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### **Vibrio species are predominantly intracellular within cultures of Neoparamoeba perurans, causative agent of Amoebic Gill Disease (AGD)**

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1 **\*Colour print requested for figure 2 and figure 3\***

2 ***Vibrio* species are predominantly intracellular within cultures of *Neoparamoeba perurans*,**  
3 **causative agent of Amoebic Gill Disease (AGD)**

4

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18

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20

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23

24

25 **Abstract**

26

27 *Neoparamoeba perurans* is a free-living protist that can cause Amoebic Gill Disease (AGD) in  
28 a number of teleost fish species and is responsible for substantial losses of farmed Atlantic  
29 salmon in various locations world-wide. The intimate relationship of the amoeba with  
30 bacteria can present challenges for its laboratory culture and drug discovery programmes.  
31 Herein, we report our findings on the bacteria that live in close association with *N. perurans*.  
32 These include the presence of various marine bacteria, including those of the  
33 *Pseudoalteromonas*, *Halomonas*, *Cellulophaga* and *Mesonnia* genera. However, next  
34 generation sequencing (NGS) identified a substantial proportion of sequences that matched  
35 with the *Vibrio* genus in filtered amoebae and not in the medium suggesting an intimate  
36 association between this genus and *N. perurans*. Fluorescence *in-situ* hybridization (FISH)  
37 revealed that *Vibrio* species are predominantly found within *N. perurans*. This information is  
38 important in the management and control of AGD as bacteria associated with *N. perurans*  
39 may have relevance to virulence and advancement of disease.

40

41 **1. Introduction**

42

43 *Neoparamoeba perurans* is a free-living protist that can cause Amoebic Gill Disease (AGD) in  
44 a wide range of teleost fishes, and is responsible for substantial losses of farmed Atlantic  
45 salmon (*Salmo salar*), with incidence of *Neoparamoeba* infection detected in Australia, New  
46 Zealand, Japan, Chile, USA, Scotland, Ireland, France, Spain and Norway (Bustos et al., 2011;  
47 Crosbie et al., 2010; Oldham et al., 2016; Steinum et al., 2008; Young et al., 2008b). The impact  
48 of AGD may also be accentuated by the consequences of climate change, such as sea  
49 temperature, supply of nutrient, harmful algal blooms (Foyle et al., 2020). *N. perurans*  
50 colonizes the fish gill epithelium, and causes structural changes in this tissue, which can  
51 increase morbidity and mortality of its host (Cano et al., 2019). This is problematic for the  
52 aquaculture industry, as it requires costly ongoing management and treatment of the disease,  
53 as the infection recurs due to the limited immune response (Young et al., 2008a). Despite  
54 these difficulties, currently relatively little is known about this opportunistic pathogen, and  
55 the disease it causes.

56

57 *Neoparamoeba perurans* and AGD research is complicated by the inability to develop axenic  
58 cultures (Figure 1) (Collins et al., 2017). The sustained presence of bacteria in *Neoparamoeba*  
59 cultures is problematic, as the presence of bacteria can affect the ability to perform high-  
60 throughput screening of drug compounds against *Neoparamoeba* and to understand its  
61 metabolic pathways. In addition, the presence of bacteria may also hinder the outcomes of  
62 colorimetric or other biochemical cell-based assays, necessitating manual cell counting  
63 methods to assess potential inhibitory compounds, which is time consuming. Furthermore,  
64 bacteria within the culture may be pathogenic to the amoeba, leading to partial or total  
65 collapse of the culture; any treatment used to reduce bacterial loads could further interfere  
66 with any assays conducted.

67

68 While the bacterial contamination may be problematic from a research point of view, it is  
69 important to consider the basic predator phagocytic relationship and also the potential  
70 symbiotic relationship between the amoebae and the bacteria, as bacteria are known to  
71 produce compounds which can be utilised by eukaryotes. Examples of the latter include the  
72 interactions between bacteria such as *Flavobacterium*, *Roseobacter*, and *Sulfitobacter* spp.  
73 and diatoms (Amin et al., 2012) or the suspected symbiotic relationship between algae and  
74 bacteria, where algae, including seaweed species acquire Vitamin B<sub>12</sub> from bacteria to  
75 produce methionine (Croft et al., 2005). On the other hand, bacteria may also benefit from  
76 these relationships, such as protection from environmental risks and (Thomas et al., 2010) as  
77 is the case with *Candidatus Legionella jeonii* and *A. proteus* (Park et al., 2006). It is therefore  
78 possible that some species of bacteria may have a symbiotic relationship with *Neoparamoeba*  
79 spp., providing compounds required for amoeba growth, or acting as a food source for the  
80 amoeba. There has previously been focus on the bacterial microbiome associated with *N.*  
81 *perurans*, such as the recent study of the effects of temperature on *N. perurans* and the  
82 microbial community within *in vitro* cultures of the pathogen (Benedicenti et al., 2019). While  
83 this study did not elucidate the relationship between the bacteria and *N. perurans*, it is  
84 important this relationship is further refined in order to develop potential new control  
85 measures, to improve culture techniques and to further our understanding of symbiosis.

86

87 Bacteria can also be fish pathogens and their association with other pathogens, such as *N.*  
88 *perurans* may be relevant to emergence of complex gill disease (CGD), a multi-pathogen

89 disease used to describe a nonspecific gill condition. The aim of this study is to describe the  
90 variety of different bacteria associated with *N. perurans*, which may impact on virulence of *N.*  
91 *perurans* and its role in CGD (Herrero et al., 2018).

92  
93  
94

## 2. Methods

### 2.1 *Neoparamoeba perurans* culture

96 *Neoparamoeba perurans* was utilised from a long-term culture originally isolated from  
97 farmed Atlantic salmon from the west coast of Scotland. It was cultured under sterile  
98 conditions under a class II safety cabinet to avoid any environmental contamination in T75  
99 vented-cap tissue culture flasks (VWR, Leicestershire, England) in Malt-Yeast Broth (MYB)  
100 [0.1g/l Malt Extract (Oxoid™, ThermoFisher Scientific, Renfrew, Scotland), 0.1g/l Yeast  
101 Extract (Oxoid™, ThermoFisher Scientific), and filtered 35 PSU Peacock Salt Seamix Artificial  
102 Sea Water (J C Peacock & Co Ltd)]. The amoebae were incubated at 18°C, and the medium  
103 was changed weekly, in order to prevent overgrowth of bacteria present in the culture. In  
104 order to maintain healthy amoebae, the cells were subcultured by mechanically detaching  
105 them from the surface of the flask and transferring to a new T75 flask containing medium as  
106 described above. The MYB culture medium was changed the following day, once cells had  
107 adhered to the surface.

108

109 *2.2 Isolation and Identification of bacterial species*

110 Bacterial species were isolated from the *N. perurans* cultures by suspending the amoeba  
111 cultures in MYB and performing 1 in 10 serial dilutions. The serial dilutions were cultured on  
112 Luria Bertani (LB) Agar + 75% seawater plates [35g/l LB agar powder (Oxoid™, ThermoFisher  
113 Scientific), 750ml/L 35 PSU artificial seawater, 250ml/L distilled H<sub>2</sub>O] and incubated at 25°C  
114 and 37°C. Individual colonies were selected for Gram staining and culture in LB broth + 75%  
115 seawater [20g/l LB broth powder (Oxoid™, ThermoFisher Scientific), 750ml/L 35 PSU artificial  
116 seawater, 250ml/L distilled H<sub>2</sub>O]. The isolated cultures were used to perform genomic DNA  
117 (gDNA) extraction, to allow identification of the individual bacterial isolates. Gram staining  
118 was performed to observe the cells under a microscope and identify their morphology. Stocks  
119 of the bacterial isolates were preserved through cryopreservation (500µl of culture was  
120 added to 500µl of 50% glycerol w/LB broth + 75% seawater) and stored at -80°C.

121

122 *2.3 Genomic Nucleic Acid Extraction*

123 DNA extraction was performed on the isolated bacteria and *N. perurans* filtered culture. *N.*  
124 *perurans* was collected by centrifugation for 8 minutes at 3000 × *g* to pellet the cells and  
125 remove the MYB culture media. The cells were suspended in 35 PSU filtered artificial  
126 seawater, then the centrifugation step and wash were repeated an additional two times,  
127 discarding the supernatant each wash. The cells were resuspended in 5ml of 35 PSU filtered  
128 artificial seawater and passed through a 0.45µm pore size filter, selected as there is evidence  
129 that a significant proportion of the seawater bacterial microbiome can pass through a 0.45µm  
130 pore size (Denner et al., 2002), allowing the *N. perurans*, which is 10-20µm in diameter, to be  
131 retained.

132 For the bacteria, single colonies were grown in LB broth with 75 % artificial seawater at 25°C,  
133 with shaking at 225rpm for 24 to 48 hours. The bacteria were harvested at 3000 × *g* for 10  
134 minutes, and the supernatant was discarded. DNA extraction was performed using the tri-  
135 reagent® (Life Technologies, Renfrew) phenol-chloroform separation method according to  
136 the tri-reagent® protocol. The method was modified to omit the addition of EDTA, which can  
137 interfere with the PCR reaction (Huggett et al., 2008). The gDNA was then quantified on a  
138 NanoDrop 1000 spectrophotometer (Thermofisher Scientific), and stored at 4°C for future  
139 use.

140

#### 141 2.4 Polymerase Chain Reaction (PCR)

142 PCR reactions consisted of 12.5µl DreamTaq (2x PCR mastermix) (Thermofisher Scientific,), 50  
143 pmol forward and reverse 16S universal oligonucleotide primers S-D-Bact-0341-b-S-17 (5'-  
144 CCTACGGGNGGCWGCAG -3'), S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC -3')  
145 (Klindworth et al., 2013) (Standard Oligos, Thermofisher Scientific,), 11µl nuclease-free water  
146 (Thermofisher Scientific), and 1µl of template. The PCRs were performed with an initial  
147 denaturation at 94°C, followed by 40 cycles of denaturation at 94°C, annealing at 55°C for 16S  
148 universal oligonucleotide primers (template extracted bacterial genomic DNA) and extension  
149 at 72°C, with a final extension at 72°C. The PCR products were visualised on a 2% agarose gel,  
150 run for 45 minutes at 125v, 400mA and viewed with a transilluminator following ethidium  
151 bromide (EtBr) (Sigma-Aldrich Company Ltd, Irvine, Scotland) staining.

152

153

#### 154 2.5 Sequencing

155 PCR-amplified DNA fragments were isolated from EtBr stained agarose gels via PureLink™  
156 Quick Gel Extraction Kit (Thermofisher Scientific,). The purified PCR amplified products were  
157 ligated into the pCR®4-TOPO® vector, using the TOPO™ TA Cloning™ Kit for Sequencing  
158 (Thermofisher Scientific) according to the manufacturer's instructions. Competent DH5α  
159 were transformed with 5µl of the ligation reaction using the heat shock method, as described  
160 in the manufacturers protocol. Transformed cells were then spread evenly onto LB agar  
161 (Oxoid™, ThermoFisher Scientific), which had been previously coated with 100 µg/ml  
162 ampicillin, and incubated overnight at 37°C. Successful transformants underwent plasmid  
163 purification using the PureLink™ Quick Plasmid Miniprep Kit (Thermofisher Scientific,),  
164 according to the manufacturer's instructions. Sanger sequencing of PCR amplified products  
165 was achieved using the M13 uni (-21) or T7 primers, and was carried out commercially by  
166 Eurofins Genomics, Ebersberg, Germany. Nucleotide BLASTn was performed to identify the  
167 bacteria isolated from the *N. perurans* culture.

168

#### 169 2.6 Next Generation Sequencing (NGS)

170 Cultures of *N. perurans* were prepared to allow the collection of DNA to be performed at  
171 various stages and culture conditions. These conditions were Day 0 (day of subculture), Day  
172 1 of culture, Day 1 with 1% Penicillin-Streptomycin (PS) (Thermofisher Scientific), Day 7, 1

173 month of culture, and filtered amoebae from the day 1 culture (Bottle top vacuum filtration  
174 systems, PES, VWR). All samples were collected in TRI Reagent® (Thermofisher Scientific,).  
175 DNA extraction was performed using the TRI Reagent® phenol-chloroform separation  
176 method, suspended in nuclease-free water, and the extracted DNA was quantified using the  
177 NanoDrop 1000 spectrophotometer. 50µl of DNA samples at a concentration of 50ng/µl were  
178 then loaded into designated wells of a 96-well plate and transferred to Eurofins Genomics  
179 (Ebersberg, Germany) for illumina sequencing of the V1-V3 region of the 16S ribosomal RNA  
180 gene (Allen et al., 2016) and bioinformatic analysis.

181

### 182 2.7 Fluorescence In Situ Hybridisation (FISH)

183 A total of  $3 \times 10^5$  *N. perurans* were collected by centrifugation as described in paragraph 2.3.  
184 The cells were resuspended in 1ml of 35 PSU filtered artificial seawater and pipetted into a  
185 24-well plate (333µl per well) (Ibidi µ-Plate 24-well plates treated with ibiTreat, Thistle  
186 Scientific, Glasgow, Scotland). The cells were left to adhere to the surface of the plate at room  
187 temperature for 1 hour. Once attached, the cells were fixed using a 4% formaldehyde with  
188 artificial seawater mix and incubated at room temperature for 15 minutes. Following the  
189 incubation, the cells were washed with artificial seawater. The artificial seawater was  
190 discarded, followed by 3-minute room temperature incubations using 50%, 80%, and 96%  
191 ethanol (EtOH) with artificial seawater. The 96% ethanol supernatant was removed, and the  
192 cells were washed with hybridisation buffer [20mM Tris (pH 9.0), 100mM NaCl, 0.5% SDS,  
193 0.2µm filtered]. The hybridisation buffer wash was removed and replaced with hybridisation  
194 buffer containing 200nM cyanine-5 labelled *Vibrio* 16S-1 PNA probe (5'-AGGAGCTTCGCTTGC-  
195 3')(Zhang et al., 2015) (Biomers, Germany). The negative control comprised hybridisation  
196 buffer without the PNA probe. The plate was incubated in the dark at 55°C for 1 hour. The  
197 hybridisation solutions were removed and replaced with pre-heated (55°C) wash solution  
198 [10mM Tris, 1mM EDTA, 0.2µm filtered]. The plate was incubated in the dark, at 55°C for 10  
199 minutes. The wash solution was discarded, and the plate was dried at 70°C. 300µl of 1x Tris-  
200 buffered saline (TBS; 150 mM NaCl, 50 mM Tris-HCl, pH 7.6) was added to each well, with  
201 gentle agitation to cover the bottom surface of the well. A drop of NeoMount Fluo with DAPI  
202 and PG (Fluorescent mounting medium) (Neobiotech, Generon, Slough, England) was added  
203 to each well with gentle agitation, then incubated in the dark at room temperature for 10  
204 minutes. Following the incubation, the suspension was removed from each well, and washed



205 with TBS. Images of the amoeba were recorded using an inverted fluorescent microscope  
206 (Olympus IX71 Inverted Microscope, Olympus TH4-200 Light Power Supply, Olympus U-  
207 HGLGPS Fluorescence Light Source, Prior Proscan III Motorised Stage Controller) with DAPI  
208 and cyanine 5 filters to detect the staining.

209

210

211 **3. Results**

212 *3.1 Culture and 16S sequencing identify three classes of bacteria associated with N. perurans*

213 Sequencing was successfully achieved for 16 out of the 17 bacterial colonies sampled from LB  
214 seawater agar with no filtration, resulting in the identification of at least 9 different Gram-  
215 negative species (Table 1 and Table A in Supplementary data) of three classes  $\alpha$ -  
216 proteobacteria,  $\gamma$ -proteobacteria and *Flavobacteria*. Most colonies identified as belonging to  
217 a specific genus, but 16S sequencing did not allow sub-genus classification.

218

219

220 3.2 Next Generation Sequencing (NGS) reveals that *Vibrio* species are the most abundant  
221 genus associated with fresh *N. perurans* cultures.  
222

223 With the successful isolation and identification of bacteria associated with *N. perurans* growth  
224 on LB agar, it was important to identify bacteria non culturable by this method by using NGS  
225 techniques. Proteobacteria was the predominant phylum of the sequences identified in all  
226 the culture conditions. A small proportion of sequences were associated with the phylum  
227 Bacteroidetes, with the highest proportion identified at day 7 and 1 month of amoeba culture.  
228 The filtered amoeba collection predominantly consisted of Proteobacteria (Figure 2).

229  
230 Analysis of microbes at the genus level of nucleotide sequences indicated that the *Vibrio*  
231 genus was found in high proportion for all non-filtered samples at early time points, including  
232 in those treated with penicillin and streptomycin, and in the filtered amoebae (Figure 2; Table  
233 2). Various genera within the *Flavobacteriaceae* family, including the genera *Cellulophaga*,  
234 *Flaviramulus*, *Mesonina*, and *Muricauda* and a variety of *Rhodobacteriaceae*, including the  
235 genera *Labrenzia* and *Pacificibacter* were also identified.

236

237 3.3 *Vibrio* species is predominantly located inside *N. perurans*

238 The presence of *Vibrio* inside *N. perurans* was further explored through *FISH* with a *Vibrio*  
239 specific probe. *FISH* confirmed that *Vibrio* is predominantly found inside the amoebae and  
240 very few were identified in the extracellular medium. This is in contrast to other bacteria that  
241 can be identified through DAPI staining (Figure 3).

242

#### 243 4. Discussion

244 *Neoparamoeba perurans* is a free-living marine amoeba that can cause Amoebic Gill Disease  
245 (AGD), which is responsible for substantial economic loss to the aquaculture industry. To date  
246 *N. perurans* remains an elusive microorganism in terms of its biology and biochemistry. Drug  
247 treatments for AGD rely on *N. perurans* characterisation and the ability to perform high  
248 throughput assays *in vitro* before validating any drug treatment *in vivo*. Research into control  
249 methods for other diseases caused by amoebae, such as *Acanthamoeba* keratitis, and primary  
250 amoebic encephalitis, caused by *Naegleria* species, have benefited from the development of  
251 axenic cultures that are free from obvious bacterial growth (Visvesvara et al., 2007). In drug

252 development axenic culture allows for a detailed analysis of the effect of the drug on the  
253 amoeba itself avoiding the effect of confounding factors associated with allied flora and  
254 fauna. The development of an axenic culture for *N. perurans* is challenging and despite several  
255 attempts to separate the *N. perurans* from its bacteria, it is likely that live, viable bacteria are  
256 important to *N. perurans* viability *in vitro*. Since efforts to separate bacteria from the *N.*  
257 *perurans* have not succeeded, it is likely that *P. perurans* is highly dependent on this  
258 relationship. Therefore, in this study we aimed to characterize the bacteria intimately  
259 associated with *N. perurans* to further the understanding of its lifestyle.

260

261

262 The presence of the bacteria identified in the *N. perurans* cultures may not be casual as  
263 studies have reported possible beneficial relationships between these species and  
264 eukaryotes. For example, *Pseudoalteromonas* and *Alteromonas* spp. may be associated with  
265 the surface of eukaryotes, and are known to produce extracellular inhibitory compounds that  
266 inhibit other bacterial growth, such as the AlpP antimicrobial protein. This relationship has  
267 been considered as a potential symbiotic mechanism for defence against biofouling in  
268 multicellular eukaryotes (such as macroalgae) (Rao et al., 2005). *Halomonas* spp. is known to  
269 produce Vitamin B<sub>12</sub>, which has been demonstrated to be enhanced by adding the algal  
270 extract fucoidan (Amin et al., 2012; Croft et al., 2005), suggesting that it be an exogenous  
271 source of the vitamin for eukaryotes such as algae.

272

273

274 NGS was performed to determine the presence of any unculturable bacteria. One sample was  
275 extracted from a culture supplemented with penicillin-streptomycin. This antibiotic cocktail  
276 is widely used to reduce the possibility of bacterial contamination in cell culture. It was used  
277 in *N. perurans* culture during the axenization attempts. However, it was observed that  
278 supplementation had a detrimental effect on amoeba growth. We originally hypothesised  
279 that this may be due to the antibiotic reducing viable bacteria but NGS revealed that this is  
280 not likely to be the case. NGS confirmed the presence of the bacteria already identified using  
281 classical 16s amplification method, but interestingly also identified the presence of non-  
282 culturable *Vibrio* spp. in all culture conditions, and this genus was responsible for a substantial  
283 proportion of the microbiome. In our study the identification was limited to genus

284 identification with some suggested species annotated. FISH revealed that *Vibrio* species are  
285 predominantly found inside the amoeba.

286

287 Previous work suggests a potential for *Vibrio* to be an endosymbiont. *V. harveyi* has been  
288 identified as an endosymbiont of *Cryptocaryon irritans*, which causes the parasitic disease of  
289 marine cryptocaryonosis and *Vibrio cholerae* is reported to survive within the free-living and  
290 opportunistic pathogenic amoeba *Acanthamoeba* spp. and may be released from the amoeba  
291 vacuoles by exocytosis (Van der Henst et al., 2016).

292

293 *Vibrio* spp. including *Vibrio tasmaniensis* and *Vibrio splendidus*, have been isolated from  
294 Atlantic salmon (Thompson et al., 2003). These are two particular species are likely to be  
295 within the microbiome of the *N. perurans* culture (Table B in Supplementary Data), and their  
296 isolation from the Atlantic salmon suggest *Neoparamoeba* and *Vibrio* species may interact  
297 within the Salmon host. It is interesting to speculate that the gill tissues may be a source of  
298 *Vibrio* spp. to the amoeba and that this in turn may contribute to the host providing a suitable  
299 environment for *N. perurans*.

300

301 Given the strong association with the *in vitro* culture of *N. perurans*, and the *Vibrio* spp.  
302 detected by microbiome sequencing, it is possible there is a symbiotic relationship between  
303 the bacteria and the *N. perurans* that may influence the development or pathogenicity of  
304 AGD, particularly as viable bacteria are noted within the amoeba cytoplasm. The symbiotic  
305 relationship does not necessarily have to be beneficial to individual amoeba, as amoebae may  
306 act as vectors for maintaining the high proportion of *Vibrio* spp. within the bacterial  
307 microbiome leading to increased bacterial loads on the fish host, or the *Vibrio* may be  
308 beneficial to the overall maintenance and survival of the *N. perurans* population by providing  
309 metabolites or cellular functions to its amoeba host. Further investigation into the  
310 relationship between *Vibrio* spp. and *N. perurans* is required to determine their relationship  
311 and determine whether this could contribute to the pathogenesis of AGD. If a positive  
312 relationship is determined, methods could be developed to target the bacteria within the  
313 amoeba, thus reducing the overall pathogenicity of the amoeba to its fish host.

314

315 We speculate that bacteria present within the same environment, on the surface, or  
316 intracellularly within the amoeba may be a symbiont of *Neoparamoeba* spp. and contribute  
317 directly, or indirectly to AGD pathogenesis. This potential interaction and contribution is  
318 supported by the emergence of complex gill disease (CGD), a multi-pathogen disease  
319 associated with *N. perurans* infection, and various other pathogens, including a  
320 paramyxovirus, pox virus, phytoplankton, and *Candidatus Branchiomonas cisticola* (Boerlage  
321 et al., 2020). AGD and CGD occurrences have coincided, which demonstrates there may  
322 multifactorial contributors to AGD and gill health (Herrero et al., 2018). Future work in AGD  
323 could focus on the impact of *Neoparamoeba*-bacteria interactions in the emergence of gill  
324 disease.

325

326

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410



411 **Figure legends**

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414 **Figure 1.** *Neoparamoeba perurans* trophozoite and bacteria from *in vitro* laboratory culture.  
415 The image demonstrates the bacteria present in *N. perurans* culture. The amoebae and  
416 bacteria were collected from a Malt-yeast Agar culture and transferred to a glass slide for  
417 imaging on an inverted microscope.

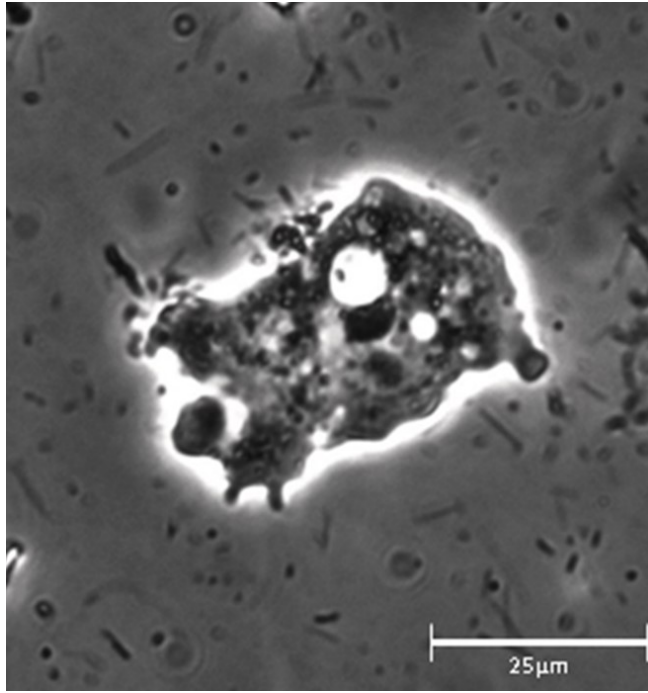
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419 **Figure 2. Proportional diversity of bacterial genera sequenced across various *P. perurans***  
420 **culture conditions.** The *Vibrio* genus occurred in the highest proportions in the sequences  
421 from day 0, day 1, day 1 PS (cultured with 125µg Pencillin-Streptomycin), and filtered  
422 amoebae (F1) samples. There was also a substantial proportion of *Vibrio* sequences in the day  
423 7 and 1-month samples. The second largest proportion of sequences were attributed to the  
424 Rhodobacteriaceae in day 0, day 1, day 1 PS, and day 7 samples. *Vibrio* was also present in  
425 the 1-month (M1) sample, with a lower proportion of sequences within this sample. There  
426 was a small proportion of the *Halomonas* genus within the day 0, day 1, day 1 PS, and day 7  
427 samples, while the 1-month sample had a larger proportion of sequences for this genus. The  
428 *Rhodospirillaceae* genus had the second largest proportion of sequences in the day 0, day 1,  
429 day 1 PS, and the largest proportion in the day 7 sample.

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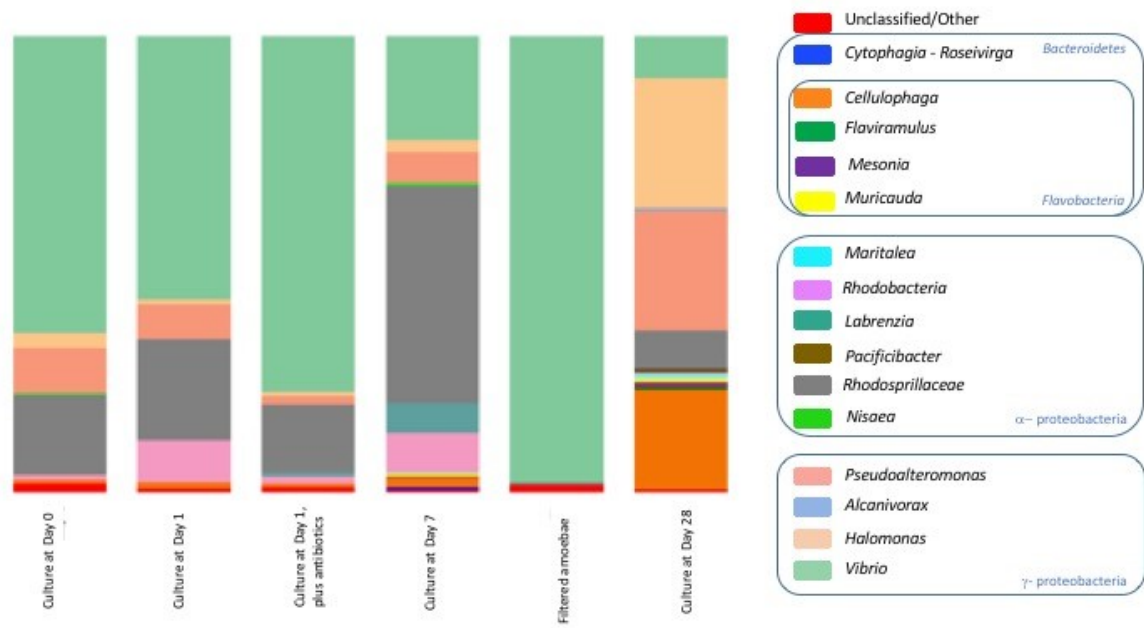
431 **Figure 3. FISH Staining of *Vibrio* in *N. perurans* using cyanine 5 labelled PNA Probe.** Cyanine  
432 5 staining (in yellow) was observed within the *N. perurans* trophozoites. The staining was  
433 primarily within *N. perurans*, with limited extracellular bacterial cyanine 5 staining, indicating  
434 that *Vibrio* is contained within the amoeba. The DAPI staining (in light blue) shows the various  
435 extracellular bacteria present on the surface of the well.

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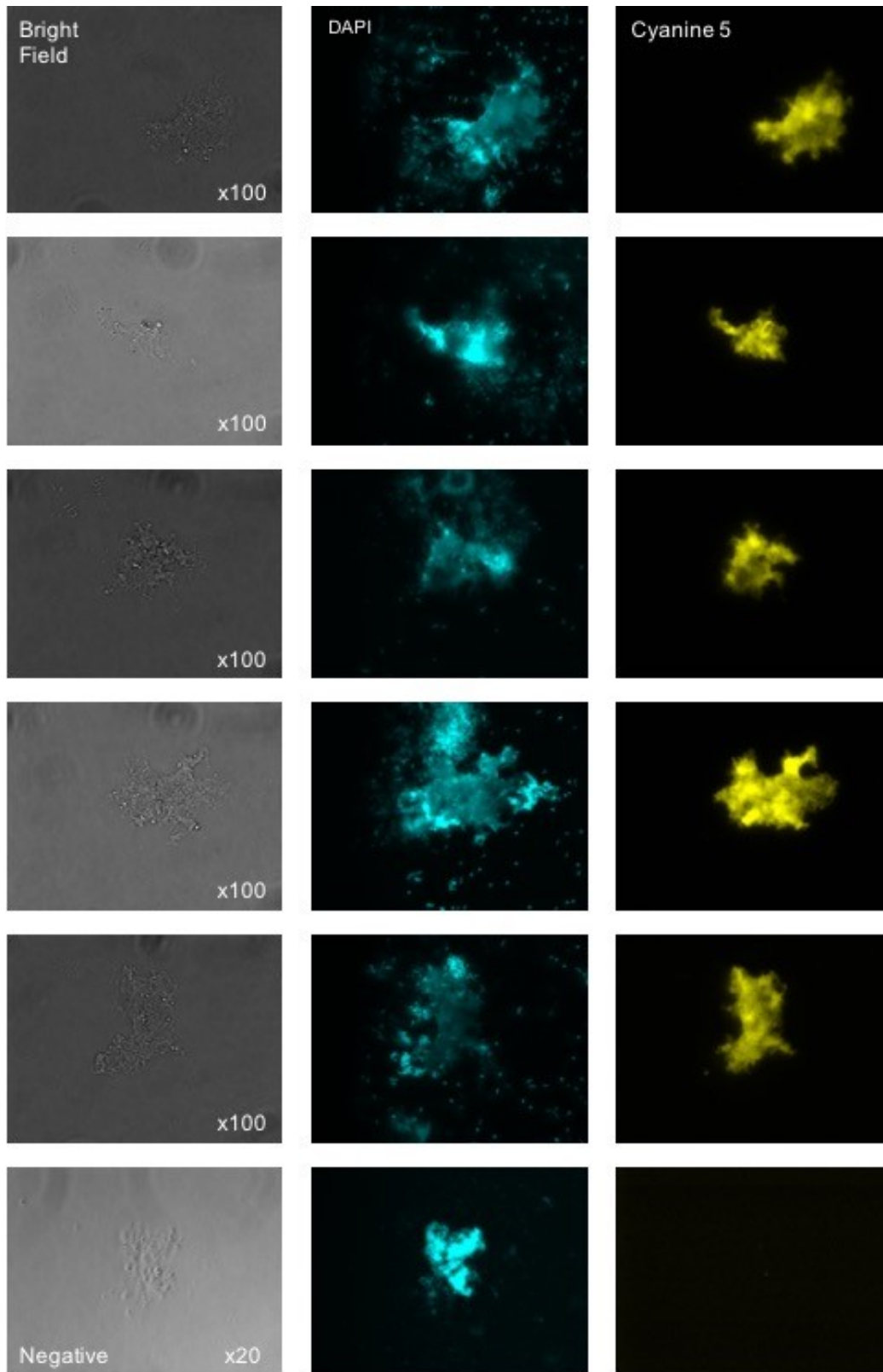
**Figure 1**



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442 **Figure 2**

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445 **Figure 3**

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450 Table 1  
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<b>Genus</b>	<b>Class</b>	<b>Habitat (reference)</b>
<i>Pseudoalteromonas</i>	$\gamma$ - proteobacteria	Marine
<i>Paraglaciecola</i> or <i>Glaciecola</i> or <i>Alteromonas</i>	$\gamma$ - proteobacteria	Antarctic sea/ marine
<i>Halomonas</i>	$\gamma$ - proteobacteria	Hypersaline environment
<i>Labrenzia</i> or <i>Polymorphum</i>	$\alpha$ - proteobacteria	Hypersaline environment
<i>Celeribacter</i> or <i>Marivita</i>	$\alpha$ - proteobacteria	Marine
<i>Cellulophaga</i>	<i>Flavobacteria</i>	Marine
<i>Thalassospira</i>	<i>Flavobacteria</i>	Marine
<i>Muricauda</i>	<i>Flavobacteria</i>	Marine
<i>Mesonina</i>	<i>Flavobacteria</i>	Marine

453 Table 2

<i>Culture time point and condition</i>	<i>% of reads</i>
<i>Day 0</i>	60.3%
<i>Day 1</i>	53.6%
<i>Day 1 (addition of Penicillin/Streptomycin)</i>	71%
<i>Day 7</i>	21%
<i>Day 1 - Filtered amoebae</i>	90%
<i>Day 28</i>	8.9%


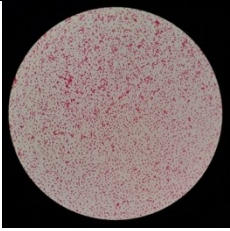
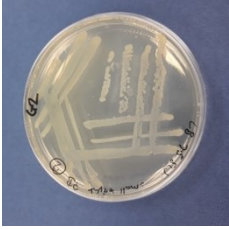
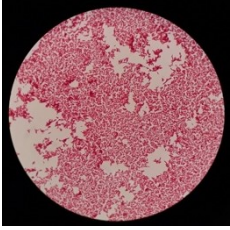

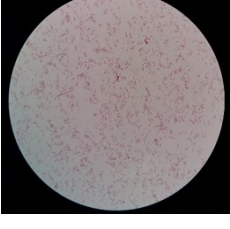

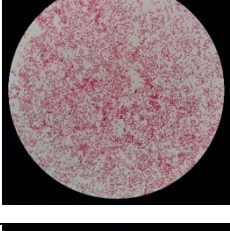
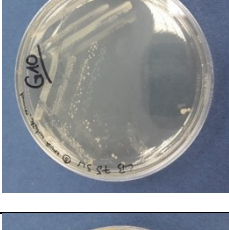
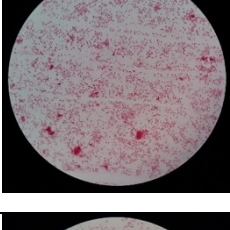
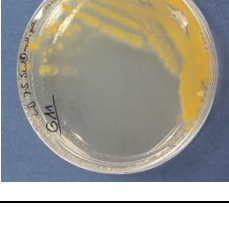
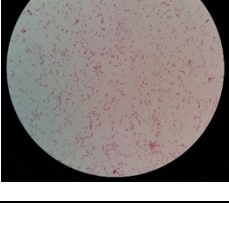
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456 **Supplementary material**

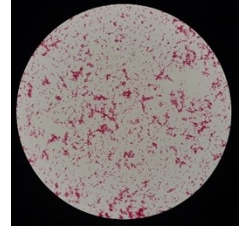
457 **Table A** Individual isolation and sequencing of bacteria present in *P. perurans* culture

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Genus	Morphology	Gram Stain
<i>Pseudoalteromonas</i>		
<i>Paraglaucicola</i> or <i>Glaucoicicola</i> or <i>Alteromonas</i>		
<i>Halomonas</i>		
<i>Labrenzia</i> or <i>Polymorphum</i>		
<i>Celeribacter</i> or <i>Marivita</i>		
<i>Cellulophaga</i>		

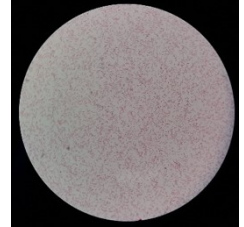
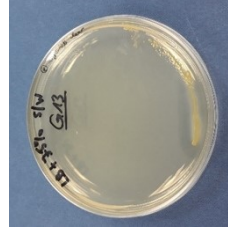
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*Thalassospira*



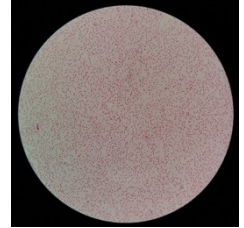
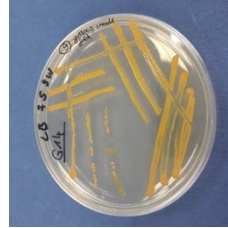
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*Muricauda*



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*Mesonina*



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**Table B**

<b>DNA Sample</b>	<b>Taxonomic Level</b>	<b>Match</b>	<b>Percentage Match (%)</b>
Filtered Sample	g	<i>Vibrio</i>	90.6
	s	<i>Vibrio splendidus</i>	7.0
	s	<i>Vibrio crassostreae</i>	0.6
	s	<i>Vibrio</i> sp.	0.4
	f	<i>Vibrio tasmaniensis</i>	0.3
	s	Rhodospirillaceae	0.2
	s	<i>Vibrio lentus</i>	0.2
	s	<i>Vibrio</i> sp. B131a	0.2
	s	<i>Pseudoalteromonas</i> sp. HG03	0.1

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