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2 **Toxoflavin secreted by *Pseudomonas alcaliphila* inhibits growth of *Legionella*

3 *pneumophila* and its host *Vermamoeba vermiformis***

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19 Running title: *P. alcaliphila* inhibits *L. pneumophila*

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21 **ABSTRACT**

22 *Legionella pneumophila* is a natural inhabitant of water systems. From there, it can be transmitted
23 to humans by aerosolization resulting in severe pneumonia. Most large outbreaks are caused by
24 cooling towers contaminated with *L. pneumophila*. The resident microbiota of the cooling tower
25 is a key determinant for the colonization and growth of *L. pneumophila*. The genus *Pseudomonas*
26 correlates negatively with the presence of *L. pneumophila*, but it is not clear which species is
27 responsible. Therefore, we identified the *Pseudomonas* species inhabiting 14 cooling towers using
28 a *Pseudomonas*-specific 16S rRNA amplicon sequencing strategy. Cooling towers free of *L.*
29 *pneumophila* contained a high relative abundance of members from the *Pseudomonas*
30 *alcaliphila/oleovorans* phylogenetic cluster. *In vitro*, *P. alcaliphila* JCM 10630 inhibited the
31 growth of *L. pneumophila* on agar plates. Analysis of the *P. alcaliphila* genome revealed the
32 presence of a genes cluster predicted to produce toxoflavin. *L. pneumophila* growth was inhibited
33 by pure toxoflavin and by extract from *P. alcaliphila* culture found to contain toxoflavin by LC-
34 ESI-MS. In addition, toxoflavin inhibits growth of *Vermameoba vermiformis*, a host cell of *L.*
35 *pneumophila*. Our study indicates that *P. alcaliphila* may be important to restrict growth of *L.*
36 *pneumophila* in water systems through the production of toxoflavin. A sufficiently high
37 concentration is likely not achieved in the bulk water but might have a local inhibitory effect such
38 as in biofilm.

39 **Keywords:** Toxoflavin, *Legionella*, *Pseudomonas*, water, biofilm

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42 ## 1. INTRODUCTION

43 Legionellosis is a human respiratory disease caused by the bacterium *Legionella* (Cunha et al.,
44 2016). *Legionella pneumophila* cause 90% of the cases; the remaining 10% of cases mostly involve
45 *L. micdadei*, *L. bozemanii*, and *L. Longbeachae* (Cunha et al., 2016). Legionellosis includes
46 Legionnaires' disease (LD), a systemic infection involving severe pneumonia, and Pontiac fever,
47 a mild, flu-like disease (Cunha et al., 2016). There is a clear upward trend in the prevalence of
48 Legionnaires' disease worldwide (Cunha et al., 2016). Such an increase in LD cases could be due
49 to densification of urban areas, improvement in diagnostic tests and reporting, a greater number of
50 persons at risk due to aging and/or increase in immunocompromised populations, aging
51 infrastructures, or climate change (Cunha et al., 2016). Importantly, a study of the impact of
52 infectious disease in Europe published in 2018 revealed that LD is the fifth most burdensome
53 disease in people older than 15 years old, after AIDS, tuberculosis, influenza and invasive
54 pneumococcal disease (Cassini et al., 2018).

55 Not long after the discovery of *Legionella*, it was established that it is transmitted to human by
56 inhalation of aerosols containing *L. pneumophila* that are generated by engineered water systems
57 (EWS) (Meyer, 1983). Several type of EWS can shed *L. pneumophila*, including water distribution
58 systems (showers and faucets), spas, fountains, and cooling towers (Heijnsbergen et al., 2015).
59 Most of the large outbreaks of LD are caused by cooling towers (Garrison et al., 2016).
60 Deficiencies in management and operation of water systems is the main cause of outbreak of LD
61 (Garrison et al., 2016; Mouchtouri et al., 2010).

62 Cooling towers contain a complex microbial ecosystem constituted by a diverse community of
63 planktonic and biofilm-associated bacteria, protozoa, and algae (Gregorio et al., 2017; Hauer et

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64 al., 2016; Llewellyn et al., 2017; Paniagua et al., 2020; Paranjape et al., 2020b, 2020a; Pereira et
65 al., 2017; Pinel et al., 2021; Tsao et al., 2019). The microbial community in cooling towers is
66 mostly found in surface attached and floating biofilms which seeds the bulk water with planktonic
67 microorganisms undefined. The bacterial community identified by different studies varies greatly,
68 but typical biofilm-forming aquatic bacteria were generally identified, such as
69 *Sphingomonadaceae, Caulobacteraceae* and *Hyphomicrobiaceae* (Gregorio et al., 2017; Paniagua
70 et al., 2020; Wang et al., 2013). Local temperature seems to influence the composition of the
71 bacterial and the protozoan community in the attached biofilm (Paniagua et al., 2020). Some
72 protozoa, such as amoeba and ciliates, prey on other microorganisms and *Legionella* has evolved
73 to hijack the endocytic pathway of a variety of these phagocytic protozoans to facilitate their own
74 growth (Boamah et al., 2017; Fields et al., 1984; Paranjape et al., 2020a; Rowbotham, 1980). In
75 fact, most, if not all, of the multiplication of *Legionella spp.* in water system occurs inside these
76 host cells (Boamah et al., 2017). Protozoal hosts also shield *Legionella* against deleterious
77 conditions (Boamah et al., 2017). Therefore, *Legionella*'s presence in engineered water systems is
78 strongly associated with the presence of host cells (Boamah et al., 2017). In multi-species biofilm,
79 *L. pneumophila* has been found associated with amoebas and other phagocytic protozoans and in
80 dense microcolonies (Taylor et al., 2013).

81

82 Several bacteria inhibit the growth of *L. pneumophila* on solid medium, including species of
83 *Aeromonas*, *Bacillus*, *Flavobacterium*, *Pseudomonas, Acinetobacter, Kluyvera, Rahnella,*
84 *Burkholderia, Staphylococcus, Stenotrophomonas or Sphingobacterium* (Corre et al., 2021, 2019;
85 Guerrieri et al., 2008; Loiseau et al., 2015; Temmerman et al., 2007; Verdon et al., 2008). The
86 active substances responsible for the inhibition were only identified for a fraction of these species.

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87 *Staphylococcus warneri* produces an antimicrobial peptide named warnericin (Verdon et al.,
88 2008). In the case of *Bacillus*, secreted proteases and surfactin were identified to have an anti-
89 *Legionella* effect (Loiseau et al., 2015). *Pseudomonas fluorescens* produces the volatile compound
90 1-undecene that inhibits the growth of *L. pneumophila* in a separate but nearby dish, a phenomenon
91 that was called aerial killing (Corre et al., 2021, 2019). These studies indicate that the cooling
92 tower microbiota likely produces a wide range of biomolecules, including proteins, antimicrobial
93 peptides, and biosurfactants, that can have deleterious effects on *L. pneumophila* and its natural
94 hosts in water systems (Berjeaud et al., 2016).
95

96 Several studies have shown that the composition of the microbial community in cooling towers is
97 influenced by the disinfection regime (Llewellyn et al., 2017; Paranjape et al., 2020b; Pereira et
98 al., 2017; Tsao et al., 2019). There is also strong inverse relationship between *L. pneumophila* and
99 *Pseudomonas* in cooling towers (Llewellyn et al., 2017; Paranjape et al., 2020b; Tsao et al., 2019).
100 These observations are consistent with our previous study that found that continuous chlorine
101 application reduced microbial diversity, decreasing the abundance of *L. pneumophila* while
102 increasing the abundance of *Pseudomonas* (Paranjape et al., 2020b). *Pseudomonas* is a large genus
103 and several species of *Pseudomonas* inhabit cooling towers (Pereira et al., 2018). Which one
104 correlates negatively with *L. pneumophila* in real cooling towers remains unknown. The goal of
105 the present study was to identify the species of *Pseudomonas* associated with the absence of *L.*
106 *pneumophila* in cooling towers and identify molecules possibly involved in this competitive
107 relationship.
108

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109 ## 2. MATERIALS AND METHODS

110 ### 2.1 Bacterial strains and cultures

111 *L. pneumophila* strains Philadelphia 1 (ATCC 33152) and lp120292, which was involved in the
112 LD outbreak in Quebec City in 2012 (Lévesque et al., 2014) and is hereafter referred to as the
113 Quebec strain, were used as test strains. Strains stored at -80°C in 10% glycerol were cultured
114 aerobically at 37°C for 3 days on buffered charcoal yeast extract (BCYE) agar supplemented with
115 0.25 mg/ml L-cysteine and 0.4 mg/ml ferric pyrophosphate. *Pseudomonas alcaliphila* strain JCM
116 10630 (CIP 108031T) was acquired from the Centre de Ressources Biologiques de l'Institut
117 Pasteur and grown on nutrient agar at 30 °C. AYE broth (BCYE without agar and charcoal) or
118 Fraquil, an approximate freshwater media (Morel et al., 1975), were used as liquid medium.

119 ### 2.2 *Pseudomonas*-specific 16S amplicon sequencing

120 The *Pseudomonas*-specific 16S amplicon sequencing strategy previously published by *Pereira et*
121 *al.* (2018) (Pereira et al., 2018) was used to identify the species of *Pseudomonas* present in 14
122 cooling towers sampled in a previous study (Paranjape et al., 2020b). A two-step PCR strategy
123 was used to amplify the V3-V4 region of *Pseudomonas* 16S rRNA and add indices using Paq5000
124 polymerase. The DNA was first amplified with the Pse434F (5'-
125 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACTTTAAGTTGGGAGGAAGGG-3')
126 and Pse665R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
127 ACACAGGAAATTCCACCACCC-3') containing 5' overhang for Illumina Nextera Indexing kit
128 (underlined). An initial denaturation step of 2 minutes at 95°C was used followed by 30 cycles
129 consisting of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C, and a final elongation step of 7 min at

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130 72°C. The amplicons were purified with AMPure XP beads as per the manufacturer's instruction.
131 Indexing PCR was then performed with the Nextera XT index kit, according to the manufacturer's
132 instruction. The amplicons were purified as above and quantified using Quant-iT PicoGreen
133 dsDNA Assay Kit (Invitrogen). The amplicons were sequenced on an Illumina MiSeq using the
134 V2 250 bp paired end reagent kit. The data are available from the Sequence Read Archive under
135 the BioProject accession number PRJNA787128.

136 The resulting sequences were processed using DADA2 (Callahan et al., 2016) implemented in
137 Qiime 2 version 2018.8 (Bolyen et al., 2019). The sequences were trimmed by 21 nt and truncated
138 to 200 nt. The dataset was rarefied to 50,000 sequences per samples. The taxonomic assignment
139 to the genus level of the resulting amplicon sequence variants (ASV) was assigned using a
140 classifier trained on the SILVA SSU database 132 (Quast et al., 2013), according to Qiime 2
141 instructions. Species-level taxonomic assignment of the ASVs was performed using BLAST+
142 against the curated 16S rRNA sequences of *Pseudomonas* as previously described (Pereira et al.,
143 2018). This dataset was then further analyzed using MicrobiomeAnalyst (Chong et al., 2020) to
144 calculate Shannon diversity and perform linear discriminant analysis effect size (LEfSe) (Segata
145 et al., 2011).

146 **2.3 *Legionella pneumophila* inhibition assay**

147 *Anti-Legionella* assay was performed using a soft agar overlay technique. Briefly, a suspension of
148 *L. pneumophila* strains, Quebec and Philliladelphia-1, and *P. alcaliphila* were prepared in AYE
149 broth and adjusted to an OD_{600nm} of 0.2. Then 200 µl of *Legionella* suspension was added to 5 ml
150 of autoclaved soft agar (0.5% of agar in ddH2O) and gently poured on the surface of solidified
151 CYE agar plate. The plates were left to solidified in a biological safety cabinet for 15 minutes.

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152 Then, a 10 µl drop of *P. alcaliphila* suspension was inoculated in the middle of the plates. Plates
153 were incubated at 25 °C, 30 °C and 37 °C for 3 days and the diameter of the *P. alcaliphila* colony
154 and the zone of inhibition was measured.

155 **2.4 Organic extraction of toxoflavin from *P. alcaliphila* supernatant**

156 Chloroform extraction was performed as previously described (Chen et al., 2012) with slight
157 modifications. *P. alcaliphila* was grown on CYE plate for 4 days at 30 °C. The bacterial cells were
158 removed from the surface of the agar using a cell scraper and the agar was cut in smaller pieces
159 using a sterile razorblade. The chopped agar was then mixed with chloroform in 1:1 (w/v) ratio in
160 50 ml falcon tube for extraction of toxoflavin. The chloroform fraction (~25 ml) was filtered
161 through Filtropur S 0.2 µm filter (Sarsted) and left to fully evaporated in the fume hood. The
162 extract was then resuspended in 200 µl of methanol. The agar from a sterile CYE plate was
163 processed the same way to serve as a negative control. Extracts were tested with the disc diffusion
164 assay described below.

165 **2.5 Disc diffusion assay**

166 The susceptibility of the test strains to commercial toxoflavin (Sigma-Aldrich) and *P. alcaliphila*
167 extracts was assessed by adapting the soft overlay agar technique previously described. Instead of
168 adding the *P. alcaliphila* suspension to the center, a sterile paper disc was placed and 10 ul of
169 extract or a range of commercial toxoflavin concentrations (0 ng/µl, 50 ng/µl, 100 ng/µl, 250 ng/µl
170 and 1000 ng/µl) was added to the disc. Plates were incubated at 30 °C for 3 days and the zone of
171 inhibition was measured.

172 **2.6 LC-ESI-MS analysis of toxoflavin**

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173 Liquid chromatography/mass spectrometry analysis was performed by using high performance
174 liquid chromatography (HPLC; Waters 2795, Mississauga, ON, Canada) equipped with a 100 ×
175 4.6 mm i.d. Kinetex C8 (Phenomenex) reversed-phase column (particle size 2.6 µm) using a
176 MeCN/H_2O gradient containing 1% acetic acid at a flow rate of 400 µl/min. The detector was triple
177 quadrupole mass spectrometer (Quattro Premier XE, Waters) equipped with a Z-spray interface
178 using electrospray ionization in positive mode. Analyses were carried out in both MS full-scan and
179 MS/MS multiple reaction monitoring (MRM) scan modes with a mass-to-charge ratio (m/z)
180 window ranging from 130–930. The capillary voltage was set at 3.5 kV and the cone voltage at 30
181 V. The source temperature was kept at 120 °C and the desolvating gas at 200 °C. Nitrogen was
182 used as the cone and desolvation gas and argon was used as collision gas at collision energies up
183 to 30 eV.

184 **2.7 Biofilm and pellicle assay.**

185 The biofilm-formation ability of *P. alcaliphila* was investigated by inoculating media with
186 bacterial. Briefly, 1 mL of trypticase soy broth, King's B, or R2-A broth was added to center four
187 wells of a 24-well plate. Surrounding wells were filled with sterile water to prevent desiccation.
188 Then, 20 µL of *P. alcaliphila* in Fraquil (OD_{600nm} = 0.1) was added to each well and incubated at
189 room temperature with or without shaking at 150 rpm. After a week, images of the wells were
190 taken. When incubated without shaking, plates were shaken at 150 rpm for 1 hour to determine if
191 pellicles could be formed.

192 **2.8 *Vermamoeba vermiformis* inhibition assay**

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193 The sensitivity of *L. pneumophila* host *Vermamoeba vermiformis* to toxoflavin was determined by
194 monitoring its growth in the presence of toxoflavin. *V. vermiformis* were grown at room
195 temperature in 75 cm^2 cell culture flasks (Sarstedt) in modified PYNFH medium (ATCC medium
196 1034) and passaged when confluence was reached. The amoebas were passaged 3 days prior to
197 exposure by adding 5 mL of culture to 20 mL of fresh modified PYNFH. Cell concentration was
198 with a Guava EasyCyte flow cytometer. To prepare samples for flow cytometer, 400 µL of culture
199 was centrifuged at 5000 g for 2 min, the supernatant discarded, and the pellet resuspended in 400
200 µL of phosphate buffered saline (PBS). The stock culture was diluted to 5×10^4 cells/mL in fresh
201 modified PYNFH and 900 µL was added to the wells of a 24-well plate. Then 100 µL of different
202 toxoflavin solutions were added to wells to give final toxoflavin concentrations of 0, 10, 25, 50 or
203 100 µg/mL. The plate was incubated at room temperature without shaking. After 2 and 4 days, 400
204 µL samples were taken from each well to measure cell concentration with flow cytometer. Each
205 condition was performed in triplicate. Results were analyzed using two-way ANOVA, with time
206 and toxoflavin concentration as factors, and Tukey's test correction for mutltiple comparison was
207 used to access significance between conditions.

208

209 **3. RESULTS**

210 **3.1 Profiling of *Pseudomonas* species in cooling towers**

211 *Pseudomonas*-specific 16S rRNA amplicon sequencing was performed on triplicate samples from
212 14 cooling towers. The microbiota of these cooling towers was previosuly studied using 16S rRNA
213 sequencing and 18S rRNA sequencing (Paranjape et al., 2020b, 2020a). For *Pseudomonas*-specific

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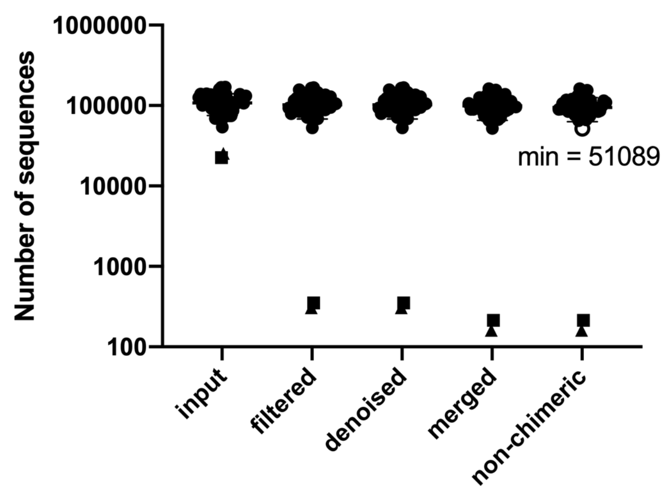
214 16S rRNA amplicon sequencing, a total of 4,680,703 reads were obtained and processed with
215 Qiime using the DADA2 pipeline (Bolyen et al., 2019; Callahan et al., 2016). A no template
216 control and DNA extracted from a blank carthridge were also included. As can be seen in Figure
217 2, these 2 control samples contained very few sequences passing quality control, indicating that
218 the amplicons from the cooling tower samples are not contaminated by spurious sequences. The
219 cooling tower samples contained a minimum of 51089 sequences passing quality control. The data
220 set was rarefied to 50000 sequences per cooling tower. All sequences were assigned to
221 *Pseudomonas*, showing the high specificity of the primers used, as previously reported (Pereira et
222 al., 2018)

223



224

225 Figure 1: Total number of sequences obtained (input) and left after each one of the processing
226 steps. Square, no template control; triangle, blank carthridge. The sample with the least number of
227 sequences is depicted by an open circle.

228

229 Considering all the cooling towers, 34 *Pseudomonas* species were found among which 14 can be
230 described as major or abundant species and the other 20 as minor species, collectively representing

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231 less than 5% of the population. Of note, this method is unable to differentiate closely related
232 species, such as *P. alcaliphila* and *P. oleovorans*. Such species are therefore grouped in clusters.
233 The highest diversity of *Pseudomonas* species was observed in cooling towers Cdq1 and Est1
234 containing 26 and 13 different species, respectively, followed by CT CN3, CN4 and MTL5 (Figure
235 2A). Gobally, the top three most abundant *Pseudomonas* species in the studied cooling towers
236 were *P. alcaliphila/oleovorans*, *P. monteilii* and *P. alcaligenes*. *P. alcaliphila/oleovorans* was
237 observed in nearly 100% of cooling towers in various proportions, but was the largely dominant
238 *Pseudomonas* species in several cooling towers including CN2, Out1, Out2, Mont1, MTL1,
239 MTL4, MTL7 and MTL8 (Figure 2A). The human pathogen *P. aeruginosa* was detected only in
240 towers CdQ1 and Est2 at a low abundance of 0.01. Next, we calculated the abundance of each
241 *Pseudomonas* species as a fraction of relative bacterial abundance (Paranjape et al., 2020b). As
242 can be seen in Figure 2B, *P. alcaliphila/oleovorans* is the most abundant *Pseudomonas* species in
243 cooling tower microbiomes dominated by *Pseudomonas*, including cooling towers Out1, Mont1,
244 and MTL1.

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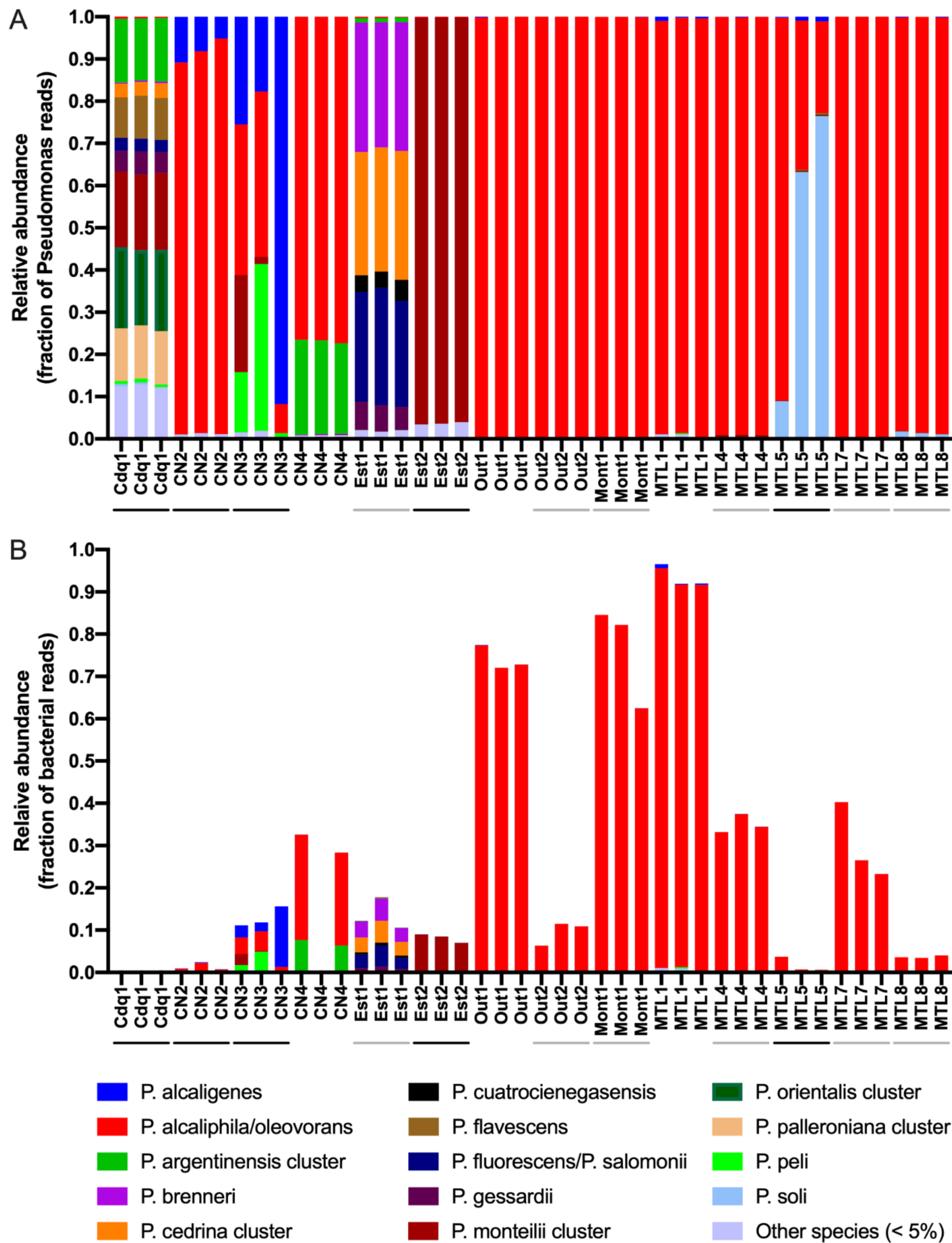

245

246 **Figure 2.** Relative abundance of *Pseudomonas* species as a fraction of *Pseudomonas* reads (A)

247 and as a fraction of bacterial reads (B). Species with a maximum relative abundance of less than

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248 5% in any towers were grouped together in the category "other species". The presence of
249 *Legionella* in each tower is depicted by a line under the cooling tower's name; a grey line indicate
250 the presence of *Legionella* species other than *pneumophila*, a black line indicate the presence of *L.*
251 *pneumophila* (Paranjape et al., 2020b).

252 Next, we sought to identify factors influencing the diversity of *Pseudomonas* within the cooling
253 towers. Shannon diversity was calculated for each tower (Figure 3A). As expected, cooling towers
254 CdQ1 and Est1 have the highest shannon diversity, while the cooling towers dominated by *P.*
255 *alcaliphila* had the lowest diversity. In our previous study, we determined that chlorine application
256 schedule had a greater impact than chlorine concentration in shaping the bacterial communities
257 (Paranjape et al., 2020b). Similarly, treatment schedule seems to affect *Pseudomonas* diversity as
258 tower treated by periodic applications showed significantly less diversity ($P = 0.03$) than cooling
259 towers treated continuously (Figure 3B). Concentration of total chlorine and residual chlorine did
260 not seem to affect *Pseudomonas* diversity. The slopes of the Shanon Diveristy against the
261 concentration of total chlorine (-0.599 ± 0.502) and against the concentration of residual chlorine
262 (-0.107 ± 0.073) were not significantly different than zero (Figure 3C and 3D).

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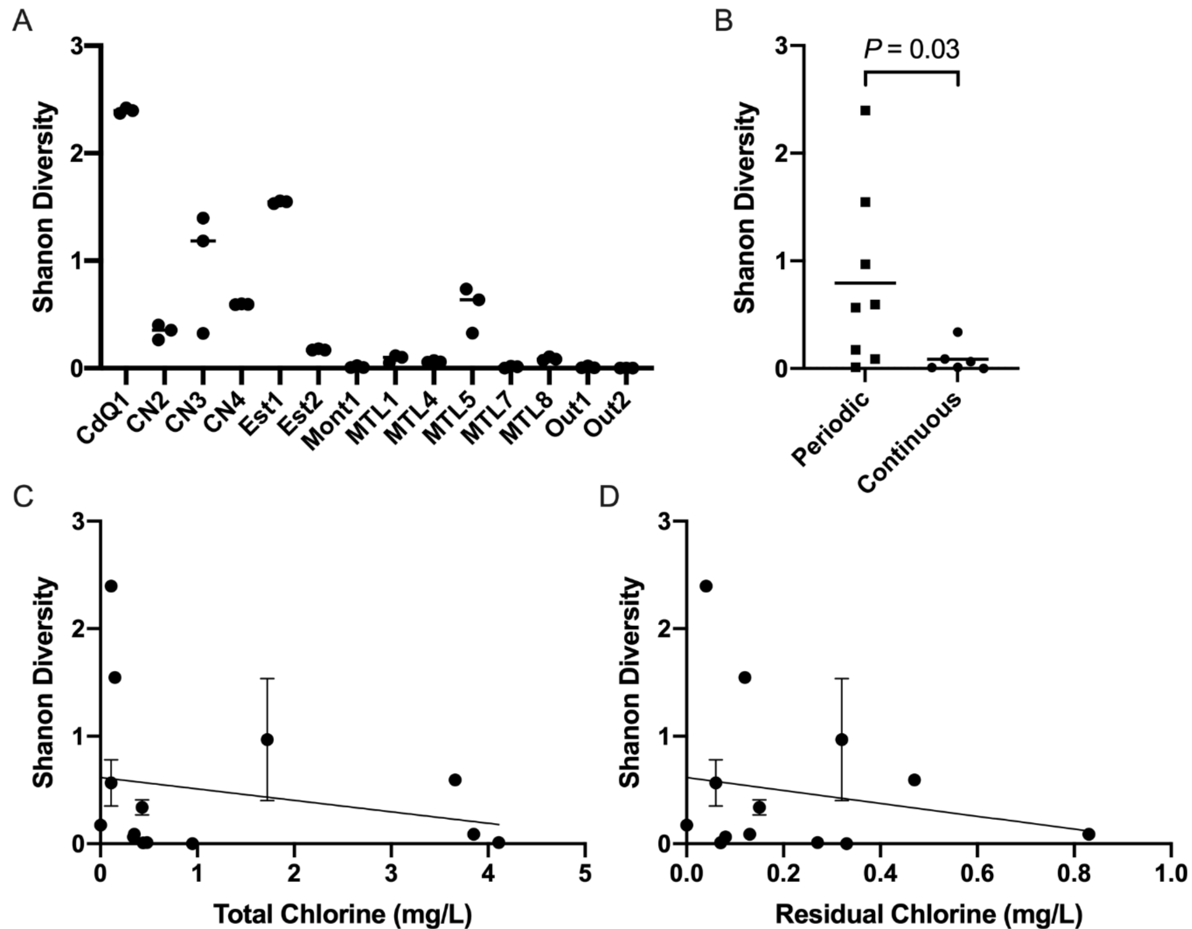

263

264 Figure 3: Treatment application schedule but not chlorine concentration affect *Pseudomonas*
265 diversity. Shanon diversity index was calculated for each cooling towers (A) to evaluate alpha
266 diversity. Individual replicate values are shown. Average shanon diversity index was calculated
267 and each tower was classified according to the treatement schedule (B). A one-tail t-test was used
268 to access statistical significance between the two groups. The average Shanon diversity index ±
269 standard deviation was plotted against total chlorine (C) and redidual chlorine concentration (D).
270 A simple linear regression model was used to determine if the slope was significantly different
271 than zero.

272

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273 We next used linear discriminant analysis effect size (LEfSe) implemented in MicrobiomeAnalyst
274 (Chong et al., 2020) to examine the differences in the abundance of *Pseudomonas* species relative
275 to the bacterial reads in cooling towers. LEfSe is an algorithm that uses a mix of statistical testing,
276 linear discriminant analysis (LDA), and effect size to identify taxa that are predictive of a particular
277 condition (Segata et al., 2011). The treatment schedule and the presence or absence of *Legionella*
278 *spp.* and *Legionella pneumophila* were considered as comparison factors (Paranjape et al., 2020b).
279 *P. alcaliphila/oleovorans* was the only species enriched in cooling towers free of *L. pneumophila*
280 and free of *Legionella spp.* (Figure 4A and B). In contrast, *P. montelli* cluster was enriched in
281 towers containing *Legionella* or *L. pneumophila*, *P. alcaligenes* was enriched in cooling towers
282 containing *L. pneumophila,* and *P. soli* was enriched in towers containing *Legionella spp.* (Figure
283 4A and B). By considering the treatment schedule, *P. alcaliphila/oleovorans* and *P. anguiliseptica*
284 were associated with continuous treatment whereas *P. monteilii* and *P. soli* were associated with
285 periodic treatment (Figure 4C).

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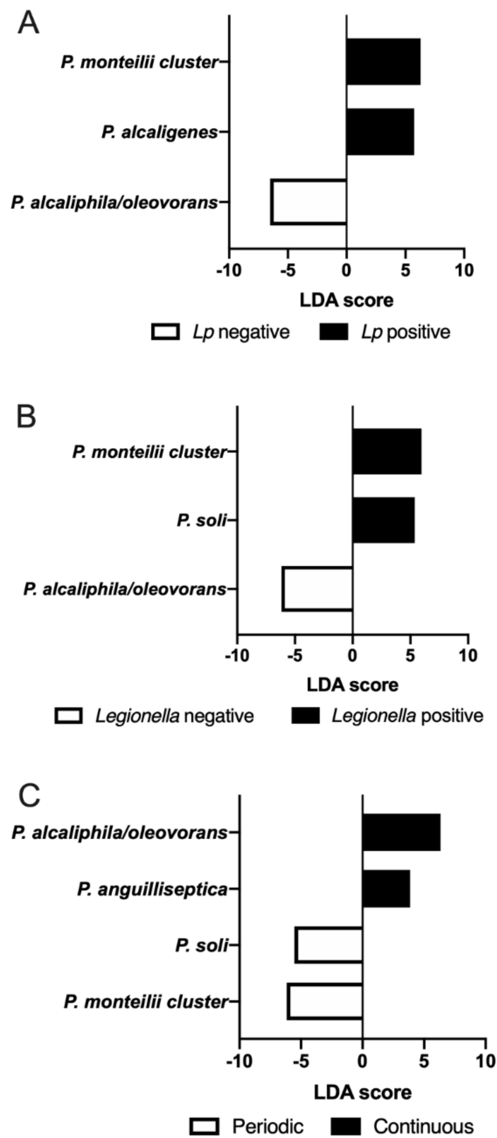

286

287 **Figure 4. *P. alcaliphila/oleovorans* is enriched in towers free of *Legionella* and associated with**
288 **continuous treatement.** LDA scores of *Pseudomonas* species associated with the presence of *L.*
289 *pneumophila* (A), the presence of *Legionella spp.* (B) and treatment schedule (C). Only species
290 with $P < 0.05$ are shown.

291

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292 **3.2 Anti-*L. pneumophila* activity of *P. alcaliphila***

293 Our results reavealed that a member of the *P. alcaliphila/oleovorans* cluster seems to be the main
294 inhibitor of *L. pneumophila* colonisation in the cooling towers we studied. Therefore, we
295 investigated if an isolate of that cluster, *P. alcaliphila* strain JCM 10630, can inhibit *L.*
296 *pneumophila* growth *in vitro* (Yumoto et al., 2001).

297 We carried out *L. pneumophila* inihibition assay at three different temperatures: 25 °C, 30 °C and
298 37 °C. After three days of incubation, *P. alcaliphila* inhibited the growth of *L. pneumophila* at 25
299 °C and 30 °C but not at 37 °C (Figure 5). A one-way ANOVA with a Tukey correction for mutltiple
300 comparison was used to access significance between conditions. The diameter of inhibition was
301 significantly larger at 25 °C than at 30 °C for both strains ($P < 0.001$). The two strain tested showed
302 similar inhibition zones at each temperature tested ($P > 0.6$). The size of the colony of *P.*
303 *alcaliphila* was significantly larger at 25 °C than at 30 °C ($P < 0.001$). The strain of *L. pneumophila*
304 seems to influence slighlty the growth of *P. alcaliphila* at 25 °C as the colony was slightly larger
305 (14.3 mm) when grown with the Quebce strain than with the Philadelphia-1 strain (13 mm, $P =$
306 0.02). There were no difference in colony size at 30 °C.

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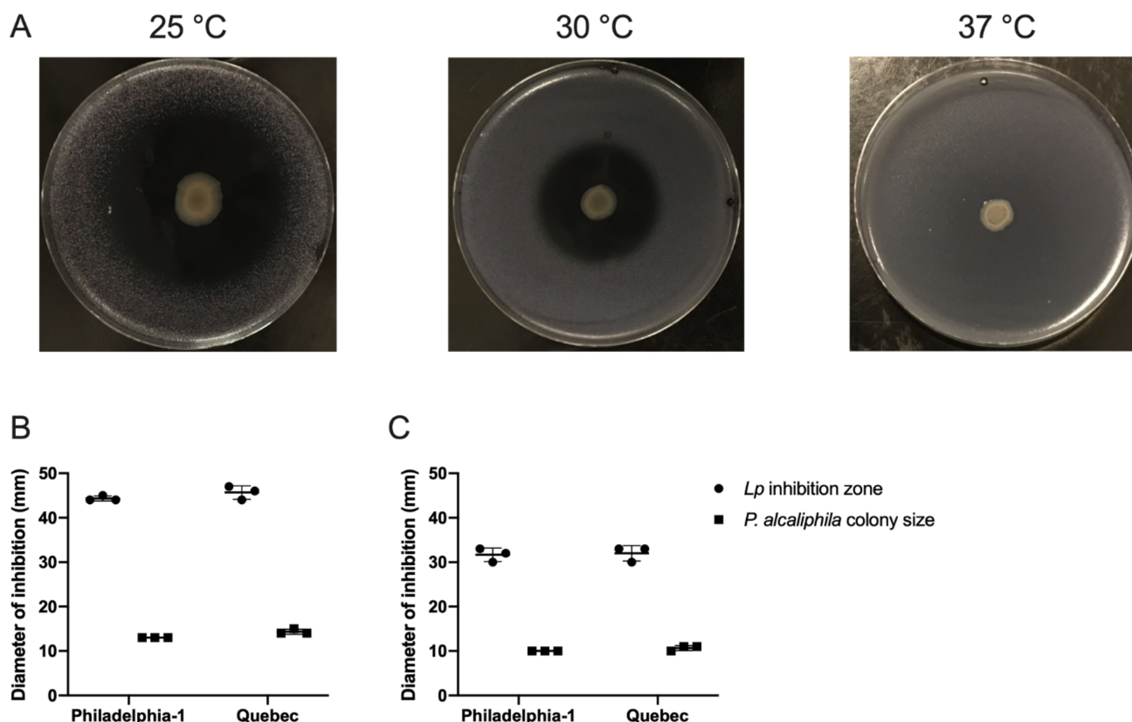

307

308 **Figure 5. *P. alcaliphila* inhibits the growth of *L. pneumophila* at 25 °C and 30 °C.** *L.*
309 *pneumophila* starin Philadelphia-1 and the Quebec strain were inoculated on CYE plates in a thin
310 layer of soft agar. Once solidified, *P. alcaliphila* was inoculated in the center of the plate. A)
311 Representative image of plates incubated at 25 °C (left panel), 30 °C (center panel) and 37 °C
312 (right panel). The diameter of inhibition (circle) and the diameter of the *P. alcaliphila* colony
313 (square) for three replicates was recorded for plates incubate at 25 °C (B) and at 30 °C (C).

314

315 **3.3 In silico analysis of P. alcaliphila genome**

316 The *P. alcaliphila* strain JCM 10630 genome was retrieved from RefSeq (GCF_900101755.1) and
317 was analysed to identify clues as to the cause of the inhibition of *L. pneumophila* growth. We first
318 used antiSMASH (Blin et al., 2019) to identify putative biosynthetic gene clusters (BCGs). Five

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319 clusters were found but none showed similarity higher than 50% with known clusters (Table 1).
320 Next, we used the Blast KOALA function of the Kyoto Encyclopedia of Genes and Genomes to
321 assign Kegg orthology annotation to the genes and predict metabolic pathways present in this
322 genome (Kanehisa et al., 2016). A cluster of genes homologous to toxoflavin synthesis cluster was
323 detected. Toxoflavin is an improtant virulence factor of the plant pathogen *B. glumae* (Suzuki et
324 al., 2004). Toxoflavin is also produced by *Pseudomonas protegens* Pf-5 (Philmus et al., 2015).
325 The *P. alcaliphila* toxoflavin cluster is most homologous to *B. glumae* cluster and organized in a
326 similar manner (Chen et al., 2012; Philmus et al., 2015; Suzuki et al., 2004). The homology of *P.*
327 *alcaliphila* genes compared to *B. glumae* varries between 69% identity to 36% identity (Table 2).
328 Our *in silico* analysis suggests that the inhibition of *L. pneumophila* growth by *P. alcaliphila* could
329 be due to the production of toxoflavin, another compound, or a mixture of several molecules.

330

331 **3.4 Toxoflavin inhibits growth of *L. pneumophila* on CYE plate**

332 The susceptibility of the two strains of *L. pneumophila* to toxoflavin was tested using a dilution
333 series of commercial toxoflavin (Sigma-Aldrich). The results showed that the size of the inhibition
334 zone proportionally increases with concentration of toxoflavin for both strains (Figure 6). The
335 diameters of the inhibition zone were 10 mm, 12 mm, 20 mm and 30 mm for concentrations of
336 0.5, 1, 2.5 and 10 µg, respectively.

337

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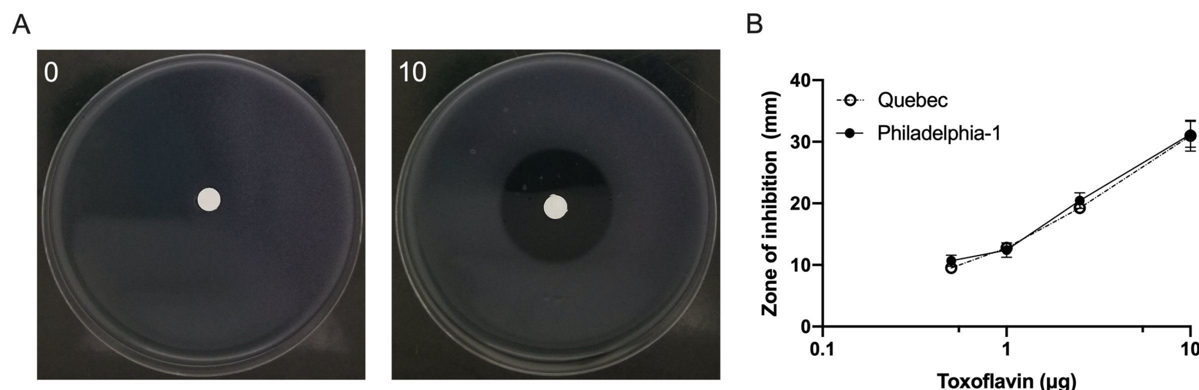

338

339 **Figure 6. Toxoflavin inhibits the growth of *L. pneumophila* on CYE plates.** A disc diffusion
340 assay was used to test the susceptibility of two *L. pneumophila* strains to 0.5, 1, 2.5 and 10 µg of
341 toxoflavin at 30°C. Representative image for *L. pneumophila* strain Philadelphia-1 at 0 and 10 µg
342 is shown in A. The average and standard deviation of the size of the zone of inhibition for each
343 concentration tested in triplicate for *L. pneumophila* strain Quebec and Philadelphia-1 is shown in
344 B.

345

346 **3.5 Toxoflavin is secreted by *P. alcaliphila* on CYE agar plate**

347 In order to confirm that *P. alcaliphila* produces toxoflavin, we performed chloroform extraction
348 from CYE plate inoculated with a pure culture of *P. alcaliphila.* Controls included an extract from
349 a sterile CYE plate and the methanol carrier alone. *L. pneumophila* growth was inhibited by the
350 extract from plates inoculated with *P. alcaliphila* (Figure 7C) slightly more than sterile CYE and
351 methanol (Figure 7A and B), with zone of inhibitions of 12, 10 and 10 mm, respectively. In order
352 to confirm that toxoflavin was present, the extracts were then subjected to LC-ESI/MS. Pure
353 toxoflavin solution produced a strong peak at m/z= 194.0 (Figure 7D). The same strong peak
354 appeared in extract from *P. alcaliphila* plate extracts (Figure 7F) while being absent in the control

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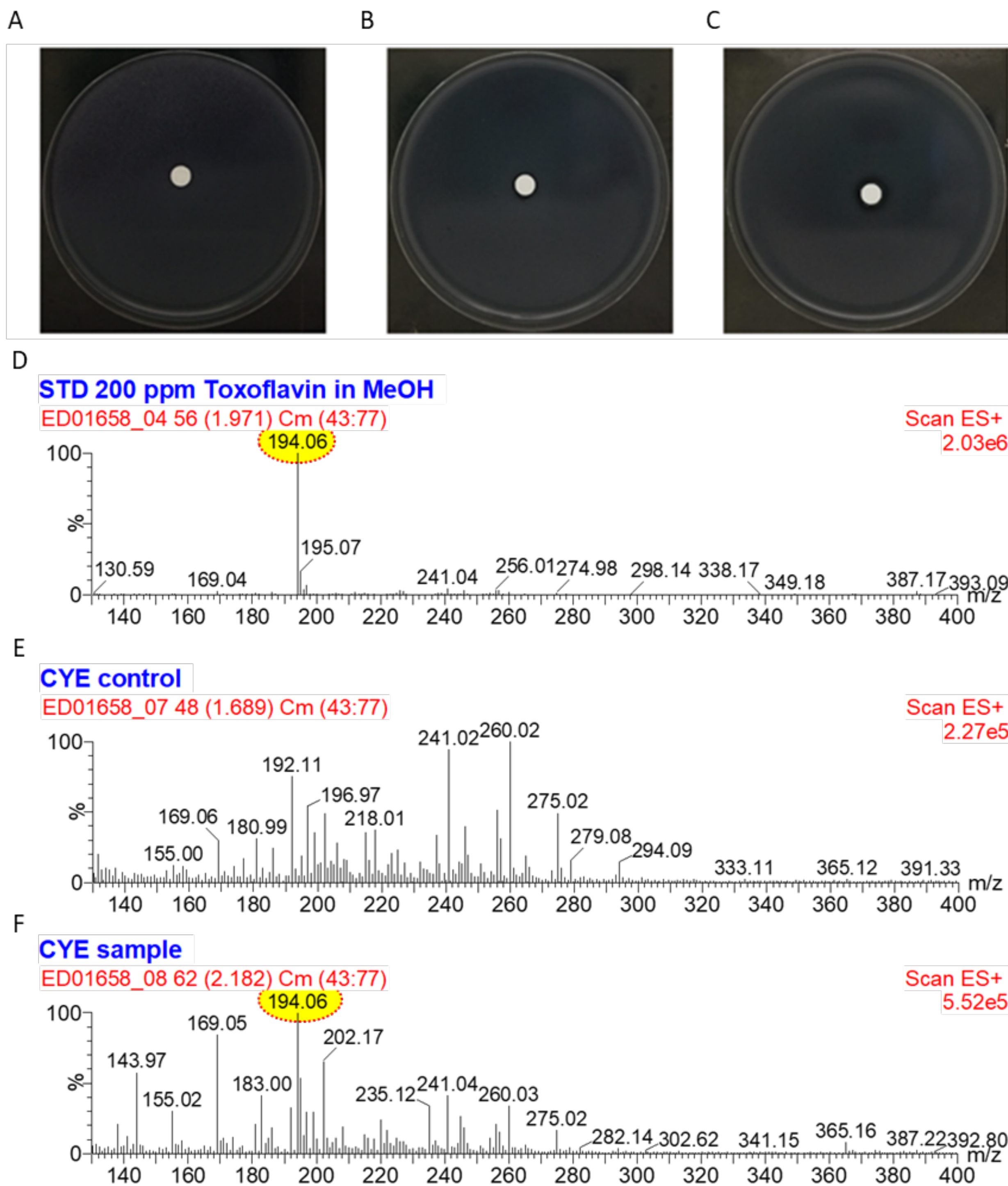
355 (Figure 7E). These results were also confirmed with LC-ESI-MS/MS in MRM mode
356 (Supplementary Figure S1).

357



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358 **Figure 7. Organic extract of *P. alcaliphila* culture inhibits *L. pneumophila* growth and**
359 **contains toxoflavin.** Disc diffusion assay was used to test the growth inhibition activity of
360 methanol (A), organic extracts from sterile CYE plate (B), and CYE plate covered by a lawn of *P.*
361 *alcaliphila* (C). The production of toxoflavin (m/z = 194.0) by *P. alcaliphila* was confirmed with
362 LC-ESI-MS by analysing the mass spectrum of pure toxoflavin (D) and comparing it to those of
363 extract from sterile CYE plate (E) and extract from CYE plate covered by *P. alcaliphila* (F).
364
365 **3.6 *P. alcaliphila* produces floating biofilm mat**
366
367 Many *Pseudomonas* species can produce attached biofilms or floating biofilm mats (Mann and
368 Wozniak, 2012). Therefore, we investigated the ability of *P. alcaliphila* JCM 10630 to form these
369 structures. First, we tested the production of attached biofilm in R2A, King's B, and in trypticase
370 soy broth at room temperature under shaking. After one week, no attached biofilm was seen,
371 however a filamentous floating mass of cells could be seen in both R2A and trypticase soy broth
372 (Figure 8A). We then tested the production of a pellicle by incubating *P. alcaliphila* in the same
373 three media but without shaking. No pellicle was formed in any of these media (Figure 8B).
374 Nevertheless, we could see a mat at the bottom of the well in all case. Shaking for 1 h dislodge the
375 mat produced in trypticase soy broth and R2A, resulting in a floating mat similar to what was seen
376 after incubation with shaking (Figure 8A). We therefore concluded that *P. alcaliphila* may
377 produces floating biofilm mats in cooling towers.
378

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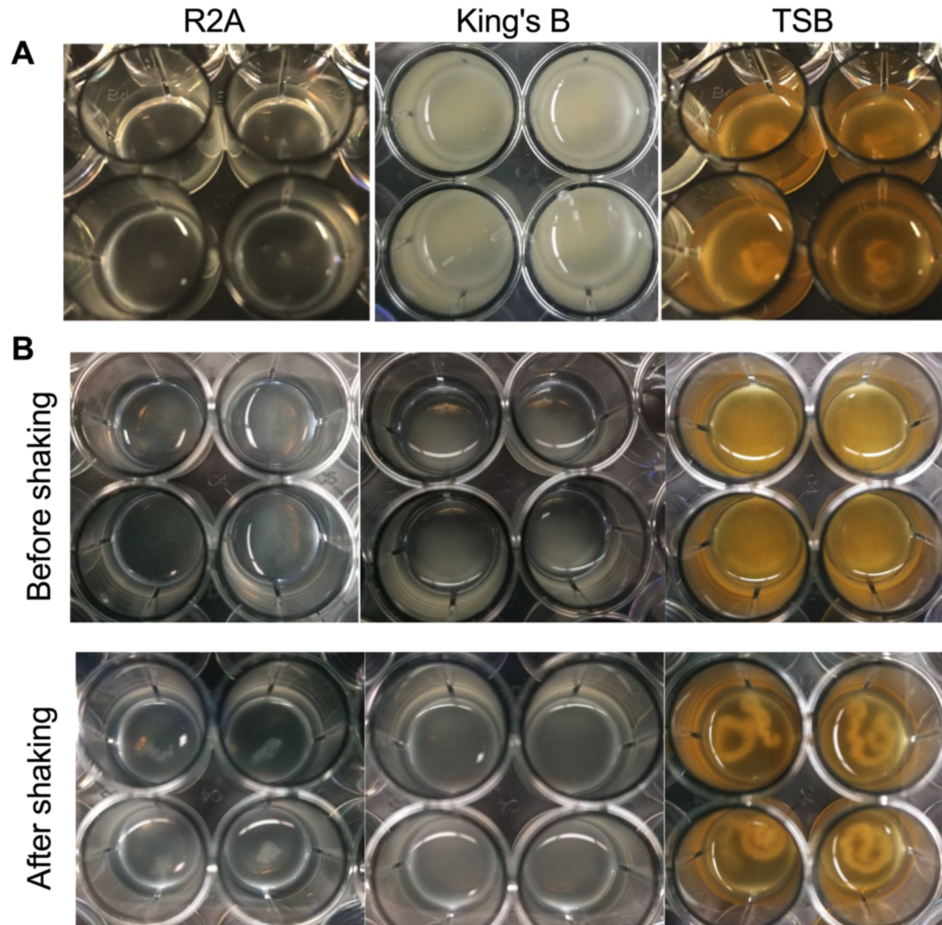

379

380 **Figure 8. *P. alcaliphila* produced floating biofilm mat.** A) R2A, King's B and trypticase soy
381 broth were inoculated with *P. alcaliphila* and incubated at room temperature with shaking (150
382 rpm). B) R2A, King's B and trypticase soy broth was inoculated with *P. alcaliphila* and incubated
383 at room temperature without shaking. After incubation, plates were shaken (150 rpm) for 1h.
384 Representative images of 4 wells of a 24-well plates are shown.

385

386 **3.7 Toxoflavin is toxic for *Vermamoeba vermiformis***

387

388 Since *P. alcaliphila* was also negatively correlated with the presence of host cells in cooling towers
389 (Paranjape et al., 2020a), we next hypothesize that toxoflavin might be toxic for amoebas typically

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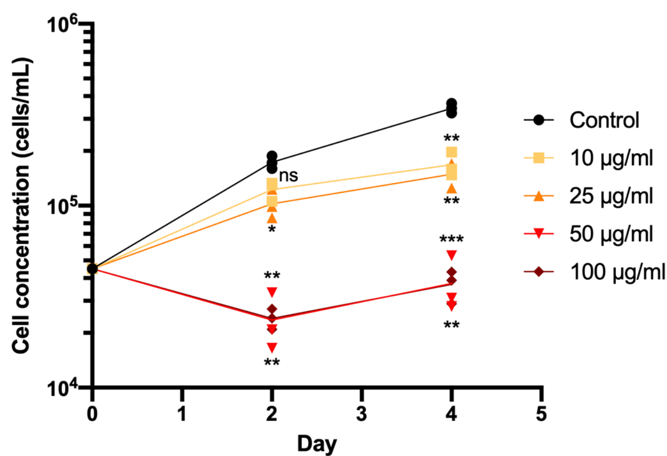
390 found in water systems. Therefore, we monitored the growth of *V. vermiformis* when exposed to
391 toxoflavin (Figure 9). Within four days, cells unexposed to toxoflavin grew by 7.6-fold. In contrast,
392 cells exposed to 10 µg/ml and 25 µg/ml, grew much less, by a factor of 3.7 and 3.3 respectively.
393 Cells exposed to higher concentration show a sharp decrease in cell number at day 2. By day 4 the
394 cells have grown back to the number of cells present at the start of the experiment, suggesting that
395 toxoflavin is metabolized by *V. vermiformis* over time.
396



397

398 **Figure 9: Toxoflavin inhibits axenic growth of *V. vermiformis*.** *V. vermiformis* was cultured in
399 PYFNH to a concentration of 50,000 cells/mL and exposed to 10 µg/ml, 25 µg/ml, 50 µg/ml and
400 100 µg/ml of toxoflavin. Cultures without toxoflavin served as a control. Cell concentration was
401 determined using flow cytometry at day 0, 2 and 4. A two-way ANOVA with a Tukey correction
402 for mutltiple comparison was used to access significance of each test conditions compared to the
403 control (ns, non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)
404

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405

406 **4. DISCUSSION**

407

408 Several previous studies have shown an inverse relationship between the presence of *L.*
409 *pneumophila* and *Pseudomonas* in cooling towers (Llewellyn et al., 2017; Paranjape et al., 2020b).
410 By using *Pseudomonas*-specific 16S rRNA amplicon sequencing approach (Pereira et al., 2018),
411 we identified that members of *P. alcaliphila/oleovoran* cluster are the main species associated with
412 the exclusion of *L. pneumophila* from cooling towers. The dominance of *P. alcaliphila/oleovorans*
413 is surprising as a previous study of a single cooling towers located in Braunschweig, Germany,
414 found a high diversity of species even when *P.alcaliphila/oleovorans* was a member of the core
415 community (Pereira et al., 2018). Differences in source water or cooling tower management could
416 explain the dominance of *P. alcaliphila/oleovorans* in our cooling tower samples compared to
417 those in Germany. The diversity and abundance of *Pseudomonas* may also be affected by seasons,
418 but this could not be investigated with our current data set. Importantly, the presence of *P.*
419 *alcaliphila/oleovorans* was strongly associated with continuous disinfectant application. This
420 strategy presumably creates conditions favorable for this group or specific members of this group.

421

422 Our analysis also revealed that some species of *Pseudomonas* may be beneficial for *L.*
423 *pneumophila*. LEfSe analysis suggest that members of the *P. monteilii* cluster and *P. alcaligenes*
424 are positively associated with *L. pneumophila* (Figure 4A). For example, it has been reported that
425 in an environment lacking critical nutrients for its growth, *L. pneumophila* can form microcolonies
426 around certain aquatic bacteria including *Flavobacterium breve* (Wadowsky and Yee, 1983) and
427 *Pseudomonas alcaligenes* (Çotuk et al., 2005).

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428

429 Unfortunately, the method used is not able to differentiate between members of the *P.*
430 *alcaliphila/oleovorans* cluster as the region targeted is identical. In addition to *P. alcaliphila* and
431 *P. oleovorans*, this cluster also contain *P. chengduensis* (Pereira et al., 2018). These three species
432 are associated with various water environment (Peix et al., 2018; Tao et al., 2014). It is possible
433 that the cooling towers studied here contain a diversity of species belonging to this cluster. The
434 water of the cooling towers included in this study was typically between 20-25 °C and pH 8
435 (Paranjape et al., 2020b). This falls within the conditions that *P. alcaliphila* JCM 10630 can thrive
436 in, having been isolated from sea water and shown to be alkali-tolerant and psychrophilic, growing
437 best at temperature between 4 and 30 °C (Yumoto et al., 2001).

438

439 In this study, we found that *P. alcaliphila* was able to inhibit *L. pneumophila* growth was, at least
440 in part, through toxoflavin production. We found that 0.5 µg of toxoflavin directly inhibit *L.*
441 *pneumophila* growth on plates, and that a concentration of 25 µg/mL inhibits the growth of *L.*
442 *pneumophila* host *V. vermiformis.* Genomic analysis revealed that *P. alcaliphila* also contains a
443 homologue of the toxoflavin biosynthetic cluster and the presence of toxoflavin was confirmed in
444 corresponding organic extracts. Inhibition appeared to be temperature-dependent since *P.*
445 *alcaliphila* inhibited *L. pneumohila* growth at 30°C but not 37 °C. Multiple mechanisms could
446 explain this phenomenon. It could be that the rate of growth of *L. pneumophila* is far greater than
447 *P. alcaliphila* at 37°C, and so outpaced the accumulation of toxoflavin. The other possibility is
448 that temperature affects toxoflavin production. To our knowledge, regulation of toxoflavin
449 production by temperature has not been reported in *Pseudomonas spp*. However, *Pseudomonas* is
450 recognized for having complex quorum sensing systems, which have been studied extensively in

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451 *P. aeruginosa* (Chadha et al., 2021). Therefore, the regulation of toxoflavin production by quorum
452 sensing could explain this result. The toxoflavin biosynthetic gene cluster has been thoroughly
453 studied in detail in *B. glumae* and in *Pseudomonas protegens* (Chen et al., 2012). In *B. glumae*,
454 toxoflavin is regulated by quorum sensing system involving TofI, encoding the *N*-octanoyl
455 homoserine lactone synthase, and the associate receptor TofR (Chen et al., 2012; Kim et al., 2004).
456 In turn, TofR induced expression of ToxJ, which induced expression of ToxR, the main regulator
457 of the toxoflavin biosynthesis and transporter loci (Chen et al., 2012; Kim et al., 2004). The *P.*
458 *alcaliphila* JCM 10630 genome contains a homologue of tofR (40% identity) but no homologue
459 of TofI could be identified. Whether or not toxoflavin production is regulated by quorum sensing
460 system in *P. alcaliphila* will require additional experiments.
461
462 It is extremely unlikely that *P. alcaliphila* can produce enough toxoflavin for it to accumulate in
463 cooling towers and reach inhibitory concentrations in bulk water. However, local concentrations
464 in biofilms or floating mats could potentially reach inhibitory or lethal concentrations for *L.*
465 *pneumophila* and/or *V. vermiformis* since *Pseudomonas* are known to form a diversity of biofilm
466 structure (Koza et al., 2020). Indeed, we showed that *P. alcaliphila* cells can aggregate in pure
467 culture to form floating mats, but not an attached biofilm. This does not eliminate the possibility
468 that *P. alcaliphila* can colonize surfaces in water system or multispecies biofilm. We also cannot
469 rule out that other compounds contributed to the inhibition of *L. pneumophila* by *P. alcaliphila*.
470 Of note, RiPP-like type biosynthetic gene clusters includes bacteriocin and other antimicrobial
471 peptide-derived molecules (Arnison et al., 2012). Similarly, it is possible that the member of the
472 *P. alacaliphila/oleovorans* cluster found in cooling towers is different from the isolate used in this

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473 study, and so other or additional BCG and corresponding molecules could be acting as *L.*
474 *pneumophila* inhibitors.
475
476
477 **5. CONCLUSION**
478
479 *Pseudonomas* specific 16S amplicon sequencing increased the species-level resolution of our data
480 which allowed us to narrow down candidate species responsible for the exclusion of *L.*
481 *pneumophila* in cooling towers. The absence of *L. pneumophila* was most strongly associated with
482 the presence of *P. alcaliphila/oleovorans*. Inspection of the genome of a member of *P.*
483 *alcaliphila/oleovorans* revealed a biosynthetic gene clusters (BCGs) homologous to toxoflavin
484 synthesis cluster of *B. glumae*. Using LC-MS/MS, toxoflavin was found in extracts from *P.*
485 *alcaliphila* agar. Toxoflavin is known to be toxic to many microorganisms, we confirmed that this
486 is also the case for both *L. pneumophila* and its host *V. vermiformis* using commercial toxoflavin.
487 To our knowledge, this is the first report of a *P. alcaliphila* strain that produces toxoflavin and
488 which inhibits the growth of *L. pneumophila*. In water systems, this inhibitory effect would be
489 strongest near or within densely populated biofilms or floating mats that *P. alcaliphila* is capable
490 of forming in different media. Finally, toxoflavin was only one of many candidate molecules, as
491 other biosynthetic gene clusters were identified in *P. alcaliphila*. Multiple molecules could be
492 contributing to inhibitory effects of *Pseudomonas* in cooling towers and will need to be
493 investigated.
494

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502

503 **COMPETING INTEREST STATEMENT**

504 The authors have no competing interest to declare.

505

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704 **TABLES**

705

706 **Table 1. Biosynthetic gene clusters identified in the genome of *P. alcaliphila* JCM 10630 with**

707 **antiSMASH.**

Location in JCM 10630	Type	Most similar cluster	Similarity
50,792 - 83,086	Betalactone	Fengycin	20 %
80,430 - 92,292	Siderophore	Putrebactin	30 %
176,284-198,514	Redox-cofactor	Lankacidin	13 %
199,774 - 214,675	NAGGN[1]	Pyoverdine	2 %
494,171 - 505,013	RiPP-like[2]	None	NA
1,579,397 - 1,632,252	Aryl polyene	Aryl polyene of *Vibrio fisheri*	45 %

708 [1] N-acetylglutaminylglutamine amide

709 [2] Ribosomally synthesised and post-translationally modified peptide product

710

711 **Table 2. Comparison of the genes in *P. alcaliphila* toxoflavin cluster with *B. glumae***

712 **homologues**

Locus tag	Protein product	Length	Annotated function	Name	*B. glumae* homologue (% identity)
PAL02S_RS12525	WP_074675559.1	388	bifunctional diaminohydroxyphosphoribosylaminopyrimidine deaminase/5-amino-6-(5-phosphoribosylamino)uracil reductase	ToxE	bglu_2g06440 (42%)
PAL02S_RS12530	WP_074675558.1	562	WD40 repeat domain-containing protein	ToxC	bglu_2g06420 (47%)
PAL02S_RS12535	WP_074675557.1	201	GTP cyclohydrolase II	ToxB	bglu_2g06410 (44%)
PAL02S_RS12540	WP_139202874.1	340	SUMF1/EgtB/PvdO family nonheme iron enzyme	ToxD	bglu_2g06430 (54%)
PAL02S_RS12545	WP_074675555.1	245	class I SAM-dependent methyltransferase	ToxA	bglu_2g06400 (68%)
PAL02S_RS12550	WP_074675554.1	302	LysR family transcriptional regulator	ToxR	bglu_2g06390 (69%)
PAL02S_RS12555	WP_074675553.1	157	DMT family transporter	ToxF	bglu_2g06380 (64%)
PAL02S_RS12560	WP_074675552.1	364	efflux RND transporter periplasmic adaptor subunit	ToxG	bglu_2g06370 (36%)
PAL02S_RS12565	WP_074675551.1	1023	MexW/MexI family multidrug efflux RND transporter permease subunit	ToxH	bglu_2g06360 (55%)

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714