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Biopurification system as a source of pesticide-tolerant bacteria able to degrade the commonly used pesticides chlorpyrifos and iprodione

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Abstract

Intensive use of pesticides applied simultaneously in field to improve the effectiveness of pest control increase the environmental contamination, affecting the soil and water quality. Some of the commonly used pesticides are the insecticide chlorpyrifos and the fungicide iprodione; being thus critically essential to develop bioremediation methods to remove these contaminants by tolerant-bacteria. In this study we selected and characterized different pesticides-tolerant bacteria isolated from a biomixture of a biopurification system that had received continuous applications of a mixture of the pesticides chlorpyrifos and iprodione. Out of the 10 isolated bacterial colonies, only six strains presented adequate growth in presence of the both pesticides at 100 mg L^{-1}. Biochemical and enzymatic characterization using API ZYM showed that all isolates (100%) were positive for esterase, leucine aminopeptidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase. According to the molecular level study of the 16S ribosomal gene and MALDI TOF/TOF MS, it was possible to determine that the isolated bacteria belong to the genera *Pseudomonas*, *Rhodococcus* and *Achromobacter*. Bacterial growth decreased proportionally ($R^2 > 0.96$) as been as both pesticide concentrations increased from 10 to 100 mg L^{-1}. *Achromobacter* sp. strain C1 showed the best chlorpyrifos removal (between 56–29%) after 120 h of incubation. On the other hand, the highest iprodione removal (between 91.2–98.9%) was observed for the *Pseudomonas* sp. strain C9, which was not detected after 48 h of incubation. According with their identification and ability to remove the contaminants, *Achromobacter* sp. strain C1 and *Pseudomonas* sp. strain C9 appear as promising microorganisms for their use in the treatment of matrices contaminated with chlorpyrifos, iprodione or their mixture. The results of this study will help to improve current technologies for the biodegradation of this commonly used insecticide and fungicide, in order to give a response to the problem of contamination by pesticides.

Keywords: isolation, tolerant-bacteria, iprodione, chlorpyrifos, biopurification.

Introduction

Different pesticides are applied simultaneously in the field to improve the effectivity of pest control, thus increasing environmental contamination and affecting the soil and water quality [1,2]. Toward minimizing pesticide point-source contamination, a preventative technology of biopurification named biobed was introduced and implemented in Sweden in the 90s by Torstensson and Castillo [3] to reduce the risk of water resources contamination. Pesticides removal by this biopurification technology is based on the adsorption and degradation capacity of an organic biologically active matrix (biomixture) composed of top soil, peat, and

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74 lignocellulosic material and a vegetal layer [4,5]. The biopurification system is highly
75 efficient in pesticide removal, achieving high degradation of different pesticides commonly
76 applied in farms, even after repeated applications [6,7]. The microbial communities are
77 considered a key factor to control the depuration capacity of the biopurification system, and
78 knowledge of the biological activity occurring in the biomixture is relevant for understanding
79 pesticide degradation and to optimize their degradation [7–9]. In this respect, some studies
80 have correlated pesticides degradation in the biomixture with microbial activities such as
81 phenoloxidase activity [10–12], respiration rate [13], and microbial community changes
82 [6,13–15].

83 A genotypically and phenotypically versatile microbial community can degrade different
84 pesticide residues at different concentrations in a biomixture [6]. A greater bacterial diversity
85 compared to fungal diversity has been reported, such that bacterial diversity increased
86 throughout the biopurification system as affected by pesticide exposure [16].

87 It is well known that microorganisms are responsible for the degradation of pesticides in soils
88 [17,18]. This is due to the extensive use of these compounds in agricultural soils, which has
89 induced mechanisms of genetic adaptation in microorganisms. These genetic adaptations
90 have led to the synthesis of enzymes that oxidize, hydrolyze, and hydroxylate pesticides,
91 allowing them to use pesticides as the sole source of carbon, nitrogen, sulfur, or phosphorus
92 and facilitating the elimination of the compound's toxicity [19]. Further, active microbial
93 populations develop in the soil with the ability to degrade persistent compounds after
94 repeated pesticide application in the same field for a certain number of years [20].

95 Although bacterial species play important roles in the transformation of pesticides, the
96 complete mineralization of pesticide residues is more likely to occur with mixed populations
97 than individual microorganisms [21]. Fungi have also been reported as good pesticide

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98 degraders. A new fungal strain Hu-01 isolated from an activated sludge sample from an
99 aerobic chlorpyrifos-manufacturing wastewater treatment plant, identified as *Cladosporium*
100 *cladosporioides*, showed high chlorpyrifos degradation activity [22].
101 Among bacteria responsible for the degradation of pesticides, the genera *Streptomyces*,
102 *Arthrobacter,* and *Achromobacter* have been isolated from contaminated soil and soil with
103 historical application and studied due to their great capacity to degrade various pesticide
104 residues, including CHL and IPR [23–26].
105 Campos *et al*. [25] reported two strains, *Arthrobacter* sp. strain C1 and *Achromobacter* sp.
106 strain C2 isolated from soil, which are able to transform IPR and its degradation metabolite
107 (3,5-dichloraniline) in different culture media. The degradation of IPR by the *Arthrobacter*
108 strain C1 proceeded rapidly in all media with complete degradation observed within 8 and
109 24 h of culture, and this strain maintained its degrading capacity in a wide range of
110 temperatures and pH. In contrast, *Achromobacter* sp. strain C2 was only able to slowly co-
111 metabolize IPR. Additionally, metabolic intermediates 3,5-dichlorophenyl-carboxamide and
112 3,5-dichlorophenylurea-acetate in the metabolic IPR pathway, produced by these soil
113 bacteria and their combination, were reported by Campos *et al*. [27].
114 Briceño *et al*. [23] reported two *Actinobacteria* isolated from an agricultural soil that had
115 received continuous applications of CHL, which were able to rapidly degrade CHL with
116 approximately 90% degradation after 24 h of incubation. These two strains were identified
117 as *Streptomyces* sp. (AC5 and AC7 strains). Despite the high CHL degradation by both
118 strains, a different behavior was observed when its main metabolite, 3,5,6-trichloro-2-
119 pyridinol (TCP), was analyzed. A lower concentration of TCP (0.46 mg L^{-1}) was produced
120 by *Streptomyces* sp. strain AC5, and its concentration decreased as a function of time, as the

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121 TCP produced was 10 times lower compared to that produced by *Streptomyces* sp. AC7 strain
122 (4.32 mg L^{-1}).
123 Further, several studies have reported the potential of indigenous microbial consortia isolated
124 from contaminated soils to degrade different pesticides and pesticide mixtures. In this
125 context, Fuentes *et al*. [28] reported a *Streptomyces* sp. consortium able to remove an
126 organochlorine pesticide mixture composed of lindane, methoxychlor, and chlordane.
127 Recently, mixed cultures of the fungus *Trametes versicolor* and *Streptomyces* spp. were used
128 to inoculate different biomixtures based on their previously demonstrated ligninolytic and
129 pesticide-degrading activities [21]. The authors demonstrated that the consortium improved
130 lindane dissipation (81–87%) or removal at 66 d of incubation in different biomixtures,
131 decreasing the lindane half-life to an average of 24 d, 6-fold less than the T50 of lindane in
132 soils. In addition, Briceño *et al*. [29] reported for the first time the removal of the
133 organophosphorus pesticides mixture composed of CHL and diazinon from different
134 environmental matrices (liquid medium, soil, and a biobed biomixture) by a *Streptomyces*
135 mixed culture.
136 The previous studies mentioned have reported the ability of selected bacteria isolated from
137 pesticide-contaminated soils to remove pesticides. However, the isolation and
138 characterization of pesticide-degrading microorganisms from a biopurification system used
139 for pesticide treatment have been scarcely studied. Therefore, the goal of this study was to
140 select and characterize bacterial species isolated from a biopurification system and with the
141 ability to degrade the fungicide IPR and the insecticide CHL.
142
143

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144 # Materials and Methods

145 ## Pesticides and culture media

146 Analytical grade (99%) iprodione (IPR), 3,5-dichloroaniline (3,5-DCA), chlorpyrifos (CHL),
147 and 3,5,6-trichoro-2-pyridinol (TCP) for chromatographic analyses by HPLC were
148 purchased from Sigma-Aldrich (St. Louis, MO). The stock solutions (1000 mg L^{-1}) in
149 acetone were sterilized by filtration through 0.22-µm pore-size membranes. For degradation
150 assays, formulated commercial CHL (Troya 4EC) and IPR (Rovral 50 WP) were purchased
151 from Agan Chemicals Manufacturers Ltd. The characteristics of the commercial products are
152 shown in Table 1. Commercial products were prepared individually in a stock solution of
153 10,000 mg L^{-1} in methanol, filtered through a 0.22-mm PTFE filter, and then stored at 4 °C
154 until their use. All other chemicals and solvents were of analytical reagent grade (Merck).

155 **Table 1.** Physicochemical characterization for the tested commercial pesticides.

Pesticide	Commercial product	Concentration	Kind	Chemical formula	Water solubility (mg L^{-1})	Molecular weight (g mol^{-1})	$T_{1/2}$ (d)	GUS	K_{oc}
Chlorpyrifos	Troya 4 EC	480 g L^{-1}	Insecticide	$C_9H_{11}Cl_3NO_3PS$	1.05	350.58	50	0.17	8151
Iprodione	Rovral 50 WP	500 g kg^{-1}	Fungicide	$C_{13}H_{13}Cl_2N_3O_3$	6.8	330.17	36.2	0.58	700

156 Solubility in water at 20 ºC; $T_{1/2}$: Time half-life, GUS: Groundwater Ubiquity Score; Koc: Adsorption coefficient.

157

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158 Mineral salts medium (MSM) broth containing (per L) 1.6 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g
159 $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2$, and 1 mL salt stock solution (2.0 g boric acid, 1.8
160 g $MnSO_4 \cdot H_2O$, 0.2 g $ZnSO_4$, 0.1 g $CuSO_4$, 0.25 g Na_2MoO_4, 1000 mL distilled water) was
161 used for pesticide degradation assay. The initial pH of the medium was adjusted to 7.0 prior
162 to sterilization by autoclaving (121 °C for 20 min). Subsequently, cycloheximide (0.05 g L^{-1}
163) was added to avoid fungal contamination. Luria Bertani (LB) broth containing (per L) 5.0
164 g NaCl, 2.5 g yeast extract, and 10.0 g casein peptone was used for routine cultivation of the
165 isolated bacteria. The pH of LB was adjusted to 7.0 prior to autoclaving. Plate count agar
166 (PCA) containing (per L) 5.0 g tryptone, 2.5 g yeast extract, 1.0 g glucose, and 15.0 g agar-
167 agar was adjusted to pH 7.2 prior to sterilization, and 0.05 g cycloheximide was added to
168 avoid fungal contamination. Finally, R2A agar containing (per L) 0.5 g casein acid
169 hydrolysate, 0.5 g yeast extract, 0.5 proteose peptone, 0.5 g dextrose, 0.5 g soluble starch,
170 0.3 g K_2HPO_4, 0.024 g $MgSO_4$, 0.3 g sodium pyruvate, and 15 g agar, pH 7.2 was used for
171 strains biochemical characterization.
172
173 **Biopurification system for isolation of pesticide-tolerant bacteria**
174 Pesticide-tolerant bacteria were isolated from a biopurification system (BPS) used during the
175 last three years for pesticide treatment (CHL and IPR at 50 mg kg^{-1} a.i. each) with re-
176 application every 30 days 1/1/0001 12:00:00 AM. The BPS consisted of a plastic tank of 1
177 m^3 capacity packed with 125 kg of biomixture (dry weight) (bulk density (ρ) 0.29 g mL^{-1}),
178 which reached a height of 60 cm. The biomixture used in BPS was prepared with top soil,
179 commercial peat, and wheat straw in a proportion of 1:1:2 (v v^{-1}), and humidity was
180 maintained at about 65–70% of water holding capacity (WHC) by addition of tap water.

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181 For strain isolation, biomixture subsamples were collected from different parts of the BPS,
182 and a composite sample (500 g) was stored at 4 °C for no longer than 12 hours.
183 Microorganisms in the biomixture were counted using the serial dilution method. For this,
184 10 g biomixture was added to 90 mL saline solution (0.9%), and the suspension was shaken
185 vigorously. Subsequently, 150-μL aliquots of each dilution were inoculated on Petri dishes
186 containing PCA medium. Incubation was performed at 28 ± 2 °C for 48 h, following which,
187 the colonies formed were counted.

188 Pesticide-tolerant bacteria were isolated by placing 10 g of the biomixture in sterile 250-mL
189 Erlenmeyer flasks containing 90 mL of MSM broth supplemented with CHL plus IPR at 10
190 mg L^{-1} a.i. each). Flasks were incubated for 7 days at 28 ± 2 °C and 130 rpm with constant
191 shaking in the dark. After this period, decimal dilutions from 1×10^{-1} to 1×10^{-4} were
192 prepared in order to obtain perfectly separated strains. For this, 65-μL aliquots of each
193 dilution were inoculated on Petri dishes with 30 mL of PCA medium. Plates were incubated
194 at 28 ± 2 °C for seven days, and morphology and coloration of the colonies were analyzed
195 for bacterial selection. The bacterial strains were maintained on LB-glycerol (70/30%)
196 medium slants at 4 °C, and they were filed at the Laboratory of Environmental Biotechnology
197 in La Frontera University.

198 To examine the ability of the strains to grow in the presence of pesticides (CHL and IPR), a
199 quantitative assay was performed. The study consisted of evaluating biomass growth in flasks
200 containing 50 mL of LB broth supplemented with each pesticide at 10 mg L^{-1} concentration.
201 The flasks were incubated at 28 ± 2 °C and 130 rpm under constant shaking during 48 h, and
202 bacterial growth was measured by measuring the absorbance at 600 nm. Thereafter,
203 absorbance values were converted to biomass dry weight (g L^{-1}) using a calibration curve (R^2
204 > 0.999).

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205 **Characterization of selected pesticide-tolerant strains**

206 The selected bacterial strains were characterized by a combination of phenotypic tests as
207 described by Krishnapriya *et al*. [30], which are based mainly on colony morphology, Gram
208 staining reaction, and colony pigmentation.

209 Visualization of pesticide-tolerant bacterial cells was performed using Scanning Electron
210 Microscopy (SEM) with variable pressure (VP-SEM) and the instrument equipped with a
211 STEM detector (Transmission Module) (SU-3500 Hitachi-Japan). The strain samples were
212 obtained from a fresh 24-h culture in PCA. The bacterial colonies were washed three times
213 with distilled water, and the pellet was re-suspended in LB medium at 0.5 McFarland with
214 distilled sterile water and acetylchlorine (0.1%). A sample of 65 µL of each strain was placed
215 in the equipment sampler and dried at 30°C, followed by microscopic observations.

216 The strains were subjected to biochemical characterization using the APIZYM kit
217 (Biomerieux, France) according to the manufacturer's instructions. This microbial
218 identification system consists of 19 substrates in a microplate, which was incubated at 28 °C
219 for up to 4 days. The enzyme activity was detected based on the intensity of color developed
220 following the addition of reagents.

221 Moreover, extracellular hydrolyzing enzyme production was screened as described by
222 Margesin *et al*. [31]. The presence of amylase, cellulase, lipase, protease, and gelatinase
223 activity was tested on R2A agar supplemented with starch (0.4% w v^{-1}),
224 carboxymethylcellulose and trypan blue (0.4% and 0.01% w v^{-1}), Tween 80 (1% v v^{-1}), skim
225 milk powder (0.4% w v^{-1}), or gelatin (1% w v^{-1}), respectively. The agar plates were prepared
226 in triplicate. After 3–10 days at 15 °C, a positive reaction was observed when transparent
227 zones around the colonies were directly visible or detected after precipitation or coloration

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228 of the non-degraded substrate. To reveal amylase and protease activities, the plates were
229 stained with Lugol's solution and Coomassie brilliant blue solution, respectively [31]. The
230 assays were performed in triplicate plates.
231

232 Bacterial identification by sequence analyses and MALDI-TOF/TOF MS

233 For identification of pesticide-tolerant bacteria, genomic DNA was extracted using the
234 UltraClean® Microbial DNA Isolation Kit (MOBIO, CA, USA) according to the
235 manufacturer's instructions. 16S rDNA was selectively amplified from genomic DNA by
236 polymerase chain reaction (PCR) using universal primers 27F (5'-
237 AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'),
238 enabling the amplification of approximately 1.500 bp of the 16S rRNA gene. PCR
239 amplification was performed in a Multigene Optimal Thermal Cycler (Labnet, USA) in 50
240 μL of PCR mix comprising 25 μL mix reaction buffer 2x (SapphireAmp Fast PCR Master
241 Mix, Takara), 22 μL ultra-pure water, 1 μL of each primer (10 μM), and 1 μL of DNA. The
242 temperature and cycling conditions were as follows: preheating at 94 ºC for 2 min; 30 cycles
243 at 94 ºC for 1 min; 55 ºC for 1 min; 72 ºC for 1.5 min; and incubation at 72 ºC for 10 min.
244 The presence of PCR products was assessed by electrophoresis on a 1% agarose gel stained
245 with gel red. Sequencing was conducted using a dye Terminator Cycle Sequencing Kit and
246 an ABI 3730XL DNA Sequencer (Applied Biosystems) by Macrogen (Korea). The nearest
247 taxonomic group was identified by 16S rDNA nucleotide sequence BLASTN
248 (http://www.ncbi.nlm.nih.gov/blast) using DDBJ/EMBL/GenBank nucleotide sequence
249 databases. The phylogenetic affiliation of bacteria in GenBank was performed using
250 MEGA7. For the MALDI-TOF/TOF MS analysis, samples of selected bacterial colonies

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251 were applied directly to the equipment sampler plate and coated with a saturated solution of
252 α-cyano 4-hydroxy cinnamic acid diluted in 50% acetonitrile with 2.5% trifluoracetic acid.
253 Mass spectra were obtained using a MALDI-TOF/TOF MS Autoflex Speed (Bruker
254 Daltonics, Bremen, Germany) equipped with a smart beam laser source (334 nm). Analyses
255 were performed in linear mode with positive polarity, acceleration voltage of 20 kV, and
256 extraction with delay of 220 ns. Each spectrum was collected as an average of 1200 laser
257 shots with enough energy to produce good spectra without saturation in the range of 2000 to
258 20,000 m/z. Analyses equipment was calibrated externally using the protein calibration
259 standard I (Bruker Daltonics, Bremen, Germany) (insulin, ubiquitin, cytochrome C and
260 myoglobin) with Flex Control 1.4 software (Bruker Daltonics, Bremen, Germany). The
261 sample analyses were performed with the MALDI Biotyper Compass 4.1 software (Bruker
262 Daltonics, Bremen, Germany) in the range of 3000–15000 m/z, compared with a library of
263 6509 spectra of bacterial identifications. According to the guidelines of the manufacturer, a
264 score of ≥ 2 depicts identification to the species level, and an intermediate log score between
265 < 2 and ≥ 1.7 for identification to the genus level. A dendrogram generated by MALDI
266 Biotyper mass spectra was performed for all strains isolated after enrichment with CHL and
267 IPR in liquid cultures.

268

269 Pesticide degradation in liquid culture

270 A pesticide degradation assay was conducted with the six selected pesticide-tolerant and
271 well-characterized strains. For obtaining inocula, the strains were re-activated on plate dishes
272 with PCA medium and incubated for 24–48 h at 28 ± 2 °C. After that, the bacteria were
273 initially grown at 28 ± 2 ºC for 48 h in Erlenmeyer flasks with LB broth supplemented with

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274 a mixture of 10 mg L^{-1} of IPR and CHL to acquire enough biomass for downstream
275 inoculation. Biomass was collected by centrifugation (6000 rpm for 10 min), washed, and re-
276 suspended using sterile NaCl (0.9%). The degradation experiments in liquid media were
277 conducted in 100-mL flasks that contained 50 mL of LB broth supplemented with each
278 individual pesticide at a concentration of 0, 10, 20, 50, and 100 mg L^{-1}. Subsequently, the
279 biomass inoculum was added at 1% (v v^{-1}), and non-inoculated flasks were run as controls.
280 The flasks were then incubated at 28 ± 2 °C on a rotary shaker at 130 rpm in the dark for 48
281 h and 120 h for IPR and CHL, respectively. Samples were taken at different times for analysis
282 of biomass growth, residual pesticide concentrations (IPR and CHL), and metabolite (3,5-
283 DCA and TCP) formation. For biomass growth and pesticide degradation, kinetics
284 parameters were calculated.

285

286 Analyses of pesticides and metabolites

287 One milliliter of each sample was taken directly from each flask, and centrifuged at 6500
288 rpm for 10 min. After that, 0.5 mL of the supernatant was diluted in 1 mL of acetonitrile
289 grade HPLC. The sample was homogenized in a vortex and filtered by filter PTFE 0.22 µm
290 before analysis of pesticide concentrations. Analysis was performed using a Merck Hitachi
291 L-2130 pump equipped with a Rheodyne 7725 injector and a Merck Hitachi L-2455 diode
292 array detector. Separation was achieved using a C18 column (Chromolit RP-8e, 4.6 µm ×
293 100 mm). The mobile phase was 70% 1 mM ammonium acetate and 30% acetonitrile injected
294 at a flow rate of 1 mL min^{-1}. The column temperature was maintained at 30 ± 1 °C; the
295 detector was set for data acquisition at 290 nm. Instrument calibrations and quantifications
296 were performed against pure reference standards (0.01–10 mg L^{-1}) for each pesticide.

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297 Average recoveries for the pesticide were: IPR, 92 ± 2.2%; CHL, 101 ± 0.7%. Limit of
298 quantification (LOQ) was determined using the smallest concentration of the analyte in the
299 test sample, which induced a signal that was ten times higher than the background noise level
300 (CHL = 0.214 mg L^{-1} and IPR = 0.238 mg L^{-1}). Limit of detection (LOD) was 0.081 for
301 CHL and 0.089 for IPR.
302

303 **Kinetics and statistical analysis**

304 Data obtained in the degradation assays were used to determine the specific growth rate for
305 the exponential phase using the following equation: μmax = dx/dt × 1/x; where μ= specific
306 growth rate (h^{-1}), x = biomass concentration (g L^{-1}), and t = time (h). The removal of IPR and
307 CHL was described using the first-order kinetic model: ln Ct/C0 e-kt, where C0 is the amount
308 of contaminant in the liquid medium at time zero, Ct is the amount of contaminant at time t,
309 and k and t are the rate constant and degradation time in hours, respectively. The time at
310 which the IPR and CHL concentrations in the liquid medium were reduced by 50% ($T_{1/2}$)
311 was calculated using the equation $T_{1/2}$= ln (2)/k. In degradation study, simple correlation
312 analysis was done to determine correlation between biomass growth and initial pesticide
313 concentration.
314 Data were averaged and the standard deviation (SD) of the means was calculated. Removal
315 percentage data were transformed using an angular transformation (arc sen √x/100) prior to
316 statistical analysis. Post hoc analysis of differences in means of the assay data was conducted
317 with the Tukey test (α=0.05). Statistical analyses were performed using SPSS statistical
318 software version 17.
319

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320 # Results

321 ## Isolated bacteria from the biopurification system

322 Pesticide-tolerant bacteria were isolated from a biomixture used in a biopurification system,
323 which in the last three years had been used to degrade a mixture of pesticides added
324 repeatedly at a concentration of 50 mg L^{-1}. To approximate the number of viable bacteria in
325 the biomixture, a plate count test was performed. The results revealed 23×10^6 UFC g^{-1} of
326 biomixture in the PCA medium.
327 In the present study, 10 different types of bacterial colonies (strains C1–C10) isolated using
328 PCA medium were obtained after enrichment with CHL plus IPR (10 mg L^{-1} a.i. each) from
329 the biomixture. Out of the 10 bacterial colonies, only six strains presented adequate growth
330 expressed as biomass concentration ≥ 1.0 g L^{-1} in LB broth, which had been supplemented
331 with a mixture of CHL and IPR (10 mg L^{-1} a.i. each). Specifically, strains C4, C9, and C10
332 showed a biomass growth of > 2.0 g L^{-1}, while other strains showed a biomass growth
333 between 1.2 and 1.9 g L^{-1}. Considering these growth results, the six previously mentioned
334 strains were used for subsequent studies.
335

336 ## Characterization of pesticide-tolerant bacteria

337 The strains selected for their tolerance and ability to grow in the presence of pesticides were
338 characterized based on some phenotypic and biochemical characteristics (Table 2).
339 According to Gram-staining analyses, all strains were Gram-negative, except strain C8. Most
340 isolates exhibited cream-colored colonies; strain C8 was dark-cream colored, and strain C7
341 had white colonies. The morphological characteristics of the bacteria were evaluated by
342 means of SEM. The presented micrographs in Fig. 1 show three different strains with

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343 representative morphological cell structures. Five of the six selected strains were bacillus,
344 while strain C8 presented a coccus shape. In general, strains C4 and C10 with a bacillus cell
345 shape and size ranging from 0.82 × 2.35 µm to 0.91 × 1.84 µm were observed, respectively.
346 All other strains were omitted due to similarities in size. For strain C8 with a coccus cell
347 shape, the sizes ranged from 0.76 to 1.26 µm in diameter.
348

349 **Fig. 1.** Electron scan micrographs of cells morphology of C4 (a), C8 (b) and C10 (c) strains
350 isolated by enrichment from a biomixture of a biopurification system treated repeatedly with
351 pesticides.
352

353 The results of biochemical and enzymatic characterization using API ZYM showed that all
354 isolates (100%) were positive for esterase (C4), leucine aminopeptidase, acid phosphatase,
355 and naphthol-AS-BI-phosphohydrolase. Enzymes, such as alkaline phosphatase and valine
356 aminopeptidase, tested positive in 83% of strains, esterase lipase (C8) in 50% of strains, and
357 lipase (C14) and trypsin in 33% strains. Strain C8 was positive for most enzymes, including
358 α-glucosidase and β-glucosidase involved in glucose metabolism. Finally, screening of
359 pesticide-tolerant strains isolated from the biopurification system showed that C4 and C9
360 strains produced five extracellular enzymes on solid R2A medium. Regarding the production
361 of lipases and amylase, the six selected strains were positive for both enzymes, while strains
362 C1, C4, C8, and C9 were positive for cellulolytic enzymes (Table 2).
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368 **Table 2.** Phenotypic features and biochemical characteristics of different pesticide- tolerant
369 bacteria isolated from biopurificaction system.

	(-)	(+)	Response of strains					
	(%)		C1	C4	C7	C8	C9	C10
Color of colonies			Cream	Cream	White	Dark-Cream	Cream	Cream
Morphology			Bacillus	Bacillus	Bacillus	Coccus	Bacillus	Bacillus
Gram staining			-	-	-	+	-	-
Enzyme*			**C1**	**C4**	**C7**	**C8**	**C9**	**C10**
Control	100	0	-	-	-	-	-	-
Alkaline phosphatase	16.7	83.3	+	-	+	+	+	+
Esterase (C4)	0	100	+	+	+	+	+	+
Esterase lipase (C8)	50	50	-	+	-	+	+	-
Lipase (C14)	66.7	33.3	-	+	-	+	-	-
Leucine aminopeptidase	0	100	+	+	+	+	+	+
Valine aminopeptidase	16.7	83.3	+	+	-	+	+	+
Cystine aminopeptidase	83.3	16.7	-	-	-	+	-	-
Trypsin	66.7	33.3	-	+	-	-	+	-
α- Chymotrypsin	83.3	16.7	-	-	-	+	-	-
Acid phosphatase	0	100	+	+	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	0	100	+	+	+	+	+	+
α-Galactosidase	100	0	-	-	-	-	-	-
β- Galactosidase	100	0	-	-	-	-	-	-
β-Glucuronidase	100	0	-	-	-	-	-	-
α- Glucosidase	83.3	16.7	-	-	-	+	-	-
β- Glucosidase	83.3	16.7	-	-	-	+	-	-
N-acetyl-β- Glucosaminidase	100	0	-	-	-	-	-	-
α- Mannosidase	100	0	-	-	-	-	-	-
α- Fucosidase	100	0	-	-	-	-	-	-
Extracellular hydrolase activity #			**C1**	**C4**	**C7**	**C8**	**C9**	**C10**
Amylolysic (starch 0.4%)	0	100	+	+	+	+	+	+
Cellulolytic (CMC 0.4%)	33.3	66.7	+	+	-	+	+	-
Lipolytic (tween 80%)	0	100	+	+	+	+	+	+
Proteolytic (milk 30%)	66.7	33.3	-	+	-	-	+	-
Proteolytic (gelatin 1%)	66.7	33.3	-	+	-	-	+	-

370 + : Positive reaction, - : Negative reaction; *Analysed by API ZYM kit; # Tested by tested on R2A
371 agar.
372

373

374

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375 **Molecular and proteomic identification of bacteria**

376 The identification of selected strains made by both 16S rDNA sequencing and MALDI-
377 TOF/TOF MS showed similar results. The strains selected for their tolerance and ability to
378 grow in the presence of pesticides CHL and IPR were identified based on 16S rDNA
379 sequence analysis as bacteria belonging to the phylum *Proteobacteria*, family
380 *Alcaligenaceae*, genus *Achromobacter* (strains C1, C7, and C10), and family
381 *Pseudomonadaceae*, genus *Pseudomonas* (strains C4 and C9). Moreover, the phylum
382 *Actinobacteria*, family *Nocardiaceae*, and genus *Rodococcus* (strains C8) was identified. A
383 comparison of the 16S rDNA sequences (entire sequence compared with available sequences
384 in GenBank) of strains C1, C4, C7, C8, C9, and C10 showed ≥ 96% similarity to those of
385 *Achromobacter spanius*, *Pseudomonas rhodesiae*, *Achromobacter deleyi*, *Rhodococcus*
386 *jialingiae*, *Pseudomonas marginalis*, and *Achromobacter kerstersii*, respectively (Table 3).
387 To identify the phylogeny of the isolates, strains from different genera were chosen to
388 construct the phylogenetic tree. Phylogenetic analysis (Fig. 2) based on the 16S rDNA using
389 MEGA7 software indicated that the isolates had higher similarity with the 16S rDNA
390 sequence from pesticide-degrading bacteria, i.e., *Pseudomonas caspiana* (strains C4 and C9),
391 *Rhodococcus jialingiae* (strain C8), and *Achromobacter spirinitus* (C1, C7 and C10).

392

393 **Fig. 2.** Phylogenetic tree constructed by the neighbor-joining method based on 16S rDNA
394 sequences of studied C1, C4, C7, C8, C9, C10 strains and related ones.

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398 **Table 3.** Phylogenetic assignment of isolated strains tolerant to chlorpyrifos (CHL) and
399 iprodione (IPR) and their best match results with 16S rDNA gene sequences and MALDI -
400 TOF TOF BioTyper.
401

Strains	Most closely related strain (NCBI accession no.)[a]	Identity (%)	Acession nº	Identification MALDI Biotyper database	Score[b]
C1	*Achromobacter spanius* (MF624722.1)	96	MK110041	*Achromobacter* sp.	2.36
C4	*Pseudomonas rhodesiae* (R_024911.1)	99	MK110043	*Pseudomonas* sp.	2.31
C7	*Achromobacter deleyi* (NR_152014.1)	97	MK110044	*Achromobacter* sp.	2.06
C8	*Rhodococcus jialingiae* (NR_115708.1)	98	MK110045	*Rhodococcus* sp.	2.54
C9	*Pseudomonas marginalis* (NR_117821.1)	99	MK110046	*Pseudomonas* sp.	2.14
C10	*Achromobacter kerstersii* (NR_152015.1)	98	MK110047	*Achromobacter* sp.	2.04

402 (a) Based on partial sequencing of 16S rDNA gene and comparison with those present in GenBank
403 database from National Center for Biotechnology Information (NCBI) by using BLAST.
404 (b) Log score value of the MALDI-TOF MS identification.
405

406 Direct analysis of intact cells by MALDI-TOF/TOF MS showed a very good spectral quality
407 with score identification of 2.04 to 2.54 (Table 3), safely allowing accurate identification to
408 the genus level. Genus identification of the different strains was in agreement with the 16S
409 rDNA sequence identification. The dendrogram constructed using the MALDI Biotyper data
410 of the six bacteria in the presence of CHL and IPR showed that *Achromobacter* sp. strains
411 C1, C7, and C10 were differentiated and grouped separately when exposed to different
412 pesticides, and a similar response was observed for *Pseudomonas* sp. strains C4 and C9 (Fig.
413 3).
414

415 **Fig. 3.** Dendrogram obtained by MALDI Biotyper Compass 4.1 software (Bruker Daltonics,
416 Bremen, Germany) of C1, C4, C7, C8, C9, C10 strains after enrichment with CHL and IPR
417 of liquid cultures.
418

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419 **Growth and degradation of pesticides in liquid cultures**

420 Biomass growth of the six tolerant-pesticides strains was evaluated at different incubation
421 times and increasing pesticide concentrations, observing that bacterial growth decreased
422 proportionally ($R^2 > 0.96$) as both pesticide concentrations increased from 10 to 100 mg L^{-1}.
423 As observed in Fig. 4, all bacteria exposed to CHL concentrations from 10 to 50 mg L^{-1}
424 showed an increase in biomass over time. However, a high inhibition of biomass growth was
425 observed in all strains cultivated in liquid medium supplemented with 100 mg L^{-1} CHL. In
426 the same way, only *Achromobacter* sp. strain C1 and *Pseudomonas* sp. strain C4 showed the
427 highest tolerance to 100 mg L^{-1} of CHL, which was compared to microbial growth observed
428 in the control treatment without pesticide. In general, the μ max for the studied strains ranged
429 from 0.18 to 0.48 h^{-1} in the treatment without pesticides, and these values decreased showing
430 a μ rate between 0.02 to 0.16 h^{-1} for CHL added at the highest concentration. *Pseudomonas*
431 sp. strain C4 showed to be the most tolerant strain to CHL in relation to the growth observed
432 in the control treatment (Table 4).

433

434 **Fig. 4.** Remaining chlorpyrifos (CHL) (%) and biomass by C1 (a), C4 (b), C7 (c), C8 (d), C9
435 (3), C10 (f) strains at initial CHL concentration of 0, 10, 20, 50 and 100 mg L^{-1}, evaluated
436 during 120 h. (●) CHL 10 mg L^{-1}; (■) CHL 20 mg L^{-1}; (x) CHL 50 mg L^{-1}; (◆) CHL 100
437 mg L^{-1}; (▲) Control without CHL. Continuous line: Remaining CHL (%); Dotted line:
438 biomass (g L^{-1}).

439

440

441

Table 4. First-order kinetics parameter for chlorpyrifos (CHL) and iprodione (IPR) removal and specific growth rate (μ) of strains C1, C4, C7, C8, C9 and C,10 in liquid medium supplemented with 0-100 mg L^{-1} of pesticide individually.

Chlorpyrifos

	10 mg L^{-1}				20 mg L^{-1}				50 mg L^{-1}				100 mg L^{-1}			
Strain	**μ max (h^{-1})**	**R (%)**	***k* (d^{-1})**	**$T_{1/2}$ (d)**	**μ max (h^{-1})**	**R (%)**	***k* (d^{-1})**	**$T_{1/2}$ (d)**	**μ max (h^{-1})**	**R (%)**	***k* (d^{-1})**	**$T_{1/2}$ (d)**	**μ max (h^{-1})**	**R (%)**	***k* (d^{-1})**	**$T_{1/2}$ (d)**
C1	0.17	56.25±0.10 **a**	0.147	4.7	0.16	53.11±0.01 **a**	0.149	4.6	0.11	34.46±0.02 **a**	0.085	8.2	0.09	29.10±0.01 **a**	0.072	9.7
C4	0.30	41.80±0.02 **b**	0.113	6.1	0.29	38.04±0.04 **b**	0.097	7.2	0.25	28.60±0.09 **b**	0.069	10.0	0.16	28.04±0.01 **a**	0.061	11.4
C7	0.26	38.34±0.02 **c**	0.108	6.4	0.22	32.56±0.00 **b**	0.084	8.2	0.16	26.89±0.07 **c**	0.066	10.6	0.02	24.34±0.07 **b**	0.054	12.9
C8	0.22	29.75±0.04 **d**	0.067	10.4	0.19	19.70±0.01 **cd**	0.041	16.8	0.15	16.89±0.02 **e**	0.036	19.4	0.08	18.98±0.04 **c**	0.042	16.4
C9	0.28	19.82±0.04 **e**	0.047	14.7	0.22	15.29±0.05 **d**	0.037	18.6	0.14	15.88±0.02 **f**	0.030	22.8	0.11	13.57±0.05 **d**	0.032	21.9
C10	0.40	37.45±0.01 **c**	0.105	6.6	0.39	24.26±0.02 **c**	0.060	11.5	0.27	20.52±0.04 **d**	0.050	13.8	0.14	19.46±0.02 **c**	0.044	15.8

Iprodione

	10 mg L^{-1}				20 mg L^{-1}				50 mg L^{-1}				100 mg L^{-1}			
Strain	**μ max (h^{-1})**	**R (%)**	***k* (h^{-1})**	**$T_{1/2}$ (h)**	**μ max (h^{-1})**	**R (%)**	***k* (h^{-1})**	**$T_{1/2}$ (h)**	**μ max (h^{-1})**	**R (%)**	***k* (h)**	**$T_{1/2}$ (h)**	**μ max (h^{-1})**	**R (%)**	***k* (h^{-1})**	**$T_{1/2}$ (h)**
C1	0.18	97.60±0.07 **b**	0.160	4	0.17	94.40±0.01 **b**	0.123	6	0.15	90.30±0.15 **b**	0.099	7	0.11	84.30±0.00 **b**	0.078	9
C4	0.26	94.20±0.02 **c**	0.121	6	0.23	92.70±0.00 **c**	0.112	6	0.22	87.50±0.00 **c**	0.089	8	0.09	81.50±0.14 **c**	0.072	10
C7	0.25	87.10±0.09 **e**	0.087	8	0.20	84.70±0.31 **d**	0.079	9	0.17	79.60±0.17 **e**	0.068	10	0.05	76.60±0.25 **e**	0.062	11
C8	0.24	88.60±0.00 **d**	0.092	8	0.22	85.60±0.15 **d**	0.082	8	0.20	81.10±0.05 **d**	0.071	10	0.12	77.80±0.10 **d**	0.065	11
C9	0.35	98.90±0.00 **a**	0.193	4	0.34	96.90±0.00 **a**	0.148	5	0.32	93.10±0.09 **a**	0.113	6	0.22	91.20±0.02 **a**	0.103	7
C10	0.43	81.10±0.70 **f**	0.070	10	0.42	77.10±0.01 **e**	0.062	11	0.36	74.20±0.09 **f**	0.058	12	0.24	70.40±0.18 **f**	0.052	13

Values of removal (% R) within a concentration with the same letter are not significantly different based on the Tukey test (α=0.05), the average values and the standard error are presented (n= 3); R (%): removal of pesticides, *k*: rate constant, $T_{1/2}$: half-life time, μ: specific growth rate.

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448 Similar to that observed for CHL, biomass of bacteria exposed to IPR increased over time,
449 up to 50 mg L^{-1} IPR concentration, where growth decreased as the pesticide concentration
450 increased (Fig. 5). In the control treatments, a biomass between 1.43 and 2.38 g L^{-1} and μ
451 max from 0.18 to 0.48 h^{-1} were observed, instead of biomass between 0.78 and 1.80 g L^{-1} and
452 μ max from 0.15 to 0.36 h^{-1} at 50 mg L^{-1} of IPR. Application of 100 mg L^{-1} IPR in the liquid
453 medium caused a marked inhibition of microbial growth with biomass ranging between 0.34
454 to 1.06 g L^{-1} and a μ max between 0.05 and 0.24 h^{-1}. *Achromobacter* sp. strain C1 and
455 *Pseudomonas* sp. strain C9 were the most tolerant strains to IPR in relation to the growth
456 observed in the control treatment (Table 4).
457

458 **Fig. 5.** Remaining iprodione (IPR) (%) and biomass by C1 (a), C4 (b), C7 (c), C8 (d), C9 (3),
459 C10 (f) strains at initial CHL concentration of 0, 10, 20, 50 and 100 mg L^{-1}, evaluated during
460 120 h. (●) CHL 10 mg L^{-1}; (■) CHL 20 mg L^{-1}; (x) CHL 50 mg L^{-1}; (◆) CHL 100 mg L^{-1};
461 (▲) Control without CHL. Continuous line: Remaining IPR (%); Dotted line: biomass (g L^{-1}
462).
463

464 With respect to CHL removal, it decreased for all bacteria strains as the contaminant
465 concentration increased from 10 to 100 mg L^{-1}. *Achromobacter* sp. strain C1 showed the best
466 CHL removal (56–29%) after 120 h of incubation, which was significant ($p \leq 0.05$) relative
467 to that by the other five strains for all CHL concentrations. In this context, the kinetic data
468 showed that CHL removal by the C1 strains were characterized by a rate constant of 0.147–
469 0.072 d^{-1} and $T_{1/2}$ 4.7–9.7 d^{-1} in liquid medium treated with 10 and 100 mg L^{-1} CHL. This
470 trend was closely followed by the *Pseudomonas* sp. strains C4, *Achromobacter* sp. strains
471 C7, and *Achromobacter* sp. strain C10 with a removal between 42–27%, 38–24%, and 37–
472 19%, respectively. However, the lowest degradation was observed for the *Pseudomonas* sp.

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473 strain C9 with CHL degradation between 19–13%, a rate constant between 0.030–0.047 d^{-1},
474 and $T_{1/2}$ 14–22 d^{-1} (Table 4).
475 With respect to IPR degradation, when concentrations increased from 10 to 100 mg L^{-1}, it
476 was removed efficiently (81–98%) by all strains, requiring only 24 h of incubation. IPR was
477 not detected at 48 h. The significantly ($p \leq 0.05$) highest IPR removal (between 91.2–98.9%)
478 was observed for the *Pseudomonas* sp. strain C9 relative to that for the other strains at all
479 IPR concentrations. According to the kinetic parameters, strain C9 showed the highest rate
480 constant of 0.193 h^{-1} for IPR added at 10 mg L^{-1} and a maximum $T_{1/2}$ of 7 h^{-1} when IPR was
481 added at a concentration of 100 mg L^{-1}. For *Achromobacter* sp. strain C10, the lowest IPR
482 removal (70–81%) was observed after 24 h of incubation and a $T_{1/2}$ ranging from 11 to 13 h^{-1}
483 (Table 4).
484 In parallel to pesticides removal from the liquid medium, the concentrations of metabolite
485 TCP and 3,5-DCA produced during CHL and IPR removal, respectively, were analyzed
486 (Table 5).
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497 **Table 5.** Production of 3,5,6-trichlo-2-pyridinol (TCP) and 3,5-DCA by strains C1, C4, C7,
498 C8, C9 and C,10 in liquid medium supplemented with 0-100 mg L^{-1} of chlorpyrifos (CHL)
499 and iprodione (IPR) individually.
500

Strains	**Time (h)**	**Chlorpyrifos**				**Iprodione**			
		10 mg L^{-1}	**20 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**	**10 mg L^{-1}**	**20 mg L^{-1}**	**50 mg L^{-1}**	**100 mg L^{-1}**
		Metabolite (mg L^{-1})	**Metabolite (mg L^{-1})**	**Metabolite (mg L^{-1})**	**Metabolite (mg L^{-1})**	**Metabolite (mg L^{-1})**	**Metabolite (mg L^{-1})**	**Metabolite (mg L^{-1})**	**Metabolite (mg L^{-1})**
C1	24	0.17±0.01	0.36±0.02	0.82±0.02	1.61±0.02	0.21±0.00	0.35±0.01	0.29±0.01	0.47±0.01
	72	0.22±0.29	0.41±0.01	0.94±0.01	1.84±0.03	0.24±0.05	0.36±0.02	0.45±0.02	0.65±0.01
	120	0.50±0.02	0.69±0.01	1.62±0.02	2.10±0.02	0.38±0.00	0.42±0.04	0.52±0.06	0.87±0.10
C4	24	0.12±0.02	0.32±0.02	0.80±0.02	1.40±0.02	0.15±0.03	0.29±0.01	0.26±0.01	0.35±0.01
	72	0.14±0.01	0.33±0.01	0.88±0.04	1.47±0.04	0.20±0.06	0.34±0.01	0.41±0.06	0.59±0.06
	120	0.34±0.01	0.52±0.00	1.48±0.08	1.50±0.04	0.34±0.01	0.41±0.07	0.49±0.02	0.86±0.06
C7	24	0.08±0.02	0.28±0.02	0.74±0.02	1.42±0.02	0.32±0.03	0.16±0.03	0.19±0.07	0.27±0.05
	72	0.10±0.00	0.30±0.01	0.77±0.02	1.46±0.04	0.18±0.02	0.26±0.04	0.29±0.04	0.41±0.07
	120	0.29±0.01	0.48±0.02	1.27±0.04	1.47±0.02	0.30±0.02	0.35±0.01	0.37±0.02	0.75±0.13
C8	24	0.08±0.02	0.27±0.02	0.66±0.02	1.38±0.02	0.15±0.02	0.20±0.03	0.22±0.04	0.35±0.01
	72	0.07±0.01	0.26±0.01	0.73±0.04	1.46±0.03	0.20±0.00	0.32±0.05	0.35±0.05	0.50±0.02
	120	0.24±0.00	0.42±0.02	0.96±0.03	1.45±0.00	0.31±0.04	0.39±0.01	0.42±0.04	0.85±0.08
C9	24	0.07±0.02	0.26±0.02	0.61±0.02	1.36±0.03	0.25±0.00	0.38±0.03	0.37±0.03	0.47±0.08
	72	0.06±0.01	0.25±0.01	0.78±0.04	1.38±0.04	0.26±0.02	0.38±0.01	0.47±0.04	0.70±0.04
	120	0.21±0.00	0.47±0.01	0.91±0.01	1.40±0.00	0.31±0.00	0.49±0.01	0.59±0.03	0.95±0.00
C10	24	0.06±0.02	0.25±0.02	0.74±0.02	1.56±0.02	0.07±0.01	0.10±0.02	0.10±0.00	0.22±0.01
	96	0.07±0.03	0.26±0.02	0.83±0.01	1.45±0.08	0.17±0.06	0.12±0.04	0.22±0.03	0.39±0.02
	120	0.28±0.00	0.47±0.01	0.98±0.01	1.38±0.00	0.21±0.05	0.25±0.00	0.35±0.00	0.65±0.05

501 The average values and the standard error are presented (n= 3)

502

503 The results showed that over time and when the pesticide concentration increased, the
504 metabolite concentrations increased. TCP production was highest when liquid media was
505 treated with *Achromobacter* sp. strain C1, detecting concentrations between 0.504–2.098 mg
506 L^{-1} after 120 h of incubation. For all other strains, TCP concentrations ranged from 0.214 mg
507 L^{-1} produced by *Pseudomonas* sp. strain C9 during the treatment of 10 mg L^{-1} CHL to 1.498
508 mg L^{-1} produced by *Pseudomonas* sp. strains C4 during the treatment of 100 mg L^{-1} CHL. In
509 relation to IPR degradation, the results showed that the metabolite began to appear in the

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510 liquid medium at 9 h of incubation. After 48 h of incubation, 3,5-DCA concentrations ranged
511 between 0.210–0.384 mg L^{-1} and 0.648–0.945 mg L^{-1} after treatment of 10 and 100 mg L^{-1}
512 IPR, respectively. Moreover, the highest 3,5-DCA concentrations were produced during the
513 treatment of 20, 50, and 100 mg L^{-1} IPR by *Pseudomonas* sp. strain C9.
514

515 # Discussion

516 Inappropriate pesticide management has resulted in their release into the environment,
517 including food. Therefore, efforts to develop technologies that guarantee effective treatment
518 of pesticide residues have been made. Due to the use of microorganisms in pesticide
519 treatment, it is extremely important to previously determine their potential for removal from
520 liquid media under optimal conditions. Screening of degrading microorganisms through an
521 enrichment procedure in the pesticides-contaminated system allows for selection of potential
522 isolates with a high tolerance and maximal degrading activity [22]. In our study, the
523 microorganism isolation matrix consisted of the biopurification system repeatedly treating a
524 biomixture of pesticides. Pesticide application in the biomixture did not cause relevant effects
525 on total cultivable bacteria; however, bacteria are active, which is associated with the high
526 efficiency of pesticides degradation shown in this biopurification system [15,32]. In this
527 context, the use of a biomixture allowed us to obtain ten tolerant strains. However, six strains
528 were selected for their high tolerance and ability to grow in the presence of CHL and IPR;
529 both contaminants were demonstrated as carbon and energy sources presumably via partial
530 transformation reactions that can occur with different chemical classes of pesticides.
531 The selected strains were characterized in different ways. Enzymatic characterization using
532 the ApiZym test showed production of different enzymes for each substrate. It should be

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533 noted that some authors have highlighted bacteria-produced enzymes obtained from
534 contaminated sites of high biotechnological, clinical, and industrial interest [26,29]. In our
535 study, strains C4, C8, and C9 presented the highest biochemical activity, being positive for
536 at least 13 of the 24 enzymes tested.

537 Tolerant and characterized strains were identified using two methods: 16S rDNA gene
538 sequencing, which is widely used to determine microorganism taxonomic positions, and
539 MALDI-TOF/TOF MS, which identifies and classifies an organism according to the spectral
540 profile of its ribosomal proteins. According to our results, both identifications were
541 concordant. The phylogenetic analysis of isolates showed a closer relation with bacteria from
542 genera *Pseudomonas*, *Achromobacter,* and *Rhodococcus*, known as metabolically active
543 microorganisms capable of degrading many pesticides, including CHL and IPR [24,27]. Use
544 of MALDI-TOF/TOF MS has led to a new era of routine and rapid identification of different
545 organisms, including environmental bacteria [11]. In our study, dendrograms generated by
546 MALDI Biotyper were able to separate or assemble different strains in the same cluster,
547 depending on pesticides exposure, which could explain effects on proteins due to
548 environmental conditions [33].

549

550 The results of bacterial growth cultured in the presence of CHL and IPR in liquid medium
551 demonstrated that pesticides produced an inhibitory effect at concentrations above 50 mg L^{-}
552 ^{1}. Although various researchers have reported that CHL and IPR in liquid media could be
553 used as a source of C and energy for growth [24,25], metabolites, which are more toxic than
554 the parent compound and with antimicrobial properties to inhibit microbial growth, can be
555 produced during microbial treatment [34]. IPR is known to inhibit DNA and RNA synthesis,
556 cell division, and cellular metabolism in fungi; however, there is limited information that IPR

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557 may inhibit environmental microbes [35]. According to our results, *Achromobacter* sp. C1
558 and *Pseudomonas* sp. C4 were the most tolerant strains to CHL, and *Achromobacter* sp. C1
559 and *Pseudomonas* sp. C9 were tolerant to IPR, which was associated with a minor difference
560 in growth relative to that for the control treatment. Consistently, both strains presented the
561 highest removal of pesticides. *Achromobacter* and *Pseudomonas* genera are both microbial
562 groups recognized by their ability to remove pesticides, e.g. *Achromobacter xylosoxidans*
563 strain CS5 removes endosulfan [36], *Arthrobacter* sp. BS2 and *Achromobacter* sp. SP1
564 degrade diuron and their metabolite 3,4-dichloroaniline [37], *Pseudomonas* sp. and
565 *Achromobacter* sp. isolated from agricultural soil degrade atrazine [38], *Arthrobacter* sp.
566 strain C1 and *Achromobacter* sp. strain C2 isolated from soil degrade IPR [25], and
567 *Pseudomonas* spp. has been described as a CHL-degrader [24]. The highest CHL removal by
568 *Achromobacter* sp. strain C1 could be explained by the presence or activity of the enzyme
569 alkaline phosphatase, as this enzyme is a phosphomonoesterase that regulates CHL
570 degradation through hydrolysis of O-P bonds [39]. Similarly, the presence of diverse
571 enzymes in *Pseudomonas* sp. strain C9 could influence fast degradation, and therefore reduce
572 $T_{1/2}$ required for pesticide reduction.
573 Previous researchers have reported that CHL removal by bacteria occurred through formation
574 of metabolites, such as CHL-oxon, 3,5,6-trichloro-2-methoxypyridine, 2-chloro-6-
575 hydroxypyridine, and TCP. We evaluated TCP as a primary metabolite at different times.
576 The results showed that product levels slightly increased over time, reaching a maximum
577 concentration of 2.098 mg L^{-1} in the liquid medium. According to our results, TCP was not
578 metabolized by any strain, resulting in its accumulation in the liquid medium. Therefore, TCP
579 accumulation and the presence of chlorine atoms on the pyridinol ring caused a toxic effect
580 on the microorganisms [39], resulting in incomplete CHL removal in the time evaluated.

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581 Nonetheless, the removal of 10 mg L^{-1} and 20 mg L^{-1} of CHL was effectively performed by
582 the *Achromobacter* sp. strain C1, probably requiring only few days more to achieve complete
583 CHL elimination. A study reported that *A. xylosoxidans* JCp4 was able to mineralize 100 mg
584 L^{-1} CHL completely after ten days with only a transient accumulation of TCP [40]. Our work
585 constitutes one of the few reports of *Achromobacter* as CHL-degraders.
586 The appearance of 3,5-DCA, recognized as the major metabolite of IPR degradation, their at
587 9 h of incubation was observed at concentrations lower than 0.5 mg L^{-1}. The appearance of
588 3,5-DCA was coincident with the fastest decrease of IPR levels. After this time, 3,5-DCA
589 concentrations were slightly increased, such that no IPR residues were found after 48 h of
590 incubation. Although IPR is a common fungicide frequently used in crops and with a
591 classification of "probable carcinogen to humans," treatment to eliminate IPR using
592 microorganisms has been poorly studied. Some studies reported IPR and 3,5-DCA
593 degradation by microorganisms isolated from soil, *Arthrobacter* sp. strains C1, and
594 *Achromobacter* sp. strains C2 from liquid medium, showing a $T_{1/2}$ of 2.3 h and 19.5 h,
595 respectively [9]. In our study, a small amount of time was required for *Achromobacter* sp.
596 strains C1 to remove 50% of the contaminant from liquid medium ($T_{1/2}$ between 4–9 h),
597 which might signify the environmental adaptation of this bacteria being exposed to continued
598 pesticide application in the biomixture used for their isolation. According to Campos *et al.*
599 [9], IPR removal could occur via initial hydrolysis to isopropylamine and metabolite I (3,5-
600 dichlorophenyl-carboxamide) and then to metabolite II (3,5-dichlorophenylurea-acetate)
601 before being hydrolyzed to 3,5-DCA and probably glycine. Similar results were reported by
602 Cao *et al*. [35] for a *Microbacterium* sp. strain CQH-1 isolated via the enrichment culture
603 technique from a soil with previous exposure to IPR.

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604 In this study, we described different bacteria isolated from a biomixture used in a
605 biopurification system that received continuous pesticide applications. These bacteria were
606 capable of degrading compounds such as CHL and IPR. Given their identification and ability
607 to remove contaminants, *Achromobacter* sp. strain C1 and *Pseudomonas* sp. strains C9
608 appear as promising microorganisms for treatment of matrices contaminated with CHL, IPR,
609 or their mixture. The results of this study will help to improve current technologies for
610 biodegradation of this commonly used insecticide and fungicide in response the problem of
611 pesticide contamination.
612

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616

617 # References

618 1. Alavanja MCR. Introduction: pesticides use and exposure extensive worldwide. Rev
619 Environ Health. 2009;24: 303–309.
620 2. Cycoń M, Mrozik A, Piotrowska-Seget Z. Bioaugmentation as a strategy for the
621 remediation of pesticide-polluted soil: A review. Chemosphere. 2017;172: 52–71.
622 doi:10.1016/j.chemosphere.2016.12.129
623 3. Torstensson L, Castillo MDP. Use of biobeds in Sweden to minimize environmental
624 spillages from agricultural spraying equipment. Pestic Outlook. 1997; 8, 24–27.

bioRxiv preprint doi: https://doi.org/10.1101/489252; this version posted December 6, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

625 4. Castillo M del P, Torstensson L, Stenstrom J. Biobeds for environmental protection
626 from pesticide use--a review. J Agric Food Chem. 2008;56: 6206–6219.
627 doi:10.1021/jf800844x
628 5. Diez MC, Elgueta S, Rubilar O, Tortella GR, Schalchli H, Bornhardt C, et al. Pesticide
629 dissipation and microbial community changes in a biopurification system: influence of
630 the rhizosphere. Biodegradation. 2017;28: 395–412. doi:10.1007/s10532-017-9804-y
631 6. Coppola L, Comitini F, Casucci C, Milanovic V, Monaci E, Marinozzi M, et al.
632 Fungicides degradation in an organic biomixture: impact on microbial diversity. New
633 Biotechnol. 2011;29: 99–106. doi:10.1016/j.nbt.2011.03.005
634 7. Karanasios E, Karpouzas DG, Tsiropoulos NG. Key parameters and practices
635 controlling pesticide degradation efficiency of biobed substrates. J Environ Sci Health
636 B. 2012;47: 589–598. doi:10.1080/03601234.2012.665753
637 8. Briceño G, Schalchli H, Mutis A, Benimeli CS, Palma G, Tortella GR, et al. Use of pure
638 and mixed culture of diazinon-degrading Streptomyces to remove other
639 organophosphorus pesticides. Int Biodeterior Biodegrad. 2016;114: 193–201.
640 doi:10.1016/j.ibiod.2016.06.018
641 9. Campos M, Karas PS, Perruchon C, Papadopoulou ES, Christou V, Menkissoglou-
642 Spiroudi U, et al. Novel insights into the metabolic pathway of iprodione by soil
643 bacteria. Environ Sci Pollut Res Int. 2017;24: 152–163. doi:10.1007/s11356-016-7682-
644 1
645 10. Castillo M del P, Torstensson L. Effect of Biobed Composition, Moisture, and
646 Temperature on the Degradation of Pesticides. J Agric Food Chem. 2007;55: 5725–
647 5733. doi:10.1021/jf0707637

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648 11. Loucif L, Bendjama E, Gacemi-Kirane D, Rolain J-M. Rapid identification of
649 Streptomyces isolates by MALDI-TOF MS. Microbiol Res. 2014;169: 940–947.
650 doi:10.1016/j.micres.2014.04.004
651 12. Urrutia C, Rubilar O, Tortella G, Castillo JM, Romero E, Azcón R, et al. Influence of
652 the rhizosphere in a biopurification system on the dissipation of a pesticide mixture. J
653 Soil Sci Plant Nutr. 2015;15: 914–927. doi:10.4067/S0718-95162015005000063
654 13. Fernández-Alberti S, Rubilar O, Tortella GR, Diez MC. Chlorpyrifos degradation in a
655 Biomix : Effect of pre-incubation and water holding capacity. J Soil Sci Plant Nutr.
656 2012;12: 785–799. doi:10.4067/S0718-95162012005000027
657 14. Castro-Gutiérrez V, Masís-Mora M, Diez MC, Tortella GR, Rodríguez-Rodríguez CE.
658 Aging of biomixtures: Effects on carbofuran removal and microbial community
659 structure. Chemosphere. 2017;168: 418–425. doi:10.1016/j.chemosphere.2016.10.065
660 15. Elgueta S, Correa A, Campo M, Gallardo F, Karpouzas D, Diez MC. Atrazine,
661 chlorpyrifos, and iprodione effect on the biodiversity of bacteria, Actinomycetes, and
662 fungi in a pilot biopurification system with a green cover. J Environ Sci Health - Part B
663 Pestic Food Contam Agric Wastes. 2017;0: 1–7. doi:10.1080/03601234.2017.1330070
664 16. Bergsveinson J, Perry BJ, Sheedy C, Braul L, Reedyk S, Gossen BD, et al. Identifying
665 the core bacterial and fungal communities within four agricultural biobeds used for the
666 treatment of pesticide rinsates. J Appl Microbiol. 2018; doi:10.1111/jam.14051
667 17. Singh SB, Lal SP, Pant S, Kulshrestha G. Degradation of atrazine by an acclimatized
668 soil fungal isolate. J Environ Sci Health B. 2008;43: 27–33.
669 doi:10.1080/03601230701735227

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670 18. Verma JP, Jaiswal DK, Sagar R. Pesticide relevance and their microbial degradation: a-
671 state-of-art. Rev Environ Sci Biotechnol. 2014;13: 429.
672 doi:https://doi.org/10.1007/S11157-014-9341-7
673 19. Simon-Delso N, Amaral-Rogers V, Belzunces LP, Bonmatin JM, Chagnon M, Downs
674 C, et al. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action
675 and metabolites. Environ Sci Pollut Res. 2015;22: 5–34. doi:10.1007/s11356-014-3470-
676 y
677 20. Hernández M, Magalí V, Avila M, Villalobos P, Miralles P, González M, et al. Novel
678 s-triazine-degrading bacteria isolated from agricultural soils of central Chile for
679 herbicide bioremediation. Electron J Biotechnol Vol 11 No 5 2008. 2008; Available:
680 http://www.ejbiotechnology.info/index.php/ejbiotechnology/article/view/v11n5-4/667
681 21. Saez JM, Bigliardo AL, Raimondo EE, Briceno GE, Polti MA, Benimeli CS. Lindane
682 dissipation in a biomixture: Effect of soil properties and bioaugmentation. Ecotoxicol
683 Environ Saf. 2018;156: 97–105. doi:10.1016/j.ecoenv.2018.03.011
684 22. Chen S, Liu C, Peng C, Liu H, Hu M, Zhong G. Biodegradation of Chlorpyrifos and Its
685 Hydrolysis Product 3,5,6-Trichloro-2-Pyridinol by a New Fungal Strain *Cladosporium*
686 *cladosporioides* Hu-01. Lin B, editor. PLoS ONE. 2012;7: e47205.
687 doi:10.1371/journal.pone.0047205
688 23. Briceño G, Fuentes MS, Palma G, Jorquera MA, Amoroso MJ, Diez MC. Chlorpyrifos
689 biodegradation and 3,5,6-trichloro-2-pyridinol production by actinobacteria isolated
690 from soil. Int Biodeterior Biodegrad. 2012;73: 1–7. doi:10.1016/j.ibiod.2012.06.002
691 24. Chishti Z, Hussain S, Arshad KR, Khalid A, Arshad M. Microbial degradation of
692 chlorpyrifos in liquid media and soil. J Environ Manage. 2013;114: 372–380.
693 doi:10.1016/j.jenvman.2012.10.032

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694 25. Campos M, Perruchon C, Vasilieiadis S, Menkissoglu-Spiroudi U, Karpouzas DG, Diez
695 MC. Isolation and characterization of bacteria from acidic pristine soil environment able
696 to transform iprodione and 3,5-dichloraniline. Int Biodeterior Biodegrad. 2015;104:
697 201–211. doi:10.1016/j.ibiod.2015.06.009
698 26. Khatun MA, Hoque MA, Zhang Y, Lu T, Cui L, Zhou N-Y, et al. Bacterial Consortium-
699 Based Sensing System for Detecting Organophosphorus Pesticides. Anal Chem. 2018;
700 doi:10.1021/acs.analchem.8b02709
701 27. Campos M, Karas PS, Perruchon C, Papadopoulou ES, Christou V, Menkissoglou-
702 Spiroudi U, et al. Novel insights into the metabolic pathway of iprodione by soil
703 bacteria. Environ Sci Pollut Res. 2017;24: 152–163. doi:10.1007/s11356-016-7682-1
704 28. Fuentes MS, Raimondo EE, Amoroso MJ, Benimeli CS. Removal of a mixture of
705 pesticides by a Streptomyces consortium: Influence of different soil systems.
706 Chemosphere. 2017;173: 359–367. doi:10.1016/j.chemosphere.2017.01.044
707 29. Briceño G, Vergara K, Schalchli H, Palma G, Tortella G, Fuentes MS, et al.
708 Organophosphorus pesticide mixture removal from environmental matrices by a soil
709 Streptomyces mixed culture. Environ Sci Pollut Res. 2017; 1–12. doi:10.1007/s11356-
710 017-9790-y
711 30. Krishnapriya S, Venkatesh Babu DL, G PA. Isolation and identification of bacteria to
712 improve the strength of concrete. Microbiol Res. 2015;174: 48–55.
713 doi:10.1016/j.micres.2015.03.009
714 31. Margesin R, Labbe D, Schinner F, Greer CW, Whyte LG. Characterization of
715 hydrocarbon-degrading microbial populations in contaminated and pristine Alpine
716 soils. Appl Environ Microbiol. 2003;69: 3085–3092.

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717 32. Diez MC, Leiva B, Gallardo F. Novel insights in biopurification system for dissipation
718 of a pesticide mixture in repeated applications. Environ Sci Pollut Res Int. 2018;25:
719 21440–21450. doi:10.1007/s11356-017-0130-z
720 33. Maldonado N, Robledo C, Robledo J. La espectrometría de masas MALDI-TOF en el
721 laboratorio de microbiología clínica. Infectio. 2018;22: 35–45.
722 34. Kharabsheh HA, Han S, Allen S, Chao SL. Metabolism of chlorpyrifos by
723 *Pseudomonas aeruginosa* increases toxicity in adult zebrafish (*Danio rerio*). Int
724 Biodeterior Biodegrad. 2017;121: 114–121. doi:10.1016/j.ibiod.2017.03.024
725 35. Cao L, Shi W, Shu R, Pang J, Liu Y, Zhang X, et al. Isolation and characterization of a
726 bacterium able to degrade high concentrations of iprodione. Can J Microbiol. 2018;64:
727 49–56. doi:10.1139/cjm-2017-0185
728 36. Li H, Zheng Z, Cong-xin X, Bo H, Chao-yuan W, Gang H. Isolation of cellulose-
729 producing microbes from the intestine of grass carp (Ctenopharyngodon idellus).
730 Environ Biol Fishes. 2009;86: 131–135. doi:10.1007/s10641-008-9384-0
731 37. Devers M, Pesce S, Rouard N, Martin F. Evidence for cooperative mineralization of
732 diuron by *Arthrobacter* sp. BS2 and *Achromobacter* sp. SP1 isolated from a mixed
733 culture enriched from diuron exposed environments. Chemosphere. 2014;117: 208–
734 215.
735 38. Tonelli AF, Braz VS, Bauermeister A, Rizzato JA, Lopes NP, Stehling EG. Degradation
736 of atrazine by *Pseudomonas* sp. and *Achromobacte*r sp. isolated from Brazilian
737 agricultural soil. Int Biodeterior Biodegrad. 2018;130: 17–22.
738 doi:10.1016/j.ibiod.2018.03.011
739 39. Singh BK, Walker A. Microbial degradation of organophosphorus compounds. FEMS
740 Microbiol Rev. 2006;30: 428–471. doi:10.1111/j.1574-6976.2006.00018.x

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741 40. Akbar S, Sultan S. Soil bacteria showing a potential of chlorpyrifos degradation and
742 plant growth enhancement. Braz J Microbiol Publ Braz Soc Microbiol. 2016;47: 563–
743 570. doi:10.1016/j.bjm.2016.04.009

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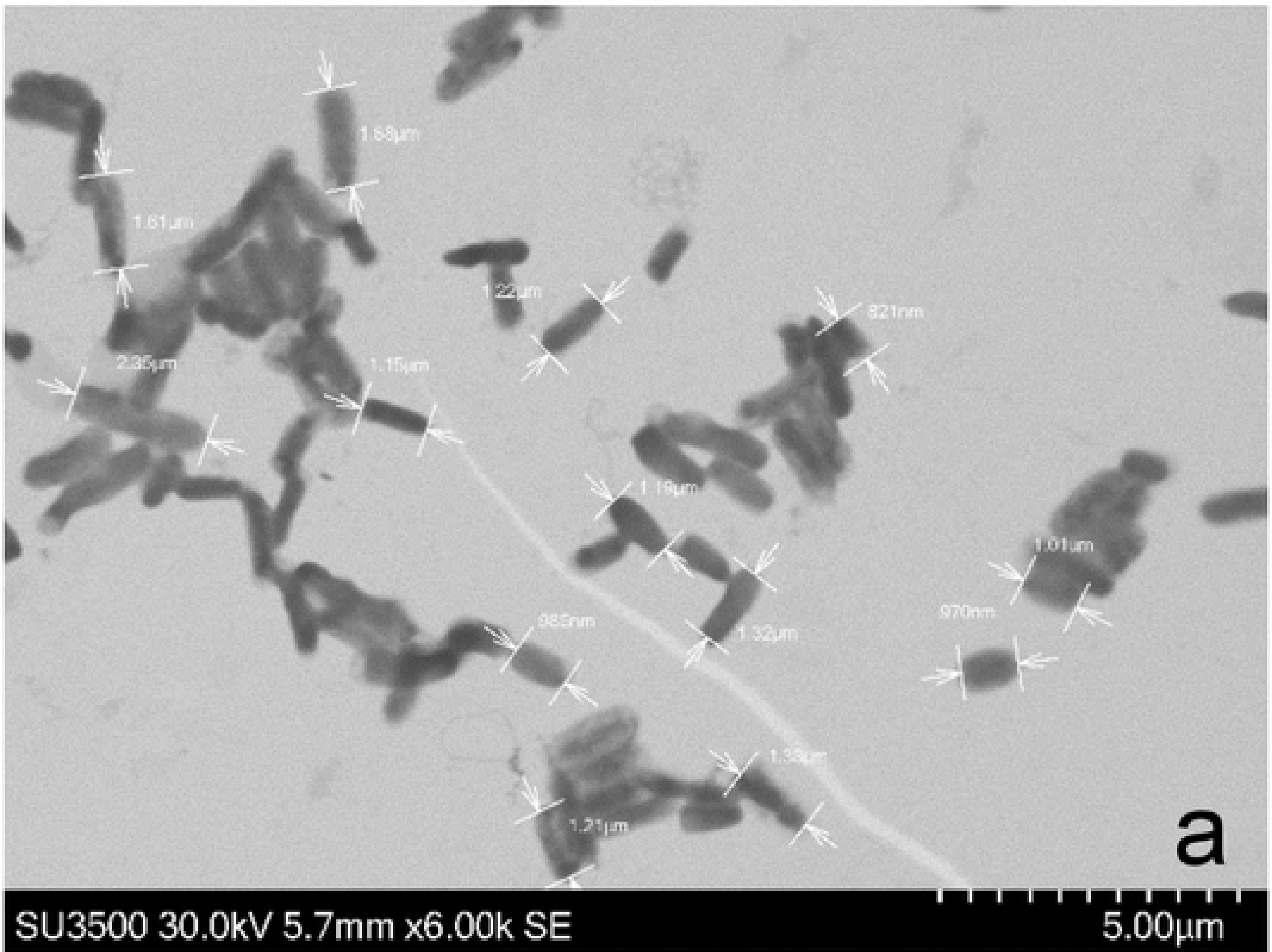

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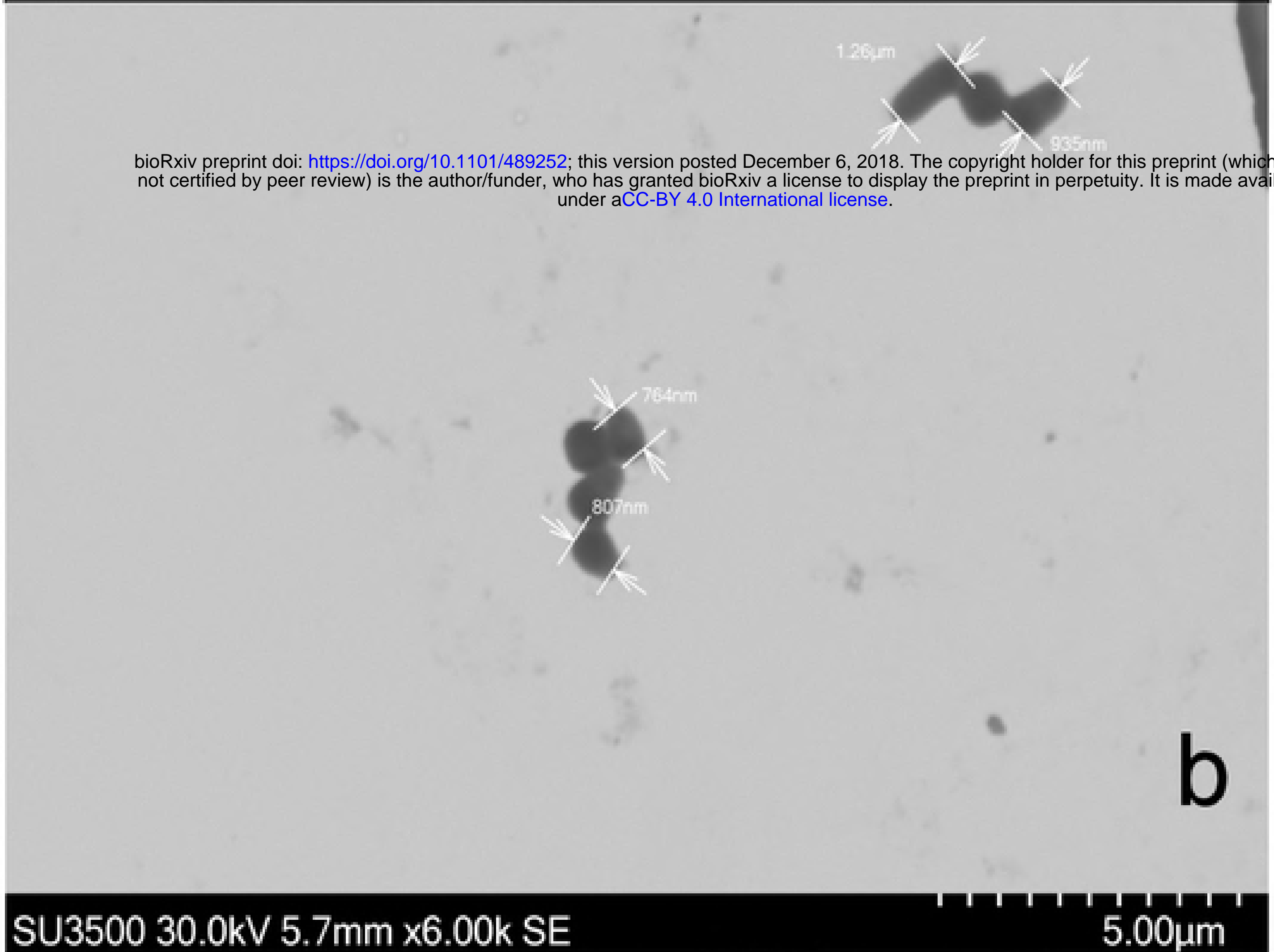

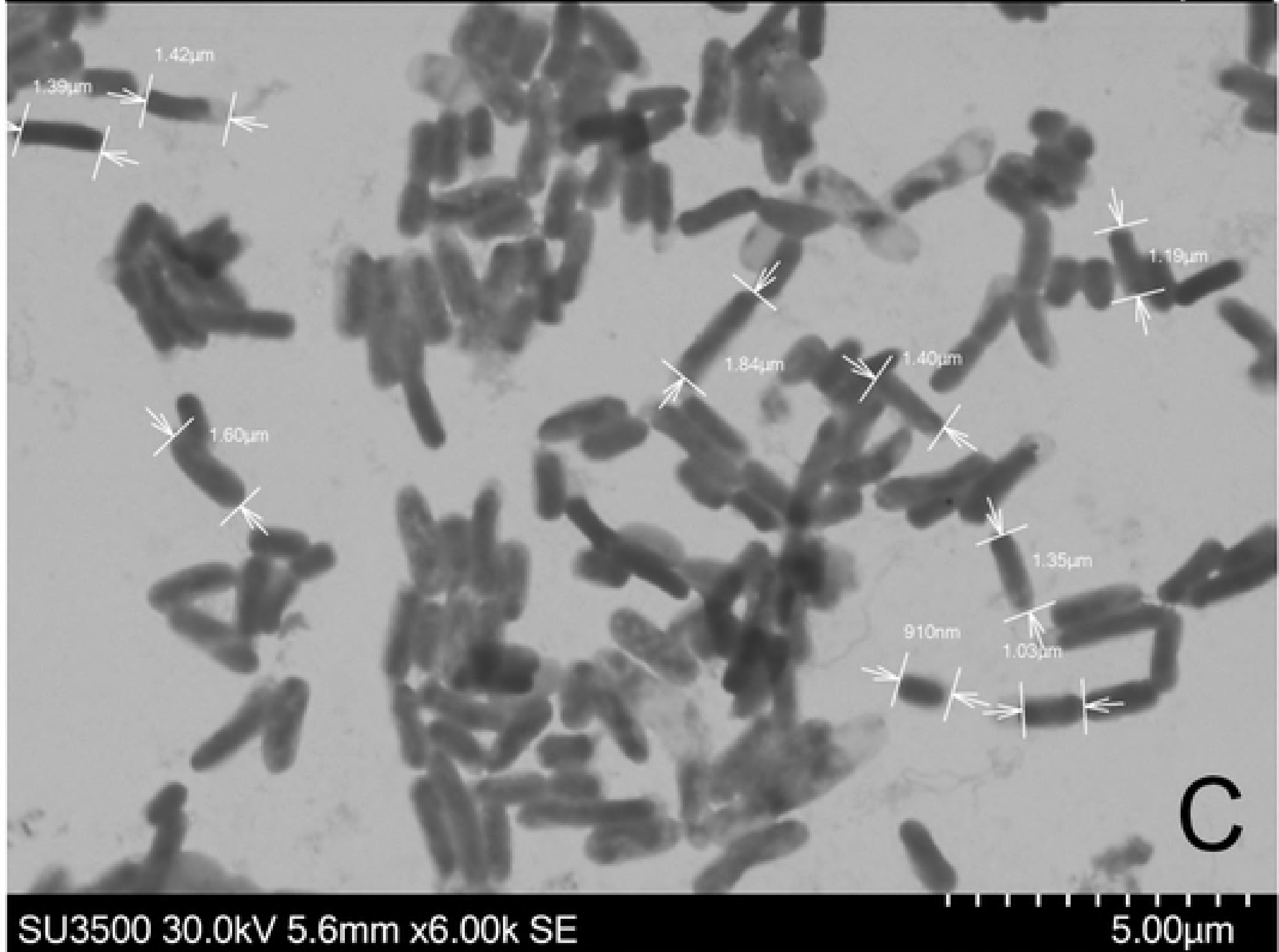

Figure 1

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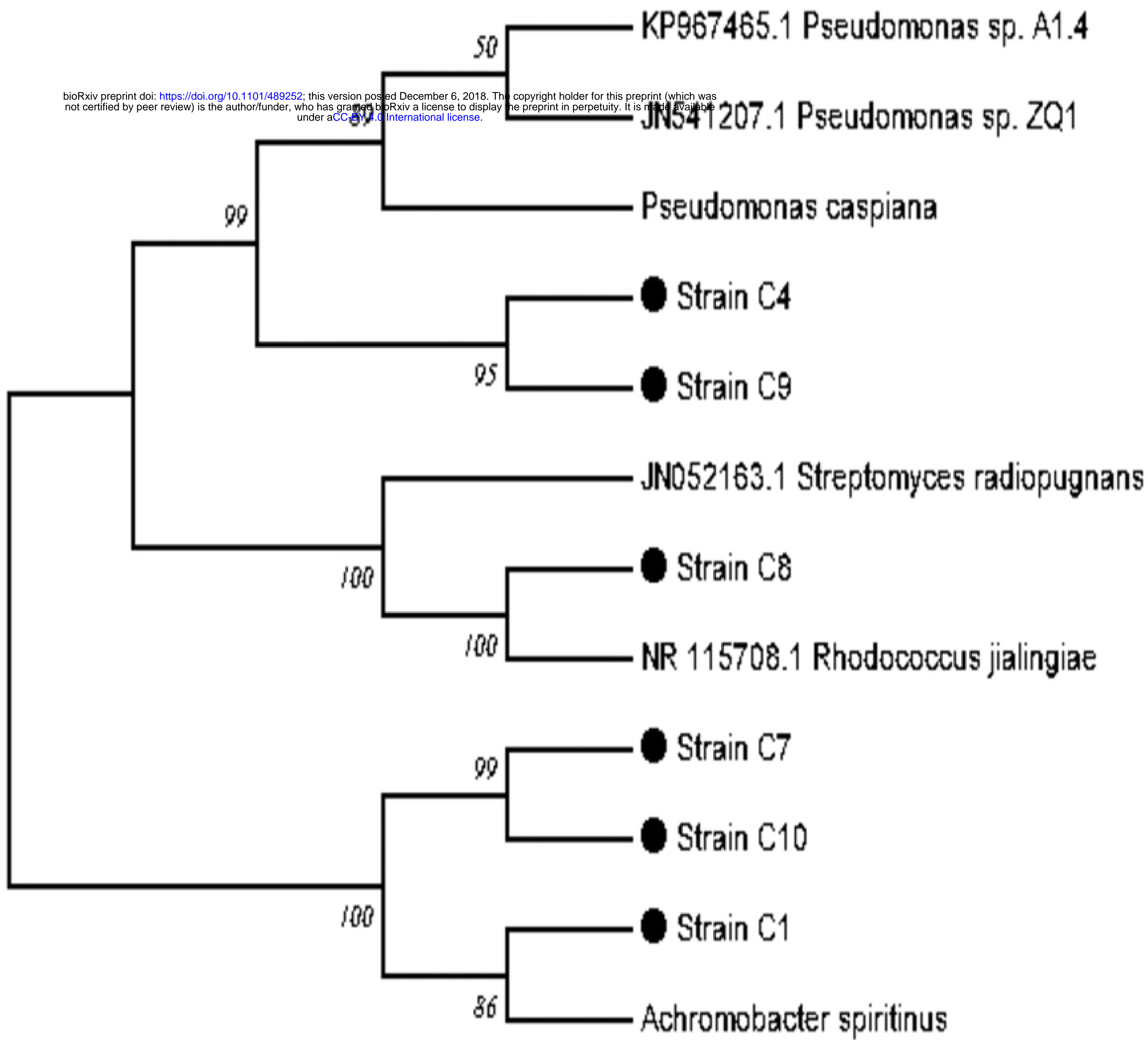
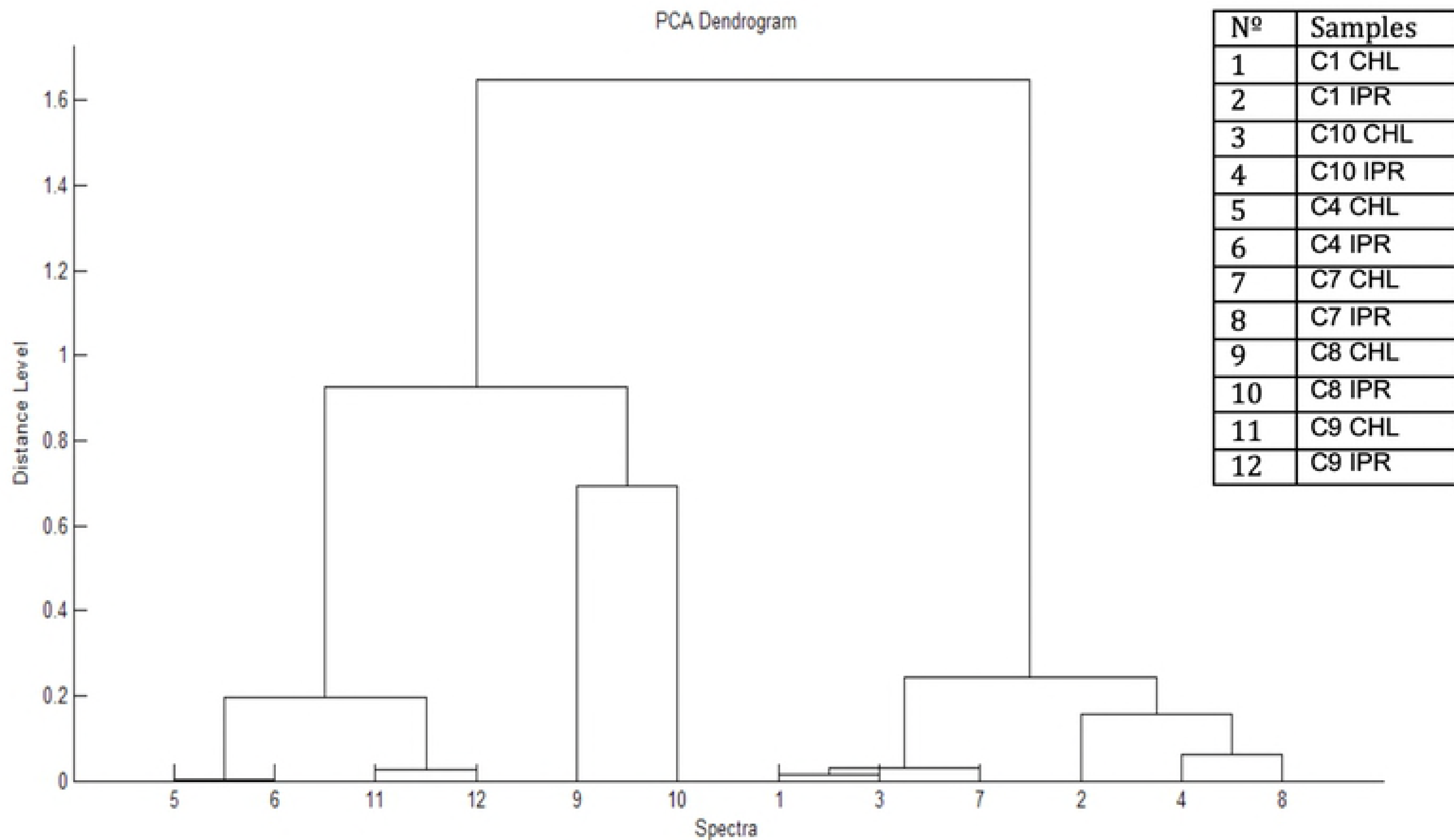


Figure 2



Nº	Samples
1	C1 CHL
2	C1 IPR
3	C10 CHL
4	C10 IPR
5	C4 CHL
6	C4 IPR
7	C7 CHL
8	C7 IPR
9	C8 CHL
10	C8 IPR
11	C9 CHL
12	C9 IPR

Figure 3

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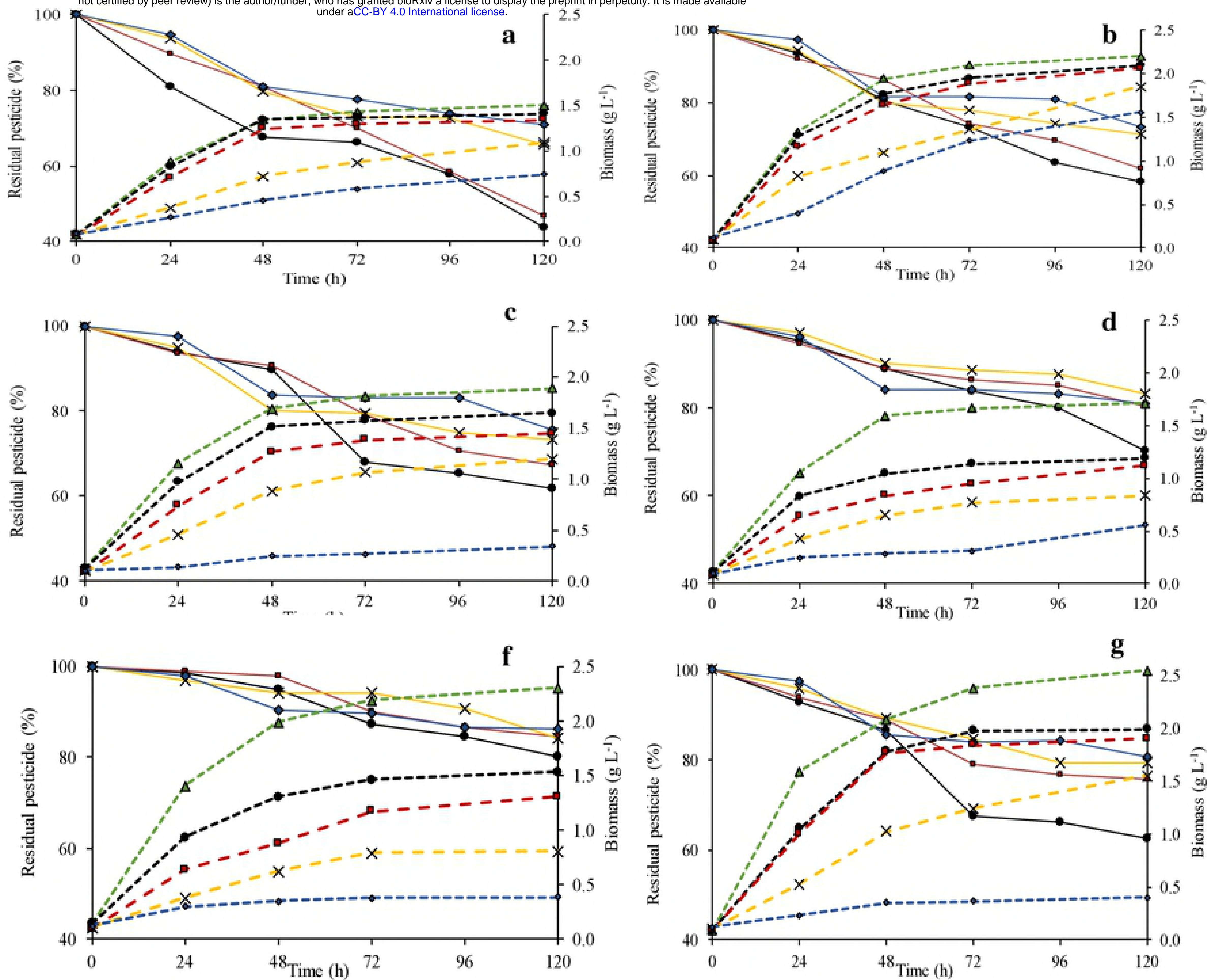

Figure 4

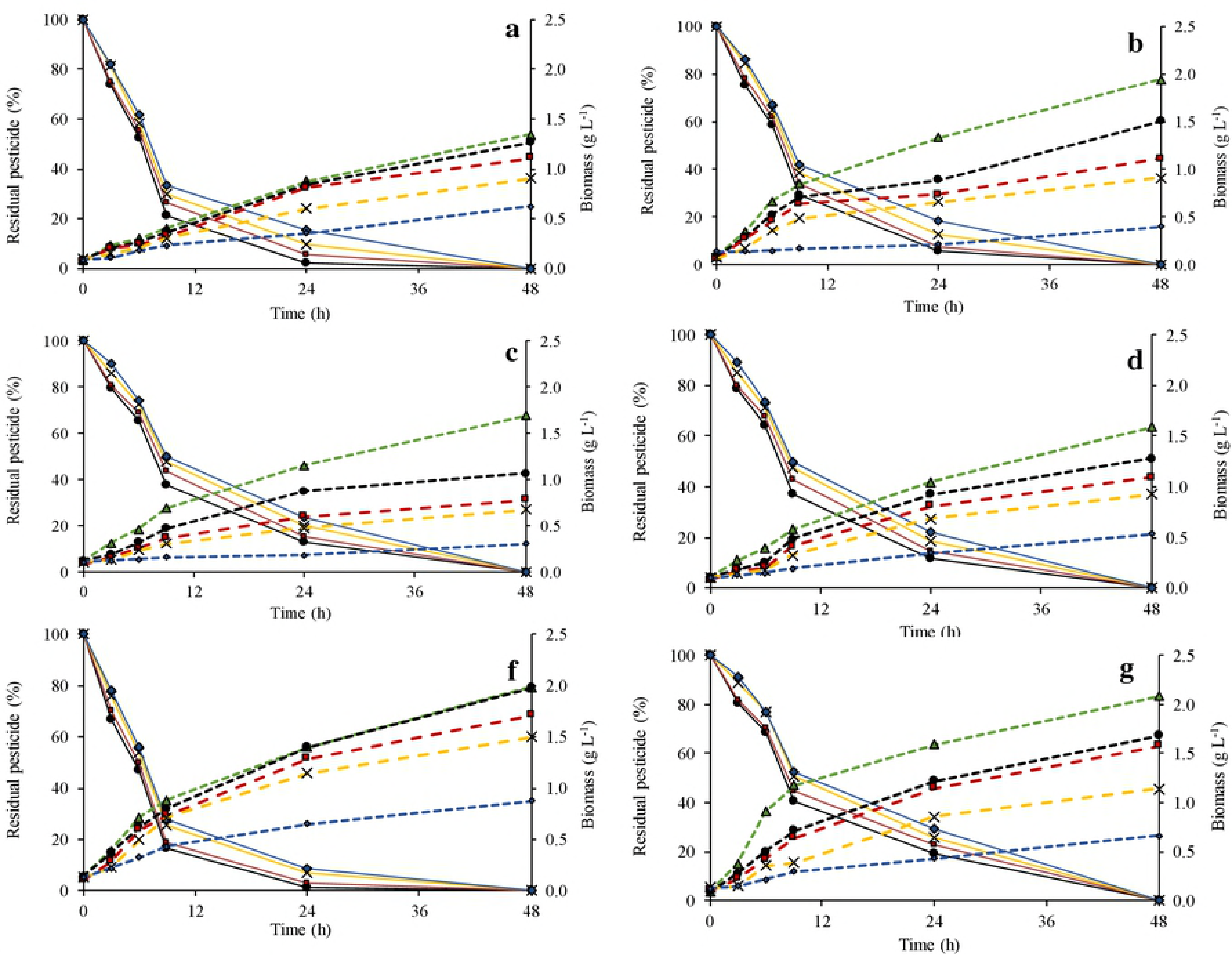

Figure 5