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Can duckweed be used for biomonitoring of textile effluents?

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1 Abstract

2 In certain countries, poorly managed freshwaters need to be seriously controlled to not lead to ecosystems
3 degradation. This control can be achieved by a biomonitoring using living organisms as ecological alert
4 indicators. In this context, biotests were undertaken on *Lemna gibba* (*L. gibba*) to assess the toxic effect of
5 textile pollutants reaching a natural ecosystem. *L. gibba* were exposed to different concentrations (5-100 mg/L)
6 of two dyes, namely Direct Red 89 (DR-89) and Vat Blue 20 (VB-20), under laboratory conditions. Our findings
7 showed that at concentrations > 50 mg/L, visible damage of toxicity (chlorosis and dislocation of fronds) were
8 appeared from the third day of toxicity tests leading to serious necrosis. However, at dye concentration ≤ 50
9 mg/L, duckweed showed no visible signs of toxicity within an exposure time of 4 days. However, these
10 concentrations exhibited a significant inhibition in *L. gibba* growth rate with a clear abatement in the
11 photosynthetic pigments contents. The dye concentration that reduces by 50% the growth rate of the plants
12 (IC50) was 36.3 and 26.9 mg/L for DR-89 and VB-20, respectively. The concentration-dependant reduction in
13 growth and photosynthetic pigments demonstrated their sensitivity in detection of dyes in aquatic systems. The
14 current findings proving physiological alterations of *L. gibba* following dye exposure, suggested that the species
15 are strongly suitable for the testing of textile effluents contaminating water bodies.

16 **Keywords:** Biomonitoring; *Lemna gibba*; dye pollutant; Photosynthetic pigments; Toxicity test.

18 1. Introduction

19 The urban industrialization and population growth have placed an escalating pressure on our environment by
20 discharging organic and inorganic pollutants in atmospheric, terrestrial and aquatic ecosystems. This fact led to a
21 threat in human and animal health and a disturbance in the ecological balance (**Khataee et al. 2012; Padmesh et**
22 **al. 2005**). Among the most dangerous effluents, the textile industry handles nearly one million tons of synthetic
23 dyes. Ten percent of these dyes are released into natural ecosystems following inefficiencies in dying or loss of
24 dye products during textile processes (**Jadhav et al. 2010**). Although the textile effluents are treated in
25 wastewater plants, the big majority of them is bio-recalcitrant and can easily escape to reach the nature. They can
26 contaminate the water bodies because of the poorly managed plants in certain developing countries (**Mangadze**
27 **et al. 2019**). The colored effluents are a serious ecological concern because of their potential toxicity to aquatic
28 life with eventual carcinogenic and mutagenic effects (**Ventura-Camargo et al. 2016**). Additionally, these non-
29 biodegradable compounds can contribute to strong coloration of water even at low concentration reducing

30 sunlight penetration into the water. Moreover, dying effluents can enter terrestrial systems and contaminate the
31 agriculture lands leading to serious health implications as recently reported (**Raziq et al., 2017**). It is therefore
32 essential to ascertain the toxicity threshold of dye products in order to detect and assess their impact on biotic
33 systems.

34 For pollutant analysis purposes, a wide range of approaches is adopted including the direct chemical analysis and
35 physical detection. The direct chemical analysis has several disadvantages such as complex procedures for the
36 preparation of samples, the expensive chemicals requirement and the interference caused by the secondary
37 pollutants during the analysis (**Park et al., 2012**). In addition, this approach does not take into account the
38 temporal changes of the exposure or interactive effects of pollutants (**Kumar and Han, 2010**). On the other
39 hand, physical methods require strong and often expensive and heavy equipment. To compensate these
40 limitations, various biological tests have been developed with standardization of the hazard and risk assessment
41 to identify exposure value and adverse effects of pollutants on human health and the environment (**Hund-Rinke**
42 **et al., 2016**). Particularly, plant-based bioassays have gained remarkable popularity among the toxicological
43 assessment procedures. Those bioassays include the use of aquatic plants that can provide information on the
44 toxic effects of certain pollutants (**Siddiqui et al., 2011; Eullaffroy and Vernet, 2003**). In certain
45 Mediterranean countries, such as Algeria where the management of aquatic systems is still difficult, aquatic
46 macrophytes have attracted the interest of researchers in recent years for elaborating toxicity tests (**Alonso et al.,**
47 **2018; Bck et al., 2013; Wang, 1990**). Among these aquatic organisms, duckweed may perhaps be used as
48 ecological alert indicator to measure the phytotoxicity of synthetic dyes (**Bck et al., 2013; Cleuvers and**
49 **Ratte, 2002**).

50 The duckweed *Lemna gibba* is a small floating plant belonging to the family of *Lemnaceae*. It is found on the
51 surface of eutrophic backwater or slow flows of fresh and brackish waters forming green dense carpets (**Driever**
52 **et al., 2005**). *Lemna* is a very appropriate species for the toxicity tests with its simple structure, rapid growth and
53 high tolerance to different stresses and part of an international standard protocol (**OECD, 2006**). Duckweed
54 species have been previously used in toxicity tests and applied as bioindicator for detecting several pollutants in
55 aquatic environment (**Trker et al., 2016; Khellaf and Zerdaoui, 2010; Sinha et al., 1995**). Studies detailing
56 the effect of dyes effluents on these aquatic species warrant to be deepened to complete data reported in the
57 literature which are still insufficient since they concern only few pollutants and few plant species. The first
58 objective of the present work is to ascertain the toxicity effects of two dyes on the morphology and physiology
59 of *L. gibba* in order to detect and assess their impact on biotic systems. The selected dyes were Direct Red 89

(DR-89) and Vat Blue 20 (VB-20). The effect of these two pollutants was determined from the concentration that reduces by 50% the growth rate of the plants (IC50), the lowest observed effect concentration (LOEC) and the no-observed effect concentration (NOEC). Additionally, the degree of pollutant inhibition on the concentration of photosynthetic pigments (chlorophyll-a, chlorophyll-b and total carotenoids) was evaluated.

2. Experimental

2.1. Plant selection and culture conditions

The species used in this study was *L. gibba* obtained from a natural pond located in Northeastern Algeria. The plants were transported to the laboratory in plastic containers filled with water from the pond. The healthy fronds with roots characterized by a green color were selected for conducting the different experiments. The plants were gently rinsed with tap water and distilled water to eliminate debris then placed in a wide aquarium containing a nutrient medium (Bajaj et al., 2008). The culture medium contained macronutrients (NH₄NO₃, KNO₃, KH₂PO₄, MgSO₄.7H₂O, NaCl, K₂HPO₄ and Ca(NO₃)₂. 4H₂O) and micronutrients (MnSO₄.H₂O, H₃BO₃, ZnSO₄.7H₂O, CuSO₄.5H₂O, Na₂MoO₄.2H₂O, NiCl₂.6H₂O, FeSO₄.7H₂O). The aquatic plants were left in growth for three days before the beginning of the tests. The laboratory conditions were as follows: pH = 6.1±0.01, T = 21±1 °C, photoperiod = 12 h. A system of continuous aeration was used to provide oxygen for the *Lemna* fronds and prevent fungal diseases (Kamal et al., 2004).

2.2. Chemicals

Two textile dyes were selected as organic pollutants: Direct Red 89 (DR-89) and Vat Blue 20 (VB-20). Some of their physicochemical characteristics and chemical structures are given in Table 1. The other chemicals (HCl, NaOH and nutrients) were purchased from Merck. All the solutions were prepared using distilled water (pH = 6.5, $\lambda = 1.76 \mu\text{S/cm}$). Stock solutions of DR-89 and VB-20 were prepared by dissolving 1 g of dye in 1 L distilled water and were kept in an incubator at a temperature of 4 °C. The concentration of the two dyes was analyzed using a UV-vis Spectrophotometer (Screw SECOMAM Prim Light V9B S/N 2836). Dye concentration was evaluated by measuring the absorbance at the maximal wavelength of each dye (Table 1) and extrapolation onto the calibration curve that was carried out by using solutions with known DR-89 and VB-20 concentrations in the range of 0-15 mg/L.

2.3. Toxicity tests

88 The protocol of toxicity tests was derived from a draft directive Standard 221 for a 4-days growth inhibition
 89 (OECD, 2006). This test mimics a small artificial ecosystem. Nine to twelve fronds of *Lemna* (2 to 3 colonies)
 90 were gently placed in crystallizing cups (7 cm in height and 5 cm in diameter) containing 100 mL of nutrient
 91 medium with different initial dye concentrations (5-100 mg/L) frequently detected in water samples from
 92 WWTPs. They were immersed in a thermostated bath and placed in conditions similar to those of plant culture
 93 but without aeration as shown in **Figure 1**. The control tests (without dye) were carried out to compare the
 94 results and to calculate the growth rate of the plants according to **Equation 2**. All the biological tests
 95 (corresponding to ten initial concentrations) were carried in triplicates. The cups were randomized within the
 96 bath to avoid location effects. The dyes concentration range was fixed after observing morphological and
 97 toxicity behavior of the *L. gibba* on a daily bases (Khellaf and Zerdaoui, 2010). These visual symptoms of
 98 toxicity may appear in different ways such as chlorosis, dislocation of fronds and necrosis. Dye toxicity on
 99 duckweed may be also manifested as a decrease in the biomass, the growth rate and/or the photosynthetic
 100 pigments.

2.4. Data analysis and toxicity assessment

103 The estimation of duckweed growth was based on the fronds number; more specifically, all visible fronds were
 104 counted manually at initial (t = 0) and final time (t = 4 days) during the test period. The average growth rate
 105 (AGR) was calculated according to the guidelines of OECD (2006) as illustrated by the following equation (Eq.
 106 1):

$$AGR = \frac{\ln N_j - \ln N_i}{\Delta t} \quad (\text{Eq. 1})$$

108 The percentage of inhibition of the growth rate (% Ir) was determined using **Eq. 2** (Gür et al., 2016):

$$\% Ir = \frac{AGR_c - AGR_t}{AGR_c} \times 100 \quad (\text{Eq. 2})$$

110 Where N_i and N_j are numbers of fronds at time i and j , respectively; Δt is the period of experience (4 days);

111 AGR_c and AGR_t are the average growth rates of the control and the treatment, respectively.

112 The concentration inhibiting 50% of the growth rate of *L. gibba* in the presence of the two dyes is deduced from
 113 figure representing the percentage of inhibition of the growth rate versus the logarithmic concentrations. The
 114 validity of the present test is subject to value of the doubling time of frond number (T_d). In the control, this value

115 test must be less than 2.5 days according to the test protocol of OECD (2006). T_d was assessed by the following
116 equation (Eq. 3):

$$117 \quad T_d = \frac{\ln 2}{AGRc} \quad (\text{Eq. 3})$$

118
119 In order to illustrate the physiological state of the used *L. gibba*, chlorophyll and carotenoid pigments were
120 quantified. 0.1 g of fresh material was grounded and homogenized in 100% acetone (100 ml), centrifuged at
121 2000 g for 10 min and the absorbance of the supernatant was measured by a UV-vis spectrophotometer (Screw
122 Secomam Prim Light V9B S/N 2836) at 470, 661 and 664 nm (Vafaei et al., 2012). Chlorophyll-a, chlorophyll b
123 and carotenoids were calculated using the following equations (Eq. 4-9) as previously described by Hartmut et
124 al. (2001).

$$125 \quad \text{Chla (mg / L)} = 11.24 \times A_{661} - 2.04 \times A_{664} \quad (\text{Eq. 4})$$

$$126 \quad \text{Chla (mg / g)} = \text{Chla (mg / L)} \times (0.01 \text{ L acetone} / 0.2 \text{ g frond}) \quad (\text{Eq. 5})$$

$$127 \quad \text{Chlb (mg / L)} = 20.13 \times A_{664} - 4.19 \times A_{661} \quad (\text{Eq. 6})$$

$$128 \quad \text{Chlb (mg / g)} = \text{Chlb (mg / L)} \times (0.01 \text{ L acetone} / 0.2 \text{ g frond}) \quad (\text{Eq. 7})$$

$$129 \quad C_{X+C} \text{ (mg / L)} = (1000 A_{470} - 1.90 \text{ Chla} - 63.14 \text{ Chlb}) / 214 \quad (\text{Eq. 8})$$

$$130 \quad C_{X+C} \text{ (mg / g)} = C_{X+C} \text{ (mg / L)} \times (0.01 \text{ L acetone} / 0.2 \text{ g frond}) \quad (\text{Eq. 9})$$

131 **2.5. Statistical analysis**

132 Three independent experiments were performed for each of the ten dye concentrations. Error bars given in the
133 figures represent standard deviation of the means. The results were analyzed using a one-way analysis of
134 variance (ANOVA). Comparison between control and treatments was statistically analyzed and the validity of
135 investigation was expressed as probability value of $p < 0.05$.

136 **3. Results**

137 **3.1. Symptoms of toxicity**

138 To determine the effect of DR-89 and VB-20 on *L. gibba*, morphological symptoms of toxicity were identified.
139 The fronds were daily observed, and the results show that the two dyes caused visible damages on the plants as
140 shown in **Figure 2** at concentrations > 50 mg/L. These damages were visible from the second day of exposure

141 for DR-89 and from the third day of exposure for VB-20. Additionally, DR-89 exhibited more sign of toxicity
142 than VB-20. The signs of toxicity consisted on a dislocation of fronds followed by a reduction in biomass for
143 concentrations > 50 mg/L. At high concentrations (100 mg/L), a rapid chlorosis of some fronds was observed
144 where their color changed from green to yellow for both dyes. These signs progressed to necrosis of small fronds
145 at the end of toxicity tests.

146 **3.2. Dose-Growth rate relationship**

147 The toxic effects of the used dyes in this study were evaluated by the average growth rate inhibition calculated
148 according to **Eq. 1**. The doubling time of the frond number in the control test was 2.4; this value is less than 2.5
149 days validating the toxicity tests on *L. gibba*. **Figure 3** shows that VB-20 and DR-89 caused a decrease in the
150 average growth rate of duckweed for all the selected concentrations used in this work. The growth rate decreased
151 with a monotonous decline without reaching a plateau value. At concentrations ≤ 50 mg/L, biomass and growth
152 rate were affected without visible signs of toxicity (chlorosis, frond disconnection and necrosis). However, at
153 concentrations > 50 mg/L, the plants exhibited a reduction in biomass demonstrating chlorosis that progressed to
154 necrosis (dead fronds). The average growth rate in the control treatment was 0.286 day⁻¹. The most important
155 decrease in growth rate was at maximum dye concentration of 100 mg/L with a value of 0.085 and 0.048 day⁻¹
156 for VB-20 and DR-89, respectively (indicated by the broken lines on **Figure 3**). This concentration reduced the
157 value of AGR by 69.6 (VB-20) and 82.5% (DR-89), compared to the control.

158 **Fig. 3**

159 **3.3. Growth inhibition parameters**

160 The growth inhibition parameters were deduced from the insert in **Figure 3** illustrating the growth-rate inhibition
161 percentage versus the logarithmic concentration. The calculated concentrations that lead to a reduction of 50% in
162 the plant growth (IC50) were 36.31 and 26.92 mg/L for VB-20 and DR-89, respectively. The minimum dye
163 concentrations having an inhibitory effect on *L. gibba* (LOEC) were also estimated (**Table 2**). The coefficients
164 of determination (R²) were also determined. The no-observed effect concentration (NOEC) could not be
165 determined under the selected conditions of the present work; further measurements are required (especially at
166 low dye concentrations) in order to calculate the NOEC.

167 **Table 2**

168 **3.4. Effect of VB-20 and DR-89 on the content of chlorophyll and carotenoid pigments**

169 The concentrations of photosynthetic pigments, namely chlorophyll-a (Ch-a), chlorophyll-b (Ch-b) and total
170 carotenoids (Cx,c) (xanthophylls and carotenes) were analyzed quantitatively in order to measure the response of
171 *L. gibba* to the stress induced by the two dyes ($C_0 = 10-50$ mg/L). **Figure 4** shows the alteration of the different
172 pigment content as a function of dye concentration. The results show that chlorophyll-a content was significantly
173 affected by the presence of the dyes. It decreased by 30% and 32% after 4 days of treatment with 50 mg/L of
174 VB-20 and DR-89, respectively. The amount of chlorophyll-b content also decreased significantly by 36% and
175 54% at the same conditions for VB-20 and DR-89, respectively. Carotenoids content exhibited a reduction of
176 26% and 35% in response to VB-20 and DR-89 exposure, respectively.
177 To fit the relation between pigment content and initial dye concentration, we used linear regression equations
178 that showed linearity with coefficients of determination varying from 0.938 to 0.983 as shown in **Table 3**.

179 **Fig. 4**

180 **Table 3**

181 **4. Discussions**

182 The present study was undertaken with the specific aims to evaluate the degree of dye inhibition to *L. gibba*
183 growth and to investigate the deleterious effects of the pollutants on chloroplastic pigments in order to test the
184 feasibility of using duckweed as contaminant bioindicator. Biochemical and physiological effects of chemical
185 pollutants on several duckweed species were extensively studied (**Singh et al., 2018; Sree et al., 2018;**
186 **Appenroth et al., 2010; Khellaf and Zerdaoui, 2010**) and literature on the effect of organic pollutants such as
187 synthetic dyes deserves to be enriched. The results of the present study show that, at dye concentration > 50
188 mg/L, morphological symptoms of toxicity were induced. The signs consisted on a dislocation of fronds and/or a
189 rapid chlorosis of some fronds. These signs progressed to necrosis of small fronds at the end of the treatment
190 tests. Several researchers asserted that visible symptoms of toxicity in living organisms could be an efficient
191 indicator to assess the deleterious effects of some mineral and organic pollutants (**Costa et al., 2018; Song et al.,**
192 **2015; Coronado-Posada et al., 2013; Henke et al., 2011**). Furthermore, some researchers suggested to take
193 into account the importance of identifying the suitable endpoints of toxicity assessment and to consider the
194 intrinsic differences between species when evaluating the toxicological effect of pollutants (**Song et al., 2015**).
195 Several published studies define plant growth such as the increase in an observable parameter like the frond
196 number, total frond area, dry weight or the chlorophyll content (**Mkandawire et al., 2006; Mazur et al., 2018**).
197 In this project, the growth was expressed as average growth rate based on fronds number. The different assays

198 confirmed that the aquatic plant reacts to the two dyes by changes in the growth rate. Inhibition of the growth
199 was observed in all experiments as shown in **Figure 2**. This may be caused by an inhibition of cell division
200 resulting from oxidative stress or alteration of the plant photosystem following a reduction in the electron
201 transport of chloroplasts (**Sree et al., 2018**). **Vafaei et al. (2012)** attributed the reduction of growth-rate to the
202 dye inhibitory effect of photosynthesis and/or the synthesis of growth proteins. Some studies reported the effect
203 of various pollutants on several species of aquatic plants. **Khataee et al. (2012)** found that the relative growth
204 rate (RGR) of *L. minor* was negatively influenced by the increase of the concentration of Acid Blue 92.
205 Additionally, RGR of the same species decreased with an increase in the initial concentration of Methylene Blue
206 (**Reema et al., 2011**). Similar results reported by **Khataee et al., 2013** indicated that the exposure of *Azolla*
207 *filiculoides* to Acid Blue 92 resulted in a growth reduction with the increase of the dye concentration (10 and 20
208 mg/L). **Vafaei and al. (2012)** demonstrated that the exposure of this plant species to the dye Basic Red 46 (10
209 and 20 mg/L) reduced the value of RGR by 26.83 and 32.05%, respectively compared to the control. In the
210 present study, it was found that 36.31 mg/L of VB-20 and 26.92 mg/L of DR-89 reduced the RGR of *L. gibba* by
211 50%; additionally, photosynthetic pigments content exhibited a significant reduction in response to VB-20 and
212 DR-89 exposure. The intensity level of toxicity varies from one species to another, which could be due to their
213 behavioral responses and their origin.

214 In the present study, some differences were found between *L. gibba* development patterns during contact with
215 the two pollutants. The red dye had a more aggressive impact on the duckweed development; indeed, the growth
216 rate was inhibited by 80 and 65% in the presence of 50 mg/L of DR-89 and VB-20, respectively. The results also
217 demonstrated that concentrations of 26.92 mg/L DR-89 and mg/L 36.31 VB-20 in the culture medium reduced
218 the growth by 50%. These values can be used as indicator indices in biomonitoring of water textile pollution.

219 The inhibitory effect of the two pollutants on the photosynthetic pigments was also highlighted. Low chlorophyll
220 and carotenoid pigments in the polluted media were observed compared to the control. This indicates the relative
221 damages to the photosynthetic cellular machinery (**Vafaei et al., 2012**). The decrease of photosynthetic pigment
222 levels under different dyes exposure is a direct reason for the reduction in the biomass and the relative growth
223 rate of *L. gibba*. Several studies have reported similar results. **Türker et al. (2016)** asserted that the
224 concentration of photosynthetic pigments (Ch-a, Ch-b and C_{x+c}) decreased in *L. gibba* exposed to boron
225 concentration of 8 mg/L. Another study carried out by **Khataee et al. (2013)** on *Azolla filiculoides* exposed to 10
226 and 20 mg/L Acid Blue 92 solutions showed that Ch-a, Ch-b and C_{x+c} concentrations decreased in both cases
227 compared to the control. The critical interference on the chloroplastic pigments could be due to the inhibition of

228 the photosynthetic electron transport and decomposition of the chloroplast membrane (Sandmann and Böger,
229 1980). According to Sree et al. (2015), a pollutant present in the culture medium inhibits the involved enzymes
230 in the synthesis of chlorophyll intermediates like 5-aminolevulinic acid and protoporphyrin.
231 Regarding the physiological changes following dyes exposure, a relationship was found between initial pollutant
232 concentration and the physiological affecting parameter (e.g. growth and photosynthetic pigments) using linear
233 regression models. These models are beneficial to verify the validity and reproducibility of the toxicity tests.
234 Additionally, these models should provide scientific support to reduce uncertainties of the risk assessment of
235 dyes; this can help risk manager to act in the interaction of exposure, effects, risk and uncertainties. Finally, it
236 can be concluded that morphological and physiological changes in the duckweed *L. gibba* following exposure to
237 pollutants can be used in testing of dye(s) pollution.

238 **Conclusion**

239 In this work, we studied the morphological and physiological behavior of *L. gibba* in the presence of dyes. We
240 found that the duckweed *L. gibba* could be used for testing dye-contaminated water. Visually, the duckweed
241 tolerated the two textile dyes (DR-89 and VB-20) up to 50 mg/L in a nutrient medium without manifesting
242 significant symptoms of toxicity. Although no visible damage was observed on the morphological aspect of the
243 macrophyte at concentration ≤ 50 mg/L, there was a significant decrease in the growth rate and reduction in the
244 photosynthetic pigments content (chlorophyll and carotenoid) after an exposure of 4 days. *Lemna gibba* could be
245 taken as potential indicator of dye pollutants. These results must be combined with field investigations to prove
246 that this indicator species could be a useful management tool to determine the quality of freshwater system
247 impacted by textile industry or agro-alimentary factories releasing dyes to the environment.

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252 **Conflict of interest statement**

253 On behalf of all authors, the corresponding author states that there are no conflicts of interest.

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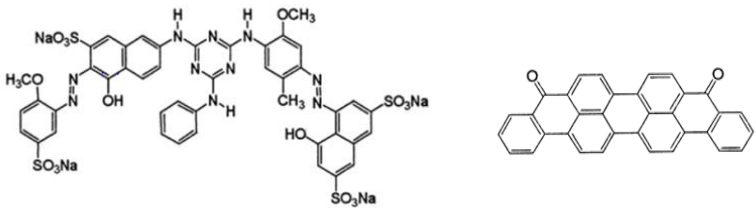
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346

347 **Table 1** Physicochemical data and chemical structures of the two dyes under study

348	Color index name	Direct Red 89 (DR-89)	Vat Blue 20 (VB-20)
349	Molecular formula	$C_{44}H_{32}N_{10}Na_4O_{16}S_4$	$C_{34}H_{16}O_2$
350	Chemical class	Azoic dye	Anthraquinone dye
351	Molecular weight	1372	456
352	λ_{max} (nm)	494	580
353	Chemical structure		
354			

360 **Table 2** Growth inhibition parameters of *L. gibba* in the presence of the dyes

361	Dye	IC50	R ²	LOEC	NOEC
362		(mg/L)	(/)	(mg/L)	(mg/L)
363	DR-89	26.92	0.88	5	*
364	VB-20	36.31	0.98	5	*

365 * Not observed

367 **Table 3** Linear regression models: pigment content versus initial dye concentration

368	Pigment	Regression equations	R ²	
369	DR-89	Chl-a	$Y = -0.029x + 0.517$	0.963
370		Chl-b	$Y = -0.027x + 0.281$	0.957
371		Carotenoid	$Y = -0.009x + 0.151$	0.938
372	VB-20	Chl-a	$Y = -0.032x + 0.542$	0.958
373		Chl-b	$Y = -0.018x + 0.270$	0.987
374		Carotenoid	$Y = -0.007x + 0.157$	0.983

375 Y, X and R² represent the pigment content (mg/g FW), the initial dye concentration (mg/L)

376 and the coefficient of determination, respectively

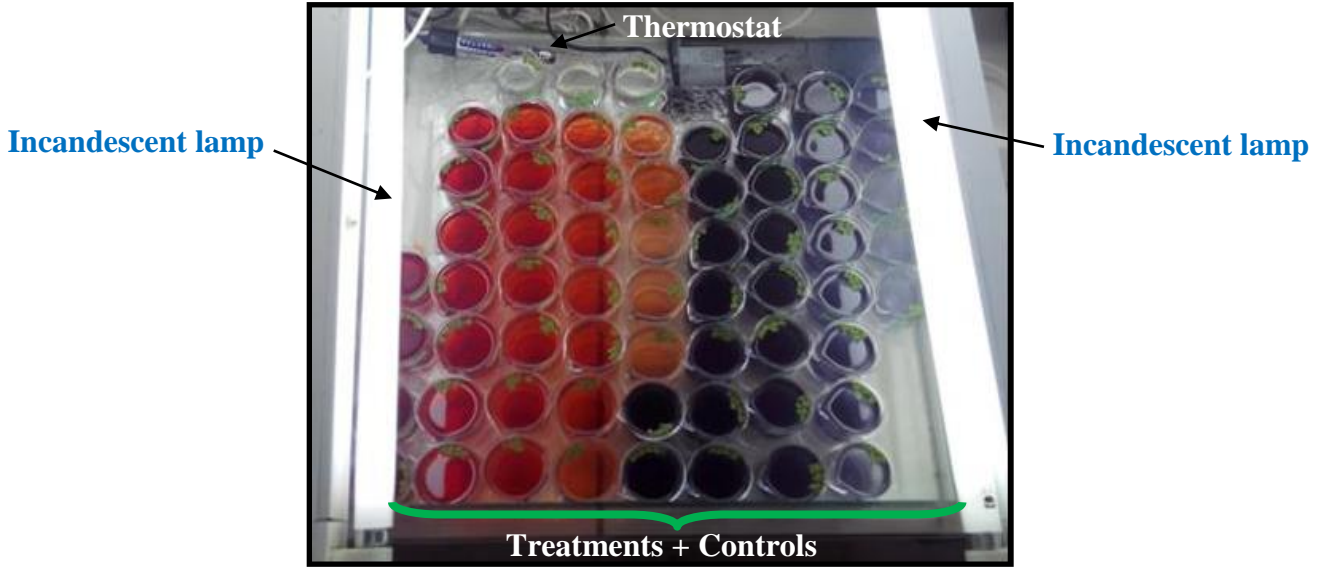


Fig. 1 Experimental setup for the phyto-toxicity tests

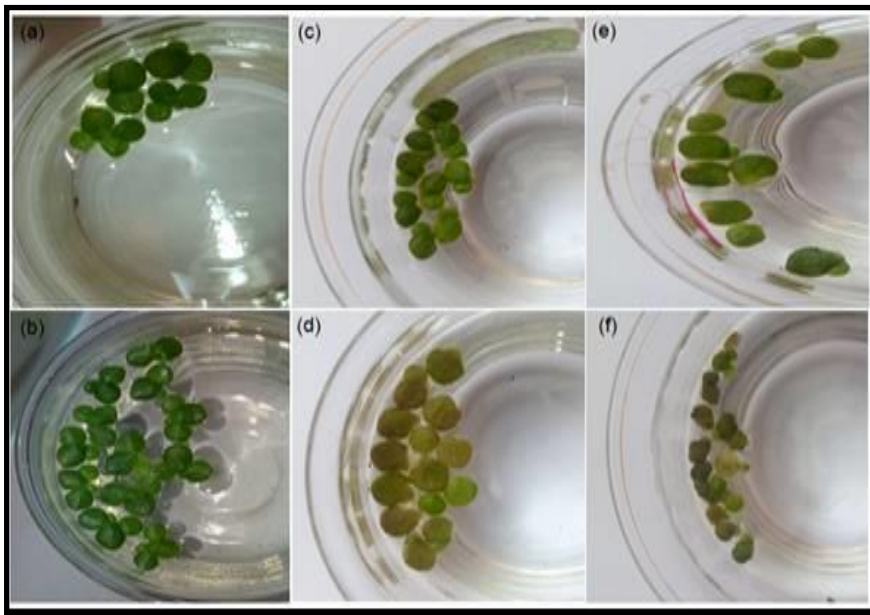


Fig. 2 Visible signs of toxicity in *L. gibba* following a 4-days exposure to DR-89 for concentrations > 50 mg/L

(a) Control , (b) Control at t = 4 days , (c) Biomass reduction , (d) Chlorosis , (e) Dislocation of fronds , (f) Necrosis

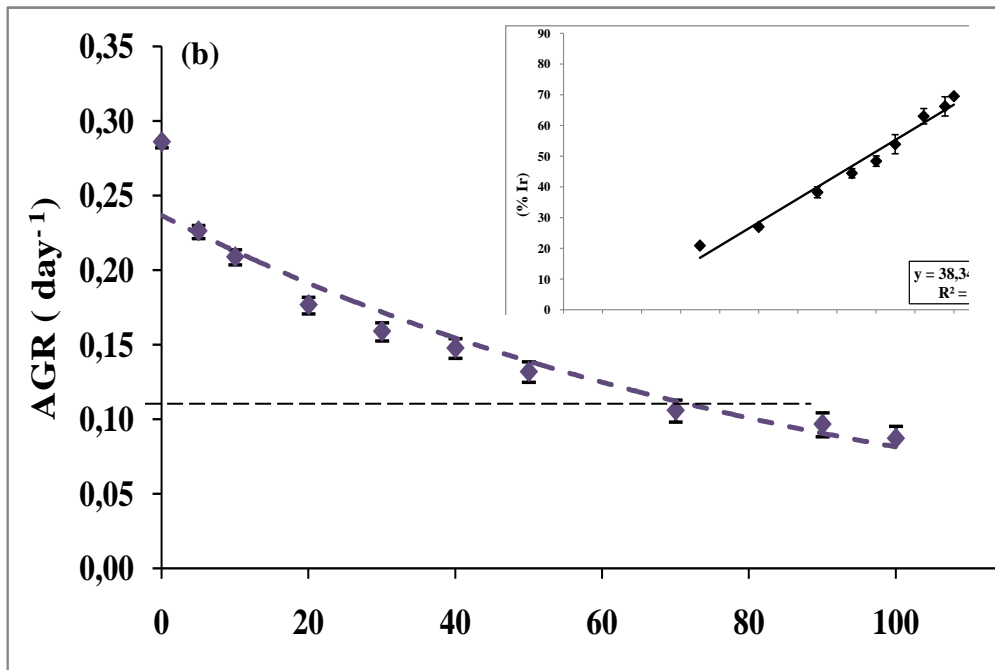
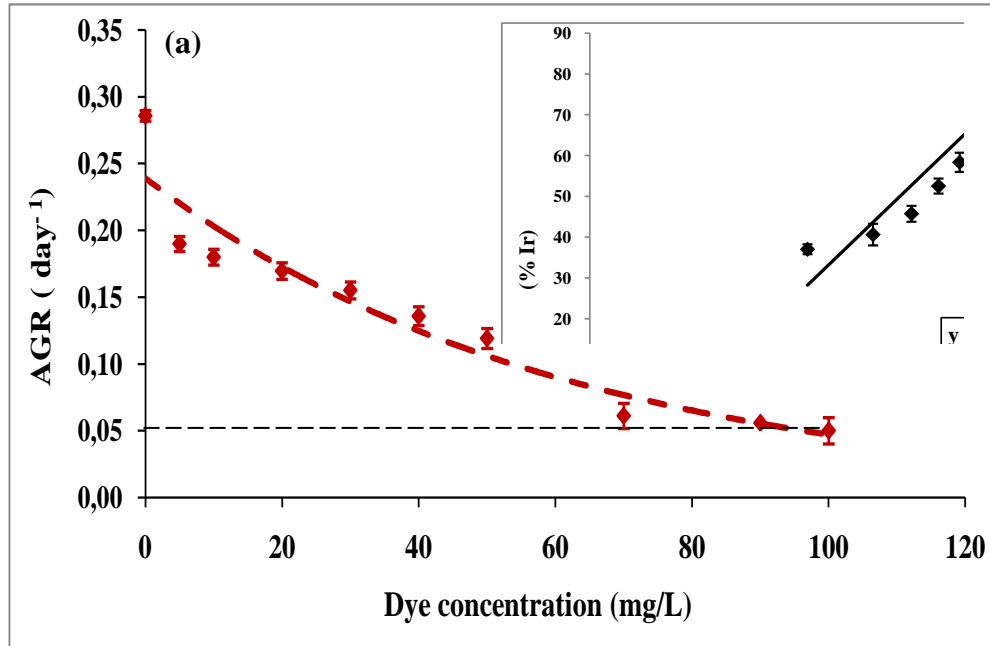


Fig. 3 Average growth rate of *L. gibba* with different concentrations of (a) DR-89 and (b) VB-20. Inserted curves showed percent inhibition of the growth rate versus the logarithmic dye concentration. Broken line indicate minimal growth rate. Vertical bars indicate standard deviation, n = 3

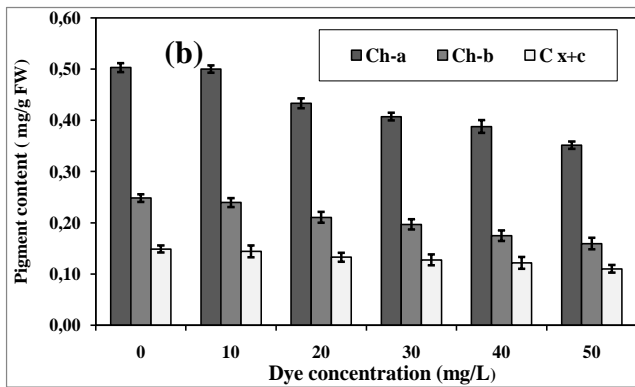
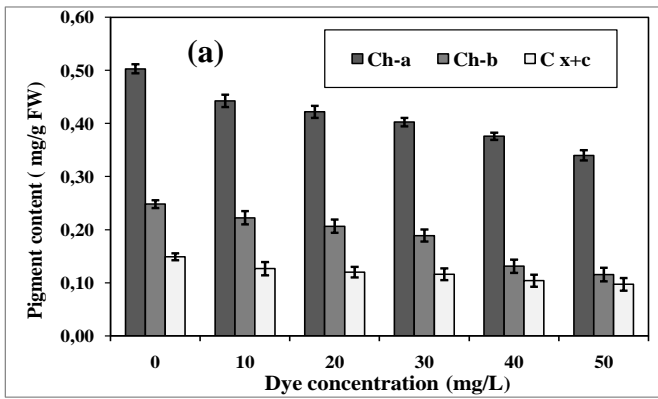


Fig. 4 Variation of chlorophyll and carotenoid pigments with increasing concentration of (a) DR-89 and (b) VB-

20. Vertical bars indicate standard deviation, n = 3.