Stronger together: harnessing natural algal communities as potential probiotics for inhibition of aquaculture pathogens

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Running title: Algal microbiomes as probiotics against fish pathogens

Keywords: algal microbiome, probiotics, *Vibrio, Isochrysis galbana, Tetraselmis suecica,* aquaculture, pathogen antagonism

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ABSTRACT (250 words)

Intensive fish rearing in aquaculture is challenged by infectious diseases, and although vaccines have been successfully developed for mature fish, alternative disease control measures are needed for fish larvae and juveniles. Probiotics offer a promising alternative to antibiotics, with the potential to reduce the risk of antibiotic resistance. Probiotics are typically isolated and used as pure cultures, however, in natural environments it is the concerted effort of the complex microbiome that keeps pathogens at bay. Here, we developed an *in vitro* assay to evaluate the anti-pathogen efficacy of mixed algal microbiomes from the live-feed microalgae *Tetraselmis suecica* and *Isochrysis galbana.* The inhibition of a GFP-tagged *Vibrio anguillarum*, a key fish pathogen, by microbial communities, was measured and quantified as reduction in fluorescence. The *Isochrysis galbana* microbiome was more inhibitory to *V. anguillarum* than the *Tetraselmis suecica* microbiome. During co-culture with the pathogen, the bacterial density of the *Isochrysis* microbiomes increased whilst the diversity was reduced as determined by metataxonomic analyses. Bacteria isolated from the fully inhibitory microbiomes were members of *Alteromonadaceae, Halomonadaceae, Rhodobacteraceae, Vibrionaceae, Flavobacteriaceae,* and *Erythrobacteraceae*. Although some strains individually inhibited the pathogen, these were not the key members of the microbiome and 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, and that bacterial co-cultures may offer synergistic advantages over monocultures as probiotics, highlighting their promise for aquaculture health strategies.

IMPORTANCE

Aquaculture is the fastest growing food protein producing sector and sustainable disease control measures are required. Probiotics have gained interest as a promising solution for combating fish pathogens and using mixtures of microorganisms rather than pure cultures may represent a more stable pathogen control. We developed an assay using GFP-tagging of a fish pathogen, enabling the quantitative assessment of the anti-pathogen effects of complex microbiomes. We show that the efficiency of pathogen suppression can be increased with co-cultures compared to monocultures, thus emphasising the potential in using mixtures of bacteria as probiotics.

INTRODUCTION

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

Many commercially valuable marine fish species require live feed, such as microalgae, at the larval stages since suitable artificial feed formulations are not available (7, 8), however, live feed can serve as the infection vector for pathogenic bacteria in larval rearing, leading to swift and widespread infections and complicating disease management (8, 9). Historically, antibiotics have been used to control these infections among larvae and juveniles, but since this may lead to spread of antimicrobial resistance (AMR) of both clinical and agri- and aquacultural relevance, alternative approaches are urgently needed (10). A promising strategy to mitigate bacterial pathogen proliferation in live feed and fish larvae is the use of probiotic bacteria which can inhibit pathogenic bacteria (11–13). Probiotic microorganisms, as defined by FAO and WHO, are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (14). As reviewed in Sonnenschein *et al.* (15), probiotics can be an environmentally sustainable and economically viable approach to counteract economic losses due to bacterial pathogens in aquaculture, and have been intensively studied in the past decades (2, 16). However, the vast majority of such studies have focused on utilising pure cultures of single probionts (17) which may pose challenges in terms of prolonged establishment of a probiont in live feed microbiomes (18). In contrast to traditional single-isolate probiotics, complex microbiomes may offer a more holistic approach by capitalising on the synergistic interactions among multiple microorganisms, including bacteria, to effectively combat pathogens (19). Several studies have focused on use of probiotics in larviculture and have suggested that deriving potential probiotic cultures from the aquaculture system offers an ecological advantage when they are re-introduced (11, 15, 20, 21).

89

90 Microalgae, such as *Tetraselmis suecica* and *Isochrysis galbana*, that are used as live feed in many
91 larviculture productions are colonized by a microbiome that can influence the growth and
92 metabolism of the algae (22–24). The algal microbiome depends on the host species (25), is
93 taxonomically diverse and harbours a large bioactive potential (26, 27). The microbial world is
94 characterized by an intricate web of interactions some of which are mediated by small molecules
95 through which microorganisms exchange information with their neighbours, competitors, and hosts.
96 These interactions shape the microbial community and its functionality, however, monocultures lack
97 these interactions that are important for e.g. inducing expression of biosynthetic gene clusters
98 producing bioactive compounds (28–30). We therefore hypothesize that the collective microalgal
99 microbiome and not only pure cultures of probionts could serve to counteract invasion of
100 aquaculture pathogens. Using a complex microbiome to inhibit a target bacterium, *Vibrio*
101 *anguillarum*, we hope to harvest the enhanced inhibitory properties promoted by the microbiome
102 interactions.
103
104 Whilst the study of single culture inhibition is relatively simple, the high throughput screening of
105 pathogen inhibition by a complex microbiome is more challenging, and a purpose of the present
106 study was to develop a simple screening assay that would allow determining pathogen inhibition by
107 a complex microbiome. Subsequently we characterized the inhibitory microbiomes by
108 metataxonomic analyses and tested mixtures of bacteria derived from these inhibitory microbiomes
109 for their capability to inhibit the fish pathogen.
110
111
112 **MATERIALS AND METHODS**
113 **Bacterial and algal strains and culture conditions**. The fish pathogen *Vibrio anguillarum* strain NB10
114 with a fluorescent tag integrated into the large chromosome (NB10_pNQFlaC4-gfp27) (31) (later
115 referred to as *Vibrio anguillarum* NB10_gfp) was used as target bacterium in the inhibition assays.
116 The expression of GFPmut3* gene was controlled by a constitutive promoter $P_{A1/04/03}$ (32).
117 *Phaeobacter piscinae* strain S26 (21) was used as a known antagonist of *Vibrio* as a positive control
118 for establishing the fluorescence-based screening assay. *P. piscinae* and *V. anguillarum* were stored
119 at –80°C and routinely cultivated on Marine Agar (MA; Difco2216 BD) at 25°C or in Marine Broth
120 (MB; Difco2216 BD) at 25°C and 200 rpm. MA supplemented with 4 µg/mL chloramphenicol was
121 used to maintain *V. anguillarum* NB10_gfp. All bacterial cultures were stored and cultivated

122 similarly, including *V. anguillarum* NB10 wild type (WT), and bacterial isolates obtained from algal
123 cultures and enriched inhibitory microbiomes (see below).
124
125 Xenic microalgal cultures of *Tetraselmis suecica* and *Isochrysis galbana* were provided by
126 aquaculture industry partners. Axenic algae *Isochrysis galbana* CCMP 1323 and *Tetraselmis suecica*
127 CCAP 66/4 were purchased from the Bigelow National Center for Marine Algae and Microbiota
128 (NCMA) and the Culture Collection of Algae and Protozoa (CCAP), respectively, and their axenic
129 status determined by plating on MA. The algae were cultured in f/2 medium without silicate (f/2 –
130 Si; NCMA; (33, 34) made with 3% Instant Ocean (IO; Aquarium Systems Inc., Sarrebourg, France) and
131 cultivated at 18°C and constant illumination at ~50 µE m^{-2} s^{-1}. Cultures were maintained by
132 continuous subculturing every 4 weeks, transferring 1% of the culture to 25 mL fresh medium.
133
134 **Fluorescence-based screening assay for GFP-labelled target organism**. *V. anguillarum* NB10_gfp
135 was grown overnight as described above and a 10-fold serial dilution was prepared using MB as a
136 diluent. 180 µL of the dilutions were distributed to the wells of a sterile, black 96-well microplate
137 with flat, clear bottom (Perkin Elmer, 6005430) in technical triplicates. *V. anguillarum* NB10 WT was
138 included as a negative control for the fluorescence measurement, and sterile medium as a no-
139 growth negative control. Fluorescence and absorbance were measured on a BioTek Cytation™ 5
140 multi-mode microplate reader at 25°C with shaking (linear, continuous, 567 cpm). Measurements
141 were taken every 20 minutes over 40 hours, measuring fluorescence (excitation: 485/15 nm,
142 emission: 513/15 nm) and OD_{600}.
143
144 **Fluorescence-based inhibition assay by *Phaeobacter piscinae* S26**. *Phaeobacter piscinae* S26 was
145 co-cultured with *V. anguillarum* NB10_gfp in the fluorescence-based inhibition assay. Overnight
146 cultures of both bacteria were prepared, 10-fold serially diluted in sterile MB and 90 µL of each
147 dilution of both bacterial cultures was loaded into sterile, black 96-well microplates allowing crossed
148 combinations of the two strains at the different dilutions (total well-content of 180 µL). Dilutions of
149 *P. piscinae* S26 were included as a negative control for the fluorescence measurement, and sterile
150 medium as a no-growth negative control. Dilutions of *V. anguillarum* NB10_gfp were included as a
151 positive control for fluorescence. Absorbance and fluorescence of the plates were measured as
152 described above. Bacterial counts of the precultures were determined by 10-fold serial dilution and
153 plating on MA. After the assay, wells showing no fluorescent signal (the inhibitory microbiomes)
154 were 10-fold serial diluted and plated on MA supplemented with 4 µg/mL chloramphenicol to
155 confirm decreased *Vibrio* counts.

156

157 **Inhibition of *Vibrio anguillarum* by complex algal microbiomes**. Microbiomes from two xenic
158 microalgae, *Tetraselmis suecica* and *Isochrysis galbana,* were co-cultured with *V. anguillarum*
159 NB10_gfp using the fluorescence-based screening assay (Figure 1). Samples from axenic algal
160 cultures were included as controls without microbiomes. Five algal cultures were tested in the assay
161 described above. This included the two axenic cultures (AXT: axenic *Tetraselmis*; AXI: axenic
162 *Isochrysis*), two xenic cultures of *Tetraselmis suecica* (NT) and *Isochrysis galbana* (NI) that had been
163 re-cultivated in the lab since 2019 and 2020, respectively, and a newly acquired (2023) *Isochrysis*
164 *galbana* culture (NNI) from an aquaculture collaborator. Cultures (200 mL) of the two microalgae,
165 both axenic and xenic, were prepared by inoculating 10% stock culture to fresh media and
166 incubating them at 18°C and constant illumination for five days reaching approx. 6 log algal cells mL^{-}
167 ^{1}. The *V. anguillarum* NB10_gfp strain was grown overnight, and algal and bacterial counts were
168 determined at the beginning of the experiment. Bacterial counts were determined by 10-fold serial
169 dilution and plating on MA. Plates were incubated for two days for *V. anguillarum* and for four days
170 for the bacterial counts from algal cultures. All CFU counts were log-transformed before further data
171 handling and plotting. Algal cell counts were determined using microscopy and a Neubauer-
172 improved chamber. To determine if any pathogen inhibition was caused by the algae or by their
173 microbiomes, the xenic algal cultures were divided into two 100 mL fractions: the full culture (FC)
174 with algal and bacterial cells, and a filtered microbiome (FM) fraction, where algal cells were
175 removed by filtering (47-mm Ø, pore size 3-µm, mixed cellulose ester, Advantec A300A047A). Before
176 filtering, the cultures were vortexed vigorously to detach bacteria from the algal cells.

177 To confirm that marine viruses or viral particles did not influence the observed inhibition, the
178 possible pathogen inhibition was tested with a filtered microbiome (FM) and a cell-free supernatant
179 (cf-SN) of NT and NI samples. To prepare the cell-free supernatant, a 100 mL fraction of the FM
180 samples was filtered through 0.22 µm polyethersulfonate (PES) filters (Nalgene™ Rapid-Flow™).

181 To increase the bacterial density of the microbiome (target ~ 7 – 8 log CFU mL^{-1}), 100 ml of algal
182 cultures were harvested (2,445 x g, 10 min, 20°C) and resuspended in 1 mL sterile 3% IO. 10-fold
183 serial dilutions of both up-concentrated algal fractions (FC and FM; 3% IO as diluent), the cell-free
184 supernatants (cf-SN; 3% IO as diluent) and *Vibrio* cultures (MB as diluent) were prepared, and loaded
185 into sterile, black 96-well microplates. Combinations of dilutions of the pathogen (target: 3 log CFU
186 mL^{-1} and 5 log CFU mL^{-1}) and algal samples (10^{-1} to 10^{-5} dilutions) were loaded. Algal cultures with no
187 addition of the GFP tagged pathogen were included as negative control for the fluorescence
188 measurement, and sterile medium as a no-growth negative control. Different dilutions of the pure
189 culture of *V. anguillarum* NB10_gfp were included as a positive control. Fluorescence and OD_{600}

190 were measured as described above. FM samples were measured on a different instrument (BioTek
191 Synergy HTX) than all other samples, resulting in a different scale of the fluorescence measurements.
192 To confirm pathogen inhibition after the assay, wells with fluorescence below 5% of the non-
193 inhibited Vibrio control were 10-fold serial diluted and plated on chloramphenicol-supplemented
194 plates (MA). The overview of sample preparation and experimental setup are summarized in Figure
195 1.
196
197 **Characterizing the enriched inhibitory microbiomes**. During the co-culture inhibition assay, the
198 bacterial density of the algal microbiomes was increased. Several dilutions of the algal microbiomes
199 inhibited growth of *V. anguillarum* in a "dose dependent" manner and in all co-cultures, the
200 bacterial density of the algal microbiomes increased. These microbiomes which caused suppression
201 of the pathogen will be referred to as enriched inhibitory microbiomes. DNA was extracted from a
202 selection of the wells taking samples from wells exhibiting a complete inhibition and wells allowing
203 only marginal growth of *V. anguillarum*. Also, samples were taken from no-growth negative controls
204 of the experiment, resulting in a total of 41 samples. Upon completion of the assay, the plates and
205 samples were stored at -80°C until further processing, and then thawed at 4°C and samples kept on
206 ice. 2 x 200 µL lysis buffer (400 mM NaCl, 750 mM sucrose, 20 mM EDTA, 50 mM Tris-HCl, 1 mg/mL
207 lysozyme [pH 8.5]) was added to the wells. The contents of the wells were then resuspended and
208 transferred to Eppendorf tubes and incubated at 37°C for 30 min. Proteinase K was added to a final
209 concentration of 100 mg/mL and sodium dodecyl sulphate (SDS) to a final concentration of 1%
210 vol/vol, and samples were then incubated overnight at 55°C, 300 rpm (35). The order of handling of
211 samples was then randomized before extraction. DNA extraction was done by the Promega Maxwell
212 16 using the Maxwell LEV Blood DNA Kit, and the quality and purity of the DNA were measured using
213 a DeNovix 439 DS-11+ spectrophotometer (DeNovix Inc., Wilmington, DE, USA) and confirmed by gel
214 electrophoresis.
215
216 The V3-V4 region of the 16S rRNA gene of all samples was amplified by PCR as described in Roager *et*
217 *al*. (2023) (36), using the primer set 341f (59-CCTACGGGNGGCWGCAG-39) and 805r (59-
218 GACTACHVGGGTATCTAATCC-39) tagged with 30 unique octametric barcodes (Table S1). PCR
219 products were purified using Agencourt AMPure XP magnetic beads (0.8:1 bead volume to DNA
220 solution; Agencourt Bioscience Corporation, Beverly, MA, USA). Concentrations of the amplicons
221 were determined using a Qubit 2.0 Fluorometer with the high sensitivity (HS) assay kit (Qubit™
222 dsDNA HS assay; Invitrogen by Thermo Fisher Scientific Inc., Eugene, OR, USA). Samples were pooled

223 in equimolar ratios before library preparation and sequencing on an Illumina NovaSeq 6000 platform
224 with paired-end 250-bp reads (Novogene; Cambridge, United Kingdom).
225
226 **Analyses of sequencing data**. Sequencing data analyses were performed using QIIME 2 version
227 2023.5 (37) and R version 4.2.1 (38). The raw reads were imported to QIIME2, demultiplexed, and
228 primers and barcodes were removed using the cutadapt demux-paired plugin (39). Reads were then
229 denoised and dereplicated using the DADA2 package (40) and an amplicon sequence variant (ASV)
230 table was created leaving out sequences shorter than 400bp. A phylogenetic tree was constructed
231 using MAFFT (41) and FastTree (42), via the phylogeny plugin. Alpha- and beta-diversity metrics were
232 computed using the diversity plugin (43) at an even sampling depth of 26,226. A classifier trained on
233 the Silva database 138 (44) and the specific primer sequences employed as mentioned above was
234 used for taxonomic classification of the reads via the feature-classifier plugin (45, 46). ASVs which
235 were either not assigned to a phylum, classified as chloroplasts/mitochondria or had a frequency <
236 10 were filtered out before further analysis in R. Two sterility controls were excluded from the
237 dataset before further analysis. QIIME 2 artifacts were imported to R v4.2.1 using the qiime2R
238 package (47) and analysis was continued in RStudio (48) with the tidyverse (49) package. For
239 multivariate analyses using the vegan package, the ASV table was normalized by square-root
240 transformation and Wisconsin standardization (50). PERMANOVA analysis was then performed using
241 the beta-group-significance function of Qiime2 and the adonis plugin (51) in R with 999
242 permutations to test significant differences in beta diversity as a function of source culture (NI and
243 NNI), degree of pathogen inhibition, microbiome dilution and sample fraction (FC and FM), based on
244 Bray-Curtis dissimilarities calculated using the normalized ASV table. The analysis was conducted
245 both on the complete dataset and on subsets divided by samples before and after the enrichment
246 assay. Interactive effects were included in the models but were removed due to lack of significance.
247
248 **Isolation and identification of bacterial strains from *Isochrysis galbana* microbiomes***.* Culturable
249 bacterial isolates were obtained from a subset of the enriched inhibitory *I. galbana* microbiomes (NI
250 and NNI) analysed for their microbiome composition. Culture from wells where *V. anguillarum* was
251 inhibited by the microbiomes was serially diluted and plated on MA. Isolates were picked
252 representing colonies with different morphology. Also, strains were picked from the plating of the
253 algal culture before testing inhibition (native microbiome), resulting in a total of 64 bacterial isolates.
254 The strains were re-streaked for purity and glycerol stocks (20% v/v) were made and stored at -80°C.
255 The isolates were later identified by 16S rRNA gene amplification by colony PCR using universal
256 primers 27F and 1492R and subsequent Sanger sequencing by Macrogen (Amsterdam, Netherlands).

257 Sequencing results were compared against the NCBI nucleotide database using the BLASTn function
258 (52) to determine the putative bacterial taxa.
259
260 **Inhibition of *V. anguillarum* by pure cultures and co-cultures of bacterial isolates**. Antagonism of
261 pure cultures of the 64 bacterial isolates against *V. anguillarum* NB10_gfp was tested using an agar-
262 based assay (53) using 50 µL of culture to 50 ml of agar and spotting 5 µL of an overnight culture of
263 the isolates on the pathogen-embedded plates. Plates were incubated at 25°C and visually inspected
264 for clearing zones after 24 and 48 hours. In a similar manner, dual cultures of isolates from the
265 enriched inhibitory microbiome were spotted on embedded *Vibrio* and inspected for inhibition. Five
266 isolates were chosen for investigating co-culture effects as representatives of the five non-*Vibrio*
267 genera isolated from the enriched inhibitory *I. galbana* microbiomes (Table 2) with complete
268 inhibition of 5.3 log CFU mL^{-1} starting inoculum of *V. anguillarum*. The strains were grown in liquid
269 cultures (MB, 25°C, 200 rpm) for 24 hours and then mixed 1:1 in pairs. 10 µL of the mixtures were
270 spotted on the pathogen-embedded plates. The plates were incubated at 25°C and visually
271 inspected for clearing zones after 24 and 48 hours. Monocultures of the individual strains mixed 1:1
272 with MB were used as controls. To validate the results by the co-culture of isolates D2 and D3, their
273 inhibition was tested against a selection of nine strains of *Vibrio anguillarum* with different virulence
274 against fish larvae (54). Monocultures and co-cultures were spotted on the pathogen-embedded
275 plates in biological triplicates, as described above. Biological triplicates of isolate H2 (*Phaeobacter*
276 *piscinae*) were used as a positive control.
277
278 **Whole genome sequencing, annotation and analysis.** The five isolates selected for the co-culture
279 study were whole genome sequenced with Oxford Nanopore sequencing, using the GridION
280 platform. Genomic DNA was extracted from overnight cultures with the NucleoSpin® Tissue Mini kit
281 (Macherey-Nagel, 740952.5) protocol for bacteria, with three hours of incubation during pre-lysis.
282 The quality, purity, and concentration of the DNA was assessed as described for amplicon
283 sequencing. The library was prepared using the Rapid Barcoding Kit 24 V14 (SQK-RBK114.24). The
284 resulting reads were processed by the dragonflye pipeline (55). Briefly, adaptors were trimmed using
285 Porechop and reads smaller than 1000bp were removed with Nanoq. The genomes were then
286 assembled with Flye and polished with Medaka. The assemblies were then annotated with Bakta
287 v1.8.2 (56) and profiled for BGCs with antiSMASH 7.0 (57). Subsequently, the assembled genomes
288 were taxonomically classified using autoMLST (58).
289

290 **Data availability.** The sequencing data from the microbiome analysis and the assembled whole
291 genomes are available on the Sequencing Read Archive under BioProject ID PRJNA1150956.
292
293
294 **RESULTS**

295 **Fluorescence-based inhibition assay for GFP-labelled *Vibrio anguillarum***. *V. anguillarum* NB10_gfp,
296 as expected, grew well in MB reaching an absorbance of 0.929 ± 0.003 at 600 nm (in 19 hours for 5.3
297 log CFU mL^{-1} starting inoculum). The starting inocula of *V. anguillarum* NB10_gfp ranged between
298 1.3 to 9.3 log CFU mL^{-1}. In pure culture, the growth of *V. anguillarum* could be followed by the
299 fluorescent signal indicating the same lag phase, growth rate and stationary phase entry as seen on
300 the OD_{600} measurements (Figure 2A and 2B). *V. anguillarum* was then co-cultured with a dilution
301 series of a known antagonist, *Phaeobacter piscinae* S26 in biological triplicates, to provide proof-of-
302 concept that growth-inhibition of the pathogen could be detected by measuring (lack of)
303 fluorescence signal of a fluorescently tagged target organism. The starting inocula of *P. piscinae* S26
304 ranged between 9.1 to 1.1 log CFU mL^{-1}. As expected, lower starting concentrations of *Vibrio* were
305 more easily inhibited than higher concentrations (data not shown) and, conversely, higher
306 concentrations of *Phaeobacter* were more inhibitory than lower concentrations (Figure 2C).
307 Quantifying *Vibrio* on chloramphenicol-supplemented plates after the assay, from the wells with
308 fluorescent signal < 5% of the *Vibrio* positive control, confirmed inhibition of the pathogen (< 4,34 ±
309 0,63 log CFU/mL).
310

311 **Anti-pathogen inhibitory effect of algal microbiomes on *Vibrio anguillarum*.** To investigate the
312 inhibitory effect of their microbiomes on *V. anguillarum* NB10, dilutions of samples from cultures of
313 two xenic microalgae, *I. galbana* and *T. suecica,* were co-cultured with the GFP-tagged fish
314 pathogen. In the experiments, the starting inocula of *V. anguillarum* NB10_gfp was 3.1 ± 0.3 log CFU
315 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
316 counts varied between 5.2 and 6.4 log cells mL^{-1} and up-concentration resulted in approx. 1.5 log
317 increase to between 7.2 and 8.0 log cells mL^{-1}. Initial bacterial counts in the *Isochrysis* samples varied
318 between 5.7 and 6.6 log CFU mL^{-1} and in the up-concentrated FC fraction between 7.6 and 8.4 log
319 CFU mL^{-1}, whereas in the up-concentrated FM fraction between 7.6 and 8.1 log CFU mL^{-1} (Table S2).
320 Microbiomes from both *T. suecica* and *I. galbana* inhibited growth of *V. anguillarum*, with the *I.*
321 *galbana* microbiome being more potent (Figure 3A vs. 3D and 3B vs. 3E). The same inhibition pattern
322 was observed for the filtered microbiomes (algal cells removed) as for the full culture (algal and
323 bacterial cells) (Figure 3A vs. 3B and 3D vs. 3E). No inhibition was observed from samples from

324 axenic algae cultures (Figure 3C and 3F) or cell-free supernatants (data not shown), indicating that it
325 is indeed the microbiomes growing in the wells that inhibit the pathogen. Plating on
326 chloramphenicol-supplemented plates from wells with decreased fluorescent signal confirmed that
327 if the GFP signal was less than 10% of the control, the *Vibrio* was inhibited, with an abundance of <
328 5.67 ± 0.75 log CFU/mL, as opposed to approximately 9 log CFU/mL in the non-inhibited control.
329 OD_{600} measurements can be seen in Figure S1.
330
331 **Sequence based analysis of microbiomes.** In total, 70,542,064 paired end reads were obtained from
332 the 16S rRNA gene amplicons, with a mean of 1,500,894 reads per sample. After denoising, merging,
333 filtering out chloroplasts and mitochondria, removal of short sequences (< 400 bp) and low
334 abundant ASVs (total frequency < 10), this resulted in a total of 320 amplicon sequence variants
335 (ASVs), with a mean read count per sample of 209,507 (Table S3).
336
337 **Microbiome composition and diversity analyses.** The native microbiomes of *I. galbana* were
338 dominated by *Alteromonadaceae, Rhizobiaceae, Flavobacteriaceae, Rhodobacteraceae* and
339 *Hyphomonadaceae*. The microbiome composition of the two *I. galbana* cultures (NI and NNI)
340 differed significantly (PERMANOVA, $R^2 = 0.312$, $p = 0.001$), with NNI representing a relatively freshly
341 recruited microbiome and NI representing a semi-adapted microbiome, subcultured many times in
342 the laboratory over four years. The two cultures shared several genera, such as *Flavobacteriaceae,*
343 *Rhizobiaceae, Alteromonadaceae* and *Rhodobacteraceae*, but also had distinct microbiome
344 compositions. *Hyphomonadaceae* and *Cyclobacteriaceae* were unique to NI, and each culture had
345 several distinct representatives of *Flavobacteriaceae* and *Rhodobacteraceae* (Figure S2).
346
347 The enriched inhibitory microbiomes sampled after the *Vibrio* inhibition assay were largely
348 dominated by *Alteromonadaceae* and *Rhodobacteraceae*, with a higher relative abundance of
349 *Vibrionaceae* compared to the native microbiome. As expected, the relative abundance of
350 *Vibrionaceae* was highest in samples with marginal growth of the pathogen, compared to those with
351 complete inhibition of the target pathogenic vibrio. However, *Vibrionaceae* were also detected in
352 lower relative abundances in enriched cultures that fully inhibited *V. anguillarum*, suggesting these
353 sequences could represent growth of native vibrios of the algal microbiome. The relative
354 abundances of *Alteromonadaceae* and *Rhodobacteraceae* increased during the inhibition assay,
355 whereas those of *Rhizobiaceae*, *Flavobacteriaceae*, and *Hyphomonadaceae* decreased.
356

357 Community richness (Faith index), diversity (Shannon index), and evenness (Pielou's index) showed
358 no significant differences between microbiomes with different degrees of pathogen inhibition ($p >$
359 0.05). However, based on a pairwise Kruskal-Wallis analysis, significant differences in Pielou's index
360 were observed between the NI and NNI cultures ($p = 0.0041$), with the NI culture having a higher
361 diversity and evenness. PERMANOVA analysis of the full dataset indicated a significant shift in
362 microbial community composition from before (native microbiome) to after the experiment
363 (enriched inhibitory microbiome). Therefore, the data was split into two subsets for further analysis.
364
365 Post-enrichment, community composition was significantly affected by the source culture (NI vs.
366 NNI) (PERMANOVA, $R^2 = 0.253$, $p = 0.001$), degree of pathogen inhibition (PERMANOVA, $R^2 = 0.064$,
367 $p = 0.017$), microbiome dilution (PERMANOVA, $R^2 = 0.098$, $p = 0.001$), and algal fraction (full culture
368 (FC) vs. filtered microbiome (FM)) (PERMANOVA, $R^2 = 0.060$, $p = 0.025$). Residual variation accounted
369 for 52.5% of the total variation.
370
371 In conclusion, the degree of pathogen inhibition was associated with differences in the taxonomic
372 composition of the sampled *Isochrysis* microbiomes. However, most variation between microbiomes
373 was explained by differences between the two source cultures (NI and NNI).
374
375 **Isolation and identification of bacterial strains from *Isochrysis galbana* microbiomes**. A total of 64
376 isolates were obtained from *I. galbana* microbiomes either from the native algal culture (19 strains)
377 or from the enriched inhibitory microbiomes after *Vibrio* inhibition (45 strains). These were
378 tentatively identified using full-length 16S rRNA gene sequencing (Table S4). Strains from the native
379 microbiome were identified as members of the *Alteromonas* (2 isolates), *Croceibacter* (1 isolate),
380 *Mameliella* (1 isolate), *Phaeobacter* (3 isolates), *Qipengyuania* (3 isolates), *Roseovarius* (2 isolates),
381 *Ruegeria* (1 isolate) and *Sulfitobacter* (6 isolates) genera (Table 1), whereas strains from the enriched
382 inhibitory microbiomes were identified as members of the *Alteromonas* (5 isolates), *Halomonas* (1
383 isolate), *Phaeobacter* (18 isolates), *Roseovarius* (7 isolates), *Sulfitobacter* (11 isolates) and *Vibrio* (3
384 isolates) genera (Table 2).
385
386 **Inhibition of *Vibrio anguillarum* by pure cultures and co-cultures of bacterial isolates**. Pure
387 cultures of all 64 bacterial isolates were tested for their ability to inhibit *V. anguillarum* NB10 and 17
388 isolates with pronounced inhibitory activity (zone of inhibition in the embedded pathogen) were all
389 identified as *Phaeobacter* sp. (Table 3, Figure S3). The dual co-cultures of representative isolates
390 showed inhibition zones in all co-cultures that included *Phaeobacter piscinae* (isolate H2). In some

391 cases, the co-culture of *P. piscinae* (isolate H2) and *Roseovarius* sp. (isolate C7) resulted in a larger
392 clearing zone as compared to the monoculture of the *P. piscinae*, however this result was not
393 consistently reproducible. Of special interest was the co-culture of *Sulfitobacter pontiacus* (isolate
394 D3) and *Halomonas campaniensis* (isolate D2), which despite showing only slight (*Halomonas*
395 *campaniensis)* or no (*Sulfitobacter pontiacus)* inhibition in single culture, were remarkably inhibitory
396 when grown together (Figure 4). Monocultures and co-cultures of these two isolates were
397 subsequently tested against eight additional *V. anguillarum* strains with different virulence and the
398 co-culture showed varying degrees of inhibition against *V. anguillarum* 775, 4299, and PF7 (Table
399 S5). Isolate H2 showed strong inhibition against all *V. anguillarum* strains.
400
401 **Whole genome sequencing and genome mining.** The species identity of the five chosen isolates was
402 confirmed through Average Nucleotide Identity (ANI%) analysis: *Roseovarius nubinhibens* (isolate C7,
403 ANI 90.0%; referred to as *Roseovarius* sp.), *Halomonas campaniensis* (isolate D2, ANI 98.4%),
404 *Sulfitobacter pontiacus* (isolate D3, ANI 97.3%), *Alteromonas macleodii* (isolate E2, ANI 99.0%),
405 *Phaeobacter piscinae* (isolate H2, ANI 98.2%). Genome assembly of the sequencing data from the
406 five isolates yielded three closed genomes and two draft genomes (Table 4). According to
407 antiSMASH (57) analysis, Halomonas *campaniensis* D2 carries seven BGCs (NI-siderophore,
408 ranthipeptide, redox-cofactor, RIPP-like, T1PKS, betalactone, ectoine) and *Sulfitobacter pontiacus* D3
409 four BGCs (RiPP-like, betalactone, hserlactone, redox-cofactor), potentially encoding antibacterial
410 compounds (Table S6). The structure of compounds cannot be accurately predicted due to the low
411 similarity compared to the known BGCs in MIBiG (Table S6).
412

413 **DISCUSSION**

414 The rapid spread of pathogenic bacteria within intensive aquaculture systems remains a significant
415 challenge, especially during the vulnerable larval development stage, and disease outbreaks often
416 result in substantial economic losses. In this study, we investigated the probiotic potential of the
417 microbiomes from two algae typically employed as live feed in marine larviculture, *Tetraselmis*
418 *suecica* and *Isochrysis galbana*. By identifying microbiomes with disease suppression, we aim to
419 provide a novel approach to disease control in aquaculture, reducing reliance on antibiotics and the
420 risk of spread of AMR.
421
422 Determining the inhibitory effect of a mixed microbiome against a particular pathogenic bacterium
423 requires specific detection and quantification of the pathogen. Previous works on fish pathogens
424 have used several approaches for determining inhibition in a mixed microbial population. For

425 instance tagging the pathogen with an antibiotic resistance marker enabling specific quantification
426 by culturing on antibiotic containing media (11, 20, 31), using semi-selective media and distinct
427 colony morphology in defined co-cultures (8, 59, 60), using flow cytometry to determine
428 concentrations of a GFP-tagged pathogen (20), or developing specific primers and designing a qPCR
429 approach has been a strategy for specific detection (8). Both the culturing approaches as well as the
430 qPCR method are labour-intensive and time-consuming. To enable a faster screen for pathogen-
431 inhibitory effect of a mixed microbial community, we used a GFP-tagged pathogen and
432 demonstrated that the fluorescent signal is a robust indication of growth of the pathogen, and that it
433 can be used to detect its inhibition by other microorganisms. Thus, we developed a simple
434 methodology for screening mixed microbiomes for their anti-pathogenic potential, thereby offering
435 a valuable tool for developing sustainable disease management strategies also outside of the
436 aquaculture sector. The method facilitates identification and selection of microbiomes with the
437 greatest potential for disease control, contributing to the sustainable growth of aquaculture and the
438 global effort to combat AMR.
439
440 We used the method to demonstrate that both the native, complete microbiomes and diluted
441 microbiomes of two microalgae used in aquacultures were able to inhibit the growth of fish
442 pathogen *Vibrio anguillarum*. In contrast to findings from previous studies where cultures of
443 *Isochrysis* sp. and *Tetraselmis suecica* have themselves been inhibitory to fish pathogenic *Vibrio*
444 species (59–61), we did not find that *V. anguillarum* was inhibited by axenic *I. galbana* or *T. suecica,*
445 nor the cell-free supernatants of either culture. Thus, in our study, it was clearly the microorganisms
446 associated with the algae that inhibited growth of the pathogen, emphasising the crucial role of
447 microbial communities associated with live feed in larviculture for disease control. These findings
448 highlight the potential of harnessing natural microbial consortia to enhance disease resistance in
449 aquaculture settings. Furthermore, in our experiments the *Isochrysis* microbiome inhibited growth
450 of the pathogen (RFU < 10% of control) at 1:10 (*Vibrio*: *Isochrysis* microbiome) starting inoculation
451 ratio, showing a more pronounced inhibitory potential as compared to that of a monoculture of *P.*
452 *piscinae* S26, where it took an inoculation ratio of 1:100 (*Vibrio*: *P. piscinae* S26) to reach similar
453 reduction of the RFU, supporting our hypothesis that a mixed microbiome may be more inhibitory to
454 a pathogen than individual pure cultures.
455
456 Sequence-based analysis of *I. galbana* microbiomes found that *Rhizobiaceae*, *Alteromonadaceae*,
457 and *Flavobacteriaceae* family members dominated the native *I. galbana* microbiome. This is in line
458 with a number of previous studies reporting these families as members often found in the core

459 microbiome of microalgae (36, 62, 63). The native microbiome also harboured members of the
460 *Rhodobacteraceae*, which is a family commonly associated with microalgae and algal blooms (36,
461 62–65). The enriched inhibitory microbiomes were dominated by *Alteromonadaceae*,
462 *Rhodobacteraceae*, and *Vibrionaceae* members, with decreased relative abundances of *Rhizobiaceae*
463 and *Flavobacteriaceae* compared to the native microbiome. These shifts are likely heavily influenced
464 by transferring the algal microbiome to a nutrient rich assay medium (MB) favouring fast-growing,
465 heterotrophic bacteria. Interestingly, the degree of pathogen inhibition by the enriched inhibitory
466 microbiomes is not explained by differences in the community richness, diversity, and evenness.
467 However, it does correlate with distinct patterns in the overall community composition, suggesting
468 that the inhibitory potential is influenced by the structure of the microbiome and synergistic
469 interactions within a diverse community. Our findings also imply that complete pathogen inhibition
470 can be achieved by a range of different enrichment cultures.
471
472 Pure cultures of *Phaeobacter piscinae* H2 isolated from the *I. galbana* microbiome inhibited *V.*
473 *anguillarum* when tested in an agar-based assay, and this is consistent with numerous previous
474 studies showing that several *Phaeobacter* species can inhibit *Vibrio* species (20, 21, 66–68). Notably,
475 all isolates that produced an inhibition zone of the embedded *V. anguillarum* were identified as
476 *Phaeobacter* species, however, this genus was not among the ten most abundant genera of the
477 enriched inhibitory microbiomes. This could indicate that instead of the potent antagonistic effect of
478 particular genera, it may be a synergistic effect of the microbiome members that inhibits the
479 pathogen.
480
481 The co-culture of *Sulfitobacter pontiacus* D3 and *Halomonas campaniensis* D2, isolated from the *I.*
482 *galbana* microbiome, was inhibitory to *V. anguillarum* NB10, despite neither isolate showing
483 pathogen inhibition as pure cultures. The co-culture also showed varying degrees of inhibition
484 against three additional *V. anguillarum* strains, belonging to different phylogenetic clades based on
485 MLSA, including a faint inhibitory effect of the highly virulent *V. anguillarum* PF7. This indicates that
486 the observed inhibition is not limited to low-virulence strains but also that the effect may not be
487 universal and that some pathogens are not affected. While it is beyond the scope of the current
488 study to unravel the molecular and chemical mechanism underlying this co-culture-induced
489 inhibition, previous research has demonstrated that biosynthetic gene clusters (BGCs) encoding
490 antibiotic compounds can be elicited by co-cultivation of bacteria (28–30, 69). According to
491 antiSMASH analyses, the genomes of both bacteria harbour several BGCs. Therefore, future
492 metabolomic analyses of the mono- and co-cultures could provide insight into the co-culture-

493 induced antimicrobial mechanism. Moreover, several *Halomonas* species have been reported to
494 produce an array of hydrolytic enzymes, extracellular polysaccharides (EPS) (70), antibiotics (71),
495 antifungals (72), and pigments (73). *Halomonas campaniensis* D2, in particular, carries seven
496 biosynthetic gene clusters potentially encoding antibacterial compounds. This combined with the
497 lack of inhibition by the monoculture could suggest that the presence of *Sulfitobacter pontiacus* D3
498 may trigger the inhibitory effect. *Sulfitobacter* species, often associated with marine algae and corals
499 (74–76), possess the ability to metabolise sulphite (77–79) and dimethylsulfoniopropionate (DMSP)
500 (74). The breakdown of DMSP produces potentially antimicrobial compounds such as acrylate (80,
501 81) and dimethyl sulphide (DMS), which could indicate that the sulphur metabolism capabilities of
502 *Sulfitobacter* may contribute to pathogen inhibition. Several *Sulfitobacter* species have also been
503 reported to inhibit fungal pathogens (82) and produce EPS (83) and extracellular cyclodipeptides
504 (84).
505
506 Beiralas et. al reported that *Sulfitobacter pontiacus* is a key species of the *Emiliania huxleyi*
507 microbiome, protecting its algal host from bacterial pathogens even at low initial abundance (76).
508 While the mechanism of the algal protector phenotype was not determined, the authors suggest
509 that harbouring *Sulfitobacter* species in the algal microbiome presents the host with an advantage
510 and that *Sulfitobacter* species may play a key role in influencing population dynamics. Building on
511 these findings, we speculate that even a low abundance of *Sulfitobacter pontiacus* in a community or
512 co-culture may induce synergistic effects that enhance pathogen inhibition, potentially mediated
513 through the production of bioactive compounds. These insights highlight the importance of
514 microbial interactions in community functionality and stability.
515
516 In conclusion, by introducing an efficient methodology for screening mixed microbiomes, our study
517 offers a promising approach for identifying candidate microbiomes for sustainable disease
518 management. Our findings highlight the potential of harnessing natural microbial consortia to
519 enhance disease resistance in aquaculture and support the importance of microbial co-cultures as
520 potential disease control strategies.
521
522 **ACKNOWLEDGEMENTS**
523 We thank our aquaculture partners for providing algal cultures. This project has received funding from
524 the European Union's Horizon 2020 research and innovation programme under Grant Agreement no.
525 101000392 (MARBLES), the Novo Nordisk Foundation (NNF20OC0064249) and the Danish National
526 Research Foundation (DNRF137) for the Center for Microbial Secondary Metabolites. This output

reflects only the author's view, and the Research Executive Agency (REA) cannot be held responsible for any use that may be made of the information contained therein. Figure 1 was created with BioRender.com.

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LEGENDS FOR TABLES AND FIGURES

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

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

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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 microbiome and the enriched inhibitory microbiome of *I. galbana* (NI, NNI) were tested for inhibition of *V. anguillarum* in an agar-based assay (E). (Created by BioRender).

801 **Figure 2.** *Vibrio anguillarum* NB10_gfp at different initial concentrations: 10^6 (●), 10^5 (□), 10^4 (▲), 10^3 (
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803 of 485/15 nm and emission of 513/15 nm, and the (C) inhibition profile of *V. anguillarum* NB10_gfp
804 (initial concentration: 10^4 CFU mL^{-1}) by antagonist *Phaeobacter piscinae* S26 at different initial
805 concentrations: 10^7 (△), 10^6 (●), 10^5 (□), 10^4 (▲), 10^3 (○), 10^2 (■) CFU mL^{-1}, as measured by fluorescence
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807
808 **Figure 3.** Inhibition by *Isochrysis galbana* (left) and *Tetraselmis suecica* (right) microbiomes of *Vibrio*
809 *anguillarum* NB10_gfp (starting inoculum of 3.1 ± 0.3 log CFU mL^{-1}), as measured by fluorescence (GFP,
810 excitation: 485/15 nm, emission: 513/15 nm). The inhibitory effect of serial dilutions (10^{-1} (△), 10^{-2} (●
811), 10^{-3} (□), 10^{-4} (▲), 10^{-5} (○)) of different fractions of the algal cultures have been tested: full culture (FC;
812 3A and 3D), with algal and bacterial cells; filtered microbiome (FM; 3B and 3E), where algal cells have
813 been removed; and axenic (AX; 3C and 3F) cultures, where the algae cells are free of bacteria. FM
814 samples were measured on a different instrument, hence a different scale of RFU.
815
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818 *Roseovarius* sp., D2: *Halomonas campaniensis*, D3: *Sulfitobacter pontiacus*, E2: *Alteromonas*
819 *macleodii*, H2: *Phaeobacter piscinae*.
820
821 **Figure 5.** Microbial composition (top 10 most abundant genera) of the native (left) and enriched
822 inhibitory (right) microbiomes based on the 16S rRNA amplicon sequencing results. Samples from
823 the enriched inhibitory microbiome are grouped by their inhibition profile: marginal growth of
824 pathogen (*), medium inhibition of pathogen (**), full inhibition of pathogen (***).
825
826 **Supplementary Tables and Figures**
827
828 **Table S1.** List of thirty unique barcodes and primers used to amplify the V3-V4 region of the 16S
829 rRNA gene in DNA from the microbiomes of *Isochrysis galbana* cultures. Primers and barcodes from
830 (85).
831
832 **Table S2.** Algal and bacterial counts of different algal cultures (AXT, AXI, NT, NI and NNI) used in the
833 *Vibrio* inhibition assay, before and after up-concentration ("Before" and "After"), and from different
834 algal sample fractions (Full culture, FC and Filtered microbiome, FM). Counts are reported as mean ±

835 standard deviation. In cases where standard deviation is not reported, data originated from
836 unreplicated experimental conditions.
837
838 **Table S3**. DNA concentration after extraction, DNA amount provided for sequencing and number of
839 sequences obtained from each sample (after denoising and filtering). Sample names indicate replicate
840 ("R1", "R2") source culture ("NI", "NNI"), sample fraction (Full culture: "FC", Filtered microbiome:
841 "FM"), dilution factor of the algal microbiome (0 to -5), and starting inoculum of *V. anguillarum*
842 NB10_gfp ("V3", "V5" for 3 or 5 log CFU mL^{-1}) for samples obtained after the inhibition assay. The algal
843 cultures were sampled before up-concentrating and filtering ("raw", triplicates), and each fraction (FC
844 and FM) was sampled before the enrichment assay ("bf"). Sterile media controls were included ("MB-
845 ctrl").
846
847 **Table S4.** 64 bacterial strains isolated from the native („Raw culture", 19 strains) or enriched inhibitory
848 („Full culture": FC, or „Filtered microbiome": FM, 45 strains) microbiome of *Isochrysis galbana*, and
849 tentatively identified using full-length 16S rRNA gene sequencing. The isolates originate from two
850 different *I. galbana* cultures (NI and NNI), acquired at different times from the same aquaculture unit.
851
852 **Table S5.** Inhibition of nine *V. anguillarum* strains by the co-culture of *Halomonas campaniensis*
853 (isolate D2) and *Sulfitobacter pontiacus* (isolate D3). *Phaeobacter piscinae* isolate H2 was used as
854 positive control. Clearing zone of different degrees (+, ++, +++), a faint clearing zone (*), or no clearing
855 zone (-) after 24 hours of incubation. Virulence ranks as defined by Rønneseth et al (54).
856
857 **Table S6.** Biosynthetic gene clusters (BGCs) predicted by antiSMASH in the genomes of *Halomonas*
858 *campaniensis* D2 and *Sulfitobacter pontiacus* D3.
859
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862 by absorbance at 600 nm. The inhibitory effect of serial dilutions (10^{-1} (△), 10^{-2} (●), 10^{-3} (□), 10^{-4} (▲), 10^{-}
863 ^{5} (○)) of different fractions of the algal cultures have been tested: full culture (FC; S1A and S1D), with
864 algal and bacterial cells; filtered microbiome (FM; S1B and S1E), where algal cells have been removed;
865 and axenic (AX; S1C and S1F) cultures, where the algae cells are free of bacteria.
866
867 **Figure S2.** Native microbiome composition (top 10 most abundant genera) of two *Isochrysis galbana*
868 cultures of different age based on the 16S rRNA amplicon sequencing results. One culture, NNI has

869 been newly provided by an aquaculture facility and has a relatively freshly recruited microbiome
870 (left). The other culture, NI has been regularly subcultured under laboratory conditions for almost
871 four years (right).
872
873 **Figure S3.** Inhibition of *Vibrio anguillarum* NB10_gfp by 64 isolates from the *Isochrysis galbana*
874 microbiome. 16 isolates showed a clearing zone of varying size (all identified as *Phaeobacter* sp.),
875 and 13 isolates showed a faint halo, or a rough agar surface around the colony (identified as
876 *Alteromonas, Sulfitobacter, Qipengyuania* and *Croceibacter* sp.).

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

878

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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882 **Table 2.** Identification (16S rRNA gene sequence and BLAST) of bacterial isolates from the enriched inhibitory *Isochrysis galbana* cultures after co-culture
883 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

884

885 **Table 3.** Bacterial strains isolated from *Isochrysis galbana* cultures with the ability to inhibit *V.*
886 *anguillarum*. All isolates characterized by a clearing zone of different sizes (+, ++, +++) or a faint
887 clearing zone (*) were identified as *Phaeobacter* sp.
888

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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899 **Table 4.** Whole genome sequencing results of five bacterial isolates from the *Isochrysis galbana*
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901 *Sulfitobacter pontiacus* D3, *Alteromonas macleodii* E2, and *Phaeobacter piscinae* H2 yielded three
902 closed genomes and two draft genomes.
903

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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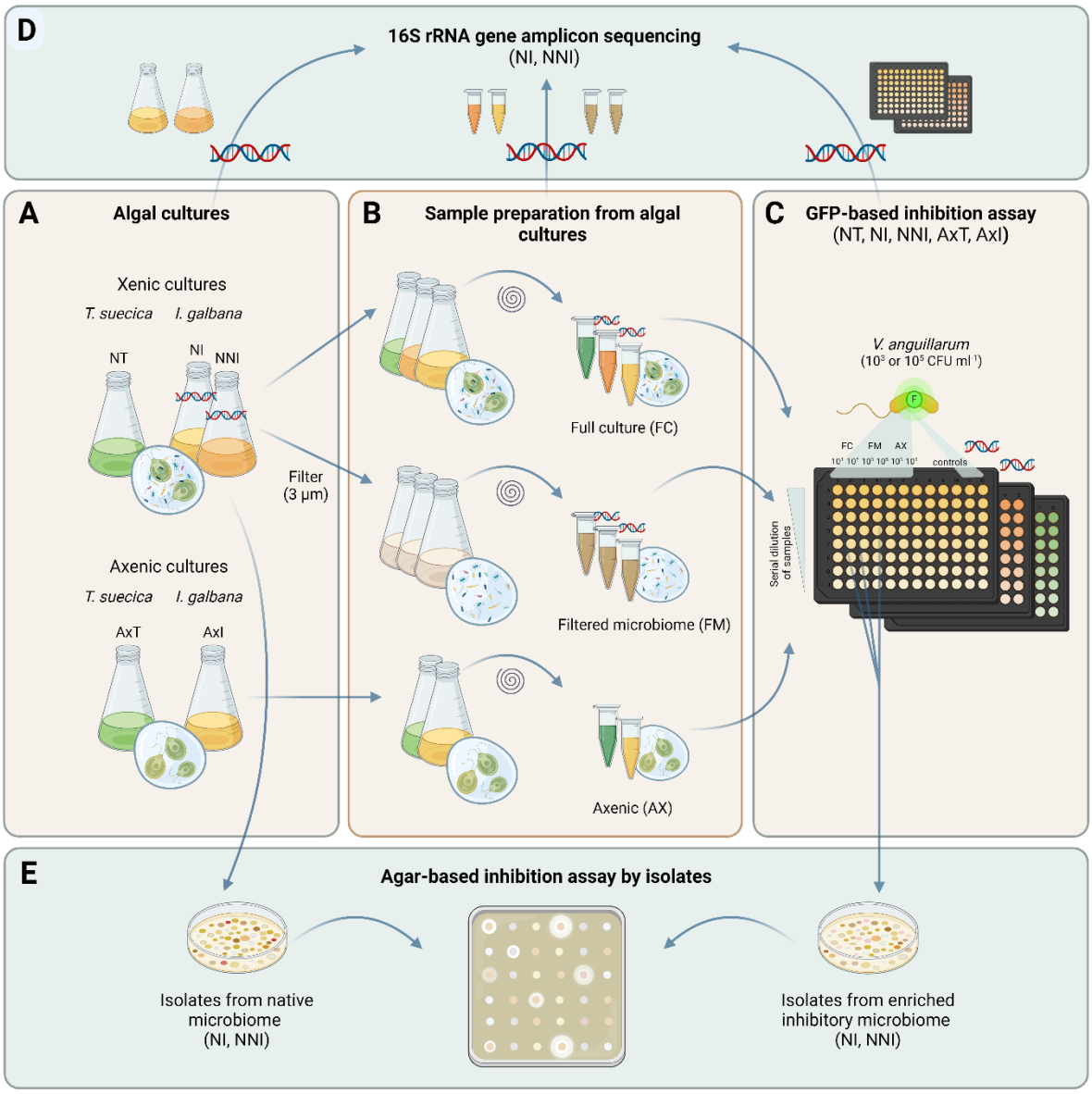

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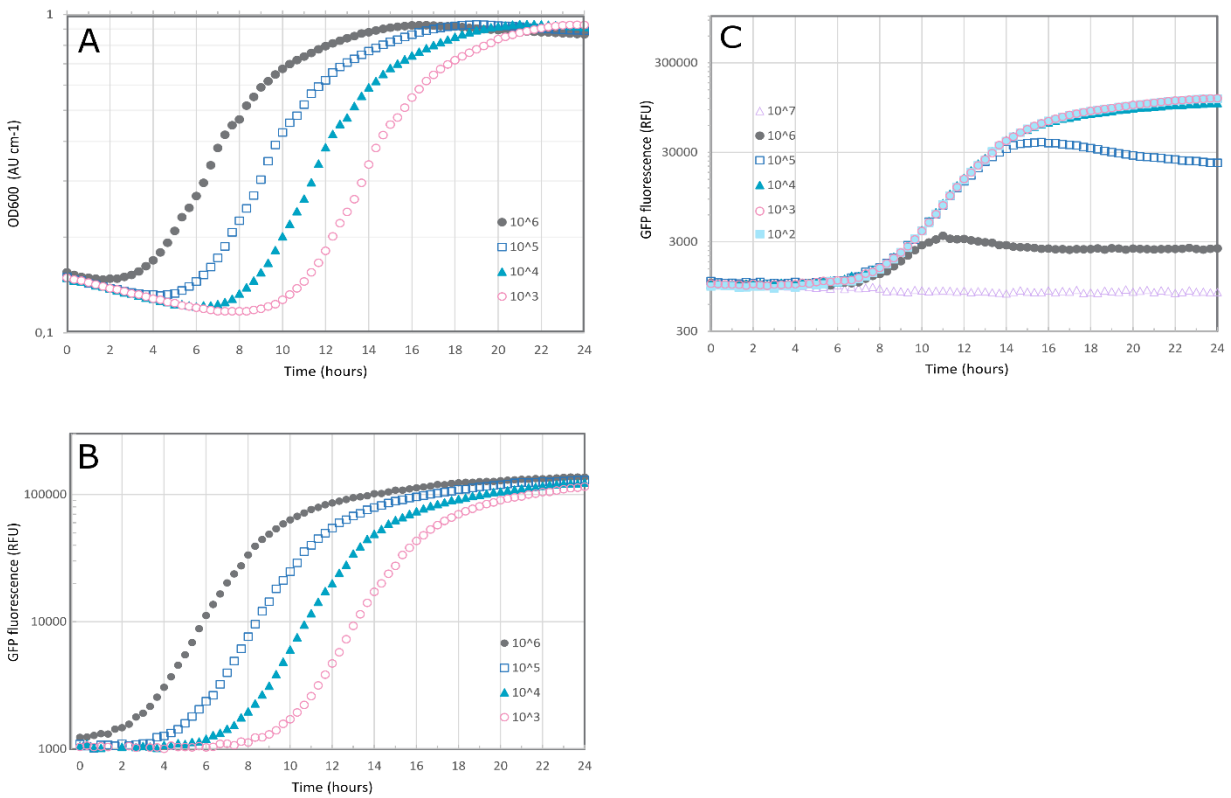

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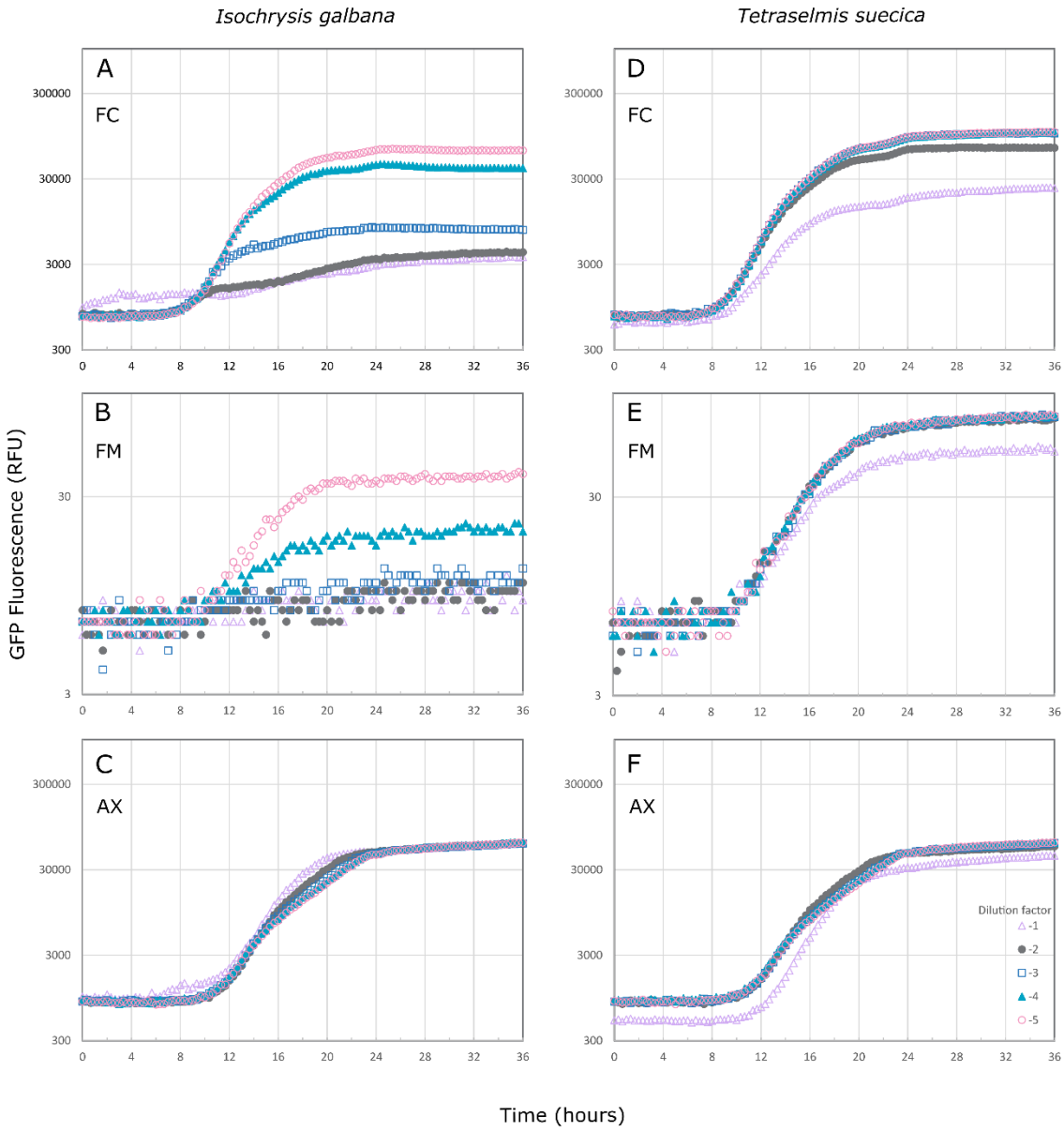

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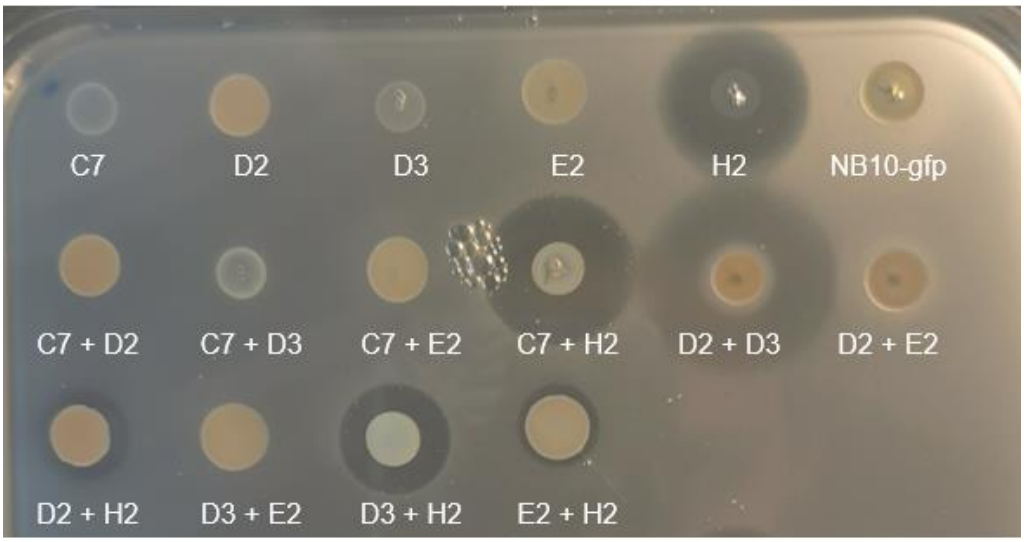

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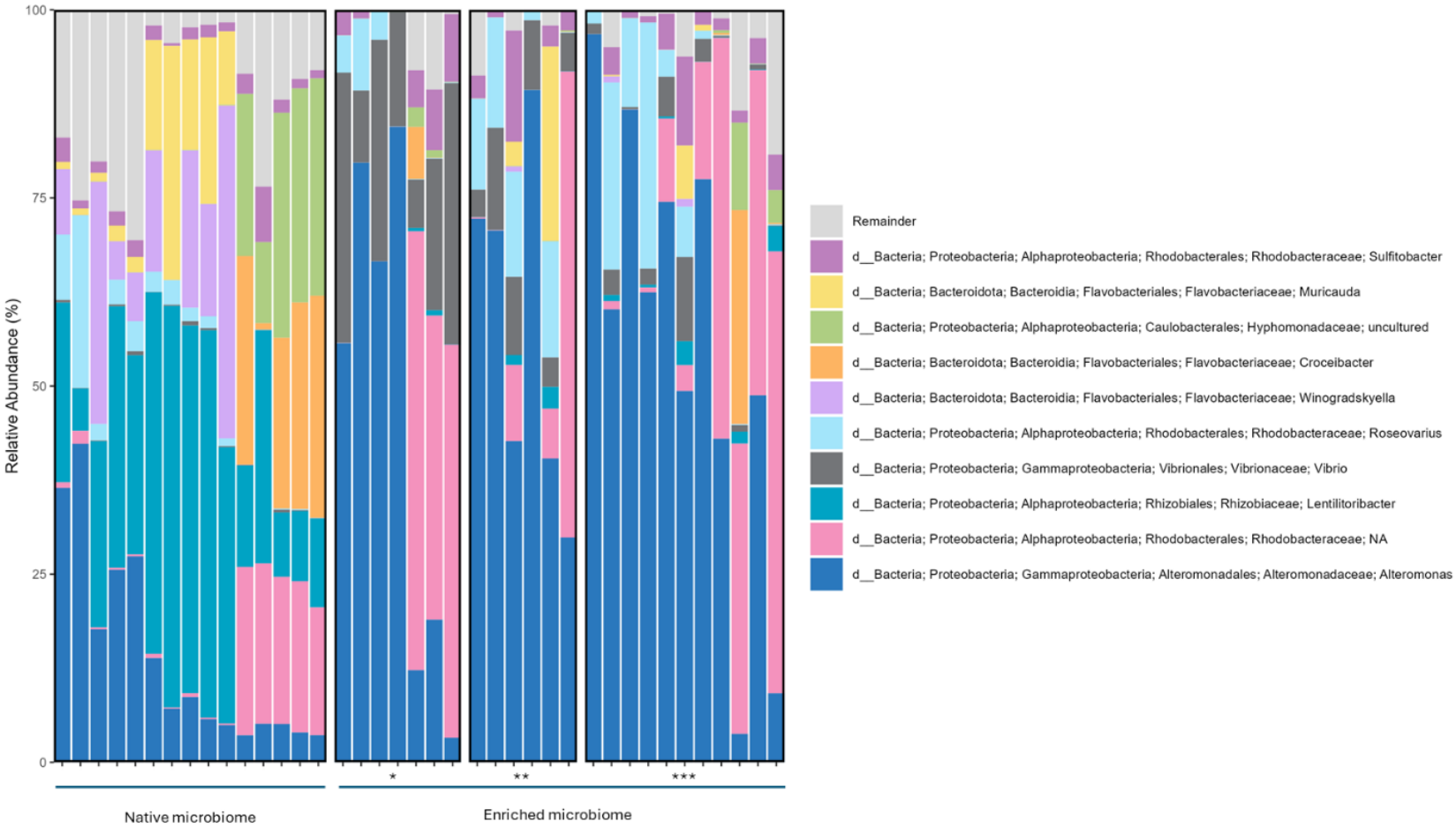

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948 gene in DNA from the microbiomes of *Isochrysis galbana* cultures. Primers and barcodes from (85).

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

949 **Table S2.** Algal and bacterial counts of different algal cultures (AXT, AXI, NT, NI and NNI) used in the
950 *Vibrio* inhibition assay, before and after up-concentration ("Before" and "After"), and from different
951 algal sample fractions (Full culture, FC and Filtered microbiome, FM). Counts are reported as mean ±
952 standard deviation. In cases where standard deviation is not reported, data originated from
953 unreplicated experimental conditions.
954

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

955
956

957 **Table S3**. DNA concentration after extraction, DNA amount provided for sequencing and number of
958 sequences obtained from each sample (after denoising and filtering). Sample names indicate replicate
959 ("R1", "R2"), source culture ("NI", "NNI"), sample fraction (Full culture: "FC", Filtered microbiome:
960 "FM"), dilution factor of the algal microbiome (0 to -5), and starting inoculum of *V. anguillarum*
961 NB10_gfp ("V3", "V5" for 3 or 5 log CFU mL^{-1}) for samples obtained after the inhibition assay. The algal
962 cultures were sampled before up-concentrating and filtering ("raw", triplicates), and each fraction (FC
963 and FM) was sampled before the enrichment assay ("bf"). Sterile media controls were included ("MB-
964 ctrl").
965

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

968 **Table S4.** 64 bacterial strains isolated from the native („Raw culture", 19 strains) or enriched inhibitory
969 („Full culture": FC, or „Filtered microbiome": FM, 45 strains) microbiome of *Isochrysis galbana*, and
970 tentatively identified using full-length 16S rRNA gene sequencing. The isolates originate from two
971 different *I. galbana* cultures (NI and NNI), acquired at different times from the same aquaculture unit.
972

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%

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975 **Table S5.** Inhibition of nine *V. anguillarum* strains by the co-culture of *Halomonas campaniensis* (isolate D2) and *Sulfitobacter pontiacus* (isolate D3).
976 *Phaeobacter piscinae* isolate H2 was used as positive control. Clearing zone of different degrees (+, ++, +++), a faint clearing zone (*), or no clearing zone (-)
977 after 24 hours of incubation. Virulence ranks as defined by Rønneseth et al (54).

***V. anguillarum* strain**	**Virulence rank**	**Inhibition by *H. campaniensis* D2 monoculture**	**Inhibition by *S. pontiacus* D3 monoculture**	**Inhibition by co-culture of *H. campaniensis* D2 and *S. pontiacus* D3**	**Inhibition by *P. piscinae* H2**
90-11-286	High	-	-	-	+++
DSM21597	High	-	-	-	+++
PF7	High	-	-	*	+++
PF4	High	-	-	-	+++
9014/8	Medium	-	-	-	+++
S2 2/9	Medium	-	-	-	+++
4299	Low	*	-	++	+++
NB10	Low	*	-	++	+++
775	N/A	+	-	+	+++

978

979 **Table S6.** Biosynthetic gene clusters (BGCs) predicted by antiSMASH 7.0 in the genomes of *Halomonas*
980 *campaniensis* D2 and *Sulfitobacter pontiacus* D3.

Strain	Cluster	Type of BGC	Most similar known BGC in MIBiG	Similarity
H. campaniensis D2	1	NI-siderophore	BGC0001572	66%
	2	ranthipeptide	BGC0000413	2%
	3	redox-cofactor	BGC0001131	25%
	4	RiPP-like	/	/
	5	T1PKS	/	/
	6	betalactone	BGC0001103	20%
	7	ectoine	BGC0000859	75%
S. pontiacus D3	1	RiPP-like	/	/
	2	betalactone	/	/
	3	hserlactone	/	/
	4	redox-cofactor	/	/

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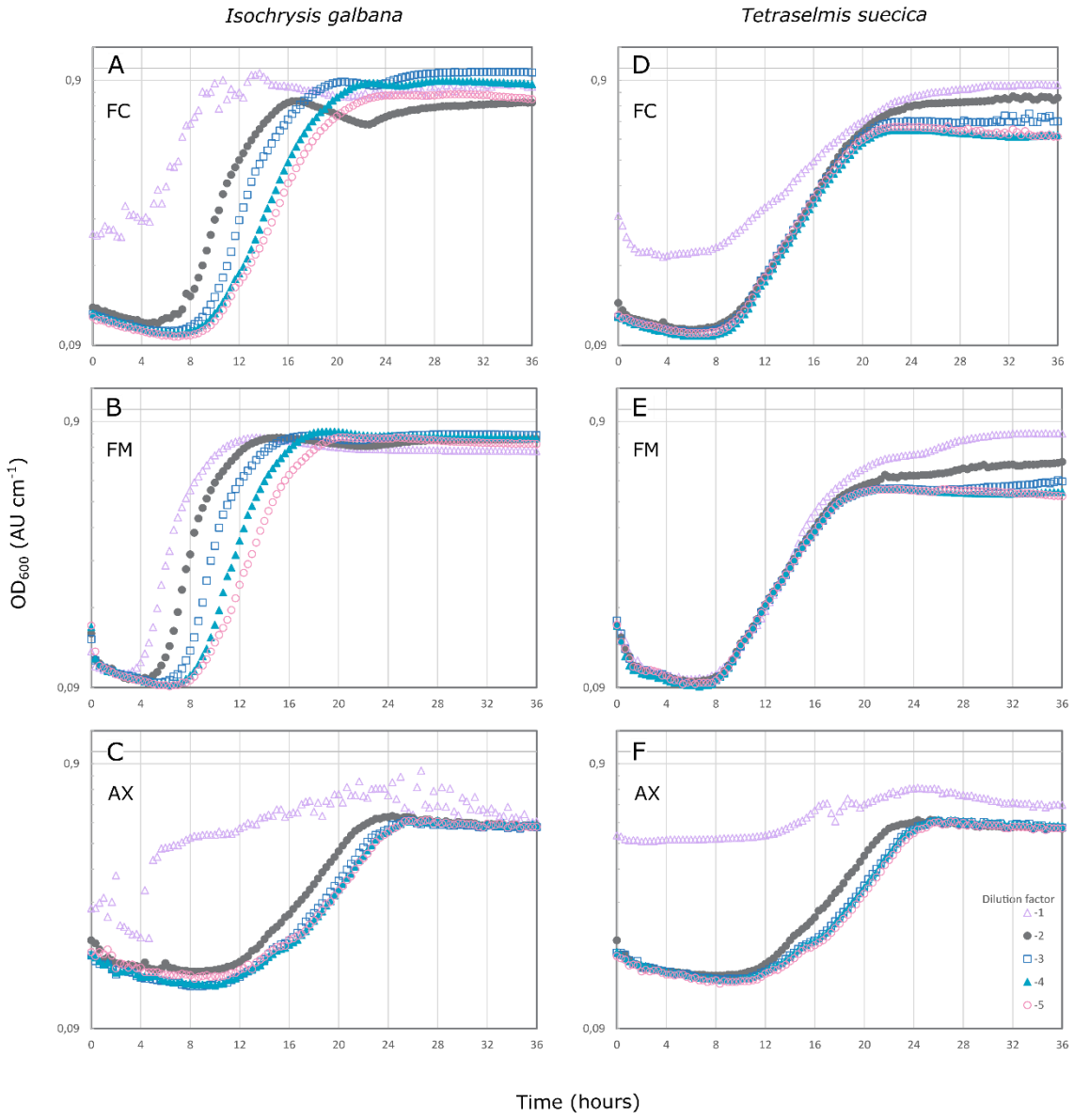

983

984 **Figure S1.** Inhibition assay by *Isochrysis galbana* (left) and *Tetraselmis suecica* (right) microbiomes
985 against *Vibrio anguillarum* NB10_gfp, at a starting concentration of 3.1 ± 0.3 log CFU mL^{-1}, as measured
986 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
988 algal and bacterial cells; filtered microbiome (FM; S1B and S1E), where algal cells have been removed;
989 and axenic (AX; S1C and S1F) cultures, where the algae cells are free of bacteria.

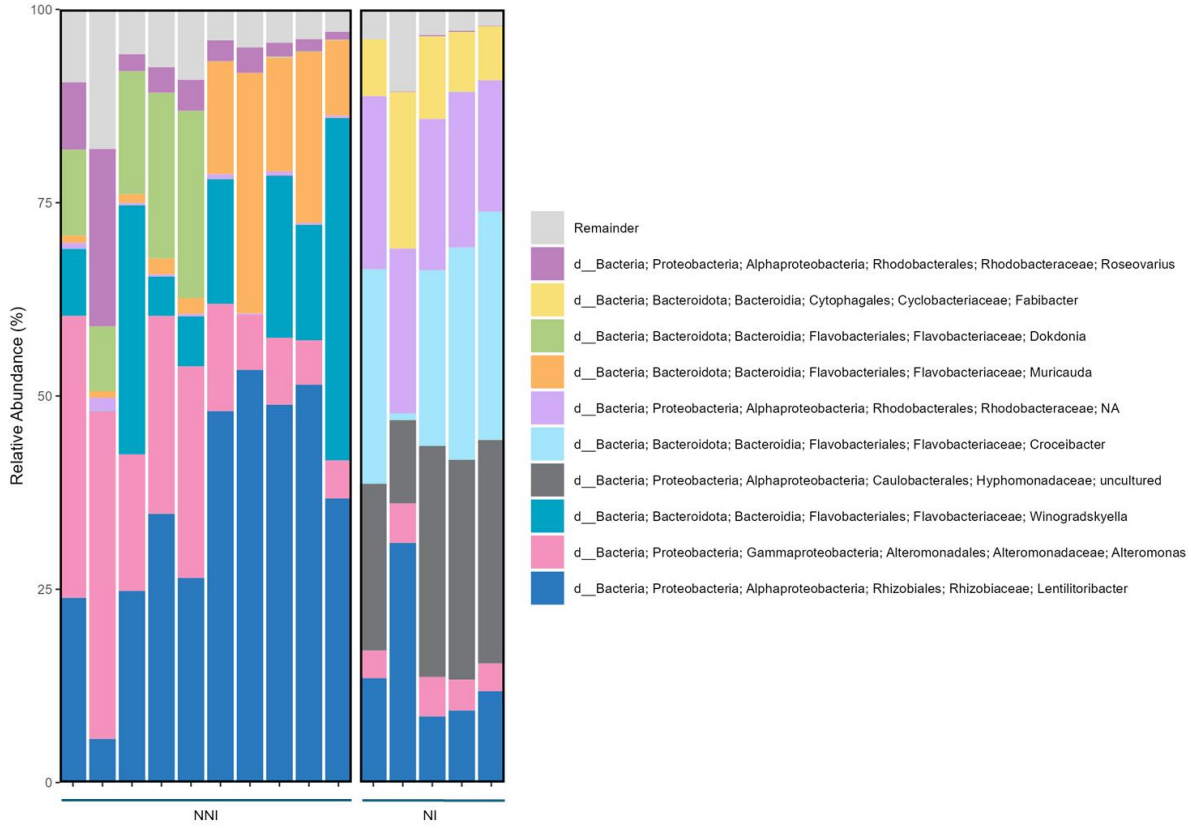

Figure S2. Native microbiome composition (top 10 most abundant genera) of two *Isochrysis galbana* cultures of different age based on the 16S rRNA amplicon sequencing results. One culture, NNI, has been newly provided by an aquaculture facility and has a relatively freshly recruited microbiome (left).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

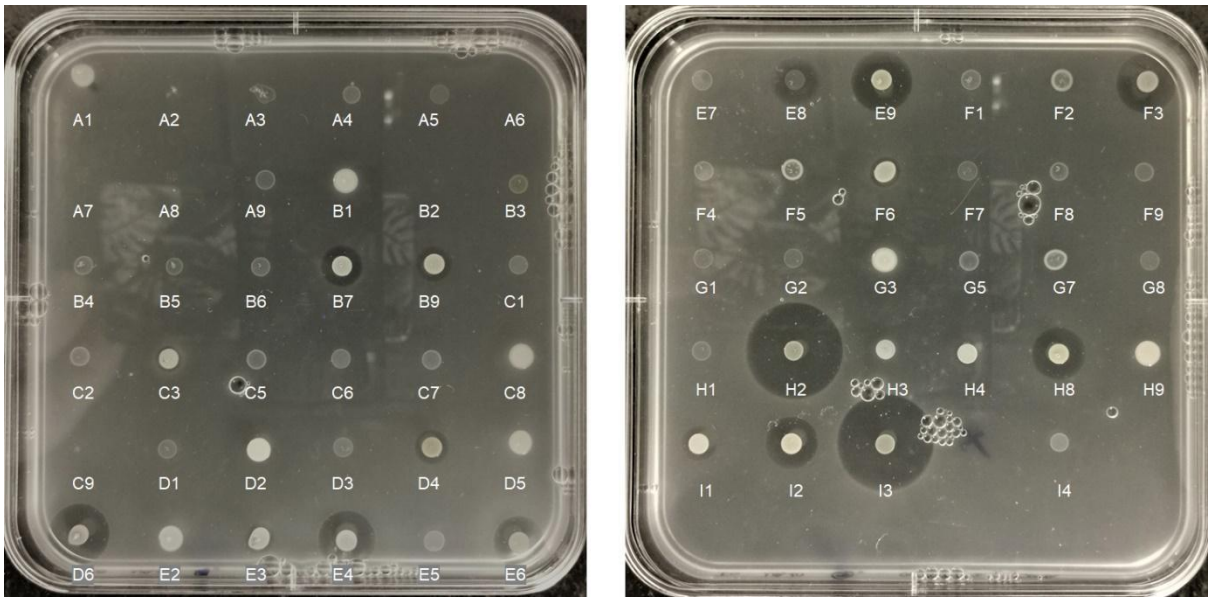

Figure S3. Inhibition of *Vibrio anguillarum* NB10_gfp by 64 isolates from the *Isochrysis galbana* microbiome.