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Complete List of Authors:	Benedicenti, Ottavia; Centro de Investigación en Sanidad Animal, Instituto Nacional De Investigaciones Agrarias, Fish Immunology Secombes, Chris; University of Aberdeen, Biological Sciences; Collins, Catherine; Marine Scotland Science, Aquaculture
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Effects of temperature on *Paramoeba perurans* growth in culture and the associated microbial community

O. BENEDICENTI *[a,b], C.J. SECOMBES [a] *and* C. COLLINS *[b]

[a] *Scottish Fish Immunology Research Centre, Institute of Biological and Environmental Sciences, University of Aberdeen, Tillydrone Avenue, Aberdeen AB24 2TZ, UK*

[b] *Marine Scotland Science Marine Laboratory, 375 Victoria Rd, Aberdeen AB11 9DB, UK*

*Corresponding authors:

Ottavia Benedicenti
Centro de Investigación en Sanidad Animal,
Instituto Nacional De Investigaciones Agrarias,
Carretera Algete-El Casar de Talamanca, Km. 8,1,
28130 Valdeolmos, Madrid, Spain
E-mail: ottavia.benedicenti@gmail.com
Tel: +34 916202300

Catherine Collins
Marine Scotland Science Marine Laboratory
375 Victoria Rd,
Aberdeen AB11 9DB, UK
E-mail: Catherine.Collins@gov.scot
Tel : +44 (0)1312 444271

SUMMARY

Population growth, *in vitro*, of three *Paramoeba perurans* cultures, one polyclonal (G) and two clonal (B8, CE6, derived from G), previously shown to differ in virulence (B8>G>CE6), was compared at 10°C and 15°C.B8 showed a significantly higher increase in attached and in suspended amoebae over time at 15°C and 10°C, respectively. CE6 and G also had significantly higher numbers of suspended amoebae at 10°C compared to 15°C at experiment termination. However, in contrast to B8, numbers of attached amoebae were significantly higher at 10°C in CE6 but showed a similar trend in G at the end of the experiment. Numbers of both suspended and attached amoebae were lower in B8 compared to CE6 and G. Significant differences in bacterial community composition and/ or relative abundances were found, between cultures, between temperatures, and between the same culture with and without amoebae, based on 16S rRNA Illumina MiSeq sequencing. Bacterial diversity was lower in B8 and CE6 compared to G, possibly reflecting selection during clonal isolation. The results indicate that polyclonal *P. perurans* populations may contain amoebae displaying different growth dynamics. Further studies are required to determine if these differences are linked to differences seen in the bacterial communities.

Keywords: temperature, *Paramoeba perurans*, amoebic gill disease, 16S rRNA.

KEY FINDINGS

- Individual *Paramoeba perurans* cultures had significant differences in growth at 10°C and 15°C.
- Higher levels of microbiota diversity was found in a poly- versus monoclonal *P. perurans* cultures.
- Significant differences in presence/ abundance of bacterial genera were seen between amoeba cultures.
- The abundance, but not presence, of bacterial genera differed in amoeba cultures between 10°C and 15°C.

1. INTRODUCTION

The causative agent of amoebic gill disease (AGD) in Atlantic salmon is *Paramoeba perurans*, an amphizoic amoeba (15-40 µm diameter) that has successfully fulfilled Koch's postulates (Crosbie et al. 2012). Molecular tools, such as real time reverse transcription polymerase chain reaction (real time RT-PCR) and *in situ* hybridization (ISH), have been established to quantify and identify the presence of *P. perurans* both in the marine environment and in gill swabs and gill tissue (Bridle et al. 2010; Crosbie et al. 2012; Downes et al. 2015; Fringuelli et al. 2012; Young et al. 2008). However, since many early studies reported on *P. pemaquidensis* as the putative aetiological agent of AGD, relatively little is known about the biology of *P. perurans* and its potential environmental risk factors and distribution in relation to AGD outbreaks. A relationship between increasing water temperature and AGD prevalence has been reported during outbreaks in Atlantic salmon farms in Tasmania (Australia), Scotland (UK), Norway, Chile and South Africa (Adams and Nowak 2003; Bustos et al. 2011; Clark and Nowak 1999; Douglas-Helders et al. 2003; Douglas-Helders et al. 2001; Douglas-Helders et al. 2005; Mouton et al. 2013; Steinum et al. 2008). For example, in Tasmania, clinical infection usually occurs when the water temperatures are in the range of 15–20°C (Munday et al. 1990), though AGD was recorded at a minimum temperature of 10.6°C and a minimum salinity of 7.2 ppt if established prior to drop in temperature and salinity (Clark and Nowak 1999). In Scotland AGD has been reported at temperatures from 7.5°C to 13.5°C (http://www.scotland.gov.uk/Resource/0039/00393037.pdf accessed 15/01/2015). Moreover, not only temperature but also environmental factors like bacterial counts and turbidity (most likely organic matter) were found to be significantly correlated with increasing *P. pemaquidensis* density (Douglas-Helders et al. 2003).

Most marine amoebae are bactivorous, although some are known to feed on other protozoans, algae or organic detritus (Bovee and Sawyer 1979; Douglas-Helders et al. 2003). Therefore, a better understanding of the role of bacterial densities and community makeup on AGD occurrence might be useful for future risk assessment of disease and management options. Previous studies in Tasmania investigated the role of salmonid gill bacteria on AGD (Bowman and Nowak 2004; Embar-Gopinath et al. 2005; Embar-Gopinath et al. 2006; Embar-Gopinath et al. 2008). A culture-independent 16S rRNA gene-based approach was used to identify gill bacteria associated with AGD in Atlantic salmon infected in the laboratory or obtained from commercial sea cages (Bowman and Nowak 2004). These

samples were found to be dominated by marine Gram-negative bacteria of the genus *Psychroserpens* spp. (family Flavobacteriaceae, phylum Bacteroidetes). These bacteria were not detected in any of the AGD-negative samples nor in one of two AGD-positive samples obtained from fish subjected to temporary freshwater immersion, suggesting that *Psychroserpens* spp. is a potential opportunistic genus associated with salmonid AGD in Tasmania (Bowman and Nowak 2004). However, a second study, using a culture-dependent approach, did not find the genus *Psychroserpens* spp. in AGD-positive samples, but rather *Winogradskyella* spp. (family Flavobacteriaceae, phylum Bacteroidetes) and *Staphylococcus* spp. (family Staphylococcaceae, phylum Firmicutes) were present (biochemical tests, cluster analysis and 16S rRNA gene-based approaches) (Embar-Gopinath et al. 2008). Moreover, fish experimentally infected with a *Winogradskyella* sp. strain developed increased numbers of AGD lesions following *Neoparamoeba* spp. infection relative to those exposed to a *Staphylococcus* sp. strain (Embar-Gopinath et al. 2005; Embar-Gopinath et al. 2006). Amoebae cultures at the Marine Scotland Science (MSS) Marine Laboratory, UK, are maintained in a non-axenic environment and, thus, a number of bacterial species are present, of which six have been isolated from an amoeba polyclonal *in vitro* culture using single colony purification, and identified to genus level by biochemical testing and 16S rRNA sequencing (McCarthy et al. 2015). These bacterial isolates were related to the genera *Pseudomonas* spp. (closest Genbank BLAST matches: JX144945, KF317743, KJ769212, FJ210842), *Marinomonas* sp. (*Marinomonas foliarum*, NR116234), and *Flavobacterium* sp. (AJ244702) (McCarthy et al. 2015).

In this study, the effect on *P. perurans* population growth, *in vitro*, at two different temperatures relevant to Scottish salmon aquaculture, 10°C and 15°C, was assessed using two different amoeba clonal cultures (i.e., B8, CE6) and a polyclonal (G) culture (Collins et al. 2017) maintained at the MSS Marine Laboratory. In addition, due to the potential role that the associated bacterial community can play in amoebae growth, an attempt was also made, by applying a 16S rRNA gene MiSeq analysis to the samples of the temperature experiment, to resolve the complex microbiota composition present in the different cultures, and the effect if any, of maintenance at 10°C and 15°C. These data could potentially inform further experiments on functional relationships of the microbiota with *P. perurans* growth.

2. MATERIALS AND METHODS

2.1. Amoeba culture

P. perurans polyclonal and monoclonal amoebae cultures were originally isolated from gills of infected farmed Atlantic salmon from the west coast of Scotland as described in Collins et al. (2017). The amoebae were cultivated at 10°C and 15°C in small petri dishes containing a 5 ml underlay of malt yeast agar (MYA) (0.05 g malt extract, 0.05 g yeast extract, 10 g bacteriological agar, 500 ml of 35 ppt filtered seawater), with approximately 7 ml overlay of 35 ppt filtered sterilized seawater. Stericup® Filter Units (© EMD Millipore Corporation, Billerica, MA, USA, 2014) with a 0.22 µm pore size were used to filter the seawater, sourced from the North Sea (ca. 35 ppt salinity). Cultures were maintained in a non-axenic environment containing different bacterial strains probably isolated with amoebae from gills during culture establishment, as previously described by Collins et al. (2017), and no antibiotics were used during the culture.

2.2. Effect of temperature on P. perurans population growth in vitro

The *in vitro* experiment was performed with three different *P. perurans* cultures, a polyclonal culture named "G" and two derived clonal cultures "B8" and "CE6" (Collins et al. 2017). These clonal cultures showed differences in virulence, in terms of number of gill lesions, in a previous study (B8 generated higher average gill score, CE6 a lower score) (Collins et al. 2017). The amoeba cultures, which were prior maintained for two years at 15°C, were acclimatised at the two different temperatures used in the *in vitro* growth study (10°C and 15°C) for three months before starting the experiment. Four passages of the cultures were performed over this time to maintain their survival until the start of the experiment. The choice of number of replicates (n = 8) and vessel (T-25 cell culture flasks, Greiner CELLSTAR®, Sigma-Aldrich®, UK) was based on the results of two preliminary experiments to enable power analysis. Amoeba cultures of the same clonal type were initially pooled and mixed in a larger T-175 cell culture flask (Greiner CELLSTAR®, Sigma-Aldrich®, UK) to avoid differences that might have arisen during the three months of acclimatisation, one flask for each culture and temperature respectively. The amoebae were then distributed to each technical replicate (n = 8) with a starting number of ca. 10 amoebae per T-25 flask (n = 10, biological replicates). The same medium (MYA 35 ppt) and filtered seawater (35 ppt) were used for preparing all the flasks for the experiment. Counts of attached amoebae and amoebae suspended in the seawater overlay, in flasks maintained at 10°C and 15°C, were taken on a

daily basis for 21 days. To count the amoebae in suspension (un-attached amoebae), flasks were first gently agitated to mix suspended amoebae but to minimise detaching amoebae from the agar surface, and then placed vertically. From each flask 100 µl samples were taken in triplicate for counting in 96-well plates. The neutral red assay was used to determine viability of suspended (non-attached) amoebae. Briefly, 0.35 µl of filtered neutral red (Sigma-Aldrich®, UK) solution (25 mg/ml in PBS) was added to the 100 µl aliquots of amoeba cultures. After 30 min to allow neutral red uptake, amoebae were centrifuged at 2,200 x g for 10 min, the supernatant was removed and amoebae re-suspended in 100 µl of sterilized seawater (35 ppt). Counts of viable amoebae were performed in triplicate in 96-well plates at a 1:10 dilution in 100 µl under a microscope (10X). The counting of amoebae attached to the MYA was performed on pictures captured with a camera mounted on the microscope (10X, camera Olympus model CKX41 with adaptors - Canon EOS 700D mounted on the trinocular tube with an Olympus U-TV1X-2 mount and U-TMAD and T-EOS), which were taken immediately after the 100 µl aliquot was collected, and was always performed in the same 3 cm^2 region at the centre of the flasks, for accurate comparisons. Every two days, 200 µl of sterile seawater (35 ppt) was added into the flasks to compensate for evaporation and samples taken for counting, to maintain a constant volume of seawater over the experimental period.

2.3. Analysis of count data

The null-hypothesis for count data analyses aimed to test for the absence of a difference in growth of each clonal culture between 10°C and 15°C over 21 days. Each culture count dataset was analysed separately for the attached and un-attached amoebae, using the R Statistical Environment (Ihaka and Gentleman 1996). The Poisson generalized linear model (GLM) is typically used in regression analysis of count data in R. However, due to an increase of the mean of the variance of response variables over time (overdispersion), a generalised linear model with a negative binomial distribution in the `MASS` library was applied to all the growth rate analyses with the function `glm.nb` (R software, software 3.0.1). The negative binomial distribution was determined to be the more appropriate model for all the count data analyses based on outcomes from the following downstream analyses: 1) the comparison of the nested models using the `anova` command, which applies a series of analysis of deviance tests; and 2) the validation of the models with a figure containing diagnostic plots. Models were validated when 1) the residuals against fitted values graph did

not represent a structure or pattern in the plot (heteroscedasticity); 2) the normal quantile-quantile plot graph was a straight line if the errors were normally distributed; 3) the square root of the standardized residuals against the fitted values graph did not represent a structure or pattern in the plot, similar to the first plot; and 4) standardized residuals as a function of leverage, along with Cook's distance (combination of leverage and residuals in a single measure) for each of the observed values of the response variable, did not show a pronounced pattern (Crawley 2007). The logarithmic link function (`link="log"`), used in both the quasi-Poisson GLM and the negative binomial GLM for results' comparisons, ensured that all the fitted values were positive.

2.4. DNA extraction for characterisation of microbial communities

To better interpret any differences in growth rate found among amoeba cultures, it was decided to characterise the microbial communities present in the different cultures and at the two different temperatures (10°C and 15°C). At the beginning of the *in vitro* amoeba population growth experiment, a "negative control" was also set up for each temperature and each amoebae culture, i.e., flasks containing the respective culture medium filtered through a 3.0 µm pore size Cyclopore™ Track Etched Membrane (GE Healthcare, Whatman, UK), in order to separate out the amoebae but retain the culture bacteria. Amoeba cultures and the "negative control" were cultured in the same way to have a similar volume/ dilution of medium to each flask. Four replicates were used to verify the homogeneity of microbial communities among samples of the same culture type and temperature. At the end of the *in vitro* experiment, 200 µl of well mixed amoeba culture containing bacteria (hereafter referred to as "samples") (n=8 per culture x temperature combination) and 200 µl of "negative control" medium (hereafter referred to as "medium") (n=4 per negative control x temperature combination) were taken for the characterisation of the microbial communities in the amoeba cultures. Samples and medium aliquots were centrifuged at 2,200 x g for 10 min, the supernatant was removed and the pellet stored at -80°C until DNA extraction. Four out of eight different technical replicates of the "samples" and all four technical replicates of the "medium" per culture and temperature were used for DNA extraction and downstream analyses. Briefly, 0.5 ml of pre-heated (65°C) sodium dodecyl sulphate (SDS) lysis buffer (0.7M NaCl, 0.1M Na_2SO_3, 0.1M Tris HCl pH 7.5, 0.05M EDTA pH 8, 1% SDS, autoclaved) was added to the pellet. After gentle mixing, the SDS extraction buffer and the pellet were transferred into 2 ml tubes containing lysing matrix B (MP Biomedicals) and 0.5 ml of

UltraPure™ phenol:chloroform:isoamyl alcohol (25:24:1, v/v) (ThermoFisher Scientific, UK) was added to the tubes. The tubes were then placed in a Hybaid Ribolyser™ Cell Disrupter (Hybaid) at a speed setting of 4.0 for 30 s and cooled on ice for 1 min. Cell debris was removed by centrifugation (11,000 x g for 15 min at 4°C), and the extracted aqueous layer was mixed with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). After centrifugation at 16,000 x g for 15 min at 4°C, the aqueous layer was transferred to a fresh tube and the DNA was precipitated by adding two volumes of 30% polyethylene glycol (PEG) 6000 solution in 1.6M NaCl (sterilized by autoclaving) and 2 µl of linear acrylamide (5mg/ml, ThermoFisher Scientific, UK). Samples were incubated for 3 h on ice and then centrifuged at 11,000 x g for 30 min at 4°C. The pellet was washed with 1 ml ice cold 70% ethanol and re-suspended in 20 µl of Tris-EDTA buffer (Sigma-Aldrich®, UK) and the purity was checked on a NanoDrop ND-1000 Spectrophotometer (PEQLAB GmbH, Germany).

2.5. Next-generation sequencing (NGS) of microbial communities

Illumina MiSeq sequencing of the bacterial 16S rRNA genes was performed for samples and medium (four technical replicates each) for the three cultures (B8, CE6, G) kept at the two different temperatures (10°C and 15°C) for 21 days. In total 48 DNA samples were sequenced. Genomic DNA samples were submitted to the Research and Testing Laboratory Genomics (RTL Genomics, Lubbock, TX, USA) for next-generation Illumina MiSeq sequencing of the bacterial 16S rRNA genes. A detailed protocol is given in the supplementary materials. For this study, it was chosen to amplify the V1-V2 region using the 28f (5' - GAG TTT GAT CNT GGC TCA G - 3') (Handl et al. 2011) and the 388r (5' – TGC TGC CTC CCG TAG GAG T - 3') (Francés et al. 2004) primers based on available assays at RTL Genomics, and on their advice based on their annotated internal database to classify sequences. Briefly, amplifications were performed in 25 µl reactions with Qiagen HotStar Taq master mix (Qiagen Inc., Valencia, CA, USA), 1µl of each 5uM primer, and 1µl of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, CA, USA) using the following thermal profile: 95°C for 5 min, then 25 cycles of 94°C for 30 s, 54°C for 40 s, 72°C for 1 min, followed by 1 cycle of 72°C for 10 min and a 4°C hold. A second PCR was also performed and the amplification products visualized with eGels (Life Technologies, Grand Island, New York). Generated sequences were processed and quality checked by the RTL Genomics data analysis pipeline. The sequence reads were then sorted by length from the longest to the shortest and clustered into operational

taxonomic units (OTUs) at a 4% divergence using the USEARCH clustering algorithms (Edgar 2010) to prefix dereplication. OTU selection was performed using the UPARSE OTU selection algorithm (clustering method in USEARCH) (Edgar 2013) to classify the large number of clusters into OTUs and chimera checking was performed using the UCHIME chimera detection software (Edgar et al. 2011). Taxonomic identifications were made by comparing the OTU sequences against a database of high quality sequences derived from the NCBI database using the Ribosomal Database Project (RDP) Classifier (Wang et al. 2007). The term "unknown" was assigned when the algorithm was not able to make a confident determination of the taxonomic classification at a certain level (number of matching taxonomic level/ number of total taxonomic level > 51%). The data were analysed by RTL Genomics using R software (software 3.0.1). Generation of a rarefaction curve plot of the number of OTUs versus the number of sequences and the Chao1 Richness and Shannon Diversity indices were performed. Measures of diversity were screened for group differences using ANOVA. Individual OTUs were examined for significant changes between genera and barplots were generated using OTU relative abundances with a sum to 100%. A heatmap was generated to visualize the relative abundances of the most predominant bacterial genera, where the samples and bacterial genera were sorted according to Euclidean metrics and weighted (a quantitative measure suited to revealing community differences that are due to changes in relative taxon abundance) using the phylogenetic distances (Unifrac method). Multivariate differences among groups were evaluated using the "Permutational Multivariate Analysis of Variance Using Distance Matrices" function `adonis`, where distances among samples were calculated using un-weighted (presence/ absence of OTUs) or weighted (relative abundance of OTUs) UniFrac distances (Lozupone et al. 2011) using the `phyloseq` package (McMurdie and Holmes 2013) in R (software 3.0.1). Principal Coordinate Analysis (PCoA) was conducted and plotted from weighted and un-weighted UniFrac distances.

3. RESULTS

3.1. Effect of temperature on P. perurans population growth in vitro

The null-hypothesis was to test whether a difference in growth of each culture was seen between 10°C and 15°C over 21 days and to test this hypothesis each culture was analysed separately for the attached and un-attached amoebae count data (see supplementary materials for models' details). At the end of the *in vitro* experiment the number of B8 attached amoebae was higher at 15°C than at 10°C (Fig. 1A). An opposite trend was shown with the

number of B8 un-attached amoebae, where higher values were detected at 10°C than at 15°C (Fig. 1B). B8 attached and un-attached amoebae count data results showed a significant difference between the two temperatures ($p \leq 0.001$ and $p \leq 0.05$, respectively, $n = 128$). Based on the attached and un-attached CE6 amoebae count data, the model output again showed significant differences in population growth between the two temperatures (10°C and 15°C) over 21 days ($p \leq 0.001$, $n = 128$) (Figs. 1C & D). For the attached G count data, the model output showed a significant difference between the two temperatures ($p \leq 0.001$, $n = 128$) that was greater than the relationship between the two temperatures over time ($p \leq 0.05$, $n = 128$). In fact, the graph shows a similar trend of count data of attached amoebae for both temperatures (Fig. 1E). While, the best fitting model for the G culture un-attached count data (Fig. 1F) showed a significant difference between the two temperatures over time ($p \leq 0.001$, $n = 128$).

Lastly, it was decided to test that there was no difference in growth among B8, CE6 and G cultures at 10°C and 15°C over 21 days, analysing separately attached and un-attached amoebae as done in the previous growth rate analyses. The model output for 10°C attached amoebae count data showed that B8 and CE6 growth rates were significantly different ($p \leq 0.01$, $n = 128$), CE6 and G growth rates were significantly different ($p \leq 0.001$, $n = 192$), and B8 and G growth rates were significantly different over time ($p \leq 0.01$, $n = 192$). At 15°C attached amoebae count data showed a similar trend for B8 and G cultures which increased over time even if at a different magnitude, while CE6 count data numbers started to decrease after 15 days. The model output for 15°C attached amoebae count data showed significant differences between the CE6 and G count data and between the G and B8 count data ($p \leq 0.001$, $n = 192$), while the B8 and CE6 count data showed a significant difference only over time ($p \leq 0.001$, $n = 192$). Un-attached amoebae count data showed an increasing trend at 10°C over time for all cultures with significant differences among the different cultures ($p \leq 0.001$ between B8 and CE6 count data and between B8 and G count data, $p \leq 0.01$ between CE6 and G count data, $n = 192$), while at 15°C un-attached count data numbers started to decrease after 10 days in all the different cultures. The model output for 15°C un-attached amoebae count data showed a significant difference between the G and CE6 count data ($p \leq 0.001$, $n = 192$) and between the G and B8 count data ($p \leq 0.001$, $n = 192$), whereas the difference between the CE6 and B8 count data was significant only over time ($p \leq 0.001$, $n = 192$).

313 *3.2 Next-generation sequencing (NGS) of microbial communities*

314 Firstly, the species richness was assessed from the results with rarefaction curves using the
315 UPARSE pipeline (Edgar 2013). The rarefaction curves demonstrated that when the number
316 of sequence reads increased, the species richness rose considerably with each sample (Suppl.
317 Figure S1). The alpha diversity (within sample diversity) demonstrated by the rarefaction
318 curves also showed that the culture samples and medium reached an asymptote, indicative of
319 adequate reads to robustly report on species composition of each sample, allowing for the
320 comparison of the experimental samples.
321 Individual OTUs were used to examine for significant changes in genera/ families, and the
322 relative abundances of OTUs were used to determine the 30 most predominant genera/
323 families in all samples (Fig. 2). A heatmap was generated to visualize the relative abundances
324 of the 30 most predominant bacterial genera/ families (Fig. 3). The heatmap and the barplots
325 showed that the four technical replicates (per sample/ medium) had similar profiles in terms
326 of genera composition, reflecting the limited source of variation related to the random noise
327 associated with different flasks and environmental bacterial contamination (Figs. 2 & 3). The
328 heatmap also showed that most of the sample/ medium for each amoeba clonal culture did not
329 closely group together in a cluster analysis, with the exception of the B8 sample/ medium at
330 15°C and CE6 sample/ medium at 10°C (Fig. 3). These clustering differences reflected a
331 change between the culture and the medium for some cultures and it also clearly showed
332 differences between temperatures in the same cultures. The most abundant phyla of the
333 overall microbiome were assigned to Proteobacteria, Bacteroidetes and Actinobacteria.
334 *Marinomonas* sp. (Proteobacteria), Flammeovirgaceae/ "unknown" (Bacteroidetes), *Joostella*
335 sp. (Bacteroidetes), *Balneola* sp. (Bacteroidetes) and *Marinobacter* sp. (Proteobacteria) were
336 the most abundant genera/ families found in the microbiome among all cultures and
337 temperatures, indicating a predominance of Gram-negative bacteria in the amoeba cultures
338 (Figs. 2 & 3). The most abundant Gram-positive bacteria were represented by the
339 Microbacteriaceae family (*Salinibacterium* sp. and *Microbacterium* sp., phylum
340 Actinobacteria) (Figs. 2 & 3).

341 Differences in the microbiota between sample and medium of the two clonal cultures, which
342 showed lowest and highest differences in virulence in the Collins et al (2017) study were as
343 follows: compared to their respective medium controls at 10°C *Marinobacter* sp. and
344 Flammeovirgaceae had higher abundance in CE6 and B8 cultures respectively, whereas

Oceanospirilaceae and *Marinomonas* sp. in the CE6 samples, and *Joostella* sp., Oceanospirilaceae and *Balneola* sp. in the B8 samples, showed lower abundance. Compared to their respective medium controls at 15°C, *Salinibacterium* sp., Microbacteriaceae, *Reichenbachiella* sp., *Balneola* sp., Flammeovirgaceae and *Marinomonas* sp. abundance all increased in the CE6 cultures, while *Joostella* sp., Sneathiellaceae, Erythrobacteraceae, *Pseudomonas* sp., Flavobacteriales and Oceanospirilaceae decreased. As for 10°C, in the B8 cultures Flammeovirgaceae increased and, *Joostella* sp. and *Balneola* sp. abundance was seen to decrease compared to medium controls. *Cytophagales* sp. abundance was also seen to decrease at 15°C in the B8 cultures compared to medium controls not containing amoebae.

Diversity measures were examined in terms of 1) overall richness (i.e., number of distinct organisms expressed as OTUs present within the microbiome) quantified using the Chao1 richness estimator (Fig. 4A), and 2) overall diversity (i.e., the distribution of abundance among distinct taxa) expressed as Shannon diversity index (*H'*) (Fig. 4B). The Chao1 richness estimator showed highest levels of overall richness in the G culture at both temperatures, while the B8 clonal culture showed the lowest overall richness (Fig. 4A), indicating a higher number of different bacterial genera/ families in the polyclonal culture compared to the B8 and the CE6 clonal cultures. In terms of overall diversity, a lower *H'* was shown by the B8 clonal culture at both temperatures, while the G culture had the highest *H'* index at 10°C and the CE6 clonal culture at 15°C (Fig. 4B). This indicates that the relative abundance of different bacterial genera/ families was more evenly distributed in the CE6 and G cultures, compared to the B8 culture where most of the bacteria belonged to fewer dominant genera/ families. ANOVA results showed that difference in terms of Chao1 richness and *H'* index among cultures were significant (CE6, B8 and G) ($p \leq 0.001$, $n = 48$). The B8 clonal culture was the only one showing a significant difference in Chao1 richness between the two temperatures ($p \leq 0.01$, $n = 16$) but not between culture types (samples/ medium) ($p > 0.05$, $n = 16$). The *H'* index was statistically significant between the two temperatures and culture type for the B8 ($p \leq 0.001$, $n = 16$) and the CE6 cultures ($p \leq 0.01$, $n = 16$), while the G culture showed a significantly different *H'* index just between the two temperatures ($p \leq 0.001$, $n = 16$). The results indicate that presence of bacterial genera/ families in all cultures grow preferentially at 10°C or 15°C, with changes in their relative abundance between the two temperatures. The differences in the *H'* index between sample and medium indicate that the presence of amoebae changed the relative abundance of certain bacterial genera/ families.

UniFrac, coupled with standard multivariate statistical techniques including PCoA, identifies factors explaining differences among microbial communities (Lozupone et al. 2011). PCoA transforms the distance matrix into a new set of orthogonal axes where the first axis (axis 1) can be used to explain the maximum amount of variation present in the dataset, followed by the second axis (axis 2). PCoA based on un-weighted UniFrac distances (not considering the relative abundance of OTUs) revealed that all cultures (CE6, B8 and G) formed distinct groups (Fig. 5A). Results of the multivariate analysis with the function `adonis` showed a significant difference among cultures ($p \leq 0.001$, $n = 48$) but not in the interaction between temperature (10°C and 15°C) and culture types (medium and samples) in each culture ($p > 0.05$) indicating that differences existed in the bacterial genera/ families between the different cultures and that temperature and *P. perurans* presence did not significantly change the presence of different genera/ families within the different cultures. Weighted (taking relative abundance of OTUs into account) UniFrac distances showed distinct groups among all culture types and temperatures (Fig. 5B), which was also reflected in the `adonis` results, including the interaction terms ($p \leq 0.001$, $n = 48$), indicating that temperature and presence of amoebae both significantly affected the relative abundance of bacterial genera/ families in the cultures.

4. DISCUSSION

In this study, an *in vitro* approach was used to gain a better understanding of the effect on *P. perurans* population growth of two different temperatures, 10°C and 15°C, relevant to the European Atlantic salmon industry to inform future risk assessments. Three different cultures were used and counts were performed on amoebae attached to the MYA and on un-attached amoebae to better mimic a natural situation on the gills (i.e., amoebae attached to the gills or suspended in adjacent seawater). All cultures (G, B8 and CE6) showed a significant difference in amoebae numbers between the two temperatures at 21 days post culture seeding; both for the attached and un-attached amoebae count data. Moreover, a comparison among the different cultures also showed significant differences for the attached and un-attached amoebae at both temperatures.

The results of a previous *in vivo* challenge study (terminated at 21 days post infection) showed difference in virulence for the amoeba cultures used here, in terms of the ability to generate gill lesions on the host, with B8 showing the highest virulence and CE6 the lowest (Collins et al. 2017), suggesting that these differences in virulence between the amoeba cultures may be linked to factors influencing amoebae numbers, such as growth rate and/ or attachment ability. However, in the current *in vitro* study, B8 attached and un-attached amoebae showed lower count values in comparison to G and CE6 cultures at the two temperatures. Nevertheless, attached and un-attached amoebae counts were stable or increasing *in vitro* at 21 days post seeding in the B8 culture, at 10°C and 15°C, while decreasing at 15°C for both attached and unattached CE6 culture amoebae at day 21. Reasons for differences in B8 and CE6 amoebae numbers, and associated virulence, seen between the *in vivo* and *in vitro* studies are not known. The CE6 amoebae numbers were decreasing by day 21 in the *in vitro* study, and perhaps this represents a falloff in CE6 numbers at day 21 *in vivo* also, as *in vivo* sampling in the Collins et al. (2017) did not occur prior to day 21. It has been shown that *P. perurans* cultures can also change over time (Bridle et al. 2015), therefore different results might have been generated if freshly isolated or older cultures were used for both *in vitro* and *in vivo* experiments. Alternatively, if the *in vitro* experimental period was extended, a decrease in amoebae numbers may also have been observed in the B8 culture due to toxic metabolites building up in the enclosed flask system. Growth characteristics *in vitro* of different cultures also may not be representative of grow characteristics *in vivo*, in the presence of host nutrients/ environment, at least for some *P. perurans* strains. The differences in virulence between B8 and CE6 cultures (Collins et al. 2017) may also be due to factors or

activities particular to B8, not related to attachment or increase in amoebae numbers. What is clear both from the study here and that of Collins et al. (2017), is that *P. perurans* clones/ cultures can differ in their growth and virulence properties, but the reasons for these differences are complex (Bridle et al. 2015; Douglas-Helders et al. 2005).

One factor which may have influenced both growth and virulence of *P. perurans* cultures is their associated microbial community (Burgess and Petri 2016; Jellett and Scheibling 1988; Paniagua et al. 2001), as *P. perurans* cultures were maintained in the laboratory in non-axenic conditions. To study this, next-generation Illumina MiSeq sequencing was performed at the end of the *in vitro* experiment for samples and filtered medium of the three cultures (G, B8 and CE6) to investigate the complexity of the microbial community in each culture. A predominance of Gram-negative bacteria was found in the amoebae cultures as also seen by McCarthy et al. (2015). In Tasmania, a culture-independent 16S rRNA gene-based approach identified the genus *Psychroserpens* spp. (family Flavobacteriaceae, phylum Bacteroidetes) as gill bacteria associated with AGD in Atlantic salmon (Bowman and Nowak 2004), while a study using a culture-dependent approach found the genera *Winogradskyella* spp. (family Flavobacteriaceae, phylum Bacteroidetes) and *Staphylococcus* spp. (family Staphylococcaceae, phylum Firmicutes) associated with AGD (Embar-Gopinath et al. 2008). In the current study other genera belonging to the Flavobacteriaceae family were found (*Joostella* sp. and *Muricauda* sp.), potentially showing a difference in microbial community dependent on the seawater area of sampling.

Chao1 richness estimator showed highest levels of overall richness in the G culture at both temperatures, while richness in the B8 clonal culture was lowest. B8 and CE6 are clonal cultures isolated from the polyclonal G (Collins et al. 2017) culture; therefore, the single amoeba cell sorting may have also reduced the selection of the bacterial community. These results could offer one explanation for the lower amoebae numbers in the B8 attached and un-attached amoebae count data in comparison to G and CE6 cultures at both temperatures, in that preferred bacterial prey species may be absent or in lower abundance in this culture (e.g., *Balneola* sp. in comparison to G and CE6 cultures). Chao1 richness ANOVA results were not significantly different between sample and filtered medium, indicating that the microbial community present was not dependent on presence or absence of *P. perurans* for their survival. The B8 clonal culture was the only one showing a significant difference in richness between the two temperatures with a lower value for 15°C, indicating a possible presence of bacterial genera/ families unable to survive or grow at this temperature, which in turn may have contributed to changes in B8 amoebae growth. However, the Shannon diversity index

(*H'*) (combining measures of richness and abundance) ANOVA results showed significant differences between the two temperatures and between the culture type (sample/ medium) for B8 and CE6, while G sample/ medium cultures showed a significantly different *H'* index for temperature only – i.e., the presence of *P. perurans* did not influence relative abundance of bacteria. A differential effect of temperature (10°C and 15°C) alone on the growth of certain bacterial genera/ families, was observed in all cultures, as evidenced by significant changes in their abundance (*H'* index) between the medium samples at the end of the experimental period. This could underlie the significant differences in *P. perurans* population numbers over 21 days found between temperatures for each culture, in both attached and un-attached count data, as a preferential distribution of abundance among distinct bacterial taxa (representing prey or inhibitors/ enhancers of amoebae growth) related to temperature. Interestingly, the results of the multivariate analysis of variance (`adonis`) showed significant differences among all culture types (sample/ medium) and temperatures only in the weighted UniFrac distance analysis, while the un-weighted UniFrac distance analysis showed significant differences among cultures but not in the interaction between temperature and culture type (for each culture). The results of un-weighted UniFrac distance analysis may again reflect the potential isolation of different bacterial taxa with single amoebae cells when establishing clonal cultures or it may reflect specific interactions with preferred bacterial genera/ families by the different clonal *P. perurans* strains, which have changed bacterial compositions in culture over time. The results of weighted UniFrac distance analysis, using abundance as well as relatedness, indicate that the isolated bacterial genera/ families have preferential growth temperatures – i.e., not all growing uniformly, and that the *P. perurans* amoebae have differential effects on the abundance of bacterial genera/ families within their environment, either through preferential choice of prey species, or factors which inhibit or enhance certain bacteria. Therefore, the differences in virulence of these amoeba cultures shown by Collins et al. (2017) may be linked to the observed differences in bacterial composition between the *P. perurans* cultures, which in turn might have influenced amoebae number in the different *in vitro* conditions in terms of growth rate and attachment ability. *Balneola* spp. were seen to differ between the CE6 and B8 cultures (of the top 30 most prominent bacterial genera/ families detected), across both temperatures, in terms of decreasing abundance in samples with amoebae. However, it should be noted that some of the observed differences between sample and medium, i.e. absence/ lower abundance in medium compared to sample, may not be due to an effect of amoebae presence/ absence, but due to

loss of bacterial species which form clumps under the *in vitro* culture conditions, excluded during the filtering step.

In conclusion, this study showed that all *P. perurans* cultures showed a significant difference in population growth dynamics between the two temperatures over time, both for the attached and un-attached count data. It also showed that different clonal cultures, isolated from the same polyclonal culture, can differ significantly in their growth dynamics, and from the overall growth dynamics of the originating polyclonal culture. Their different growth dynamics under different conditions highlights the possibility of seasonal or regional selection of different amoeba strains, though other environmental factors may also be involved. Significant differences were found among the bacterial communities in the isolated *P. perurans* cultures used, which were reflected by significant differences in relative abundance of the 30 most prevalent bacteria genera/ families. Their relative abundances changed with temperature and also with presence of amoebae, indicating an interaction with the amoebae. Further targeted *in vivo* studies are required to help elucidate relationships, if any, between environmental factors (e.g., different temperatures, microbial environment) and *P. perurans* pathogenicity and AGD prevalence.

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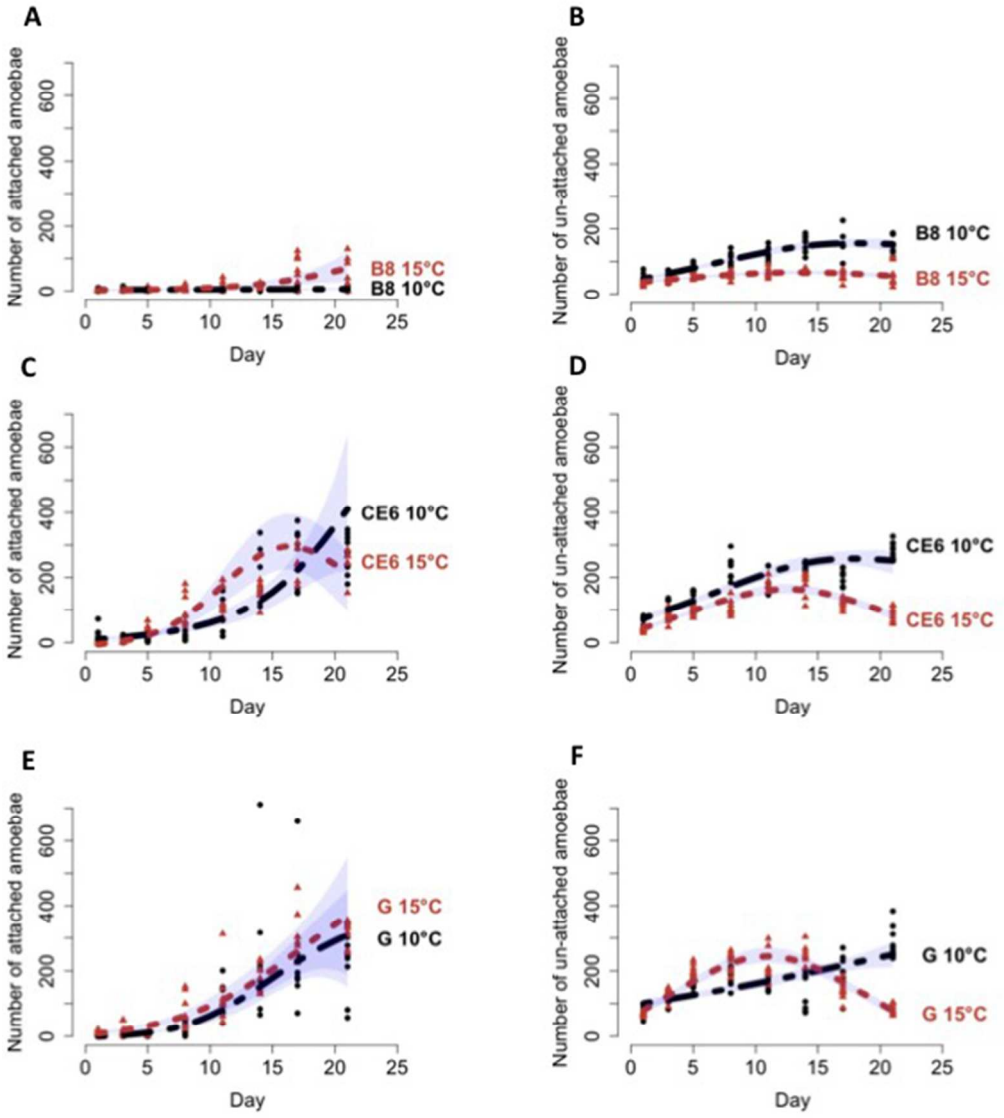

Fig. 1. Growth curves of *P. perurans* culture. Model interpretation using a predict function on the original scale for the B8, CE6, G culture at 10 °C (dark black line) and 15 °C (light red line) ± 95% C.I. (blue polygon). The number of attached amoebae were counted in an area of 3 cm^2 (A, C, E). The number of viable un-attached amoebae were counted in a 1:10 dilution in 100 µl (B, D, F), viability assessed using the neutral red assay.

51x58mm (300 x 300 DPI)

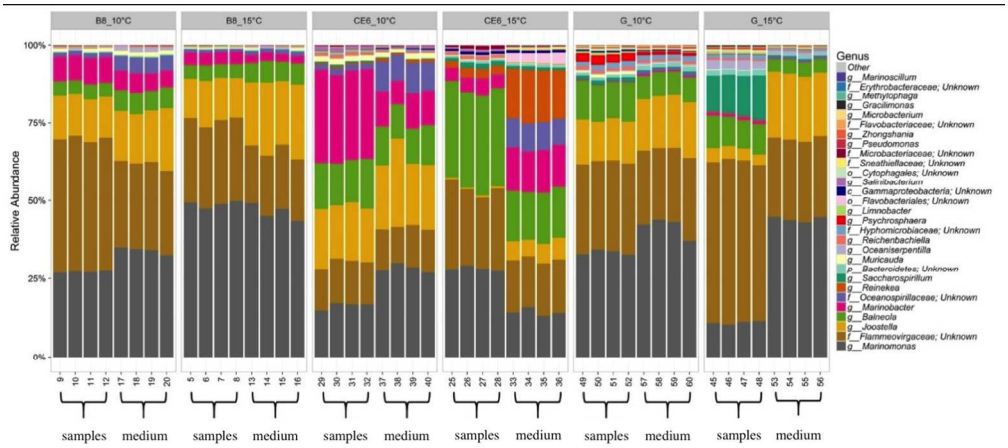

Fig. 2. Relative abundance of the 30 most dominant genera in all samples, grouped by culture type (medium and samples) and temperature (10°C and 15°C). The "unknown" taxonomic information was assigned when the algorithm was not able to make a confident determination of the taxonomic classification at a certain level (number of matching taxa/ number of total taxa > 51%). Sample: culture containing amoebae. Medium: culture without amoebae ("negative control").

127x56mm (300 x 300 DPI)

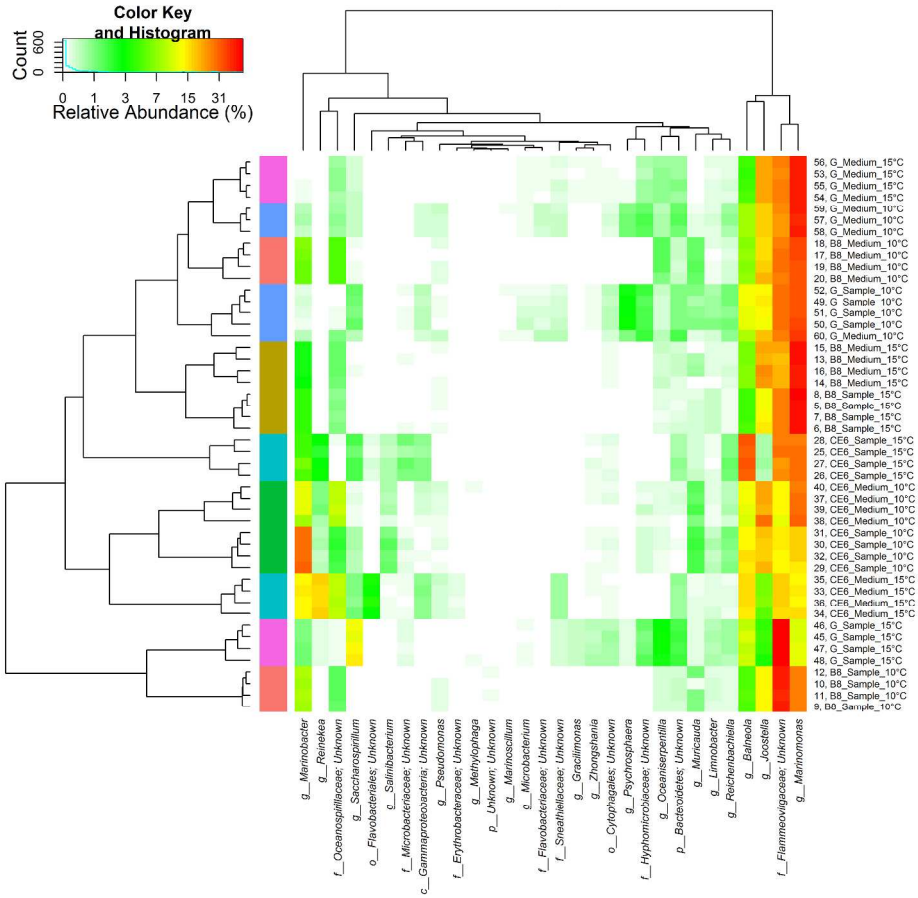

Fig. 3. Heatmap of relative abundances of the 30 most dominant bacterial genera by type. Samples/ medium and bacteria were sorted based on weighted UniFrac and Euclidean distances, respectively. Sample: culture containing amoebae. Medium: culture without amoebae ("negative control"). The x axis shows the relative abundance (%) of the different genera in each sample/ medium for each culture and temperature; on the y axis the different colours show the clustering differences between the cultures and the samples/ medium for each culture and temperature.

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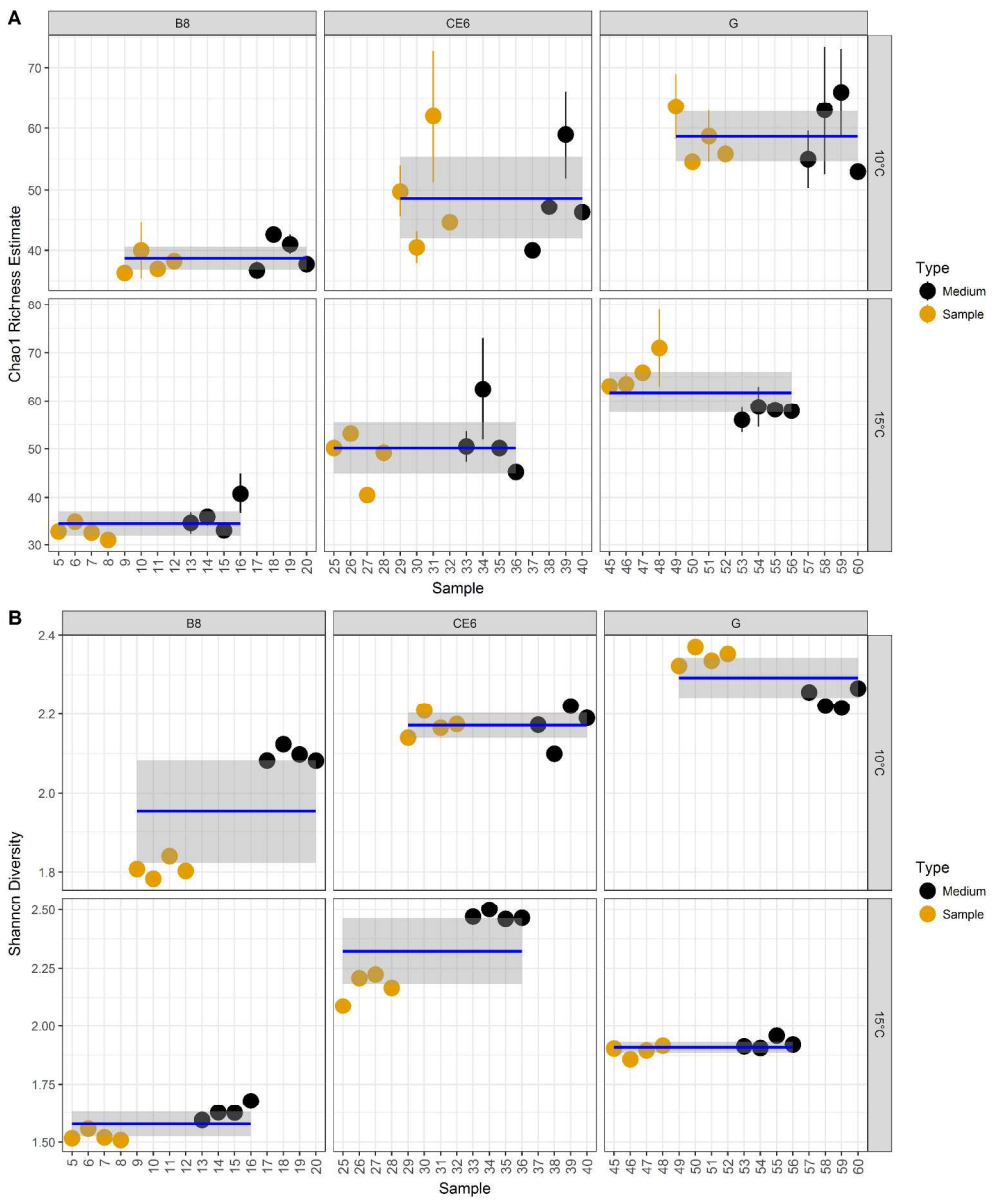

Fig. 4. Measures of richness and diversity. A) Chao1 richness within the total microbiome data, coloured by type, grouped by temperature and culture type (mean value ± 95% C.I.). B) Shannon diversity within the total microbiome data, coloured by type, grouped by temperature and culture type (mean value ± 95% C.I.). Sample: culture containing amoebae. Medium: culture without amoebae ("negative control").

338x406mm (300 x 300 DPI)

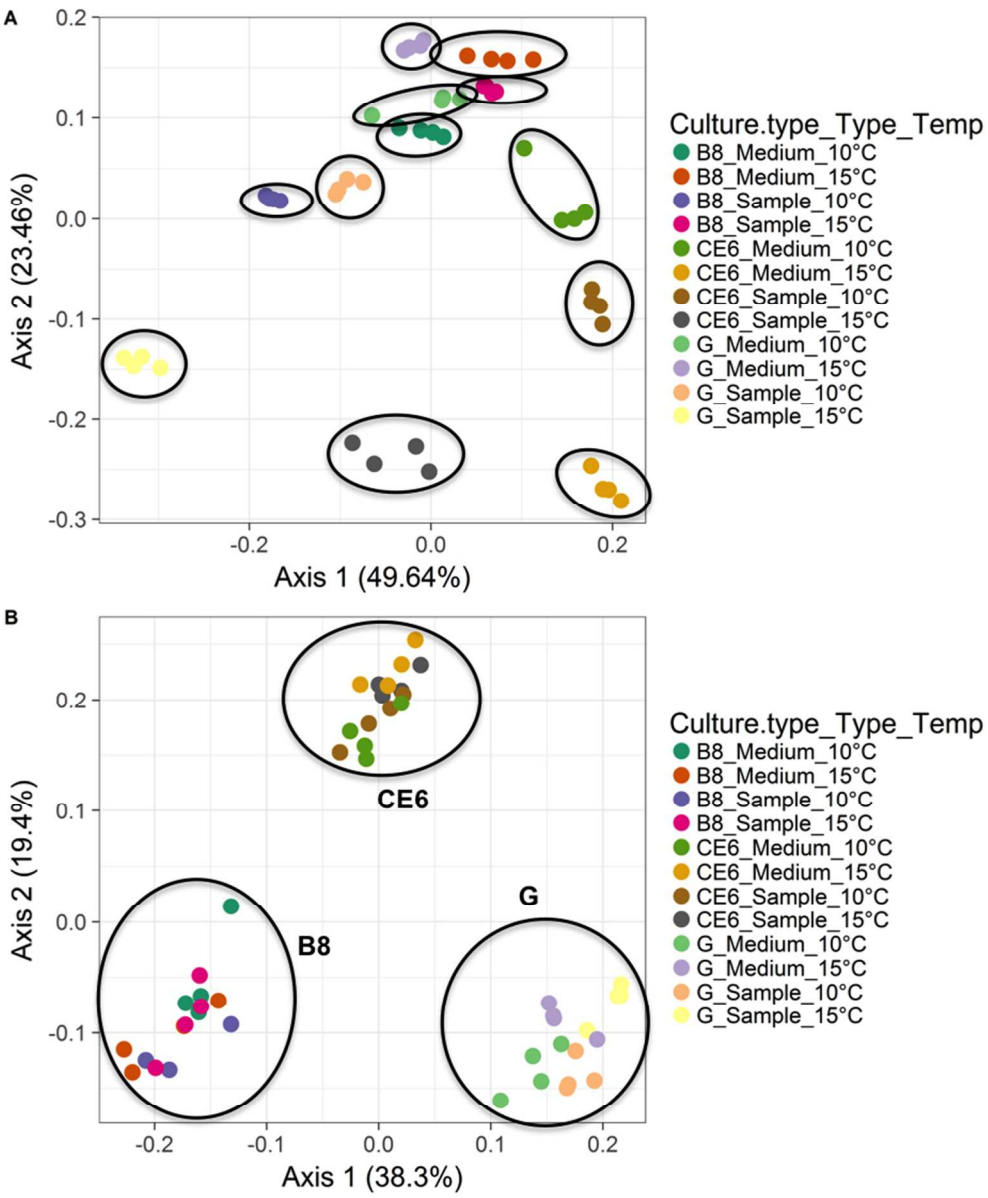

Fig. 5. Principal Coordinates Analysis (PCoA) of beta diversity based on un-weighted Unifrac distances (A) and on weighted Unifrac distances (B). Sample: culture containing amoebae. Medium: culture without amoebae ("negative control"). A) PCoA based on un-weighted UniFrac distances (not considering the relative abundance of OTUs) revealed that all cultures (CE6, B8 and G) formed distinct groups (circles), and were significantly different ($p \leq 0.001$, $n = 48$). B) PCoA based on weighted (taking relative abundance of OTUs into account) UniFrac distances showed distinct groups among all culture types and temperatures (circles), and were significantly different ($p \leq 0.001$, $n = 48$).

79x95mm (300 x 300 DPI)

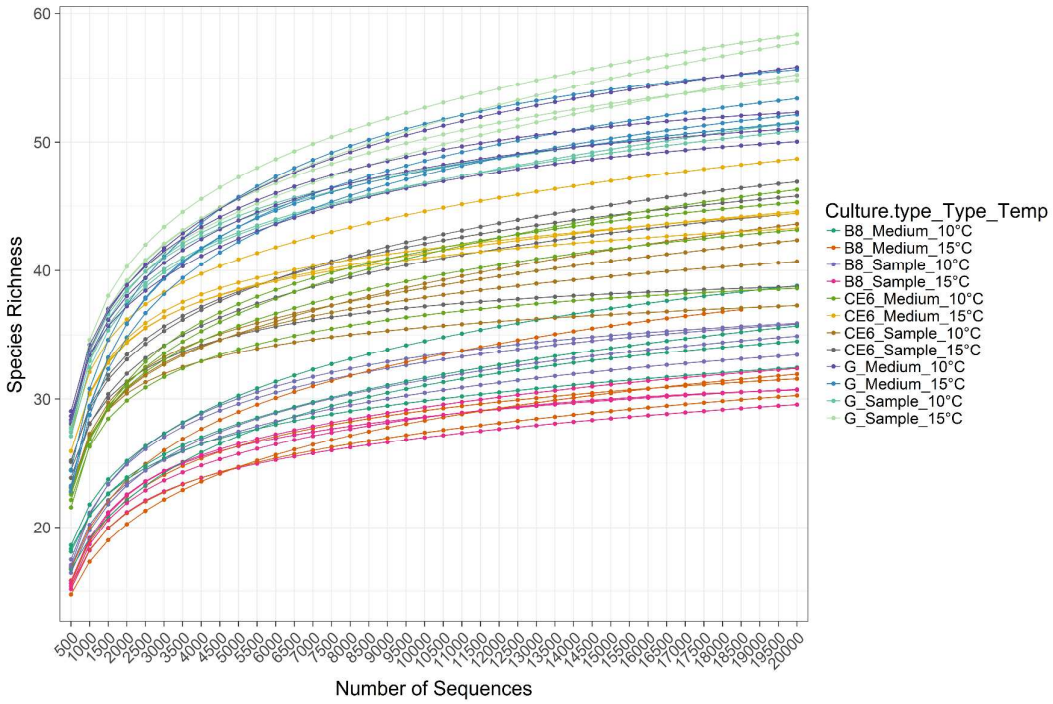

Fig. S1. Rarefaction plot of the OTUs (species richness) versus the number of sequences, subsampling from 500 to 20,000 reads in increments of 500 reads. Sample: culture containing amoebae. Medium: culture without amoebae ("negative control").

Detailed protocol of NGS:

Samples were amplified for sequencing at RTL Genomics in a two-step process. The forward primer was constructed with the Illumina i5 sequencing primer (5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG -3') and the 28f primer. The reverse primer was constructed with the Illumina i7 sequencing primer (5' – GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G – 3') and the 388r primer. Amplifications were performed in 25 µl reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), 1 µl of each 5µM primer, and 1µl of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, California) under the following thermal profile: 95°C for 5 min, then 25 cycles of 94°C for 30 s, 54°C for 40 s, 72°C for 1 min, followed by one cycle of 72°C for 10 min and 4°C hold. Products from the first stage amplification were added to a second PCR based on qualitatively determined concentrations. Primers for the second PCR were designed based on the Illumina Nextera PCR primers as follows: forward - AATGATACGGCGACCACCGAGATCTACAC[i5index]TCGTCGGCAGCGTC and reverse - CAAGCAGAAGACGGCATACGAGAT[i7index]GTCTCGTGGGCTCGG. The second stage amplification was run as for the first stage except that 10 cycles were used instead of 25. Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled in equimolar amounts and each pool was size selected in two rounds using SPRIselect (BeckmanCoulter, Indianapolis, Indiana) at a 0.7 ratio for both rounds. Size selected pools were then quantified using the Quibit 2.0 Fluorometer (Life Technologies) and loaded on an Illumina MiSeq (Illumina, Inc. San Diego, California) 2 x 300 flow cell at 10pM and sequenced. Generated sequences were processed by the RTL Genomics data analysis pipeline consisting of two major stages, 1) the denoising and chimera detection stage for quality checking to remove failed sequence reads, sequences with low quality tags, and sequences that were less than half the expected amplicon length; and 2) the microbial diversity stage. The process of denoising is used to correct errors in reads and usually observed error rates generated by Illumina MiSeq are less than 0.4%. Briefly, paired sequences were merged using the PEAR Illumina paired-end read merger and then reads were run through a RTL internal trimming algorithm.

Detailed protocol for the models in Results 3.1:

The final model for the **B8 attached amoebae** was as follows: `model.nb<-glm.nb(attached_amoebae ~ temperatureF * day, data = taskA, link="log"`, while the best fitting model for the **B8 un-attached amoebae** count data included the interaction term and the quadratic term (turning a linear regression model into a curve) as follows: `model4.nb<-glm.nb(unattached_amoebae ~ temperatureF *( day + I(day^2) ), data = taskA, link="log")`.

Final model for the **CE6 attached and un-attached amoebae**: `model4.nb<-glm.nb(amoebae ~ temperatureF *( day + I(day^2) ), data = taskA, link="log"`. Based on the attached CE6 amoebae count data, the model output showed significant differences in population growth between the two temperatures (10°C and 15°C) over 21 days, also including the day quadratic term of a non-linear relationship ($p \leq 0.001$, $n = 128$). The best fitting model for the CE6 un-attached count data showed a significant difference between the two temperatures not only over time ($p \leq 0.001$, $n = 128$) but also in the model output which included a single temperature parameter ($p \leq 0.001$, $n = 128$). Moreover, the interaction term and the day quadratic term were also significantly different in both model outputs ($p \leq 0.001$, $n = 128$).

The best model for both the **attached and un-attached G (polyclonal) culture** count data included the interaction term and the day quadratic term of a non-linear relationship as follows: `model4.nb<-glm.nb(amoebae ~ temperatureF *( day + I(day^2) ), data = taskA, link="log")`.

Difference in growth among B8, CE6 and G cultures at 10°C and 15°C over 21 days. The best fitting model for all these analyses resulted in a negative binomial distribution which included the interaction between the two temperatures and the quadratic term for a non-linear relationship, as follows: `model4.nb<-glm.nb(amoebae ~ clone *( day + I(day^2) ), data = taskA, link="log")`. The model output for 10°C attached amoebae count data showed that B8 and CE6 growth rates were significantly different ($p \leq 0.01$, $n = 128$), CE6 and G growth rates were significantly different ($p \leq 0.001$, $n = 192$) including also the interaction between the two cultures over time ($p \leq 0.01$, $n = 192$) and the quadratic term ($p \leq 0.05$, $n = 192$), and B8 and G growth rates were significantly different over time ($p \leq 0.01$, $n = 192$). At 15°C attached amoebae count data showed a similar trend

for B8 and G cultures which increased over time even if at a different magnitude, while CE6 count data numbers started to decrease after 15 days. The model output for 15°C attached amoebae count data showed significant differences between the CE6 and G count data and between the G and B8 count data ($p \leq 0.001$, $n = 192$), while the B8 and CE6 count data showed a significant difference only over time, including also the quadratic term ($p \leq 0.001$, $n = 192$). Un-attached amoebae count data showed an increasing trend at 10°C over time for all cultures with significant differences among the different cultures ($p \leq 0.001$ between B8 and CE6 count data and between B8 and G count data, $p \leq 0.01$ between CE6 and G count data, $n = 192$), while at 15°C un-attached count data numbers started to decrease after 10 days in all the different cultures. The model output for 15°C un-attached amoebae count data showed a significant difference between the G and CE6 count data ($p \leq 0.001$, $n = 192$) and between the G and B8 count data ($p \leq 0.001$, $n = 192$), whereas the difference between the CE6 and B8 count data was significant only over time and also including the quadratic term ($p \leq 0.001$, $n = 192$).