Effects of Burkholderia thailandensis rhamnolipids on the unicellular algae Dunaliella tertiolecta

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1   **Effects of *Burkholderia thailandensis* rhamnolipids on the unicellular algae**

2   ***Dunaliella tertiolecta***

3

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Burkholderia thailandensis E264

SPE

RLs extraction & characterization

Dunaliella tertiolecta culture (algal biotest)

RLs-mediated effects

Algal growth rate

24h

48h

72h

96h

Photosynthetic apparatus

Graphical abstract

Highlights

- The effects of *B. thailandensis* rhamnolipids on *D. tertiolecta* were investigated.
- *B. thailandensis* predominant RL congener is the di-rhamnolipid Rha-Rha-C_{14}-C_{14}
- Algal growth and photosynthetic parameters, using the JIP test, were tested.
- *B. thailandensis* rhamnolipids do not affect algal growth rate.
- RLs showed no significant effects on algae photosynthetic ability
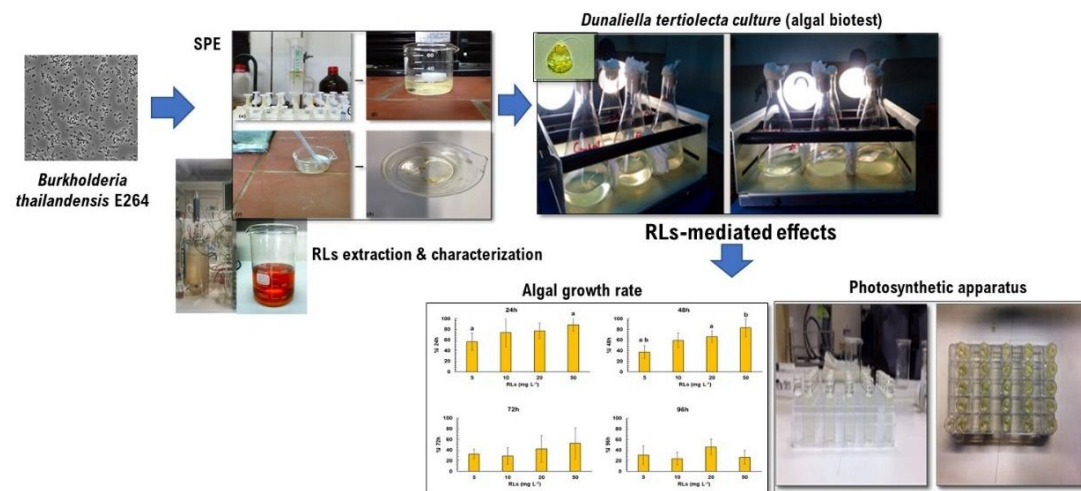
Effects of *Burkholderia thailandensis* rhamnolipids on the unicellular algae *Dunaliella tertiolecta*

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24   Abstract

25   The effects of rhamnolipids (RLs) produced and further purified from *Burkholderia*

26   *thailandensis*, on the unicellular microalgae *Dunaliella tertiolecta* were investigated,

27   in terms of RLs ability to affect algal growth, photosynthetic apparatus structure and

28   energy flux, round and through photosystems II and I. Specifically, 24-48h RLs-

29   treated algae (RLs at concentrations ranged from 5 to 50 mg L^{-1}) showed significantly

30   decreased levels of growth rate, while increased levels of Chl a and b were obtained

31   only in 72-96h RLs-treated algae. Similarly, although no changes were obtained in the

32   Chl a/b ratio and almost all chlorophyll fluorescence parameters over time, yields of

33   electron transport (φR_0, φE_0) and respective performance index (PI_{total}) were

34   negatively affected at 72 and 96h. Based on those findings, it seems that the inhibitory

35   effect of RLs on the algae growth rate after 24 and 48h and the gradual attenuation of

36   the phenomenon (after 72h of exposure), may indicate the initial response of the

37   organism, as well as algae ability to overcome, since RLs showed no effects on algae

38   photosynthetic ability. Those findings reveal for the first time that RLs from

39   *Burkholderia thailandensis* are not harmful for *Dunaliella tertiolecta*. However,

40   further studies with the use of more aquatic species could be essential for assessing

41   the RLs-mediated effects on aquatic biota.

42

43   **Keywords:** Algal growth, *Burkholderia thailandensis*, *Dunaliella tertiolecta*, Energy

44   flux, Photosynthetic apparatus, Rhamnolipids.

1. Introduction

During the last decades, the production of microbial surfactants or biosurfactants by microorganisms is of great interest. Those surface-active compounds are considered as promising alternatives to chemical surfactants, due to their advantageous characteristics, such as their surface activity, pH tolerance, temperature, ionic strength, their biodegradability, low toxicity and emulsifying/demulsifying ability (Elshikh et al., 2017; Vijayakumar and Saravanan, 2015). Among the major groups of biosurfactants (i.e. low molecular mass glycolipids, like trehalolipids, sophorolipids, rhamnolipids and lipopeptides, as well as high molecular mass amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins etc.), rhamnolipids (RLs) are of great importance, thus finding a wide range of applications, like functional food ingredients, detergents, fungicides and fertilizers, and also in cosmetic and pharmaceutical formulations and bioremediation (Müller et al., 2012; Kourmentza et al., 2017).

RLs are widely produced by the opportunistic pathogen *Pseudomonas aeruginosa*, due to its high production rates and space-time yields (Wittgens et al., 2011). However, the employment of such bacterial strains increases safety measures and process control requirements during fermentation, thus leading to the production of RLs from non-pathogenic strains belonging to *Burkholderia* species (Hörmann et al., 2010; Costa et al., 2011; Funston et al., 2016; Kourmentza et al. 2018). The latter process leads to the production of amphiphilic compounds comprising of one or two rhamnose molecules (mono- and/or di-RLs, respectively), linked glycosidically to one or two β-hydroxy fatty acids chains of 8 – 16 carbon atoms (Abdel-Mawgoud et al., 2010; Kourmentza et al., 2017). RLs, occurred as secondary metabolites in the form of mixtures of different congeners (both mono- and di- RLs), can reduce the surface

3

70   tension of water from 72 to 25-30 mN m^{-1} and the interfacial tension against

71   hydrocarbons up to 1 mN m^{-1}. Their critical micelle concentrations range between 10

72   – 225 mg L^{-1} and depend on the relative abundance of the congener mixtures and

73   congener structures (Dubeau et al., 2009; Hörmann et al., 2010). They also act as

74   emulsifiers, as they can form highly stable emulsions with various hydrocarbons and

75   oils (Gudiña et al., 2016a), and as antibacterial, antifungal and antibiofilm agents

76   (Borah et al., 2016; Elshikh et al., 2017).

77   The global market of RLs, and biosurfactants in general, is expected to reach

78   5.5,2 Billion USD by 2022, with the RLs segment projected to grow at the highest

79   Compound Annual Growth Rate (CAGR) during the forecast period between 2017

80   and 2022 (Markets and Markets, 2017). Moreover, the fact that the segment regarding

81   the application of agricultural chemicals is also expected to grow at the highest CAGR

82   within the same period, highlights the necessity of 'green' alternatives, such as

83   biosurfactants, used in crop control and indirect plant growth promotion. In this light,

84   the likelihood of those compounds, like RLs, to end up in aquatic environments (i.e.

85   with sewage water) as well as their potential effects is of great concern (Johann et al.,

86   2016).

87   Since reports on the environmental effects of such biosurfactants are limited,

88   studies concerning their impact on aquatic producers, such as algae, that possess key

89   position in the trophic chain via the production of high amounts of oxygen and their

90   participation in nutrient cycles (DeLorenzo, 2009; Ma et al., 2010; Perreault et al.,

91   2012) are needed. Among algae species, frequently used in biotests for assessing the

92   relative toxicity of various chemicals and/or waste discharges, the green microalgae

93   *Dunaliella tertiolecta* fulfills most of the criteria for a bioassay organism (i.e.

94   cultivation in the laboratory, rapid growth, acute response to environmental stressors)

95 and has been proposed as a standard organism for ecotoxicological tests (US EPA,

96 1974; APHA, 1989; ASTM, 1996; OECD, 2011). In fact, studies regarding the

97 investigation of osmoregulation mechanism, carotenoid production, and

98 photosynthesis under extreme conditions have been performed so far, using species of

99 the genus Dunaliella (Oren, 2005), while a lot of studies reported a battery of algal

100 growth and survival endpoints (i.e. cell density, growth rate and chlorophyll content)

101 as useful indices for assessing functional and structural effects due to environmental

102 stressors (Oren, 2005; DeLorenzo, 2009; Tsiaka et al., 2013; Tsarpali et al., 2016).

103   Given that the photosynthetic apparatus is a common target of environmental

104 stress, the determination of chlorophyll a (Chl a) fluorescence has been recognized as

105 a useful tool in sensing stress of photosynthetic organisms widely used in

106 ecotoxicological bioassays (Zhou et al., 2006; Ralph et al., 2007; Kumar et al., 2014).

107 Specifically, the impact sites can be related to simple structural characteristics such as

108 photosynthetic pigment concentrations and ratios, or to functional properties of PSII

109 and PSI such as antenna performance, electron transport efficiency etc. The most used

110 parameters are maximum and effective quantum yield (Fv/Fm and ΔF/Fm) and non-

111 photochemical quenching (NPQ) (Kumar et al., 2014). Lately, the fast fluorescence

112 induction kinetics of Chl a have been also adopted for ecotoxicology tests (Dewez et

113 al., 2008; Saison et al., 2010; Invally et al., 2017). The signal is the record of

114 fluorescence rise from its minimum in the dark-adapted state, to its maximum, after a

115 saturating pulse. The analysis of polyphasic curves (JIP-test) offers many parameters,

116 each of them related to a step of the energy flux round and between the photosystems

117 (Strasser et al., 2000, 2004). Consequently, the sensitivity of this technique is

118 expected to be high, as the impact site of a tested substance could be related to any of

119 those steps but not to the total process. To our knowledge, despite the fact that

120 chlorophyll fluorescence has been widely used for two decades in ecotoxicology

121 studies (Ralph et al., 2007; Kumar et al., 2014 and references there-in), the JIP-test

122 has been sporadically used the last years (Appenroth et al., 2001; Geoffroy et al.,

123 2003; Xia and Tian, 2009) and only in one study regarding impact of a chemical

124 surfactant on wheat plants (Sharma et al., 2018).

125 Based on the imperative need for investigating RLs complex mixtures instead of

126 single RLs components (Johann et al., 2016) into aquatic ecosystems, the present

127 study aimed to investigate the potential effects of *Burkholderia thailandensis*

128 produced and further purified RLs congeners on the unicellular microalgae *Dunaliella*

129 *tertiolecta*. In this context, algal growth and/or inhibition rates were estimated in RLs-

130 treated algae, while parameters commonly related with the photosynthetic apparatus,

131 such as Chl a, Chl b, total chlorophyll, total carotenoids, as well as chlorophyll

132 fluorescence parameters of photosynthetic systems I and II (PSI & PSII) were further

133 investigated by the JIP-test for the first time.

134

135 **2. Materials and Methods**

136 2.1. Bacterial strain and cultivation conditions

137 The production of RLs was performed as previously described by Kourmentza

138 et al. (2018). In brief, *Burkholderia thailandensis* E264 was grown on nutrient broth,

139 supplemented with 4% w/v of used cooking oil (sunflower) as the sole carbon source.

140 Cultivation took place in a 10L bioreactor with a working volume of 8L. pH was

141 controlled to 7.0 by the automatic addition of base (5M NaOH) or acid (2M HCl),

142 temperature was kept at 37 ± 0.1 °C, air supply was constant at 1 vvm, and DO level

143 was maintained at 20% of air saturation by automatically adjusting the stirring rate.

144 Foam formation, due to RLs production, was managed by mounting of a

145 polyetheretherketon (PEEK) disc to the agitator shaft that served as a mechanical

146 foam destroyer. An antifoam sensor was also installed, in case of excessive foaming,

147 that suppressed foam formation by the addition of Antifoam A agent. At the end of

148 the cultivation the culture broth was collected and further treated for RLs extraction

149 and purification.

150

151 2.2　Rhamnolipid extraction and purification

152 　　　At the end of the cultivation the culture broth was collected, and the cell-free

153 supernatant was obtained upon centrifugation (8000 × g, 25 min). The cell-free

154 supernatant was first acidified to pH 2.0, using 4M HCl, and placed at 4°C overnight

155 in order to promote RLs precipitation. The precipitate was then collected by

156 centrifugation (8000 × g, 15 min) and re-dissolved in distilled water. RLs were

157 extracted from the aqueous solution by adding an equal volume of ethyl acetate and

158 then vortexed for 5 min. The mix was left in a separation funnel until phase separation

159 was achieved, and then the organic phase, that contained the RLs, was collected. The

160 extraction of RLs from the aqueous solution was repeated three times in total, as

161 described above. The resulting organic phase collected after each extraction was

162 concentrated by rotary evaporator. Purification was performed by re-dissolving the

163 crude RL concentrate in chloroform and was then forwarded to solid phase extraction

164 using SI-1 Silica-based sorbent. RLs were eluted using a chloroform-methanol

165 solution (1:1 v/v), and finally concentrated under nitrogen atmosphere.

166

167 2.3　Rhamnolipids characterization

168 　　　RLs characterization and relative abundance between different congeners was

169 performed as previously described by Kourmentza et al. (2018). Liquid

170 Chromatography (Finningan Surveyor) equipped with a C8 reverse phase column

171 (Vydac® 208TP C8, ID 2.1 × L 150 mm, 5 μm) and a diode array detector (DAD)

172 coupled with a Thermo Finningan LCQ DECA XP MAX quadropole ion trap mass

173 spectrometer (MS), in negative electrospray ionization mode, was performed. RLs

174 mixtures of high purity (~95%), one dominant in the mono-RL C_{10}-C_{10} and another

175 one dominant in the di-RL C_{10}-C_{10}, were used for the calibration curves (R95D90/

176 R95M90, AGAE Technologies), in the same range of concentrations, and the results

177 were expressed as equivalents of these standards.

178

179 2.4   Algal biotest

180 The green algae *Dunaliella tertiolecta* (strain CCAP 19/6B, from Scottish

181 Marine Institute, Oban, Argyll, Scotland) was cultivated in *f/2* medium without Si (24

182 ±1°C, pH 8.3 ± 0.3, 86± 8.6 μE/m^2/s fluorescent lighting) (OECD, 2011). At late

183 logarithmic phase, 1×10^4 cells mL^{-1} were transferred to conical sterilized flasks,

184 containing freshly prepared culture medium (final volume 200 mL) and further treated

185 with different concentrations of RLs (5, 10, 20 and 50 mg L^{-1}) for 96 h. Those RLs

186 concentrations tested are referred as "nominal" concentrations, since there is no data

187 regarding RLs solubility into the culture medium, as well as RLs ability to bind to

188 culture medium compounds and culture flask cell walls as previously mentioned

189 (Tsarpali et al., 2016). On the other hand, the range of RLs currently tested was

190 almost like those previously reported to other species tested (see for example Sydow

191 et al., 2018; Wang et al., 2005; Gustafsson et al., 2009; Johann et al., 2016).

192 Every 24 h, algal cell number, growth rate (μ) and inhibition rate (%I) were

193 counted/determined according to well-known equations (for further details see SM

194 2.4). Two independent experiments were performed and RLs concentrations were

195 tested in duplicate per experiment.

196 In parallel, parameters commonly related with the photosynthetic ability of

197 algae, such as the contents of Chl a, Chl b, total chlorophyll, total carotenoids, as well

198 as chlorophyll fluorescence parameters indicative of the physiological status of

199 photosynthetic systems I and II (PSI & PSII) were also measured.

200

201 2.5 Determination of chlorophyll content and total carotenoids

202 10 mL of each culture (RLs- and RLs-free algal cultures) were transferred in

203 Falcon tubes every 24h. All samples were centrifuged at 4000 x g for 10 min and the

204 supernatant was carefully discarded. Packed cells were diluted initially with 1 mL

205 dimethyl-formamide (DMF) to a final volume of 4 ml. After an incubation period of

206 20 min, samples were centrifuged as mentioned above, and the supernatant was used

207 for spectrophotometric analysis (Shimadzu UV-VIS 160A Spectrophotometer,

208 Shimadzu Corporation, Tokyo) at 480, 647, 664 and 750 nm.

209 Chl a, Chl b and total carotenoids content (μg mL^{-1}) was calculated using the

210 Lambert-Beer based equations (3-5) (Wellburn, 1994) (for further details see SM 2.5).

211

212 2.6 Chlorophyll fluorescence measurements and JIP-test

213 Fast Chl a fluorescence transient was captured by a portable fluorimeter

214 (Handy-PEA, Hansatech Instruments Ltd. King's Lynn Norfolk, UK). Measurements

215 were conducted on dark adapted samples (2 mL of RLs- and RLs-free algal culture in

216 each case, 15 min adaptation time) and the filtered medium for each treatment served

217 as the blank. A bank of three red LEDs (peak at 650 nm) providing 3000 μmol m^{-2} s^{-1},

218 was used for excitation. Fluorescence was recorded from 10 μs to 2s with intervals of

9

219   10 μs, 100 μs, 1 ms, 10 ms and 100 ms between the readings, for time periods of 10-

220   300 μs, 0.3-3 ms, 3-30 ms, 30-300 ms, and 0.3-2 s, respectively. Fluorescence data

221   were then transformed in a logarithmic time scale and the derived polyphasic curve,

222   was analyzed according to JIP-test (Strasser et al., 1995) as extended to analyze

223   events around PSI (Oukarroum et al., 2009; Stirbet and Govindjee, 2011). The

224   parameters which were used for the photosynthetic analysis were: maximum quantum

225   yield of primary PSII photochemistry φP_0 = F_V/F_M; quantum yield of the electron

226   transport flux from Q_A to Q_B, φE_0; quantum yield for reduction of end electron

227   acceptors at the PSI acceptor side φR_0; $1-V_I$, a parameter related to the size of the

228   pools of final PSI electron acceptors and   potential for energy conservation from

229   exciton to the reduction of PSI end acceptors PI_{total}.

230

231   2.7   Statistical analysis

232   The estimation of RLs concentration that cause 50% inhibition of algae growth

233   (IC_{50} endpoints) and their 95% confidence intervals (CI) in each case was performed

234   with the use of Probit analysis ($p<0.05$, IBM SPSS 19 Inc. software package). After

235   checking for homogeneity of variance (Levene's test of equality of error variances),

236   the significant differences among parameters were tested with the use of Mann–

237   Whitney u-test ($p<0.05$).

238

239   **3.   Results**

240   3.1   RLs-mediated effects of *Dunaliella tertiolecta* growth rate

241   LC/MS analysis on the RLs produced (data not shown; for further details see

242   Kourmentza et al., 2018) revealed a narrow range of different RLs congeners,

243   dominant in di-RLs. Specifically, RLs consisted of four congeners; the di-RL Rha-

244   Rha-C_{14}-C_{14} with the highest abundance (71.40 %), the di-RL Rha-Rha-C_{12}-C_{14}, the

245   di-RL Rha-Rha-C_{14}-C_{16} (or Rha-Rha-C_{16}-C_{14}) and the mono-rhamnolipid Rha-C_{14}-C_{14}

246   (14.09, 7.56, and 6.94 % abundance, respectively). Based on the latter, the algal

247   bioassay showed that those RLs (at concentrations ranged from 5 to 50 mg L^{-1}), can

248   alter algal growth rates, thus inhibiting their growth at least at 24 and 48h, followed

249   by a significant attenuation of the adverse effects over time (72 and 96h) (Fig 1, Table

250   1). The latter is more obvious taking in mind the estimated 24-96h IC_{50} values (Table

251   2) that shows a significant attenuation of RLs ability to inhibit algal growth rate over

252   time.

253

254   3.2   RLs-mediated effects on photosynthetic apparatus of *Dunaliella tertiolecta*

255   Algae treated with RLs for 96h showed significant increase of Chl a, Chl b, total

256   Chl and carotenoids levels, irrespectively of the RLs concentration (Fig 2a-b, 3-4).

257   The increase of each photosynthetic pigment content per cell was of the same

258   magnitude, therefore, Chl a/b and Car/Chl ratios did not change (SM Fig 1, 2). The

259   elevated values in the presence of RLs were not detected at 24, 48 and 72 h. In fact,

260   pigment contents doubled their concentrations in both control and treatments at 48 h,

261   while at 72 h, control values were partly reduced only in control and the significant

262   differences between treatments and control revealed at 96 h.

263   The fluorescence measurements in the present study (Table 3), indicated that

264   yield (φE_0) related to electron transport up to Q^{-}_A as well as the pool of end electron

265   acceptors of PSI (1-V_I) were decreased by the highest concentration of RLs at 96 h.

266   Yield for reduction of end electron acceptors at the PSI acceptor side (φR_0) was

267   significantly reduced by the two highest concentrations of RLs (20 and 50 mg L^{-1}).

268 The PI$_{total}$ index showed the highest sensitivity, being reduced even at 10 mg L^{-1} of

269 RLs (Table 3).

270 **4. Discussion**

271 To our knowledge, this is the first study regarding the investigation of *B.*

272 *thailandensis* RLs effects on the unicellular microalgae *Dunaliella tertiolecta*. The

273 current non-pathogenic species has been reported to be an efficient producer of di-

274 RLs, as revealed by the abundances of di- and mono-RLs currently determined, which

275 are composed by longer chain length fatty acid moieties, instead of the opportunistic

276 pathogen *P. aeruginosa* that produces RLs congeners with the most abundant being

277 the mono- RL Rha-C$_{10}$-C$_{10}$, followed by di-RL Rha-Rha-C$_{10}$-C$_{10}$ and mono-RL Rha-

278 C$_{10}$ (Gudiña et al., 2016b). Those structural differences between RLs congeners are

279 attributed to the significant differences in the amino acid sequences of *rhlA*, *rhlB* and

280 *rlhC* genes (Funston et al., 2016). However, RLs produced by *B. thailandensis* and *P.*

281 *aeruginosa* can find different areas of applications due to their different composition

282 that affects their properties and therefore their behavior as biosurfactants, emulsifying

283 agents etc. (Kourmentza et al., 2017).

284

285 4.1 RLs-mediated effects on algal growth

286 Given that algae are considered as ideal early warning biological systems for

287 assessing any aquatic disturbances, as well as that algal biotests are preferable for

288 ethical and economic reasons (Bae and Park, 2014), the present study revealed the

289 effect of *B. thailandensis* RLs congeners on the unicellular algae *Dunaliella*

290 *tertiolecta*. The results showed for the first time that RLs at concentrations ranged

291 from 5 to 50 mg L^{-1} can cause algal growth inhibition at 24 and 48h, with a

292 concomitant recovery over time. Those RLs concentrations currently used are lower

293   than those previously used for performing algal biotests, using other algal strains

294   (Wang et al., 2005; Gustafsson et al., 2009).

295   Although studies regarding the effects of *B. thailandensis* derived RLs are still

296   lacking, the results of the present study seem to be in accordance with previous

297   studies, concerning the effects of RLs on different species. Specifically, mono-RLs

298   (Rha-C_{10}-C_{10}) were found to be less toxic than those occurred by chemical surfactants,

299   like SDS, on *Daphnia magna* (24h EC_{50} = 50 mg L^{-1}, 48h EC_{50} = 30 mg L^{-1}; 3-100

300   mg L^{-1} concentration tested) and *Danio rerio* (LC_{50} = 60 mg L^{-1}; 2-200 mg L^{-1}

301   concentration tested), (Braunbeck et al., 2005; Johann et al., 2016). Moreover,

302   *Pseudomonas aeruginosa* derived RLs showed significantly reduced levels of growth

303   rates on harmful algal blooms (HAB) species *Alexandrium minutum* and *Karenia*

304   *brevis* (Dinophyceae) even after their exposure to RLs at concentration of 5 mg L^{-1} for

305   24 h (Wang et al., 2005; Gustafsson et al., 2009), which is in accordance with the

306   results of the present study. However, according to EC Regulation 1272/2008 (OJL

307   353, 2008), *B. thailandensis* derived RLs currently tested showed high IC_{50} values

308   [i.e. 72h IC_{50} = 44.57 mg L^{-1} (25.466-212.882) and 96h IC_{50} >1000 mg L^{-1}], thus

309   indicating low harmful effects on marine biota, at least in case of algae species. The

310   latter could be due to RLs high solubility (almost negative log Kow values) and

311   degradation (Kłosowska-Chomiczewska et al., 2017), as well as to species sensitivity

312   and acclimation with time. In fact, it is known that crude RLs are soluble in aqueous

313   solutions at pH 7-7.5, while di-RLs are expected to be more soluble in water

314   compared to mono-RLs since they consist of two rhamnose molecules instead of one

315   (Abdel-Mawgoud at el., 2009). Moreover, in contrast to di-RLs, mono-RLs

316   complexes cadmium 10 times more strongly (unpublished data), is a more powerful

317   solubilizing agent, has lower water solubility, and sorbs to surfaces more strongly

13

318 (Zhang et al., 1997). In parallel with the synergistic/antagonistic effects of RLs

319 congeners previously mentioned, the obtained results (i.e. growth rate and/or %I)

320 could be over- or under- estimated in some extent, due to the absence of data

321 regarding RLs congeners solubility into the culture medium as well as their ability to

322 bind to culture medium compounds and culture flask walls, that could decrease RLs

323 effective concentration (Kramer et al., 2012; Tsarpali et al., 2016). Additionally,

324 regarding species sensitivity and acclimation, it has been reported that the presence of

325 cell wall could be linked with low algal vulnerability, while algae with no cell wall,

326 like *Dunaliella tertiolecta*, could be sensitive to surfactants and other chemical

327 substances, revealing low growth rates after a short period of exposure (Gong et al.,

328 2004), as well as growth rate recovery over time due to adaptation and metabolic

329 regulations, mainly related with detoxification and algal survival under stressed

330 conditions (Poremba et al., 1991; Maslin and Maier, 2000; Nikookar et al., 2005;

331 Zeng et al., 2007; Wen et al., 2009; Tsiaka et al., 2013). However, more studies are

332 needed for elucidating the exact mode of RLs action in algae.

333

334 4.2   RLs-mediated effects on algal photosynthetic apparatus

335   It is known that algae can adjust their intracellular concentration of chlorophylls

336 and carotenoids in response to properties of their culture medium and to

337 environmental conditions. In addition, the light intensity and nutrient availability are

338 the predominant factors influencing photosynthetic pigment concentration and as an

339 adaptive response, pigments are increasing under low light intensity or nutrient

340 transient starvation (Kana et al., 1997; Young and Beardall, 2003; da Silva Ferreira

341 and Sant'Anna, 2017). Based on the latter, the fluctuation of Chl/cell and

342 carotenoids/cell currently observed in control cells can be attributed to acclimation of

343   the algae in the new culture medium after inoculation, while the light or nutrient

344   starvation seemed to cause negligible effects, at least under such a short-term culture

345   treatment (0-96 h). On the other hand, it is therefore most likely that RLs counteracted

346   any temporal environmental pressure through modification of membrane

347   permeability, since it is known that smalls changes in RLs-treated algal surface

348   tension could lead to slight alterations of membrane permeability, preserving or even

349   stimulating pigment formation (Sharma et al., 2018), which in turn could stimulate the

350   growth of cell culture (Lowe et al. 1994).

351   Given that a surfactant can affect thylakoid membranes without affecting

352   pigments of the light harvesting complexes (Markwell and Thornber, 1982), the

353   results of the present study showed that the only negative impact of RLs on

354   photosynthetic processes carried out on thylakoid membranes was related to electron

355   transport round and between PSII and PSI (φE_0, φR_0) and the pool of the final

356   electron acceptors at PSI ($1-V_I$). The PI_{total} incorporates yields of electron transport

357   together with parameters related to flux of energy in light harvest complex and RC of

358   PSII, thus proving a sensitive tool for a variety of stresses in photosynthetic

359   autotrophs (Strasser et al. 2000; Ralph et al. 2007; Koutra et al. 2018). Actually, in the

360   present study, PI_{total} appears as the most sensitive index of the JIP-test, influenced at

361   even lower concentration of RLs, at which any effect on partial electron transport

362   processes cannot be detected.

363   According to previous studies, the most important effect of surfactants on algal

364   cell is the biolytic one. Apart from plasma membrane denaturation which leads to cell

365   lysis, they can cause partial disintegration of the membrane, changing its permeability

366   and facilitating their entrance inside the cell, where they can affect almost every

367   organelle, chloroplast ultrastructure, thylakoid organization, and chlorophyll

15

biosynthesis (Wang et al., 2005; Popova and Kemp, 2007; Vonlanthen et al., 2011).
However, the present study showed that relevant effects could be recorded only at
relatively high concentrations of RLs. In fact, the critical micelle concentration for
RLs depends on their structure and abundant congener, and may range between 10–
225 mg L^{-1} (Dubeau et al., 2009; Sobrinho et al., 2013). For the case of RLs produced
by *B. thailandensis*, that are mainly composed by Rha-Rha-C$_{14}$-C$_{14}$, the critical
micelle concentration was found to be around 225 mg L^{-1} (Kourmentza et al., 2018).
In this context, the possibility of worsening or amelioration of impact on electron
transport processes later than 96h needs further experimentation, while low
concentrations of RLs could be even protective for some aspects of acclimation of the
photosynthetic machinery.

Conclusions

The effects of RLs congeners from the bacteria *Burkholderia thailandensis* on
the growth and the photosynthetic apparatus of the green alga *Dunaliella tertiolecta*
were investigated for the first time. The 96h algal biotest currently performed using
different concentrations of RLs revealed a decrease in the growth rate of the
microalgae at 24 and 48 h, followed by a significant recovery with time (72 h and 96
h), thus indicating low RLs-mediated harmful effects. Additionally, the negligible
impact of RLs on the photosynthetic apparatus of *Dunaliella tertiolecta* revealed for
the first time, thus serving as a useful tool for assessing the applicability and usage of
B. thailandensis RLs in a battery of processes over other environmentally harmful
surfactants. Moreover, the PI$_{total}$ parameter of the JIP-test appeared as the most
sensitive index of any impact on photochemical process. However, more studies using
(a) a battery of aquatic species and (eco)toxicological tests, (b) sophisticated

393 analytical methods for the determination and prediction of the transport and fate of

394 RLs into the aquatic media, and (c) complex mixtures of RLs and environmental

395 contaminants could be of great concern for elucidating RLs environmental footprint.

401 **Conflict of interest**

402 Authors declare no conflict of interest.

403

404 **References**

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619 **FIGURE CAPTIONS**

620 **Figure 1.** Percentage of *Dunaliella tertiolecta* inhibition rate (%I) after treatment for

621 24-96h with different concentrations of RLs. The results are mean ± SDs from 2

622 independent experiments (each experiment was performed in duplicate). Values in

623 each column that share the same letter significantly differ from each other (Mann-

624 Whitney U test, $p<0.05$).

625 **Figure 2.** Determination of (a) Chl a and (b) Chl b in *Dunaliella tertiolecta* after

626 treatment for 24-96h with different concentrations of RLs. The results (expressed as

627 pg of chlorophyll per cell) are mean ± SDs from 2 independent experiments (each

628 experiment was performed in duplicate). Values in each column that share the same

629 letter significantly differ from control (Mann-Whitney U test, $p<0.05$).

630 **Figure 3.** Total chlorophyll content in *Dunaliella tertiolecta* after treatment for 24-

631 96h with different concentrations of RLs. The results are mean ± SDs from 2

632 independent experiments (each experiment was performed in duplicate). Values in

633 each column that share the same letter significantly differ from control (Mann-

634 Whitney U test, $p<0.05$).

635 **Figure 4.** Concentration of carotenoids in *Dunaliella tertiolecta* after treatment for

636 24-96h with different concentrations of RLs. The results (expressed as pg of

637 carotenoids per cell) are mean ± SDs from 2 independent experiments (each

638 experiment was performed in duplicate). Values in each column that share the same

639 letter significantly differ from control (Mann-Whitney U test, $p<0.05$).

Table 1. Algal cell number (cells/mL x 10^4) and growth rate (μ, within the parenthesis) after treatment for 24-96h with different concentrations of RLs. The results are mean ± SDs from 2 independent experiments (each experiment was performed in duplicate). Values in each column that share the same letter significantly differ from each other (Mann-Whitney U test, $p<0.05$).

RLs (mg L⁻¹)	Treatment period (h)			
	24	48	72	96
0	2.38 ±0.05[abcd]	3.29±0.23 [abcd]	6.78±0.38 [abcd]	10.12±0.29 [abcd]
	(0.87±0.02)	(0.59±0.04)	(0.64±0.02)	(0.58±0.01)
5	1.54 ±0.24[a]	2.12±0.30[aefg]	3.68±0.61[a]	5.31±1.89[a]
	(0.42±0.19)	(0.37±0.07)	(0.43±0.06)	(0.40±0.10)
10	1.29±0.32[b]	1.64±0.27[be]	4.06±1.11[b]	6.00±1.68[be]
	(0.23±0.04)	(0.24±0.08)	(0.45±0.10)	(0.44±0.07)
20	1.22±0.14[c]	1.50±0.19[cf]	3.31±1.35[c]	3.68±1.36[cef]
	0.20±0.08)	0.20±0.06)	(0.37±0.16)	(0.31±0.09)
50	1.12±0.23[d]	1.25±0.27[dg]	2.83±1.69[d]	5.68±1.67[df]
	(0.10±0.14)	(0.10±0.11)	(0.30±0.19)	(0.42±0.08)

Table 2. Evaluation of 24-96hIC_{50} values (including confidence interval values within the parenthesis) after treatment with different concentrations of RLs (Probit, $p<0.05$).

| Treatment period (h)	IC_{50} (mg L^{-1})	Hazard classification (EC Regulation 1272/2008)
24	3.011 (1.083-4.932)	
48	8.121 (5.676-10.498)	
72	44.574 (25.466-212.882)	Chronic Category 3 > 10 to ≤ 100 mg L^{-1}
96	>1000 (ne)	

ne: not evaluated due to high variability of the algal response.

Table 3. Photosynthetic parameters in *Dunaliella tertiolecta*, after treatment for a period of 24-96h with different concentrations of RLs. The results are mean ± SDs from 2 independent experiments (each experiment was performed in duplicate). Values in each column that share the same letter significantly differ from the respective control (Mann-Whitney U test, $p<0.05$).

| RLs (mg/L)	Treatment period (h)			
ΦPo or Fv/Fm	24	48	72	96
0	0.53 ± 0.15	0.65 ± 0.01	0.64 ± 0.02	0.67 ± 0.03
5	0.53 ± 0.16	0.63 ± 0.01	0.63 ± 0.01	0.63 ± 0.01
10	0.53 ± 0.16	0.64 ± 0.01	0.66 ± 0.01	0.65 ± 0.02
20	0.50 ± 0.17	0.62 ± 0.03	0.65 ± 0.02	0.64 ± 0.04
50	0.48 ± 0.15	0.62 ± 0.02	0.65 ± 0.01	0.64 ± 0.01
ΦEo				
0	0.26 ± 0.12	0.37 ± 0.02	0.36 ± 0.01	0.36 ± 0.02
5	0.26 ± 0.13	0.34 ± 0.01	0.34 ± 0.01	0.31 ± 0.02[a]
10	0.26 ± 0.13	0.36 ± 0.01	0.37 ± 0.01	0.34 ± 0.01
20	0.24 ± 0.13	0.34 ± 0.03	0.35 ± 0.02	0.33 ± 0.05
50	0.24 ± 0.13	0.33 ± 0.03	0.36 ± 0.01	0.32 ± 0.01[a]
$\varphi R0$				
0	0.10 ± 0.06	0.14 ± 0.02	0.14 ± 0.01	0.14 ± 0.01
5	0.09 ± 0.05	0.14 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
10	0.09 ± 0.06	0.14 ± 0.01	0.13 ± 0.00	0.13 ± 0.01
20	0.09 ± 0.06	0.13 ± 0.02	0.12 ± 0.00[a]	0.11 ± 0.02[a]
50	0.11 ± 0.07	0.14 ± 0.03	0.12 ± 0.00[a]	0.11 ± 0.01[a]
$1-V_I$				
0	0.17 ± 0.06	0.22 ± 0.02	0.22 ± 0.03	0.21 ± 0.02
5	0.16 ± 0.05	0.23 ± 0.02	0.20 ± 0.01	0.21 ± 0.02
10	0.16 ± 0.06	0.22 ± 0.02	0.20 ± 0.00	0.20 ± 0.01
20	0.16 ± 0.07	0.21 ± 0.02	0.18 ± 0.00	0.17 ± 0.01[a]
50	0.20 ± 0.09	0.23 ± 0.04	0.18 ± 0.00	0.17 ± 0.01[a]
PItotal				
0	0.26 ± 0.26	0.44 ± 0.15	0.39 ± 0.07	0.40 ± 0.04
5	0.22 ± 0.22	0.38 ± 0.08	0.31 ± 0.03[a]	0.29 ± 0.07
10	0.24 ± 0.24	0.39 ± 0.09	0.35 ± 0.04	0.31 ± 0.05[a]
20	0.22 ± 0.23	0.32 ± 0.14	0.30 ± 0.04[a]	0.23 ± 0.10[a]
50	0.29 ± 0.32	0.34 ± 0.14	0.29 ± 0.03[a]	0.21 ± 0.04[a]
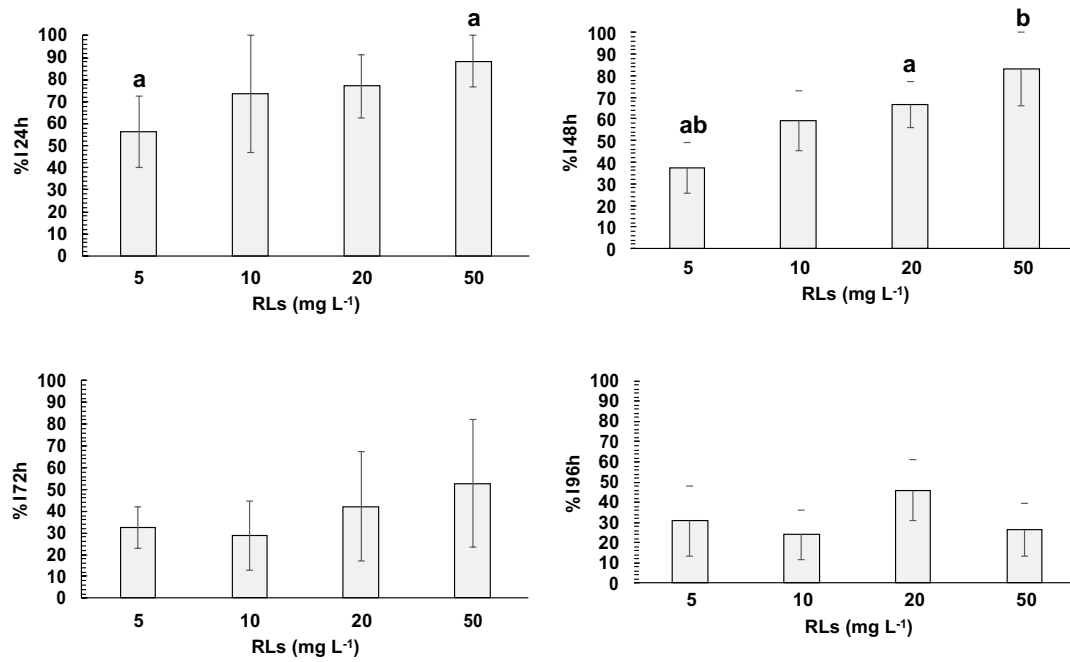
Fig 1.
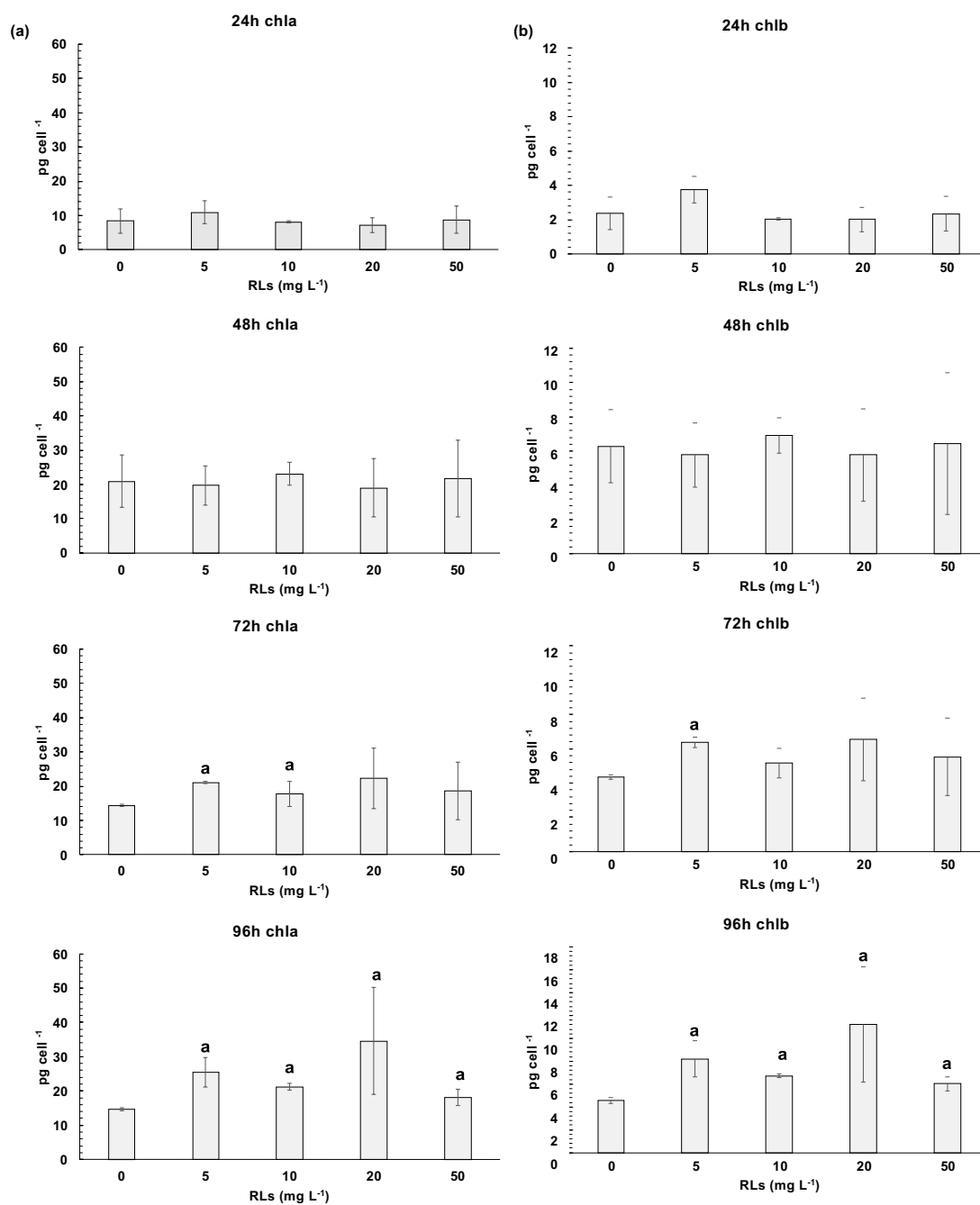
Fig 2.
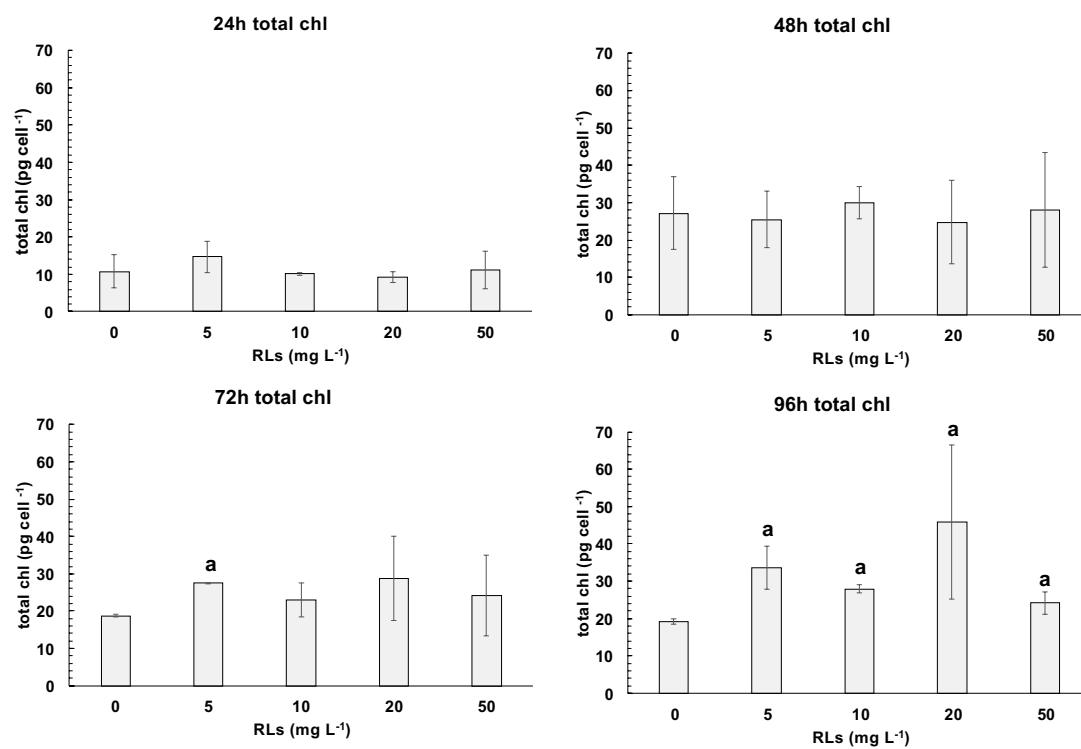
Fig 3.
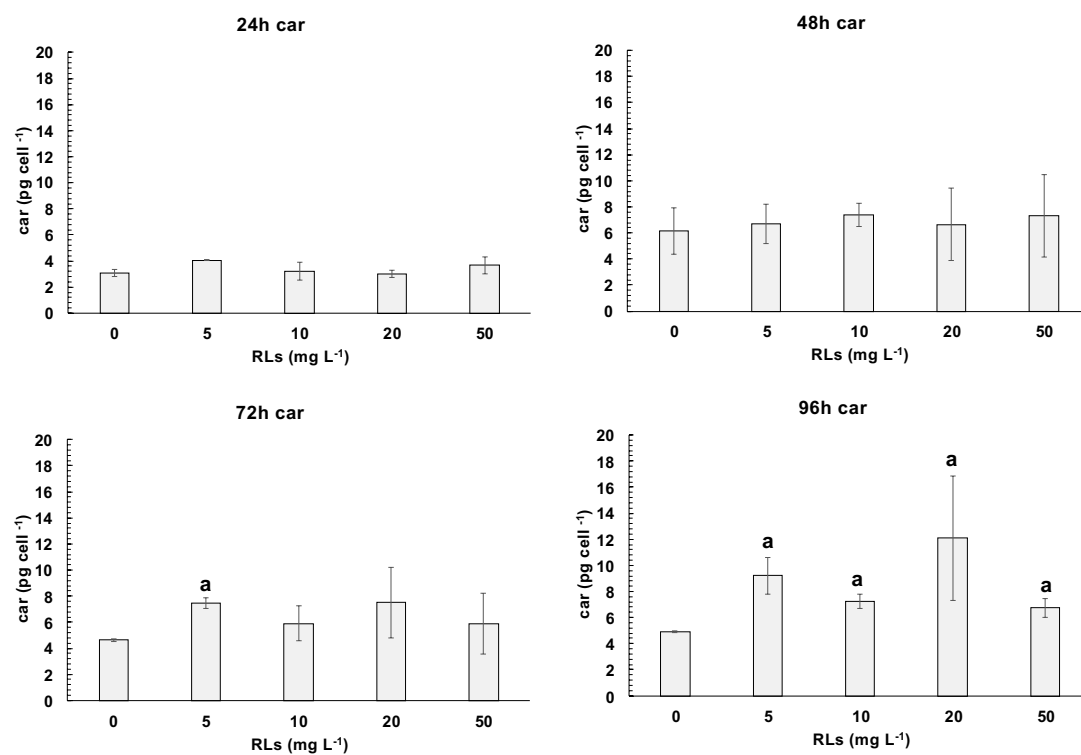
Fig 4.

Supplementary material

Effects of *Burkholderia thailandensis* rhamnolipids on the unicellular algae *Dunaliella tertiolecta*.

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SM 2.4   Algal biotest

The algal cell number was counted, using a Neubauer hemocytometer, while the growth (μ) and the inhibition rate (%I) were determined according to equations (1) and (2).

$$\mu_n = \frac{\ln X_n - \ln X_0}{t_n - t_0} \qquad (1)$$

μ_n: algal growth rate (day^{-1}) after n days (24, 48, 72 or 96h)

X_0 = number of cells/ml at time 0 (t_0)

X_n = number of cells/ml at t_n

t_0 = time of first measurement after beginning of test

t_n = time of nth measurement after beginning of test

$$\%I = \frac{\mu_c - \mu_n}{\mu_c} \times 100 \qquad (2)$$

%I: percent inhibition in average specific growth rate

μ_c: mean value for average specific growth rate (μ) in the control group

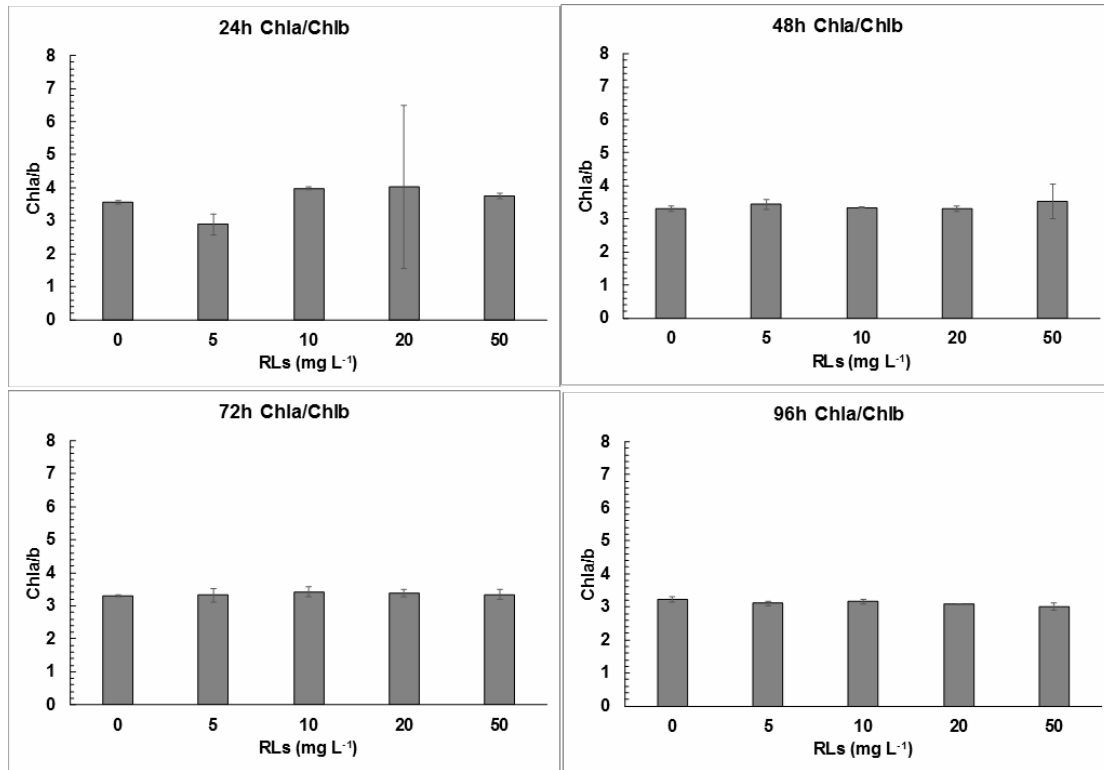μ_n: average specific growth rate for the treatment replicate.

SM 2.5   Determination of chlorophyll content and total carotenoids

Chl a, Chl b and total carotenoids content (μg mL^{-1}) was calculated using the Lambert-Beer based equations (3-5) (Wellburn, 1994).

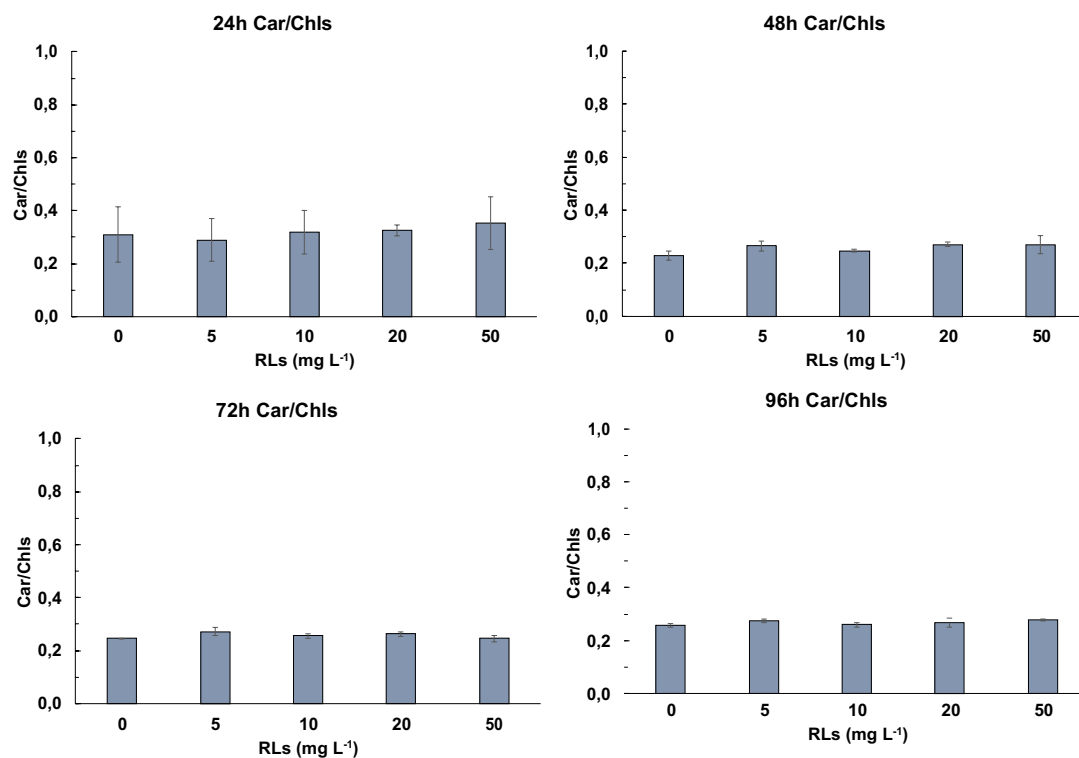$$C_a = 11.65A_{664} - 2.69A_{647} \qquad (3)$$

$$C_b = 20.81A_{647} - 4.53A_{664} \qquad (4)$$

$$C_{x+c} = (1000A_{480} - 0.89C_a - 52.02C_b)/245 \qquad (5)$$

3

SM Figure 1. Chl a/Chl b ratio in *Dunaliella tertiolecta* after treatment for 24-96h with different concentrations of RLs. The results are mean ± SDs from 2 independent experiments (each experiment was performed in duplicate).

SM Figure 2. Carotenoids/total chlorophyll ratio in *Dunaliella tertiolecta* after treatment for 24-96h with different concentrations of RLs. The results are mean ± SDs from 2 independent experiments (each experiment was performed in duplicate).