-1} starting inoculum of *V. anguillarum*. The strains were grown in liquid
270 cultures (MB, 25°C, 200 rpm) for 24 hours and then mixed 1:1 in pairs. 10 µL of the mixtures were
271 spotted on the pathogen-embedded plates. The plates were incubated at 25°C and visually
272 inspected for clearing zones after 24 and 48 hours. Monocultures of the individual strains mixed 1:1
273 with MB were used as controls.
274
275 **Whole genome sequencing, annotation and analysis.** The five isolates selected for the co-culture
276 study were whole genome sequenced with Oxford Nanopore sequencing, using the GridION
277 platform. Genomic DNA was extracted from overnight cultures with the NucleoSpin® Tissue Mini kit
278 (Macherey-Nagel, 740952.5) protocol for bacteria, with three hours of incubation during pre-lysis.
279 The quality, purity, and concentration of the DNA was assessed as described for amplicon
280 sequencing. The library was prepared using the Rapid Barcoding Kit 24 V14 (SQK-RBK114.24). The
281 resulting reads were processed by the dragonflye pipeline (59). Briefly, adaptors were trimmed using
282 Porechop and reads smaller than 1000bp were removed with Nanoq. The genomes were then
283 assembled with Flye and polished with Medaka. The assemblies were then annotated with Bakta
284 v1.8.2 (60), and profiled for BGCs with antiSMASH 7.0 (61). Subsequently, the assembled genomes
285 were taxonomically classified using autoMLST (62).
286
287 **Data availability.** The sequencing data from the microbiome analysis and the assembled whole
288 genomes are available on the Sequencing Read Archive under BioProject ID PRJNA1150956.
289
290 **RESULTS**

291 **Fluorescence-based inhibition assay for GFP-labelled *Vibrio anguillarum***. *V. anguillarum* NB10_gfp,
292 as expected, grew well in MB reaching an absorbance of 0.929 ± 0.003 at 600 nm (in 19 hours for 5.3
293 log CFU mL^{-1} starting inoculum). The starting inocula of *V. anguillarum* NB10_gfp ranged between
294 1.3 to 9.3 log CFU mL^{-1}. In pure culture, the growth of *V. anguillarum* could be followed by the

295 fluorescent signal indicating the same lag phase, growth rate and stationary phase entry as seen on
296 the OD_{600} measurements (Figure 2A and 2B). *V. anguillarum* was then co-cultured with a dilution
297 series of a known antagonist, *Phaeobacter piscinae* S26, to provide proof-of-concept that growth-
298 inhibition of the pathogen could be detected by measuring (lack of) fluorescence signal of a
299 fluorescently tagged target organism. The starting inocula of *P. piscinae* S26 ranged between 9.1 to
300 1.1 log CFU mL^{-1}. As expected, lower starting concentrations of *Vibrio* were more easily inhibited
301 than higher concentrations (data not shown) and, conversely, higher concentrations of *Phaeobacter*
302 were more inhibitory than lower concentrations (Figure 2C).
303
304 **Anti-pathogen inhibitory effect of algal microbiomes on *Vibrio anguillarum*.** To investigate the
305 inhibitory effect of their microbiomes on *V. anguillarum* NB10, dilutions of samples from cultures of
306 two xenic microalgae, *I. galbana* and *T. suecica,* were co-cultured with the GFP-tagged fish
307 pathogen. In the experiments, the starting inocula of *V. anguillarum* NB10_gfp was 3.1 ± 0.3 log CFU
308 mL^{-1} (target: 3 log CFU mL^{-1}) and 5.1 ± 0.3 log CFU mL^{-1} (target: 5 log CFU mL^{-1}). Initial algal cell
309 counts varied between 5.2 and 6.4 log cells mL^{-1} and up-concentration resulted in approx. 1.5 log
310 increase to between 7.2 and 8.0 log cells mL^{-1}. Initial bacterial counts in the *Isochrysis* samples varied
311 between 5.7 and 6.6 log CFU mL^{-1} and in the up-concentrated FC fraction between 7.6 and 8.4 log
312 CFU mL^{-1}, whereas in the up-concentrated FM fraction between 7.6 and 8.1 log CFU mL^{-1} (Table S2).
313 Microbiomes from both *T. suecica* and *I. galbana* inhibited growth of *V. anguillarum*, with the *I.*
314 *galbana* microbiome being more potent (Figure 3A vs. 3D and 3B vs. 3E). The same inhibition pattern
315 was observed for the filtered microbiomes (algal cells removed) as for the full culture (algal and
316 bacterial cells) (Figure 3A vs. 3B and 3D vs. 3E). No inhibition was observed from samples from
317 axenic algae cultures, indicating that it is indeed the microbiomes growing in the wells that inhibit
318 the pathogen (Figure 3C and 3F). OD_{600} measurements can be seen in Figure S1.
319
320 **Sequence based analysis of microbiomes.** In total, 70,542,064 paired end reads were obtained from
321 the 16S rRNA gene amplicons, with a mean of 1,500,894 reads per sample. After denoising, merging,
322 filtering out chloroplasts and mitochondria, removal of short sequences (< 400 bp) and low
323 abundant ASVs (total frequency < 10), this resulted in a total of 320 amplicon sequence variants
324 (ASVs), with a mean read count per sample of 209,507 (Table S3).
325
326 **Microbiome composition and diversity analyses.** The native microbiomes of *I. galbana* were
327 dominated by *Alteromonadaceae, Rhizobiaceae, Flavobacteriaceae, Rhodobacteraceae* and
328 *Hyphomonadaceae*. The microbiome composition of the two *I. galbana* cultures (NI and NNI)

329 differed significantly (PERMANOVA, $R^2 = 0.312$, $p = 0.001$), with NNI representing a relatively freshly
330 recruited microbiome and NI representing a selectively adapted microbiome, subcultured many
331 times in the laboratory over four years. The two cultures shared several genera, such as
332 *Flavobacteriaceae, Rhizobiaceae, Alteromonadaceae* and *Rhodobacteraceae*, but also had distinct
333 microbiome compositions. *Hyphomonadaceae* and *Cyclobacteriaceae* were unique to NI, and each
334 culture had several distinct representatives of *Flavobacteriaceae* and *Rhodobacteraceae* (Figure S2).

335 The enriched inhibitory microbiomes from the *Vibrio* inhibition assay were largely
336 dominated by *Alteromonadaceae* and *Rhodobacteraceae*, with a higher relative abundance of
337 *Vibrionaceae* compared to the native microbiome. As expected, the relative abundance of
338 *Vibrionaceae* was highest in samples with marginal growth of the pathogen, compared to those with
339 complete inhibition of the target pathogenic vibrio. However, *Vibrionaceae* were also detected in
340 lower relative abundances in enriched cultures that fully inhibited *V. anguillarum*, suggesting these
341 sequences could represent native vibrios of the algal microbiome. The relative abundances of
342 *Alteromonadaceae* and *Rhodobacteraceae* increased during the inhibition assay, whereas those of
343 *Rhizobiaceae*, *Flavobacteriaceae*, and *Hyphomonadaceae* decreased.
344 Community richness (Faith index), diversity (Shannon index), and evenness (Pielou's index)
345 showed no significant differences between microbiomes with different degree of pathogen
346 inhibition ($p > 0.05$). However, based on a pairwise Kruskal-Wallis analysis, significant differences in
347 Pielou's index were observed between the NI and NNI cultures ($p = 0.0041$), with the NI culture
348 having a higher diversity and evenness. PERMANOVA analysis of the full dataset indicated a
349 significant shift in microbial community composition from before (native microbiome) to after the
350 experiment (enriched inhibitory microbiome). Therefore, the data was split into two subsets for
351 further analysis.
352 Post-enrichment, community composition was significantly affected by the source culture
353 (NI vs. NNI) (PERMANOVA, $R^2 = 0.253$, $p = 0.001$), degree of pathogen inhibition (PERMANOVA, $R^2 =$
354 0.064, $p = 0.017$), microbiome dilution (PERMANOVA, $R^2 = 0.098$, $p = 0.001$), and algal fraction (full
355 culture (FC) vs. filtered microbiome (FM)) (PERMANOVA, $R^2 = 0.060$, $p = 0.025$). Residual variation
356 accounted for 52.5% of the total variation.
357 In conclusion, the degree of pathogen inhibition was associated with differences in the
358 taxonomic composition of the sampled microbiomes. However, most variation between
359 microbiomes was explained by differences between the two source cultures (NI and NNI.
360
361 **Isolation and identification of bacterial strains from *Isochrysis galbana* microbiomes**. A total of 64
362 isolates were obtained from *I. galbana* microbiomes either from the native algal culture (19 strains)

363 or from the enriched inhibitory microbiomes (45 strains) and tentatively identified using full-length
364 16S rRNA gene sequencing (Table S4). Strains from the native microbiome were identified as
365 members of the *Alteromonas* (2 isolates), *Croceibacter* (1 isolate), *Mameliella* (1 isolate),
366 *Phaeobacter* (3 isolates), *Qipengyuania* (3 isolates), *Roseovarius* (2 isolates), *Ruegeria* (1 isolate) and
367 *Sulfitobacter* (6 isolates) genera (Table 1), whereas strains from the enriched inhibitory microbiomes
368 were identified as members of the *Alteromonas* (5 isolates), *Halomonas* (1 isolate), *Phaeobacter* (18
369 isolates), *Roseovarius* (7 isolates), *Sulfitobacter* (11 isolates) and *Vibrio* (3 isolates) genera (Table 2).
370
371 **Inhibition of *V. anguillarum* by pure cultures and co-cultures of isolates**. Pure cultures of all 64
372 isolates were tested for their ability to inhibit *V. anguillarum* NB10 and 17 isolates with pronounced
373 inhibitory activity (zone of inhibition in the embedded pathogen) were all identified as *Phaeobacter*
374 sp. (Table 3, Figure S3). The dual co-cultures of representative isolates showed inhibition zones in all
375 co-cultures that included *Phaeobacter piscinae* (isolate H2). In some cases, the co-culture of *P.*
376 *piscinae* (isolate H2) and *Roseovarius* sp. (isolate C7) resulted in a larger clearing zone as compared
377 to the monoculture of the *P. piscinae*, however this result was not consistently reproducible. Of
378 special interest was the co-culture of *Sulfitobacter pontiacus* (isolate D3) and *Halomonas*
379 *campaniensis* (isolate D2), which despite showing only slight (*Halomonas campaniensis)* or no
380 (*Sulfitobacter pontiacus)* inhibition in single culture, were remarkably inhibitory when grown
381 together (Figure 4).
382
383 **Whole genome sequencing and genome mining.** The species identity of the five chosen isolates was
384 confirmed through Average Nucleotide Identity (ANI%) analysis: *Roseovarius nubinhibens* (isolate C7,
385 ANI 90.0%; referred to as *Roseovarius* sp.), *Halomonas campaniensis* (isolate D2, ANI 98.4%),
386 *Sulfitobacter pontiacus* (isolate D3, ANI 97.3%), *Alteromonas macleodii* (isolate E2, ANI 99.0%),
387 *Phaeobacter piscinae* (isolate H2, ANI 98.2%). Genome assembly of the sequencing data from the
388 five isolates yielded three closed genomes and two draft genomes (Table 4). According to
389 antiSMASH (61) analysis, *Halomonas campaniensis* D2 carries seven BGCs (NI-siderophore,
390 ranthipeptide, redox-cofactor, RIPP-like, T1PKS, betalactone, ectoine) and *Sulfitobacter pontiacus* D3
391 four BGCs (RiPP-like, betalactone, hserlactone, redox-cofactor), potentially encoding antibacterial
392 compounds.
393
394
395 **DISCUSSION**

396 The rapid spread of pathogenic bacteria within intensive aquaculture systems remains a significant
397 challenge, especially during the vulnerable larval development stage, and disease outbreaks often
398 results in substantial economic losses. In this study, we investigated the probiotic potential of the
399 microbiomes from two algae typically employed as live feed in marine larviculture, *Tetraselmis*
400 *suecica* and *Isochrysis galbana*. By identifying microbiomes with disease suppression, we aim to
401 provide a novel approach to disease control in aquaculture, reducing reliance on antibiotics and the
402 risk of spread of AMR.
403
404 Determining the inhibitory effect of a mixed microbiome against a particular pathogenic bacterium
405 requires specific detection and quantification of the pathogen. Previous works on fish pathogens
406 have used several approaches for determining inhibition in a mixed microbial population. For
407 instance tagging the pathogen with an antibiotic resistance marker enabling specific quantification
408 by culturing on antibiotic containing media (12, 25, 38), using semi-selective media and distinct
409 colony morphology in defined co-cultures (9, 63, 64), using flow cytometry to determine
410 concentrations of a GFP-tagged pathogen (25), or developing specific primers and designing a qPCR
411 approach has been a strategy for specific detection (9). Both the culturing approaches as well as the
412 qPCR method are labour-intensive and time-consuming. To enable a faster screen for pathogen-
413 inhibitory effect of a mixed microbial community, we used a GFP-tagged pathogen and
414 demonstrated that the fluorescent signal is a robust indication of growth of the pathogen, and that it
415 can be used to detect its inhibition by other microorganisms. Thus, we developed a robust
416 methodology for screening mixed microbiomes for their anti-pathogenic potential, thereby offering
417 a valuable tool for developing sustainable disease management strategies. This method facilitates
418 identifying microbiomes with the greatest potential for disease control, contributing to the
419 sustainable growth of aquaculture and the global effort to combat AMR.
420
421 We used the method to demonstrate that both the native, complete microbiomes and diluted
422 microbiomes of two microalgae used in aquacultures were able to inhibit the growth of fish
423 pathogen *Vibrio anguillarum* NB10. In contrast to findings from previous studies where cultures of
424 *Isochrysis* sp. and *Tetraselmis suecica* have themselves been inhibitory to fish pathogenic *Vibrio*
425 species (63–65), we did not find that *V. anguillarum* was inhibited by axenic *I. galbana* or *T. suecica*.
426 Thus, in our study, it was clearly the microorganisms associated with the algae that inhibited growth
427 of the pathogen, emphasizing the crucial role of microbial communities associated with live feed in
428 larviculture for disease control. These findings highlight the potential of harnessing natural microbial
429 consortia to enhance disease resistance in aquaculture settings.

430

431 Sequence-based analysis of *I. galbana* microbiomes found that *Rhizobiaceae*, *Alteromonadaceae*,
432 and *Flavobacteriaceae* family members dominated the native *I. galbana* microbiome. This is in line
433 with a number of previous studies reporting these families as members often found in the core
434 microbiome of microalgae (66–69). The native microbiome also harboured members of the
435 *Rhodobacteraceae*, which is a family commonly associated with microalgae and algal blooms (33,
436 66–69). The enriched inhibitory microbiomes were dominated by *Alteromonadaceae*,
437 *Rhodobacteraceae*, and *Vibrionaceae* members, with decreased relative abundances of *Rhizobiaceae*
438 and *Flavobacteriaceae* compared to the native microbiome. These shifts are likely heavily influenced
439 by transferring the algal microbiome to a nutrient rich assay medium (MB) favouring fast-growing,
440 heterotrophic bacteria. Interestingly, the degree of pathogen inhibition by the enriched inhibitory
441 microbiomes is not explained by differences in the community richness, diversity, and evenness.
442 However, it does correlate with distinct patterns in the overall community composition, suggesting
443 that the inhibitory potential is influenced by the structure of the microbiome and synergistic
444 interactions within a diverse community. Our findings also imply that complete pathogen inhibition
445 can be achieved by a range of different enrichment cultures.
446

447 Pure cultures of *Phaeobacter piscinae* H2 isolated from the *I. galbana* microbiome inhibited *V.*
448 *anguillarum* when tested in an agar-based assay, and this is consistent with numerous previous
449 studies showing that several *Phaeobacter* species can inhibit *Vibrio* species (18, 20, 25, 26, 70). We
450 anticipated this outcome also due to the distinctive brown colonies on MA agar, indicating the
451 presence of the TDA-Fe complex, and thus the production of TDA, known for its antimicrobial effects
452 (12, 16, 20, 71). Notably, all isolates that produced an inhibition zone of the embedded *V.*
453 *anguillarum* were identified as *Phaeobacter* species, however, this genus was not among the ten
454 most abundant genera of the enriched inhibitory microbiomes. This could indicate that instead of
455 the potent antagonistic effect of particular genera, it may be a synergistic effect of the microbiome
456 members that inhibits the pathogen.
457

458 The co-culture of *Sulfitobacter pontiacus* D3 and *Halomonas campaniensis* D2, isolated from the *I.*
459 *galbana* microbiome, was inhibitory to *V. anguillarum* NB10, despite neither isolate showing
460 pathogen inhibition as pure cultures. While it is beyond the scope of the current study to unravel the
461 molecular and chemical mechanism underlying this co-culture-induced inhibition, previous research
462 has demonstrated that biosynthetic gene clusters (BGCs) encoding antibiotic compounds can be
463 elicited by co-cultivation of bacteria (35–37, 72). According to antiSMASH analyses, the genomes of

both bacteria harbour several BGCs. Therefore, future metabolomic analyses of the mono- and co-cultures could provide insight into the co-culture-induced antimicrobial mechanism. Moreover, several *Halomonas* species have been reported to produce an array of hydrolytic enzymes, extracellular polysaccharides (EPS) (73), antibiotics (74), antifungals (75), and pigments (76). *Halomonas campaniensis* D2, in particular, carries seven biosynthetic gene clusters potentially encoding antibacterial compounds. This combined with the lack of inhibition by the monoculture could suggest that the presence of *Sulfitobacter pontiacus* D3 may trigger the inhibitory effect. *Sulfitobacter* species, often associated with marine algae and corals (77–79), possess the ability to metabolize sulphite (80–82) and dimethylsulfoniopropionate (DMSP) (77). The breakdown of DMSP produces potentially antimicrobial compounds such as acrylate (83, 84) and dimethyl sulphide (DMS), which could indicate, that the sulphur metabolism capabilities of *Sulfitobacter* may contribute to pathogen inhibition. Several *Sulfitobacter* species have also been reported to inhibit fungal pathogens (85) and produce EPS (86), and extracellular cyclodipeptides (87).

Beiralas et. al reported that *Sulfitobacter pontiacus* is a key species of the *Emiliania huxleyi* microbiome, protecting its algal host from bacterial pathogens even at low initial abundance (79). While the mechanism of the algal protector phenotype was not determined, the authors suggest that harbouring *Sulfitobacter* species in the algal microbiome presents the host with an advantage and that *Sulfitobacter* species may play a key role in influencing population dynamics. Building on these findings, we speculate that even a low abundance of *Sulfitobacter pontiacus* in a community or co-culture may induce synergistic effects that enhance pathogen inhibition, potentially mediated through the production of bioactive compounds. These insights highlight the importance of microbial interactions in community functionality and stability.

In conclusion, by introducing an efficient methodology for screening mixed microbiomes, our study offers a promising approach for identifying candidate microbiomes for sustainable disease management. Our findings highlight the potential of harnessing natural microbial consortia to enhance disease resistance in aquaculture and support the importance of microbial co-cultures as potential disease control strategies.

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755 **LEGENDS FOR TABLES AND FIGURES**

756

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759

760 **Table 2.** Identification (16S rRNA gene sequence and BLAST) of bacterial isolates from the enriched
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762

763 **Table 3.** Bacterial strains isolated from *Isochrysis galbana* cultures with the ability to inhibit *V.*
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767 **Table 4.** Whole genome sequencing results of five bacterial isolates from the *Isochrysis galbana*
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769 *Sulfitobacter pontiacus* D3, *Alteromonas macleodii* E2, and *Phaeobacter piscinae* H2 yielded three
770 closed genomes and two draft genomes.

771

772 **Figure 1.** Yellow panels: Overview of algal cultures used in the *Vibrio* inhibition assays. Xenic cultures:
773 *Tetraselmis suecica* (NT) and *Isochrysis galbana* (NI – continuously subcultured, NNI – freshly recruited
774 microbiome). Axenic cultures: *T. suecica* (AxT) and *I. galbana* (AxI) with no microbiome (A). Sample
775 preparation: Xenic cultures were divided into Full culture (FC, containing algal and bacterial cells) and
776 Filtered microbiome (FM, algal cells removed by filtration) (B). All samples were 100-fold up-
777 concentrated, then 10-fold serial dilutions were co-cultured with *Vibrio anguillarum* NB10_gfp
778 (starting inoculum 10^3 or 10^5 CFU mL^{-1}) in a GFP-based inhibition assay (C). Teal panels: NI and NNI
779 samples indicated by DNA symbol were analysed through 16S rRNA gene amplicon sequencing (D).
780 Bacterial isolates from the native microbiome and the enriched inhibitory microbiome of *I. galbana*
781 (NI, NNI) were tested for inhibition of *V. anguillarum* in an agar-based assay (E).

782

783 **Figure 2.** *Vibrio anguillarum* NB10_gfp at different initial concentrations: 10^6 (●), 10^5 (□), 10^4 (▲), 10^3
784 (○) CFU mL^{-1}, as measured by (A) absorbance at 600 nm and by (B) fluorescence (GFP) using
785 excitation of 485/15 nm and emission of 513/15 nm, and the (C) inhibition profile of *V. anguillarum*
786 NB10_gfp (initial concentration: 10^4 CFU mL^{-1}) by antagonist *Phaeobacter piscinae* S26 at different
787 initial concentrations: 10^7 (△), 10^6 (●), 10^5 (□), 10^4 (▲), 10^3 (○), 10^2 (■) CFU mL^{-1}, as measured by
788 fluorescence (GFP, excitation: 485/15 nm, emission: 513/15 nm).

789

790 **Figure 3.** Inhibition by *Isochrysis galbana* (left) and *Tetraselmis suecica* (right) microbiomes of *Vibrio*
791 *anguillarum* NB10_gfp (starting inoculum of 3.1 ± 0.3 log CFU mL^{-1}), as measured by fluorescence (GFP,
792 excitation: 485/15 nm, emission: 513/15 nm). The inhibitory effect of serial dilutions (10^{-1} (△), 10^{-2} (●),
793 10^{-3} (□), 10^{-4} (▲), 10^{-5} (○)) of different fractions of the algal cultures have been tested: full culture (FC;
794 2A and 2D), with algal and bacterial cells; filtered microbiome (FM; 2B and 2E), where algal cells have
795 been removed; and axenic (AX; 2C and 2F) cultures, where the algae cells are free of bacteria. FM
796 samples were measured on a different instrument, hence a different scale of RFU.

797

798 **Figure 4.** Inhibition of *Vibrio anguillarum* NB10_gfp by co-cultures of bacterial species isolated from
799 the enriched inhibitory *Isochrysis galbana* cultures, in an agar-based pathogen embedding assay. C7:
800 *Roseovarius* sp., D2: *Halomonas campaniensis*, D3: *Sulfitobacter pontiacus*, E2: *Alteromonas*
801 *macleodii*, H2: *Phaeobacter piscinae*.

802

803 **Figure 5.** Microbial composition (top 10 most abundant genera) of the native (left) and enriched
804 inhibitory (right) microbiomes based on the 16S rRNA amplicon sequencing results. Samples from
805 the enriched inhibitory microbiome are grouped by their inhibition profile: marginal growth of
806 pathogen (*), medium inhibition of pathogen (**), full inhibition of pathogen (***).

807

808 **Supplementary Tables and Figures**

809

810 **Table S1.** List of thirty unique barcodes and primers used to amplify the V3-V4 region of the 16S
811 rRNA gene in DNA from the microbiomes of *Isochrysis galbana* cultures. Primers and barcodes from
812 (88).

813

814 **Table S2.** Algal and bacterial counts of different algal cultures (AXT, AXI, NT, NI and NNI) used in the
815 *Vibrio* inhibition assay, before and after up-concentration ("Before" and "After"), and from different
816 algal sample fractions (Full culture, FC and Filtered microbiome, FM). Counts are reported as mean ±
817 standard deviation. In cases where standard deviation is not reported, data originated from
818 unreplicated experimental conditions.

819

820 **Table S3**. DNA concentration after extraction, DNA amount provided for sequencing and number of
821 sequences obtained from each sample (after denoising and filtering). Sample names indicate replicate
822 ("R1", "R2") source culture ("NI", "NNI"), sample fraction (Full culture: "FC", Filtered microbiome:

823 “FM”), dilution factor of the algal microbiome (0 to -5), and starting inoculum of *V. anguillarum*
824 NB10_gfp (“V3”, “V5” for 3 or 5 log CFU mL^{-1}) for samples obtained after the inhibition assay. The algal
825 cultures were sampled before up-concentrating and filtering (“raw”, triplicates), and each fraction (FC
826 and FM) was sampled before the enrichment assay (“bf”). Sterile media controls were included (“MB-
827 ctrl”).
828
829 **Table S4.** 64 bacterial strains isolated from the native („Raw culture”, 19 strains) or enriched inhibitory
830 („Full culture”: FC, or „Filtered microbiome”: FM, 45 strains) microbiome of *Isochrysis galbana*, and
831 tentatively identified using full-length 16S rRNA gene sequencing. The isolates originate from two
832 different *I. galbana* cultures (NI and NNI), acquired at different times from the same aquaculture unit.
833
834 **Figure S1.** Inhibition assay by *Isochrysis galbana* (left) and *Tetraselmis suecica* (right) microbiomes
835 against *Vibrio anguillarum* NB10_gfp, at a starting concentration of 3.1 ± 0.3 log CFU mL^{-1}, as measured
836 by absorbance at 600 nm. The inhibitory effect of serial dilutions (10^{-1} (△), 10^{-2} (●), 10^{-3} (□), 10^{-4} (▲), 10^{-}
837 ^{5} (○)) of different fractions of the algal cultures have been tested: full culture (FC; S1A and S1D), with
838 algal and bacterial cells; filtered microbiome (FM; S1B and S1E), where algal cells have been removed;
839 and axenic (AX; S1C and S1F) cultures, where the algae cells are free of bacteria.
840
841 **Figure S2.** Native microbiome composition (top 10 most abundant genera) of two *Isochrysis galbana*
842 cultures of different age based on the 16S rRNA amplicon sequencing results. One culture, NNI has
843 been newly provided by an aquaculture facility and has a relatively freshly recruited microbiome
844 (left). The other culture, NI has been regularly subcultured under laboratory conditions for almost
845 four years (right).
846
847 **Figure S3.** Inhibition of *Vibrio anguillarum* NB10_gfp by 64 isolates from the *Isochrysis galbana*
848 microbiome. 16 isolates showed a clearing zone of varying size (all identified as *Phaeobacter* sp.),
849 and 13 isolates showed a faint halo, or a rough agar surface around the colony (identified as
850 *Alteromonas, Sulfitobacter, Qipengyuania* and *Croceibacter* sp.).

851 **Table 1.** Identification (16S rRNA gene sequence and BLAST) of bacterial isolates from the native *Isochrysis galbana* cultures.

852

Top BLAST hit (NCBI)	Genus	Family	Number of isolates	Present in enrichment culture
Alteromonas sp.	*Alteromonas*	*Alteromonadaceae*	2	Yes
Croceibacter sp.	*Croceibacter*	*Flavobacteriaceae*	1	No
Mameliella sp.	*Mameliella*	*Rhodobacteraceae*	1	No
Phaeobacter sp.	*Phaeobacter*	*Rhodobacteraceae*	3	Yes
Qipengyuania sp.	*Qipengyuania*	*Erythrobacteraceae*	3	No
Roseovarius nubinhibens	*Roseovarius*	*Rhodobacteraceae*	2	Yes
Ruegeria sp.	*Ruegeria*	*Rhodobacteraceae*	1	No
Sulfitobacter sp.	*Sulfitobacter*	*Rhodobacteraceae*	6	Yes

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856 **Table 2.** Identification (16S rRNA gene sequence and BLAST) of bacterial isolates from the enriched inhibitory *Isochrysis galbana* cultures after
857 co-culture with *Vibrio anguillarum* NB10_gfp.

Top BLAST hit (NCBI)	Genus	Family	Number of isolates	In full (**) or marginally (*) inhibitory enrichments	
				**	*
Alteromonas sp.	*Alteromonas*	*Alteromonadaceae*	5	Yes	No
Halomonas alkaliphila	*Halomonas*	*Halomonadaceae*	1	Yes	No
Phaeobacter sp.	*Phaeobacter*	*Rhodobacteraceae*	18	Yes	Yes
Roseovarius nubinhibens	*Roseovarius*	*Rhodobacteraceae*	7	Yes	Yes
Sulfitobacter sp.	*Sulfitobacter*	*Rhodobacteraceae*	11	Yes	Yes
Vibrio sp.	*Vibrio*	*Vibrionaceae*	3	Yes	Yes

858

859 **Table 3.** Bacterial strains isolated from *Isochrysis galbana* cultures with the ability to inhibit *V.*
860 *anguillarum*. All isolates characterized by a clearing zone of different sizes (+, ++, +++) or a faint clearing
861 zone (*) were identified as *Phaeobacter* sp.
862

Isolate	Source	Best BLAST hit	ID%	Inhibition of *Vibrio anguillarum* NB10
B7	Raw culture, NI	*Phaeobacter piscinae*	99.57%	++
B9	Raw culture, NI	*Phaeobacter piscinae*	99.43%	+
C3	FC, NNI	*Phaeobacter piscinae*	99.43%	+
D4	FC, NNI	*Phaeobacter piscinae*	99.14%	+
D6	FC, NI	*Phaeobacter piscinae*	99.24%	+++
E4	FC, NI	*Phaeobacter piscinae*	99.42%	+++
E6	FC, NI	*Phaeobacter sp.* M8-4.3	98.24%	+++
E8	FC, NI	*Phaeobacter piscinae*	99.42%	++
E9	FC, NI	*Phaeobacter piscinae*	98.90%	+++
F3	FC, NI	*Phaeobacter sp.* M8-4.3	98.07%	++
F6	FM, NNI	*Phaeobacter piscinae*	98.72%	+
H2	FM, NI	*Phaeobacter piscinae*	98.96%	+++
H3	FM, NI	*Phaeobacter piscinae*	99.33%	*
H8	FM, NI	*Phaeobacter piscinae*	99.33%	++
I1	FM, NI	*Phaeobacter piscinae*	99.14%	+
I2	FM, NI	*Phaeobacter piscinae*	99.04%	++
I3	FM, NI	*Phaeobacter piscinae*	99.24%	+++

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872 **Table 4.** Whole genome sequencing results of five bacterial isolates from the *Isochrysis galbana*
873 microbiome. Whole genome sequencing of *Roseovarius* sp. C7, *Halomonas campaniensis* D2, *Sulfitobacter*
874 *pontiacus* D3, *Alteromonas macleodii* E2, and *Phaeobacter piscinae* H2 yielded three closed genomes and
875 two draft genomes.
876

Isolate	Total genome length (bp)	N50 (bp)	Number of contigs	GC content (%)	Status of genome
Roseovarius sp. C7	3,819,388	3,578,952	6	62.9	Closed
Halomonas campaniensis D2	4,408,683	4,359,254	3	52.8	Closed
Alteromonas macleodii E2	5,090,176	4,653,070	3	44.5	Closed
Sulfitobacter pontiacus D3	3,995,328	279,876	45	60.2	Draft
Phaeobacter piscinae H2	3,981,236	909,707	11	60.2	Draft

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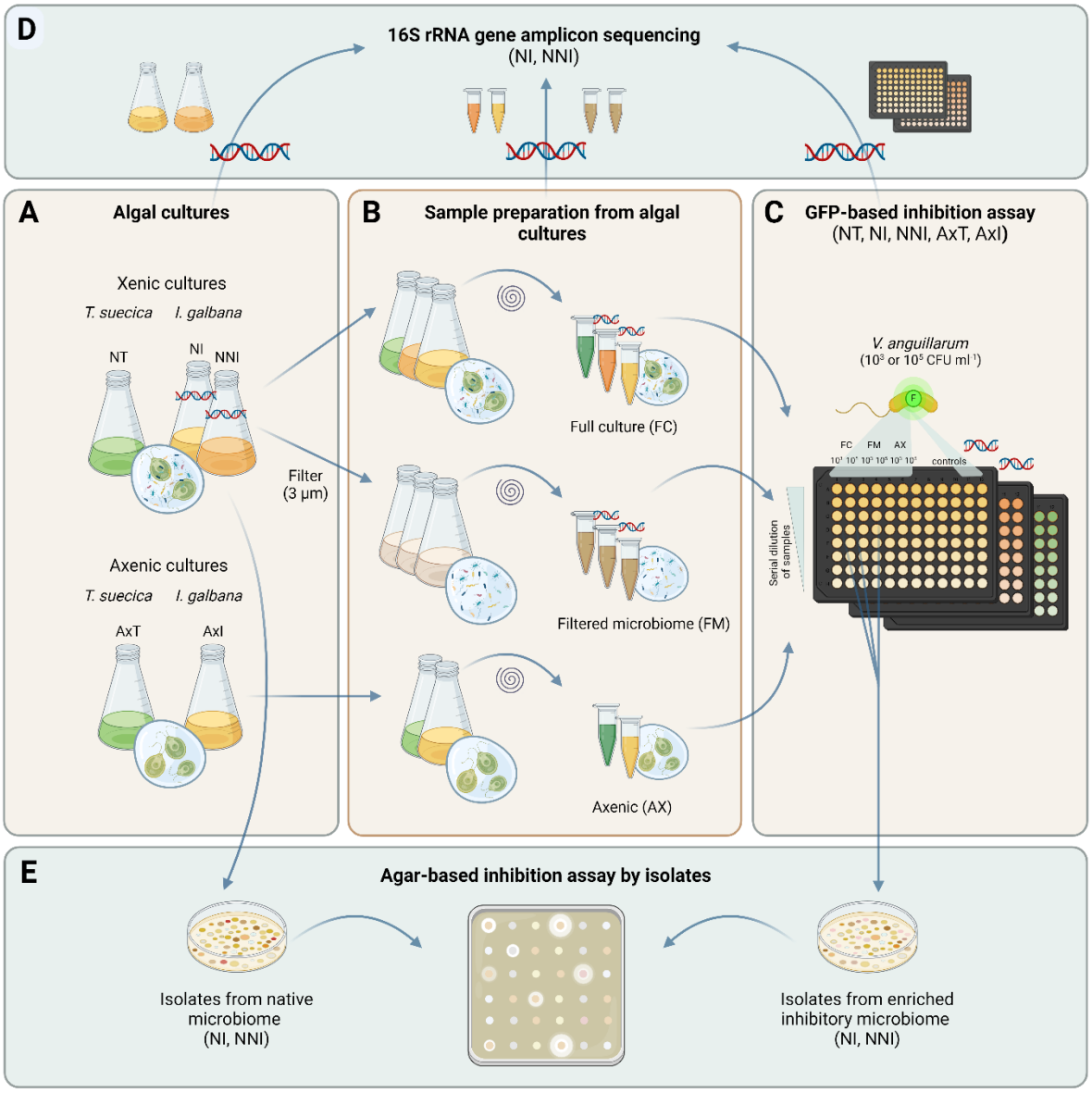

Figure 1. Yellow panels: Overview of algal cultures used in the *Vibrio* inhibition assays. Xenic cultures: *Tetraselmis suecica* (NT) and *Isochrysis galbana* (NI – continuously subcultured, NNI – freshly recruited microbiome). Axenic cultures: *T. suecica* (AxT) and *I. galbana* (AxI) with no microbiome (A). Sample preparation: Xenic cultures were divided into Full culture (FC, containing algal and bacterial cells) and Filtered microbiome (FM, algal cells removed by filtration) (B). All samples were 100-fold up-concentrated, then 10-fold serial dilutions were co-cultured with *Vibrio anguillarum* NB10_gfp (starting inoculum 10^3 or 10^5 CFU mL^{-1}) in a GFP-based inhibition assay (C). Teal panels: NI and NNI samples indicated by DNA symbol were analysed through 16S rRNA gene amplicon sequencing (D). Bacterial isolates from the native

890 microbiome and the enriched inhibitory microbiome of *I. galbana* (NI, NNI) were tested for inhibition of
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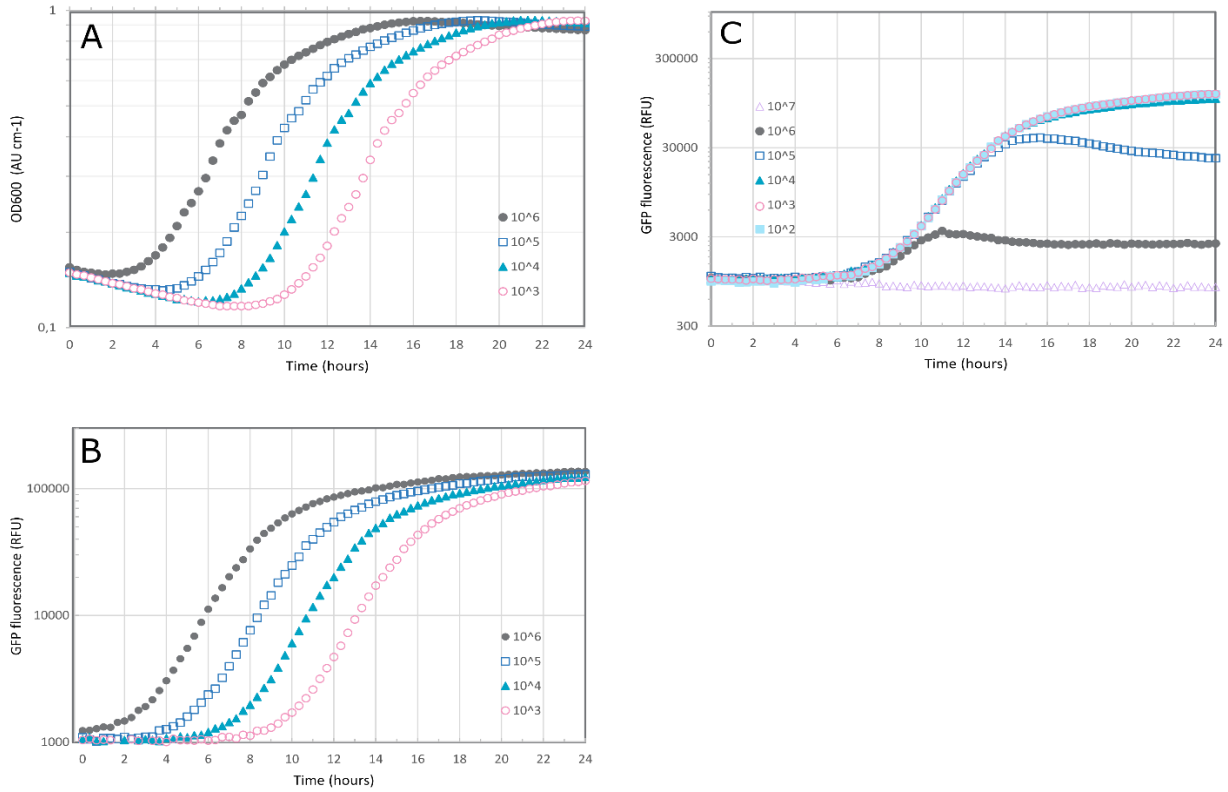

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895 485/15 nm and emission of 513/15 nm, and the (C) inhibition profile of *V. anguillarum* NB10_gfp
896 (starting inoculum: 10^4 CFU mL^{-1}) by antagonist *Phaeobacter piscinae* S26 at different initial
897 concentrations: 10^7 (△), 10^6 (●), 10^5 (□), 10^4 (▲), 10^3 (○), 10^2 (■) CFU mL^{-1}, as measured by fluorescence
898 (GFP, excitation: 485/15 nm, emission: 513/15 nm).

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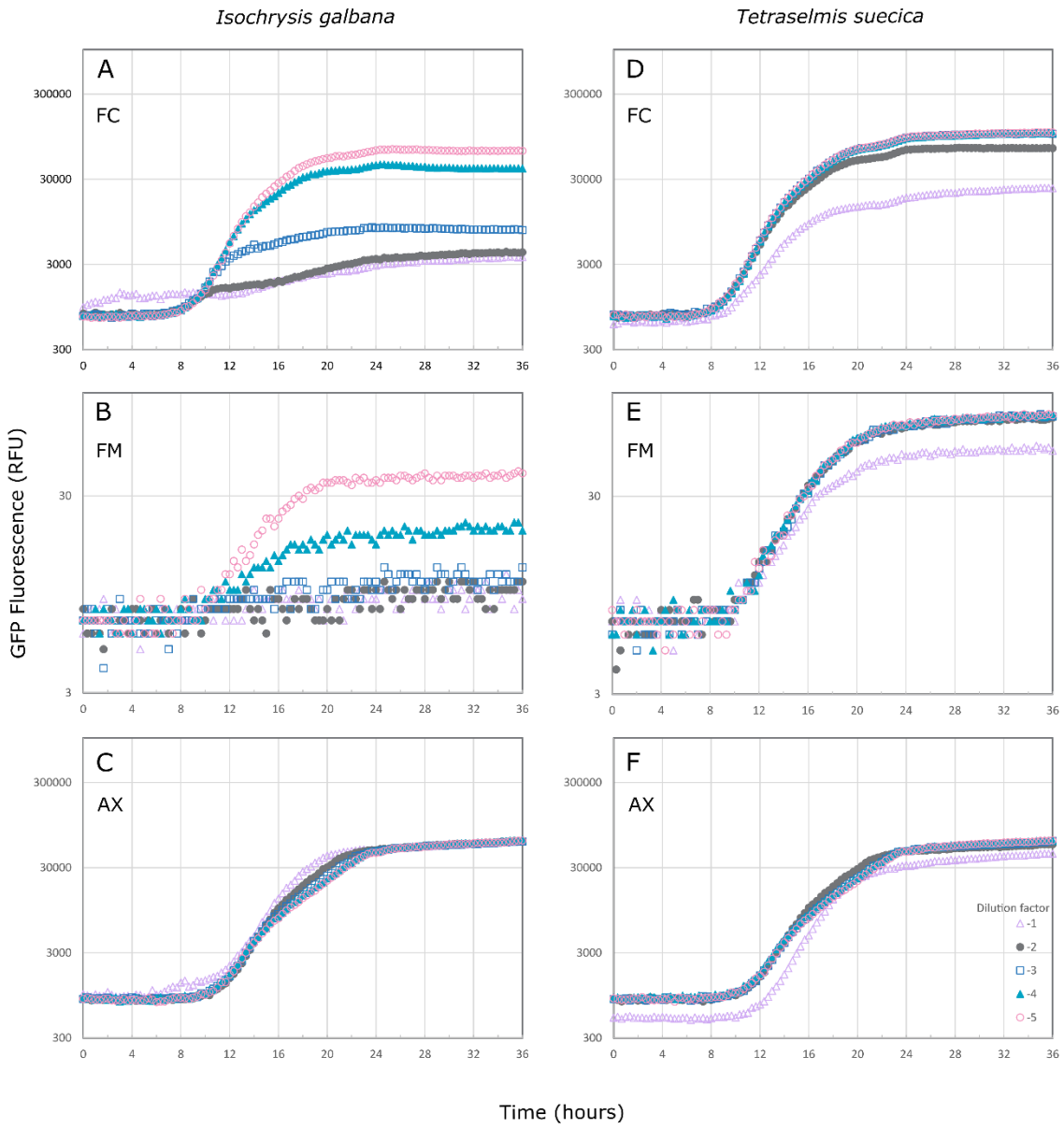
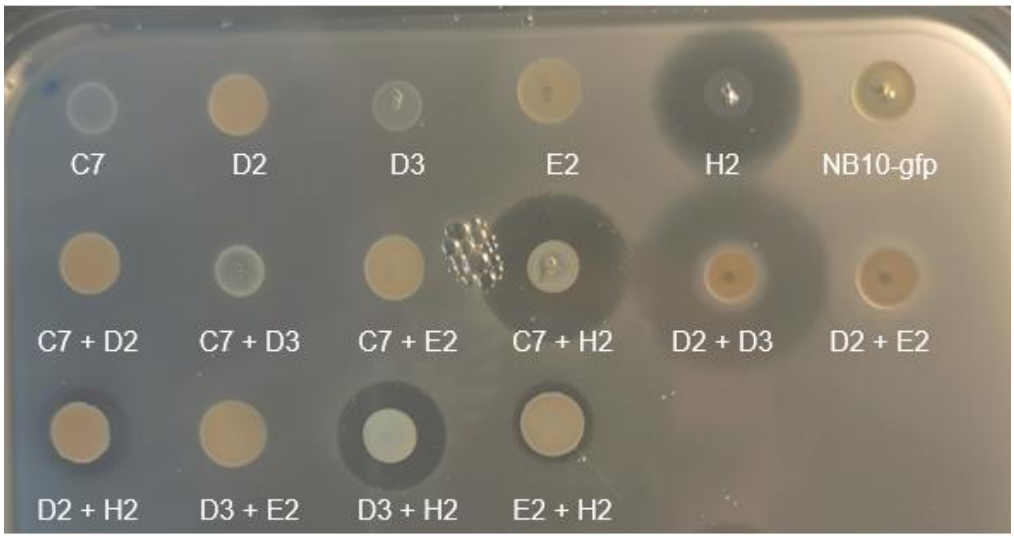


Figure 3. Inhibition by *Isochrysis galbana* (left) and *Tetraselmis suecica* (right) microbiomes of *Vibrio anguillarum* NB10_gfp (starting inoculum of 3.1 ± 0.3 log CFU mL^{-1}), as measured by fluorescence (GFP, excitation: 485/15 nm, emission: 513/15 nm). The inhibitory effect of serial dilutions (10^{-1} (△), 10^{-2} (●), 10^{-3} (□), 10^{-4} (▲), 10^{-5} (○)) of different fractions of the algal cultures have been tested: full culture (FC; 2A and 2D), with algal and bacterial cells; filtered microbiome (FM; 2B and 2E), where algal cells have been removed; and axenic (AX; 2C and 2F) cultures, where the algae cells are free of bacteria. FM samples were measured on a different instrument, hence a different scale of RFU.



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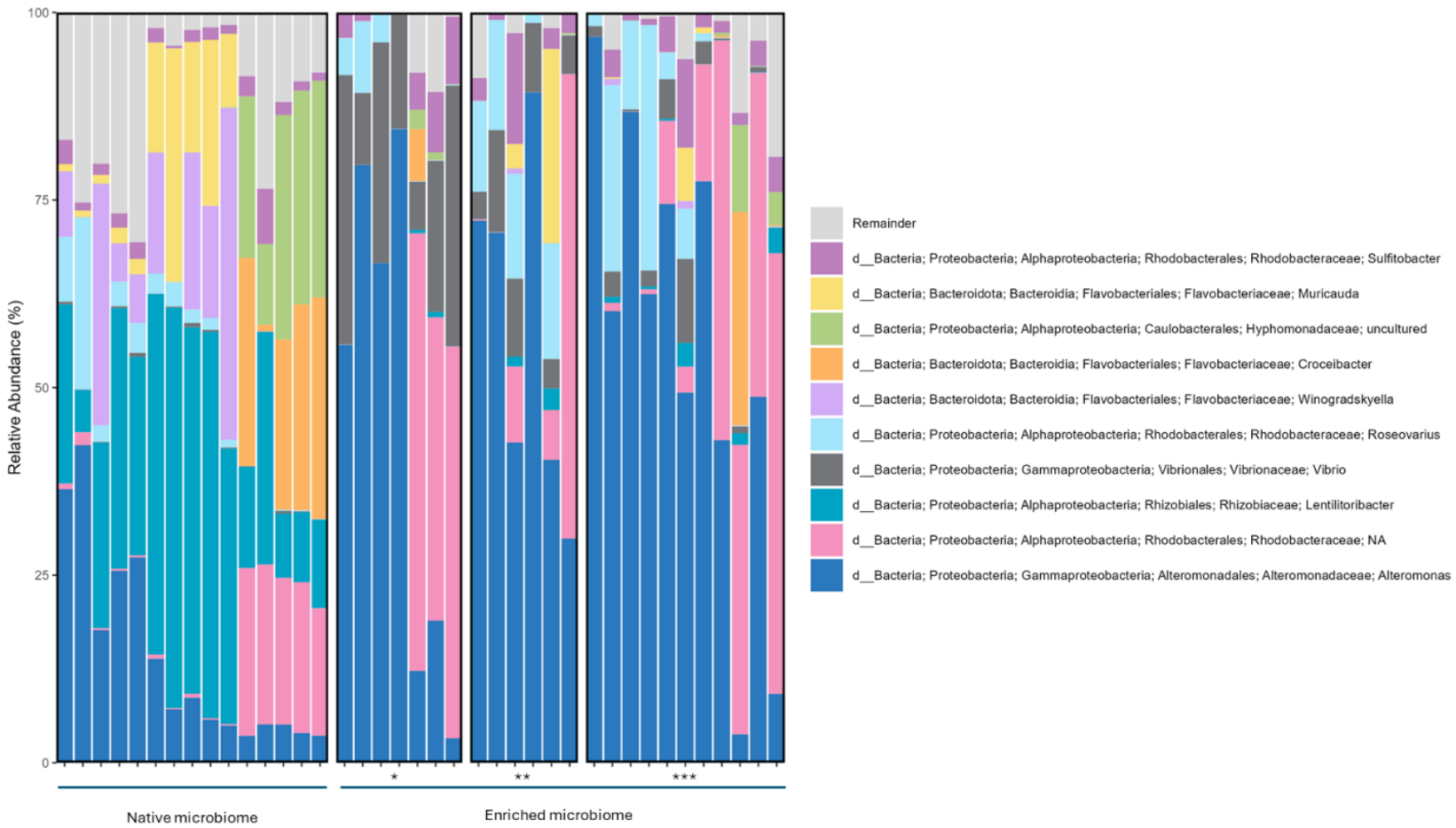

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920 **Table S1**. List of thirty unique barcodes and primers used to amplify the V3-V4 region of the 16S rRNA
921 gene in DNA from the microbiomes of *Isochrysis galbana* cultures. Primers and barcodes from (88).

Barcode no.	Barcode	Forward primer (with barcode)	Reverse primer (with barcode)
1	TTTTAATC	TTTTAATCCCTACGGGNGGCWGCAG	TTTTAATCGACTACHVGGGTATCTAATCC
2	ATAATTAG	ATAATTAGCCTACGGGNGGCWGCAG	ATAATTAGGACTACHVGGGTATCTAATCC
3	ACCAAATT	ACCAAATTCCTACGGGNGGCWGCAG	ACCAAATTGACTACHVGGGTATCTAATCC
4	CTTATCAA	CTTATCAACCTACGGGNGGCWGCAG	CTTATCAAGACTACHVGGGTATCTAATCC
5	TGATCATT	TGATCATTCCTACGGGNGGCWGCAG	TGATCATTGACTACHVGGGTATCTAATCC
6	AGAATCTA	AGAATCTACCTACGGGNGGCWGCAG	AGAATCTAGACTACHVGGGTATCTAATCC
7	TCAAGAAA	TCAAGAAACCTACGGGNGGCWGCAG	TCAAGAAAGACTACHVGGGTATCTAATCC
8	ATCGAAAT	ATCGAAATCCTACGGGNGGCWGCAG	ATCGAAATGACTACHVGGGTATCTAATCC
9	ACATTTAC	ACATTTACCCTACGGGNGGCWGCAG	ACATTTACGACTACHVGGGTATCTAATCC
10	TAGAAAAC	TAGAAAACCCTACGGGNGGCWGCAG	TAGAAAACGACTACHVGGGTATCTAATCC
11	TTATCACC	TTATCACCCCTACGGGNGGCWGCAG	TTATCACCGACTACHVGGGTATCTAATCC
12	AATAGGGT	AATAGGGTCCTACGGGNGGCWGCAG	AATAGGGTGACTACHVGGGTATCTAATCC
13	ATTGCTGA	ATTGCTGACCTACGGGNGGCWGCAG	ATTGCTGAGACTACHVGGGTATCTAATCC
14	TGAGTTCT	TGAGTTCTCCTACGGGNGGCWGCAG	TGAGTTCTGACTACHVGGGTATCTAATCC
15	GGCTATTT	GGCTATTTCCTACGGGNGGCWGCAG	GGCTATTTGACTACHVGGGTATCTAATCC
16	CAAGAGAT	CAAGAGATCCTACGGGNGGCWGCAG	CAAGAGATGACTACHVGGGTATCTAATCC
17	GGAATACA	GGAATACACCTACGGGNGGCWGCAG	GGAATACAGACTACHVGGGTATCTAATCC
18	AAGGCAAT	AAGGCAATCCTACGGGNGGCWGCAG	AAGGCAATGACTACHVGGGTATCTAATCC
19	ACAAAACG	ACAAAACGCCTACGGGNGGCWGCAG	ACAAAACGGACTACHVGGGTATCTAATCC
21	TTGAGTGA	TTGAGTGACCTACGGGNGGCWGCAG	TTGAGTGAGACTACHVGGGTATCTAATCC
22	GCTTCTGA	GCTTCTGACCTACGGGNGGCWGCAG	GCTTCTGAGACTACHVGGGTATCTAATCC
23	GGCAAGAT	GGCAAGATCCTACGGGNGGCWGCAG	GGCAAGATGACTACHVGGGTATCTAATCC
24	GTGCTTTC	GTGCTTTCCCTACGGGNGGCWGCAG	GTGCTTTCGACTACHVGGGTATCTAATCC
25	ACACACTG	ACACACTGCCTACGGGNGGCWGCAG	ACACACTGGACTACHVGGGTATCTAATCC
26	CGATTCTG	CGATTCTGCCTACGGGNGGCWGCAG	CGATTCTGGACTACHVGGGTATCTAATCC
27	GCAGAGTT	GCAGAGTTCCTACGGGNGGCWGCAG	GCAGAGTTGACTACHVGGGTATCTAATCC
30	GCTTGGTT	GCTTGGTTCCTACGGGNGGCWGCAG	GCTTGGTTGACTACHVGGGTATCTAATCC
31	ACAGGCTT	ACAGGCTTCCTACGGGNGGCWGCAG	ACAGGCTTGACTACHVGGGTATCTAATCC
40	GAGAGGGA	GAGAGGGACCTACGGGNGGCWGCA	GAGAGGGAGACTACHVGGGTATCTAATCC

922 **Table S2.** Algal and bacterial counts of different algal cultures (AXT, AXI, NT, NI and NNI) used in the
923 *Vibrio* inhibition assay, before and after up-concentration ("Before" and "After"), and from different
924 algal sample fractions (Full culture, FC and Filtered microbiome, FM). Counts are reported as mean ±
925 standard deviation. In cases where standard deviation is not reported, data originated from
926 unreplicated experimental conditions.
927

Algal culture	Algal counts (log cells/mL)		Bacterial counts (log CFU/mL)		
	Before	After	Before	After, FC	After, FM
AXT	5.2	7.2	N/A	N/A	N/A
AXI	5.9	7.2	N/A	N/A	N/A
NT	5.7	7.4	6.6	8.4	8.1
NI	6.4 ± 0.1	8.0 ± 0.1	6.0 ± 0.3	7.6 ± 0.2	7.8 ± 0.2
NNI	6.2 ± 0.6	7.5 ± 0.4	5.7 ± 0.6	7.7 ± 0.4	7.6 ± 0.3

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929
930 **Table S3**. DNA concentration after extraction, DNA amount provided for sequencing and number of
931 sequences obtained from each sample (after denoising and filtering). Sample names indicate replicate
932 ("R1", "R2"), source culture ("NI", "NNI"), sample fraction (Full culture: "FC", Filtered microbiome:
933 "FM"), dilution factor of the algal microbiome (0 to -5), and starting inoculum of *V. anguillarum*
934 NB10_gfp ("V3", "V5" for 3 or 5 log CFU mL^{-1}) for samples obtained after the inhibition assay. The algal
935 cultures were sampled before up-concentrating and filtering ("raw", triplicates), and each fraction (FC
936 and FM) was sampled before the enrichment assay ("bf"). Sterile media controls were included ("MB-
937 ctrl").
938

Sample name	DNA concentration after extraction (ng/µL)	Amount of DNA provided for sequencing (ng)	Number of sequences obtained
R1-NNI-FC_0_V5	43.2	250	172,237
R1-NNI-FC_-3_V5	60.8	250	659,903
R1-NNI-FC_-4_V5	54.8	250	141,745
R1-NNI-FC_-5_V3	36.8	250	125,582
R1-NNI-FC_bf	33.2	250	42,434
R1-NNI-FM_0_V5	100	250	37,770
R1-NNI-FM_-2_V5	66.4	250	283,545

R1-NNI-FM_-3_V5	47.8	250	211,372
R1-NNI-FM_-3_V3	77.6	250	422,921
R1-NNI-FM_-4_V3	112	250	275,217
R1-NNI-FM_bf	4.1	192,7	175,885
R1-NNI-MB-ctrl	0.834	39,198	11,352
R1-NNI-raw1	55.4	250	162,772
R1-NNI-raw2	85.4	250	54,564
R1-NNI-raw3	71.6	250	40,223
R2-NNI-FC_0_V5	58.4	250	75,491
R2-NNI-FC_-1_V5	39	250	550,497
R2-NNI-FC_-3_V3	95.4	250	106,340
R2-NNI-FC_-5_V3	73.4	250	324,687
R2-NNI-FC_bf	85	250	62,636
R2-NNI-FM_0_V5	75.4	250	238,018
R2-NNI-FM_-3_V3	120	250	162,818
R2-NNI-FM_-4_V3	48	250	294,405
R2-NNI-FM_bf	17.3	250	145,227
R2-NNI-raw1	43.4	250	25,781
R2-NNI-raw2	72.4	250	49,342
R2-NNI-raw3	72.8	250	74,725
R2-NI-FC_0_V5	81.2	250	291,271
R2-NI-FC_-2_V5	16.2	250	391,227
R2-NI-FC_-3_V3	61.6	250	297,668
R2-NI-FC_-4_V3	74.4	250	224,317
R2-NI-FC_bf	120	250	33,188
R2-NI-FM_0_V5	95.2	250	474,932
R2-NI-FM_-1_V5	61	250	265,940
R2-NI-FM_-2_V3	75	250	229,292
R2-NI-FM_-4_V3	50	250	226,214
R2-NI-FM_bf	16.4	250	367,349
R2-NI-MB-ctrl	1.98	93,06	39,985
R2-NI-raw1	86.8	250	43,457
R2-NI-raw2	92.2	250	169,394

R2-NI-raw3	73.4	250	409,195

939

940 **Table S4.** 64 bacterial strains isolated from the native („Raw culture", 19 strains) or enriched inhibitory
941 („Full culture": FC, or „Filtered microbiome": FM, 45 strains) microbiome of *Isochrysis galbana*, and
942 tentatively identified using full-length 16S rRNA gene sequencing. The isolates originate from two
943 different *I. galbana* cultures (NI and NNI), acquired at different times from the same aquaculture unit.
944

Isolate	Source	Best BLAST hit	Query Cover	E value	ID%
A1	Raw culture, NNI	*Alteromonas marina*	99%	0.0	98.14%
A2	Raw culture, NNI	*Sulfitobacter pacificus*	99%	0.0	99.17%
A3	Raw culture, NNI	*Roseovarius nubinhibens*	99%	0.0	98.61%
A4	Raw culture, NNI	*Roseovarius nubinhibens*	100%	0.0	98.88%
A5	Raw culture, NNI	*Sulfitobacter* sp.	100%	0.0	98.63%
A6	Raw culture, NNI	*Sulfitobacter pacificus*	100%	0.0	99.71%
A7	Raw culture, NNI	*Qipengyuania nanhaisediminis*	100%	0.0	99.54%
A8	Raw culture, NNI	*Qipengyuania aquimaris*	99%	0.0	98.90%
A9	Raw culture, NNI	*Ruegeria* sp.	100%	0.0	98.29%
B1	Raw culture, NNI	*Alteromonas macleodii*	100%	0.0	97.96%
B2	Raw culture, NNI	*Qipengyuania xiamenensis*	99%	0.0	99.56%
B3	Raw culture, NI	*Croceibacter* sp.	100%	0.0	99.22%
B4	Raw culture, NI	*Mameliella alba*	99%	0.0	98.43%
B5	Raw culture, NI	*Sulfitobacter* sp. LZD014	99%	0.0	99.34%
B6	Raw culture, NI	*Sulfitobacter* sp.	100%	0.0	98.82%
B7	Raw culture, NI	*Phaeobacter piscinae*	100%	0.0	99.57%
B9	Raw culture, NI	*Phaeobacter piscinae*	99%	0.0	99.43%
C1	Raw culture, NI	*Sulfitobacter* sp.	99%	0.0	99.57%
C2	FC, NNI	*Roseovarius nubinhibens*	99%	0.0	99.31%
C3	FC, NNI	*Phaeobacter piscinae*	99%	0.0	99.43%
C5	FC, NNI	*Roseovarius nubinhibens*	100%	0.0	99.37%
C6	FC, NNI	*Sulfitobacter* sp. LZD014	100%	0.0	98.86%
C7	FC, NNI	*Roseovarius nubinhibens*	100%	0.0	99.62%
C8	FC, NNI	*Alteromonas tagae*	99%	0.0	97.35%
C9	FC, NNI	*Sulfitobacter pacificus*	99%	0.0	99.24%
D1	FC, NNI	*Sulfitobacter* sp. LZD014	99%	0.0	99.52%

D2	FC, NNI	*Halomonas alkaliphila*	99%	0.0	98.89%
D3	FC, NNI	*Sulfitobacter* sp.	99%	0.0	97.53%
D4	FC, NNI	*Phaeobacter piscinae*	99%	0.0	99.14%
D5	FC, NNI	*Alteromonas* sp.	98%	0.0	98.10%
D6	FC, NI	*Phaeobacter piscinae*	100%	0.0	99.24%
E2	FC, NI	*Alteromonas macleodii*	99%	0.0	99.14%
E3	FC, NI	*Phaeobacter piscinae*	99%	0.0	99.05%
E4	FC, NI	*Phaeobacter piscinae*	100%	0.0	99.42%
E5	FC, NI	*Sulfitobacter* sp. LZD014	100%	0.0	99.33%
E6	FC, NI	*Phaeobacter* sp. M8-4.3	99%	0.0	98.24%
E7	FC, NI	*Sulfitobacter* sp. LZD014	99%	0.0	99.23%
E8	FC, NI	*Phaeobacter piscinae*	100%	0.0	99.42%
E9	FC, NI	*Phaeobacter piscinae*	100%	0.0	98.90%
F1	FC, NI	*Sulfitobacter* sp.	100%	0.0	98.82%
F2	FC, NI	*Vibrio anguillarum*	100%	0.0	97.43%
F3	FC, NI	*Phaeobacter* sp. M8-4.3	99%	0.0	98.07%
F4	FC, NI	*Sulfitobacter* sp.	94%	0.0	96.22%
F5	FM, NNI	*Vibrio* sp.	100%	0.0	98.70%
F6	FM, NNI	*Phaeobacter piscinae*	100%	0.0	98.72%
F7	FM, NNI	*Sulfitobacter* sp.	99%	0.0	99.33%
F8	FM, NNI	*Roseovarius nubinhibens*	99%	0.0	99.44%
F9	FM, NNI	*Roseovarius nubinhibens*	99%	0.0	99.71%
G1	FM, NNI	*Sulfitobacter* sp.	99%	0.0	99.43%
G2	FM, NNI	*Phaeobacter piscinae*	100%	0.0	99.23%
G3	FM, NNI	*Alteromonas* sp.	97%	0.0	97.75%
G5	FM, NNI	*Roseovarius nubinhibens*	100%	0.0	98.91%
G7	FM, NNI	*Vibrio* sp.	99%	0.0	97.46%
G8	FM, NNI	*Roseovarius nubinhibens*	99%	0.0	99.05%
H1	FM, NI	*Sulfitobacter* sp. LZD014	99%	0.0	99.61%
H2	FM, NI	*Phaeobacter piscinae*	100%	0.0	98.96%
H3	FM, NI	*Phaeobacter piscinae*	99%	0.0	99.33%
H4	FM, NI	*Phaeobacter* sp. M8-4.3	99%	0.0	98.33%
H8	FM, NI	*Phaeobacter piscinae*	99%	0.0	99.33%
H9	FM, NI	*Alteromonas macleodii*	100%	0.0	91.23%

I1	FM, NI	*Phaeobacter piscinae*	100%	0.0	99.14%
I2	FM, NI	*Phaeobacter piscinae*	99%	0.0	99.04%
I3	FM, NI	*Phaeobacter piscinae*	100%	0.0	99.24%
I4	Raw culture, NI	*Phaeobacter piscinae*	99%	0.0	98.94%

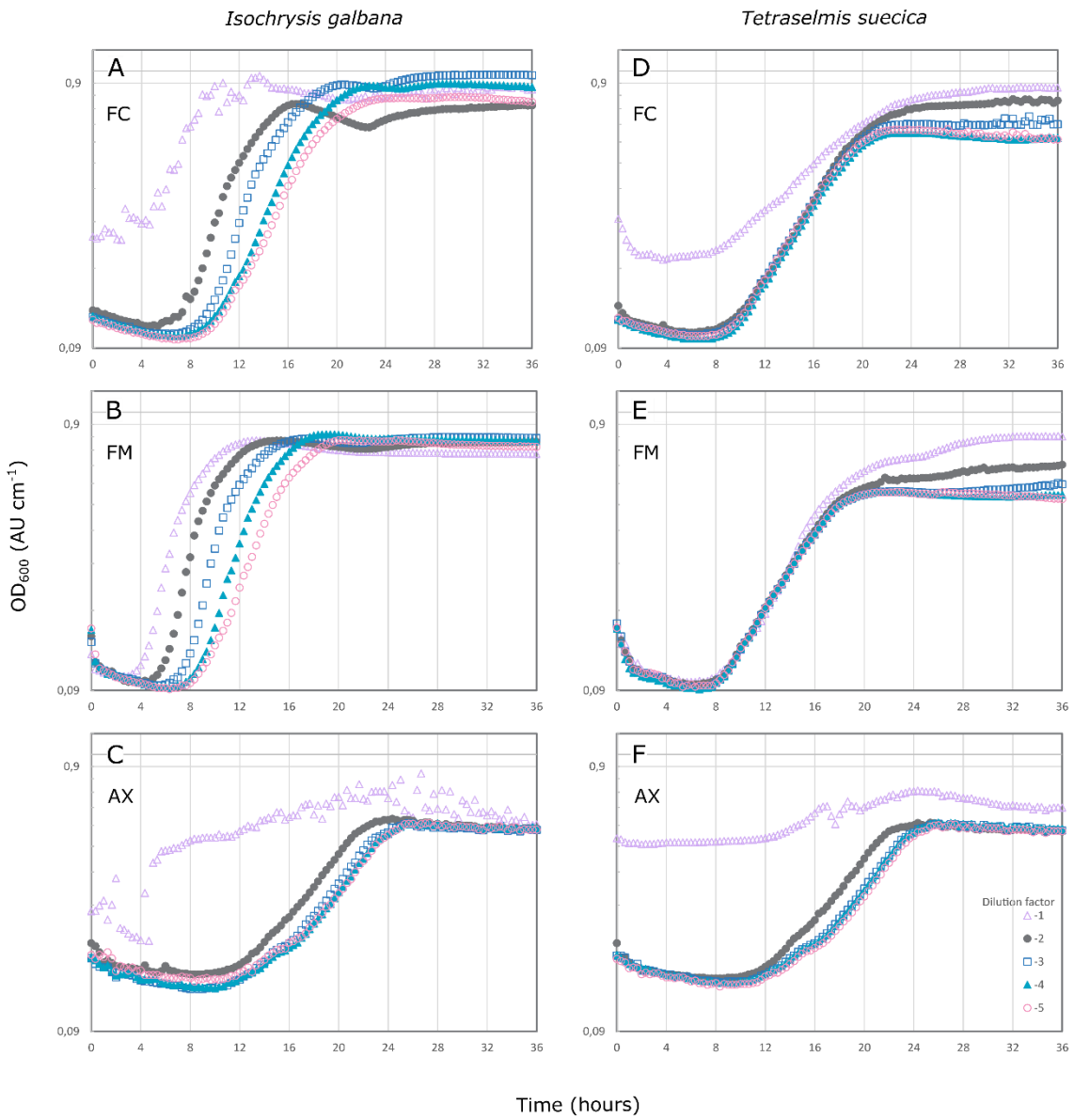

Figure S1. Inhibition assay by *Isochrysis galbana* (left) and *Tetraselmis suecica* (right) microbiomes against *Vibrio anguillarum* NB10_gfp, at a starting concentration of 3.1 ± 0.3 log CFU mL^{-1}, as measured by absorbance at 600 nm. The inhibitory effect of serial dilutions (10^{-1} (△), 10^{-2} (●), 10^{-3} (□), 10^{-4} (▲), 10^{-5} (○)) of different fractions of the algal cultures have been tested: full culture (FC; S1A and S1D), with algal and bacterial cells; filtered microbiome (FM; S1B and S1E), where algal cells have been removed; and axenic (AX; S1C and S1F) cultures, where the algae cells are free of bacteria.

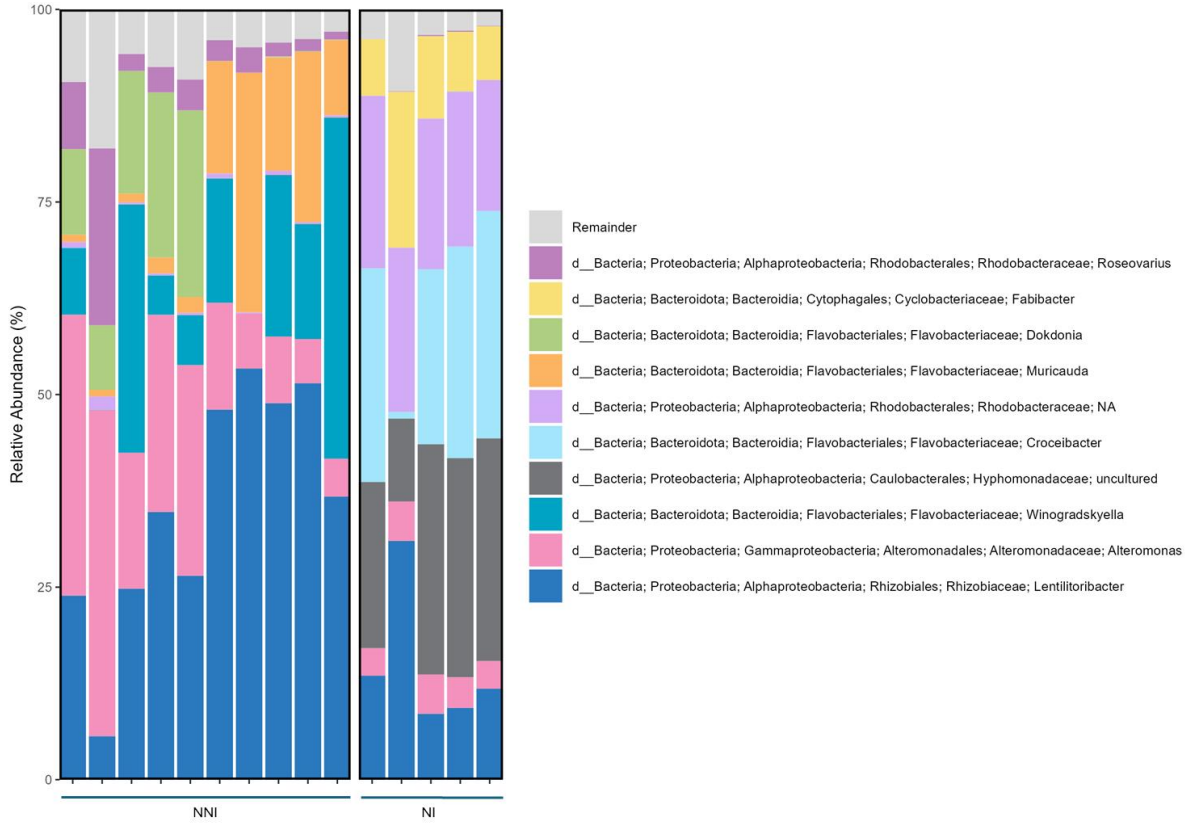

Figure S2. Native microbiome composition (top 10 most abundant genera) of two *Isochrysis galbana* cultures of different age based on the 16S rRNA amplicon sequencing results. One culture, NNI, has been newly provided by an aquaculture facility and has a relatively freshly recruited microbiome (left). The other culture, NI has been regularly subcultured under laboratory conditions for almost four years (right).

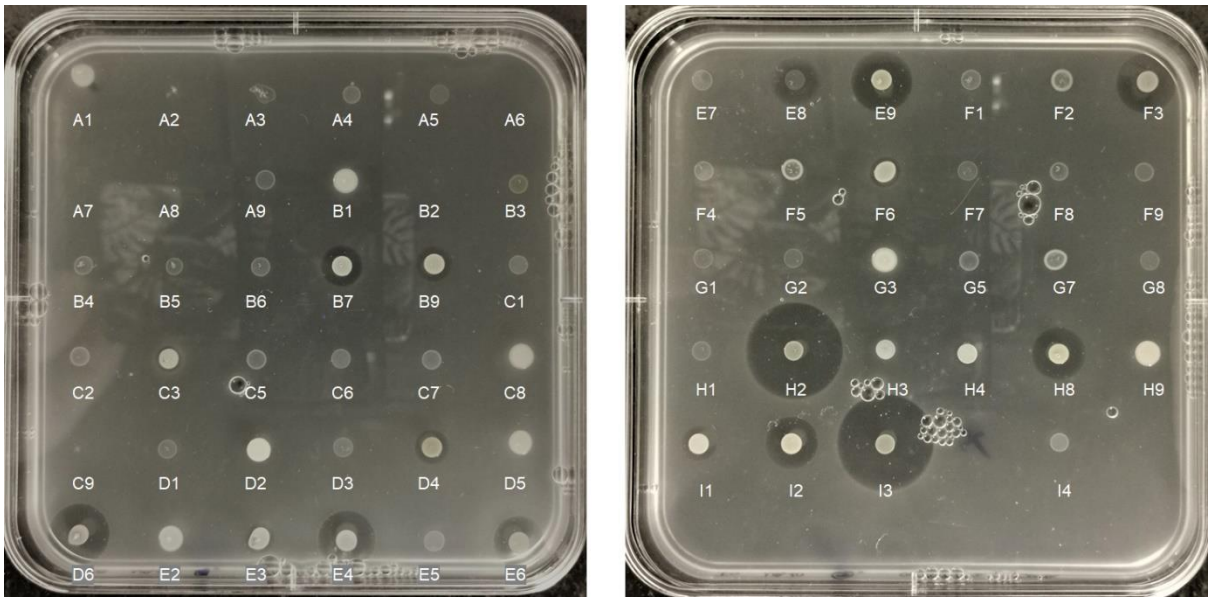

Figure S3. Inhibition of *Vibrio anguillarum* NB10_gfp by 64 isolates from the *Isochrysis galbana* microbiome.