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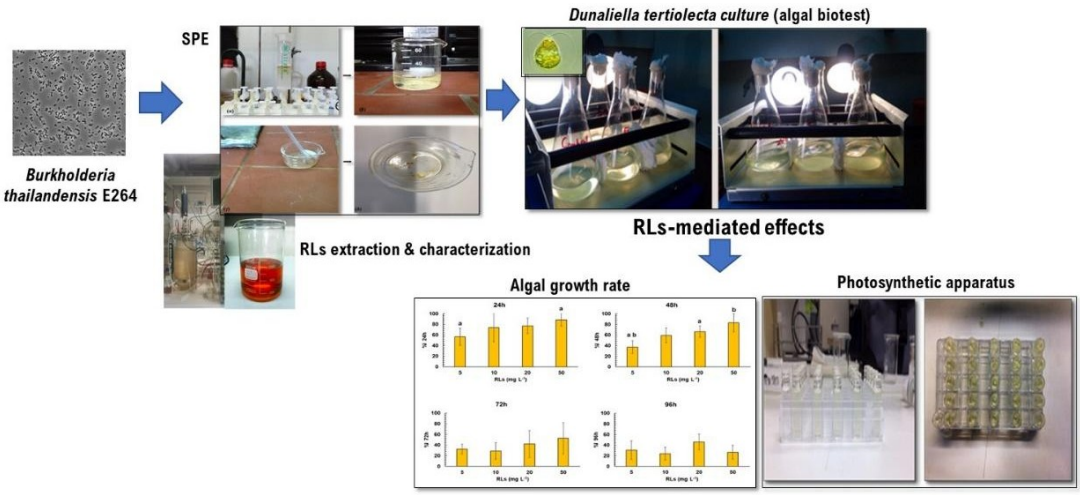

Graphical abstract

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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Abstract

The effects of rhamnolipids (RLs) produced and further purified from *Burkholderia thailandensis*, on the unicellular microalgae *Dunaliella tertiolecta* were investigated, in terms of RLs ability to affect algal growth, photosynthetic apparatus structure and energy flux, round and through photosystems II and I. Specifically, 24-48h RLs-treated algae (RLs at concentrations ranged from 5 to 50 mg L^{-1}) showed significantly decreased levels of growth rate, while increased levels of Chl a and b were obtained only in 72-96h RLs-treated algae. Similarly, although no changes were obtained in the Chl a/b ratio and almost all chlorophyll fluorescence parameters over time, yields of electron transport (φR_0, φE_0) and respective performance index (PI_{total}) were negatively affected at 72 and 96h. Based on those findings, it seems that the inhibitory effect of RLs on the algae growth rate after 24 and 48h and the gradual attenuation of the phenomenon (after 72h of exposure), may indicate the initial response of the organism, as well as algae ability to overcome, since RLs showed no effects on algae photosynthetic ability. Those findings reveal for the first time that RLs from *Burkholderia thailandensis* are not harmful for *Dunaliella tertiolecta*. However, further studies with the use of more aquatic species could be essential for assessing the RLs-mediated effects on aquatic biota.

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

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)

and has been proposed as a standard organism for ecotoxicological tests (US EPA, 1974; APHA, 1989; ASTM, 1996; OECD, 2011). In fact, studies regarding the investigation of osmoregulation mechanism, carotenoid production, and photosynthesis under extreme conditions have been performed so far, using species of the genus Dunaliella (Oren, 2005), while a lot of studies reported a battery of algal growth and survival endpoints (i.e. cell density, growth rate and chlorophyll content) as useful indices for assessing functional and structural effects due to environmental stressors (Oren, 2005; DeLorenzo, 2009; Tsiaka et al., 2013; Tsarpali et al., 2016).

Given that the photosynthetic apparatus is a common target of environmental stress, the determination of chlorophyll a (Chl a) fluorescence has been recognized as a useful tool in sensing stress of photosynthetic organisms widely used in ecotoxicological bioassays (Zhou et al., 2006; Ralph et al., 2007; Kumar et al., 2014). Specifically, the impact sites can be related to simple structural characteristics such as photosynthetic pigment concentrations and ratios, or to functional properties of PSII and PSI such as antenna performance, electron transport efficiency etc. The most used parameters are maximum and effective quantum yield (Fv/Fm and ΔF/Fm) and non-photochemical quenching (NPQ) (Kumar et al., 2014). Lately, the fast fluorescence induction kinetics of Chl a have been also adopted for ecotoxicology tests (Dewez et al., 2008; Saison et al., 2010; Invally et al., 2017). The signal is the record of fluorescence rise from its minimum in the dark-adapted state, to its maximum, after a saturating pulse. The analysis of polyphasic curves (JIP-test) offers many parameters, each of them related to a step of the energy flux round and between the photosystems (Strasser et al., 2000, 2004). Consequently, the sensitivity of this technique is expected to be high, as the impact site of a tested substance could be related to any of those steps but not to the total process. To our knowledge, despite the fact that

chlorophyll fluorescence has been widely used for two decades in ecotoxicology studies (Ralph et al., 2007; Kumar et al., 2014 and references there-in), the JIP-test has been sporadically used the last years (Appenroth et al., 2001; Geoffroy et al., 2003; Xia and Tian, 2009) and only in one study regarding impact of a chemical surfactant on wheat plants (Sharma et al., 2018).

Based on the imperative need for investigating RLs complex mixtures instead of single RLs components (Johann et al., 2016) into aquatic ecosystems, the present study aimed to investigate the potential effects of *Burkholderia thailandensis* produced and further purified RLs congeners on the unicellular microalgae *Dunaliella tertiolecta*. In this context, algal growth and/or inhibition rates were estimated in RLs-treated algae, while parameters commonly related with the photosynthetic apparatus, such as Chl a, Chl b, total chlorophyll, total carotenoids, as well as chlorophyll fluorescence parameters of photosynthetic systems I and II (PSI & PSII) were further investigated by the JIP-test for the first time.

2. Materials and Methods

2.1. Bacterial strain and cultivation conditions

The production of RLs was performed as previously described by Kourmentza et al. (2018). In brief, *Burkholderia thailandensis* E264 was grown on nutrient broth, supplemented with 4% w/v of used cooking oil (sunflower) as the sole carbon source. Cultivation took place in a 10L bioreactor with a working volume of 8L. pH was controlled to 7.0 by the automatic addition of base (5M NaOH) or acid (2M HCl), temperature was kept at 37 ± 0.1 °C, air supply was constant at 1 vvm, and DO level was maintained at 20% of air saturation by automatically adjusting the stirring rate. Foam formation, due to RLs production, was managed by mounting of a

polyetheretherketon (PEEK) disc to the agitator shaft that served as a mechanical foam destroyer. An antifoam sensor was also installed, in case of excessive foaming, that suppressed foam formation by the addition of Antifoam A agent. At the end of the cultivation the culture broth was collected and further treated for RLs extraction and purification.

2.2 Rhamnolipid extraction and purification

At the end of the cultivation the culture broth was collected, and the cell-free supernatant was obtained upon centrifugation (8000 × g, 25 min). The cell-free supernatant was first acidified to pH 2.0, using 4M HCl, and placed at 4°C overnight in order to promote RLs precipitation. The precipitate was then collected by centrifugation (8000 × g, 15 min) and re-dissolved in distilled water. RLs were extracted from the aqueous solution by adding an equal volume of ethyl acetate and then vortexed for 5 min. The mix was left in a separation funnel until phase separation was achieved, and then the organic phase, that contained the RLs, was collected. The extraction of RLs from the aqueous solution was repeated three times in total, as described above. The resulting organic phase collected after each extraction was concentrated by rotary evaporator. Purification was performed by re-dissolving the crude RL concentrate in chloroform and was then forwarded to solid phase extraction using SI-1 Silica-based sorbent. RLs were eluted using a chloroform-methanol solution (1:1 v/v), and finally concentrated under nitrogen atmosphere.

2.3 Rhamnolipids characterization

RLs characterization and relative abundance between different congeners was performed as previously described by Kourmentza et al. (2018). Liquid

Chromatography (Finningan Surveyor) equipped with a C8 reverse phase column (Vydac® 208TP C8, ID 2.1 × L 150 mm, 5 μm) and a diode array detector (DAD) coupled with a Thermo Finningan LCQ DECA XP MAX quadropole ion trap mass spectrometer (MS), in negative electrospray ionization mode, was performed. RLs mixtures of high purity (~95%), one dominant in the mono-RL C_{10}-C_{10} and another one dominant in the di-RL C_{10}-C_{10}, were used for the calibration curves (R95D90/ R95M90, AGAE Technologies), in the same range of concentrations, and the results were expressed as equivalents of these standards.

2.4 Algal biotest

The green algae *Dunaliella tertiolecta* (strain CCAP 19/6B, from Scottish Marine Institute, Oban, Argyll, Scotland) was cultivated in *f/2* medium without Si (24 ±1°C, pH 8.3 ± 0.3, 86± 8.6 μE/m^2/s fluorescent lighting) (OECD, 2011). At late logarithmic phase, 1x10^4 cells mL^{-1} were transferred to conical sterilized flasks, containing freshly prepared culture medium (final volume 200 mL) and further treated with different concentrations of RLs (5, 10, 20 and 50 mg L^{-1}) for 96 h. Those RLs concentrations tested are referred as "nominal" concentrations, since there is no data regarding RLs solubility into the culture medium, as well as RLs ability to bind to culture medium compounds and culture flask cell walls as previously mentioned (Tsarpali et al., 2016). On the other hand, the range of RLs currently tested was almost like those previously reported to other species tested (see for example Sydow et al., 2018; Wang et al., 2005; Gustafsson et al., 2009; Johann et al., 2016).

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

10 μs, 100 μs, 1 ms, 10 ms and 100 ms between the readings, for time periods of 10-300 μs, 0.3-3 ms, 3-30 ms, 30-300 ms, and 0.3-2 s, respectively. Fluorescence data were then transformed in a logarithmic time scale and the derived polyphasic curve, was analyzed according to JIP-test (Strasser et al., 1995) as extended to analyze events around PSI (Oukarroum et al., 2009; Stirbet and Govindjee, 2011). The parameters which were used for the photosynthetic analysis were: maximum quantum yield of primary PSII photochemistry $\varphi P_0 = F_V/F_M$; quantum yield of the electron transport flux from Q_A to Q_B, φE_0; quantum yield for reduction of end electron acceptors at the PSI acceptor side φR_0; $1\text{-}V_I$, a parameter related to the size of the pools of final PSI electron acceptors and potential for energy conservation from exciton to the reduction of PSI end acceptors PI_{total}.

2.7 Statistical analysis

The estimation of RLs concentration that cause 50% inhibition of algae growth (IC_{50} endpoints) and their 95% confidence intervals (CI) in each case was performed with the use of Probit analysis ($p<0.05$, IBM SPSS 19 Inc. software package). After checking for homogeneity of variance (Levene's test of equality of error variances), the significant differences among parameters were tested with the use of Mann–Whitney u-test ($p<0.05$).

3. Results

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

LC/MS analysis on the RLs produced (data not shown; for further details see Kourmentza et al., 2018) revealed a narrow range of different RLs congeners, 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 ($1\text{-}V_I$) 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

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 ($1\text{-}V_I$). 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

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

analytical methods for the determination and prediction of the transport and fate of RLs into the aquatic media, and (c) complex mixtures of RLs and environmental contaminants could be of great concern for elucidating RLs environmental footprint.

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Conflict of interest

Authors declare no conflict of interest.

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FIGURE CAPTIONS

Figure 1. Percentage of *Dunaliella tertiolecta* inhibition rate (%I) 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, $p<0.05$).

Figure 2. Determination of (a) Chl a and (b) Chl b in *Dunaliella tertiolecta* after treatment for 24-96h with different concentrations of RLs. The results (expressed as pg of chlorophyll per cell) 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 control (Mann-Whitney U test, $p<0.05$).

Figure 3. Total chlorophyll content 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). Values in each column that share the same letter significantly differ from control (Mann-Whitney U test, $p<0.05$).

Figure 4. Concentration of carotenoids in *Dunaliella tertiolecta* after treatment for 24-96h with different concentrations of RLs. The results (expressed as pg of carotenoids per cell) 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 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, $p<0.05$).

	Treatment period (h)			
RLs (mg L^{-1})	**24**	**48**	**72**	**96**
0	2.38 ±0.05^{abcd} (0.87±0.02)	3.29±0.23 ^{abcd} (0.59±0.04)	6.78±0.38 ^{abcd} (0.64±0.02)	10.12±0.29 ^{abcd} (0.58±0.01)
5	1.54 ±0.24^{a} (0.42±0.19)	2.12±0.30^{aefg} (0.37±0.07)	3.68±0.61^{a} (0.43±0.06)	5.31±1.89^{a} (0.40±0.10)
10	1.29±0.32^{b} (0.23±0.04)	1.64±0.27^{be} (0.24±0.08)	4.06±1.11^{b} (0.45±0.10)	6.00±1.68^{be} (0.44±0.07)
20	1.22±0.14^{c} 0.20±0.08)	1.50±0.19^{cf} 0.20±0.06)	3.31±1.35^{c} (0.37±0.16)	3.68±1.36^{cef} (0.31±0.09)
50	1.12±0.23^{d} (0.10±0.14)	1.25±0.27^{dg} (0.10±0.11)	2.83±1.69^{d} (0.30±0.19)	5.68±1.67^{df} (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]
φR0				
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]

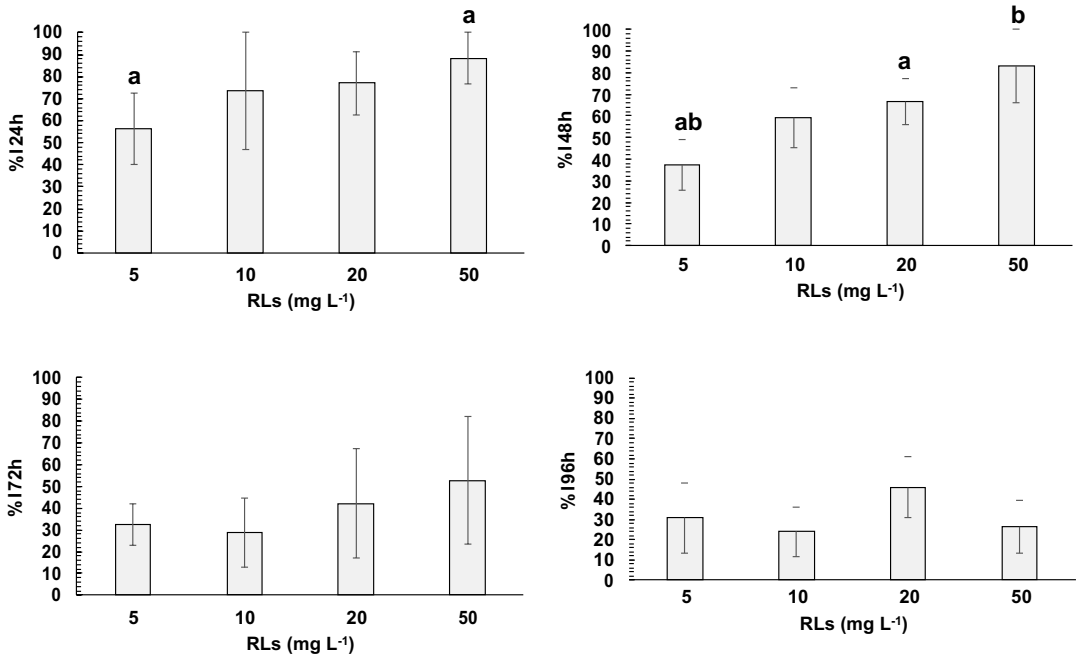

Fig 1.

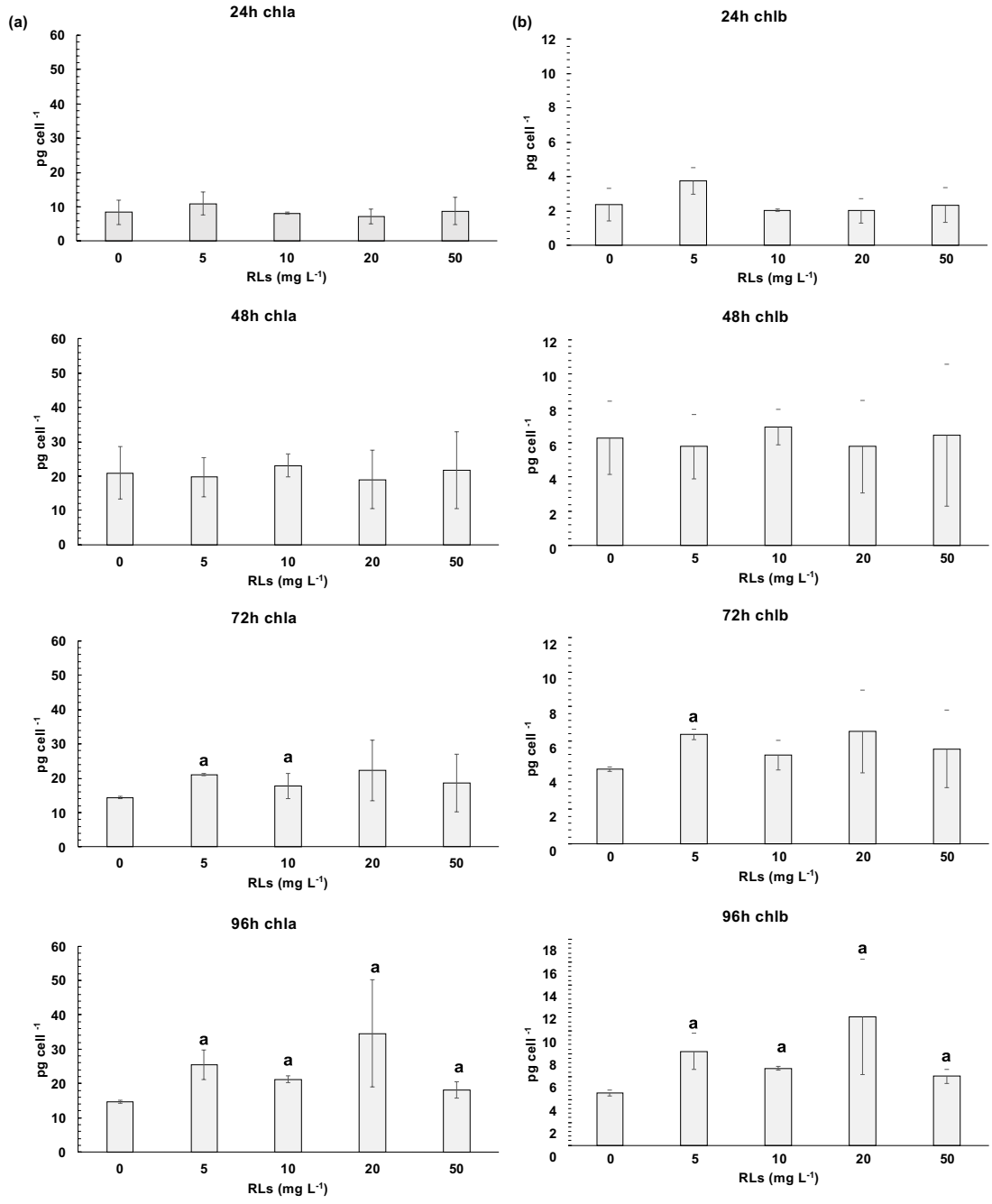
(a)
24h chla
(b)
24h chlb
48h chla
48h chlb
72h chla
72h chlb
96h chla
96h chlb
pg cell -1
RLs (mg L-1)
0
5
10
20
50
a

Fig 2.

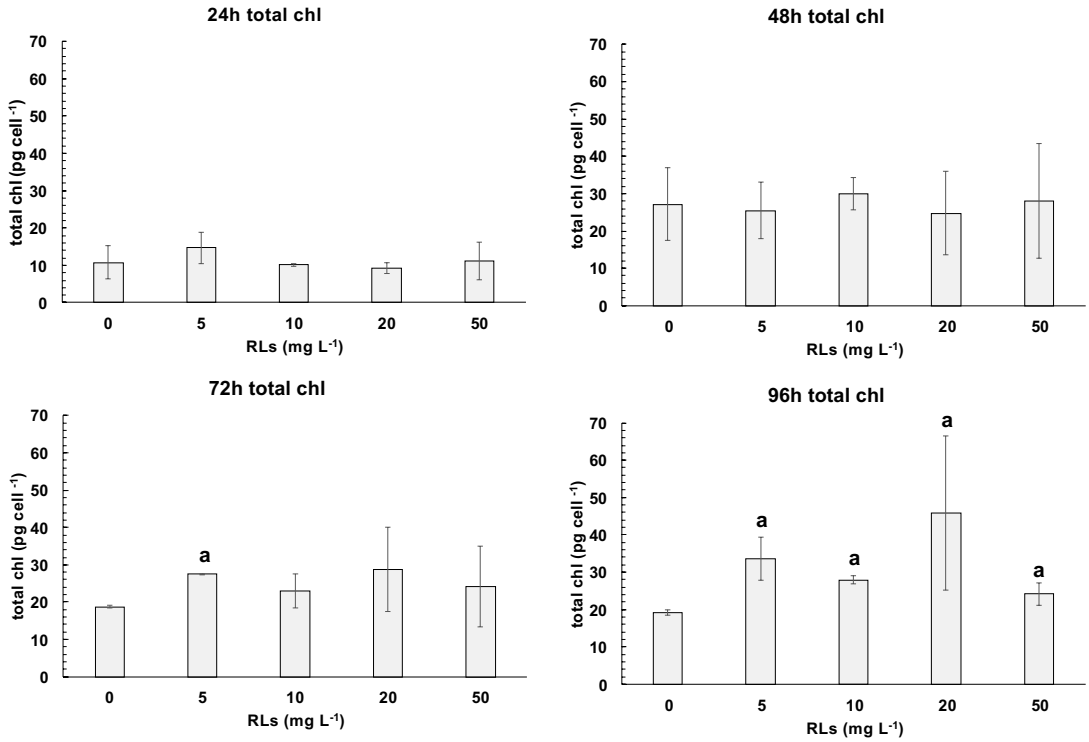
24h total chl
48h total chl
72h total chl
96h total chl
total chl (pg cell -1)
RLs (mg L-1)
0
5
10
20
50
a

Fig 3.

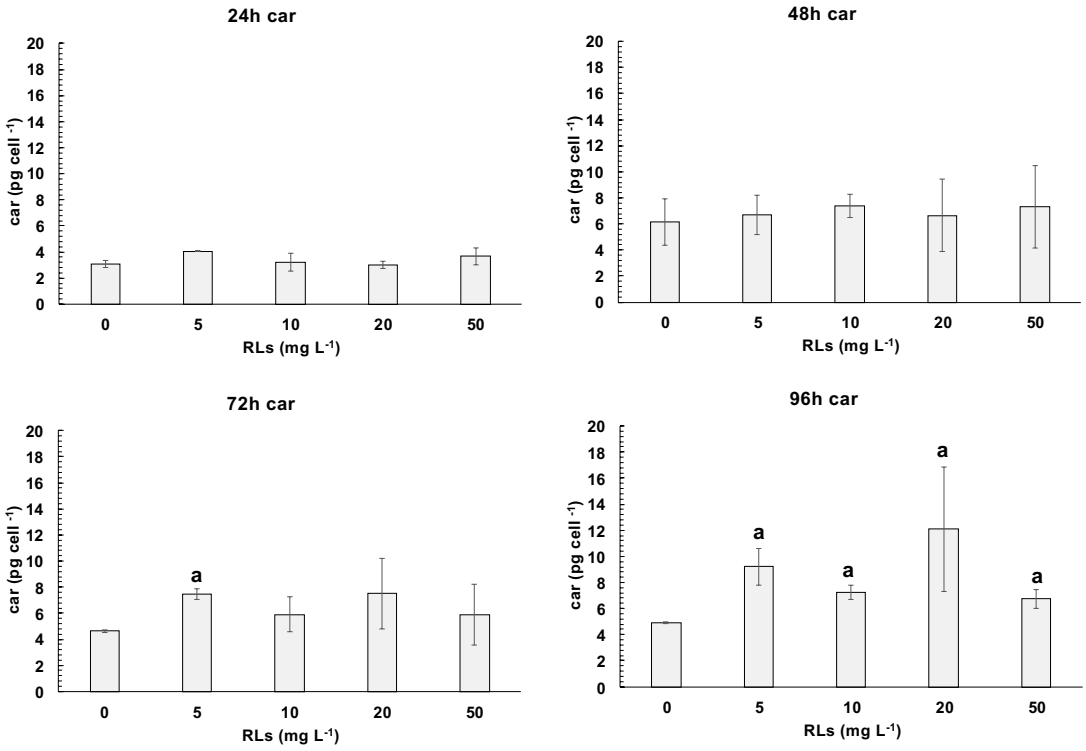
24h car
48h car
72h car
96h car
car (pg cell -1)
RLs (mg L-1)
0
5
10
20
50
a

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.

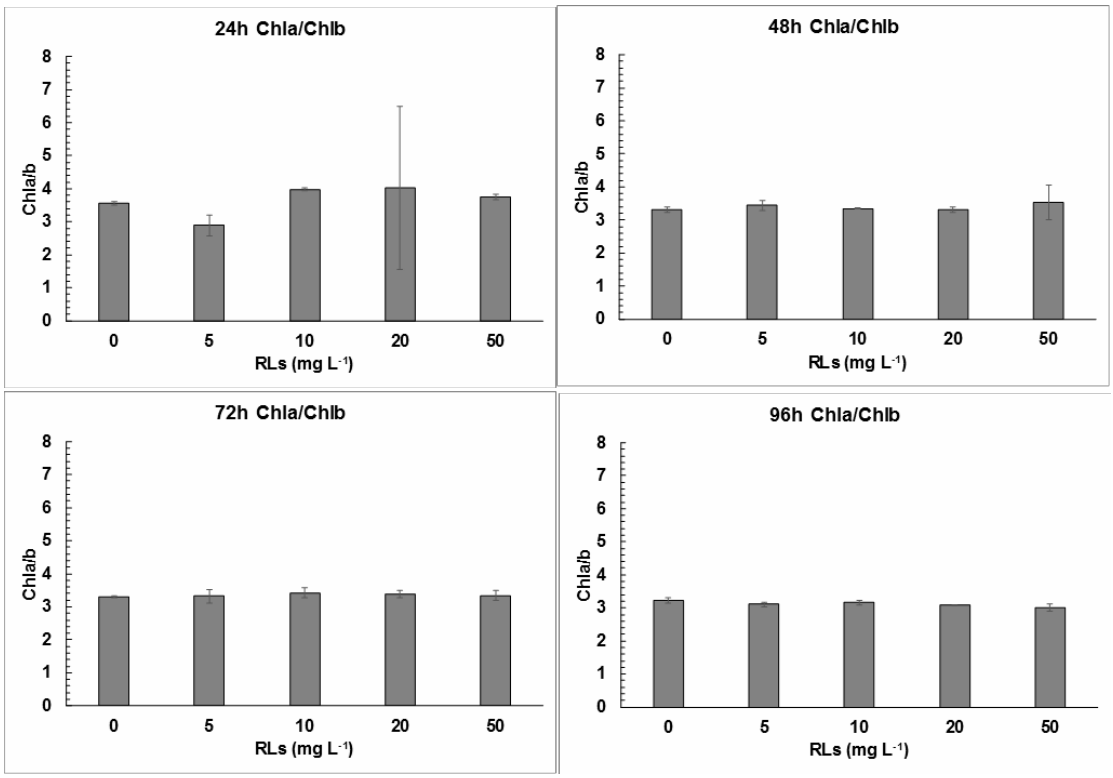
SM 2.5 Determination of chlorophyll content and total carotenoids

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

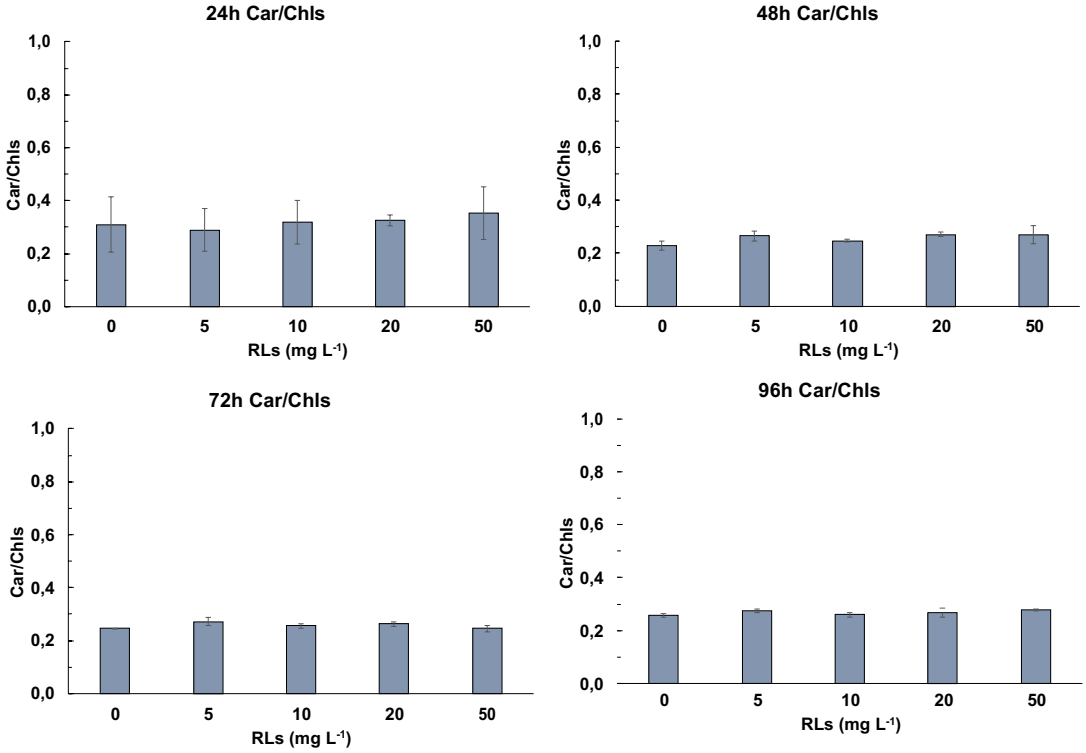
$$C_a = 11.65A_{664} - 2.69A_{647} \qquad (3)$$

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

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



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).