Can duckweed be used for the biomonitoring of textile effluents?

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Abstract

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

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

1. Introduction

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

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

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

The duckweed *Lemna gibba* is a small floating plant belonging to the family of *Lemnaceae.* It is found on the surface of eutrophic backwater or slow flows of fresh and brackish waters forming green dense carpets (**Driever et al., 2005**). *Lemna* is a very appropriate species for the toxicity tests with its simple structure, rapid growth and high tolerance to different stresses and part of an international standard protocol (**OECD, 2006**). Duckweed species have been previously used in toxicity tests and applied as bioindicator for detecting several pollutants in aquatic environment (**Türker et al., 2016; Khellaf and Zerdaoui, 2010; Sinha et al., 1995**). Studies detailing the effect of dyes effluents on these aquatic species warrant to be deepened to complete data reported in the literature which are still insufficient since they concern only few pollutants and few plant species. The first objective of the present work is to ascertain the toxicity effects of two dyes on the morphology and physiology 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 (NH_4NO_3, KNO_3, KH_2PO_4, $MgSO_4.7H_2O$, NaCl, K_2HPO_4 and $Ca(NO_3)_2$, $4H_2O$) and micronutrients ($MnSO_4.H_2O$, H_3BO_3, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $Na_2MoO_4.2H_2O$, $NiCl_2.6H_2O$, $FeSO_4.7H_2O$). 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, λ = 1.76 µS/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

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

2.4. Data analysis and toxicity assessment

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

$$AGR = \frac{\ln Nj - \ln Ni}{\Delta t} \qquad \text{(Eq. 1)}$$

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

$$\%\,Ir = \frac{AGRc - AGRt}{AGRc} \times \qquad \text{(Eq. 2)}$$

Where Ni and Nj are numbers of fronds at time i and j, respectively; Δt is the period of experience (4 days); AGRc and AGRt are the average growth rates of the control and the treatment, respectively.

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

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

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

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

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

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

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

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

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

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

2.5. Statistical analysis

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

3. Results

3.1. Symptoms of toxicity

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

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

3.2. Dose-Growth rate relationship

The toxic effects of the used dyes in this study were evaluated by the average growth rate inhibition calculated 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 days validating the toxicity tests on *L. gibba*. **Figure 3** shows that VB-20 and DR-89 caused a decrease in the average growth rate of duckweed for all the selected concentrations used in this work. The growth rate decreased with a monotonous decline without reaching a plateau value. At concentrations ≤ 50 mg/L, biomass and growth rate were affected without visible signs of toxicity (chlorosis, frond disconnection and necrosis). However, at concentrations > 50 mg/L, the plants exhibited a reduction in biomass demonstrating chlorosis that progressed to necrosis (dead fronds). The average growth rate in the control treatment was 0.286 day^{-1}. The most important decrease in growth rate was at maximum dye concentration of 100 mg/L with a value of 0.085 and 0.048 day^{-1} for VB-20 and DR-89, respectively (indicated by the broken lines on **Figure 3**). This concentration reduced the value of AGR by 69.6 (VB-20) and 82.5% (DR-89), compared to the control.

Fig. 3

3.3. Growth inhibition parameters

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

Table 2

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

The concentrations of photosynthetic pigments, namely chlorophyll-a (Ch-a), chlorophyll-b (Ch-b) and total carotenoids (C_{x+c}) (xanthophylls and carotenes) were analyzed quantitatively in order to measure the response of *L. gibba* to the stress induced by the two dyes (C_0 = 10-50 mg/L). **Figure 4** shows the alteration of the different pigment content as a function of dye concentration. The results show that chlorophyll-a content was significantly affected by the presence of the dyes. It decreased by 30% and 32% after 4 days of treatment with 50 mg/L of VB-20 and DR-89, respectively. The amount of chlorophyll-b content also decreased significantly by 36% and 54% at the same conditions for VB-20 and DR-89, respectively. Carotenoids content exhibited a reduction of 26% and 35% in response to VB-20 and DR-89 exposure, respectively.

To fit the relation between pigment content and initial dye concentration, we used linear regression equations that showed linearity with coefficients of determination varying from 0.938 to 0.983 as shown in **Table 3**.

Fig. 4

Table 3

4. Discussions

The present study was undertaken with the specific aims to evaluate the degree of dye inhibition to *L. gibba* growth and to investigate the deleterious effects of the pollutants on chloroplastic pigments in order to test the feasibility of using duckweed as contaminant bioindicator. Biochemical and physiological effects of chemical pollutants on several duckweed species were extensively studied (**Singh et al., 2018; Sree et al., 2018**; **Appenroth et al., 2010; Khellaf and Zerdaoui, 2010**) and literature on the effect of organic pollutants such as synthetic dyes deserves to be enriched. The results of the present study show that, at dye concentration > 50 mg/L, morphological symptoms of toxicity were induced. The signs consisted on a dislocation of fronds and/or a rapid chlorosis of some fronds. These signs progressed to necrosis of small fronds at the end of the treatment tests. Several researchers asserted that visible symptoms of toxicity in living organisms could be an efficient indicator to assess the deleterious effects of some mineral and organic pollutants (**Costa et al., 2018; Song et al., 2015; Coronado-Posada et al., 2013; Henke et al., 2011**). Furthermore, some researchers suggested to take into account the importance of identifying the suitable endpoints of toxicity assessment and to consider the intrinsic differences between species when evaluating the toxicological effect of pollutants (**Song et al., 2015**).

Several published studies define plant growth such as the increase in an observable parameter like the frond number, total frond area, dry weight or the chlorophyll content (**Mkandawire et al., 2006; Mazur et al., 2018**). In this project, the growth was expressed as average growth rate based on fronds number. The different assays

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

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

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

the photosynthetic electron transport and decomposition of the chloroplast membrane (**Sandmann and Böger, 1980**). According to **Sree et al.** (**2015**), a pollutant present in the culture medium inhibits the involved enzymes in the synthesis of chlorophyll intermediates like 5-aminolevulinic acid and protoporphyrin.

Regarding the physiological changes following dyes exposure, a relationship was found between initial pollutant concentration and the physiological affecting parameter (e.g. growth and photosynthetic pigments) using linear regression models. These models are beneficial to verify the validity and reproducibility of the toxicity tests. Additionally, these models should provide scientific support to reduce uncertainties of the risk assessment of dyes; this can help risk manager to act in the interaction of exposure, effects, risk and uncertainties. Finally, it can be concluded that morphological and physiological changes in the duckweed *L. gibba* following exposure to pollutants can be used in testing of dye(s) pollution.

Conclusion

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

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

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

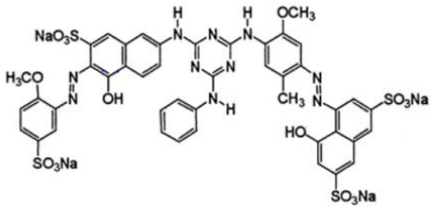
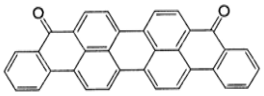
Color index name	**Direct Red 89 (DR-89)**	**Vat Blue 20 (VB-20)**
Molecular formula	$C_{44}H_{32}N_{10}Na_4O_{16}S_4$	$C_{34}H_{16}O_2$
Chemical class	Azoïc dye	Anthraquinone dye
Molecular weight	1372	456
λ_{max} (nm)	494	580
Chemical structure		

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

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

* Not observed

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

	Pigment	**Regression equations**	**R^2**
DR-89	Chl-a	Y = - 0.029x + 0.517	0.963
	Chl-b	Y = - 0.027x + 0.281	0.957
	Carotenoid	Y = - 0.009x + 0.151	0.938
VB-20	Chl-a	Y = - 0.032x + 0.542	0.958
	Chl-b	Y = - 0.018x + 0.270	0.987
	Carotenoid	Y = - 0.007x + 0.157	0.983

Y, X and R^2 represent the pigment content (mg/g FW), the initial dye concentration (mg/L) and the coefficient of determination, respectively

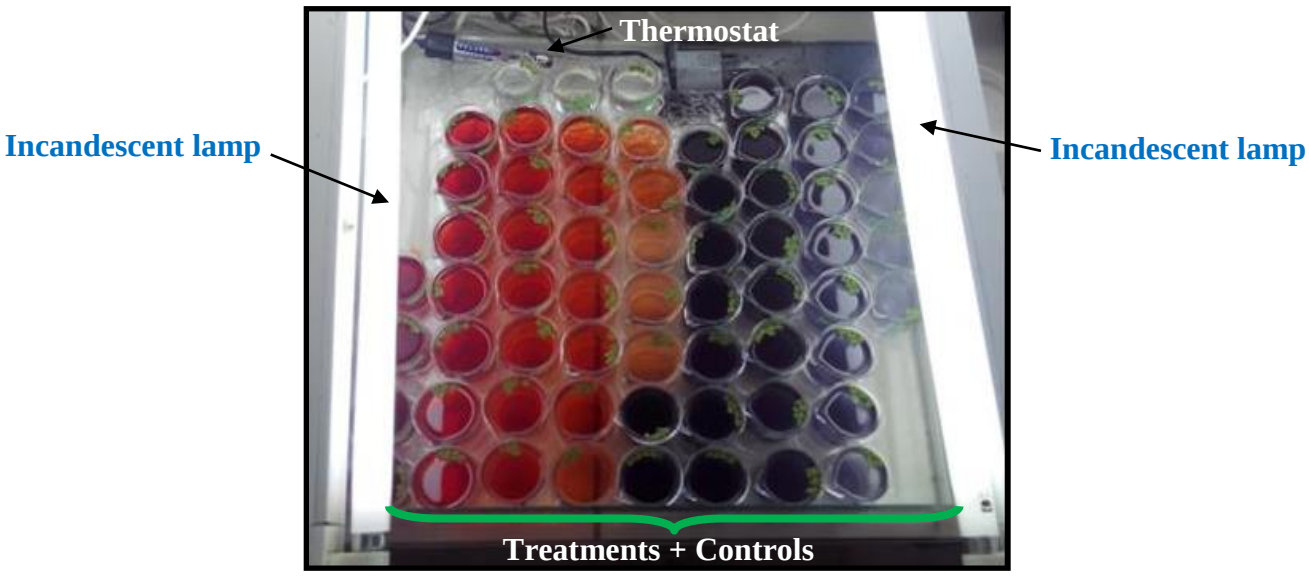


Fig. 1 Experimental setup for the phyto-toxicity tests

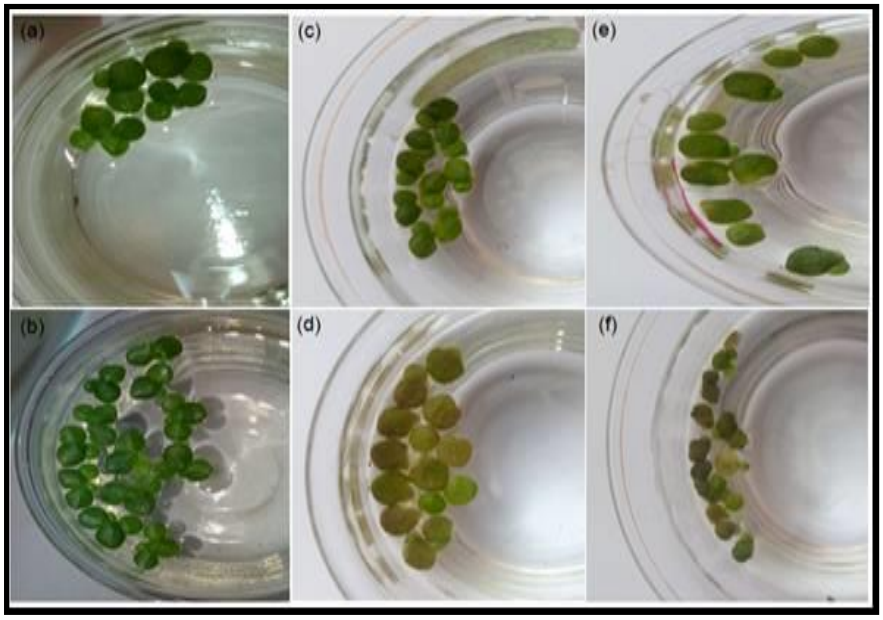
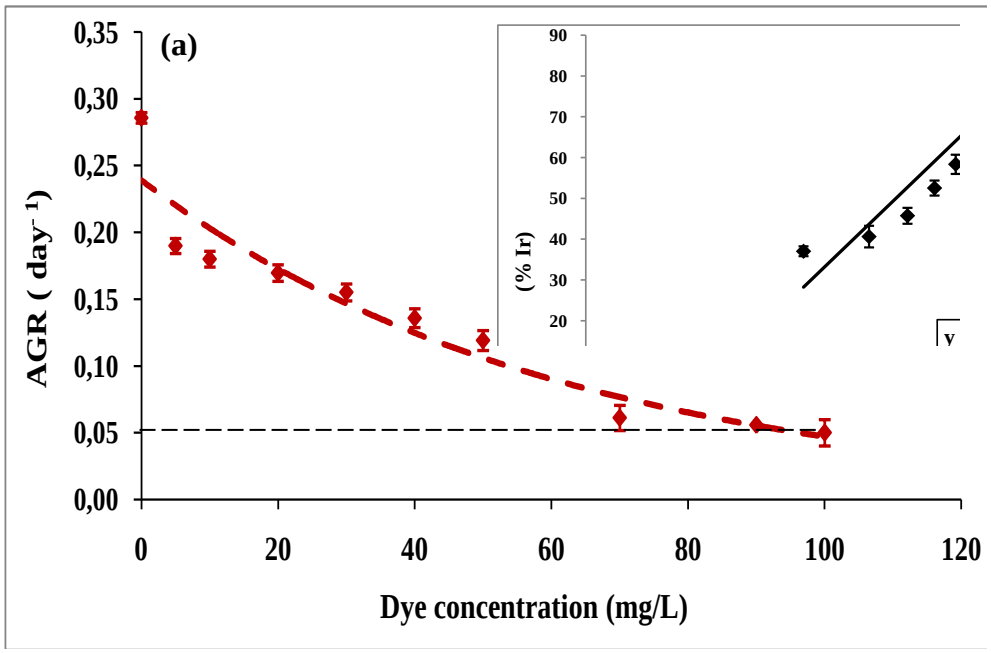


Fig. 2 Visible signs of toxicity in *L. gibba* following a 4-days exposure to DR-89 for concentrations > 50 mg/L

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



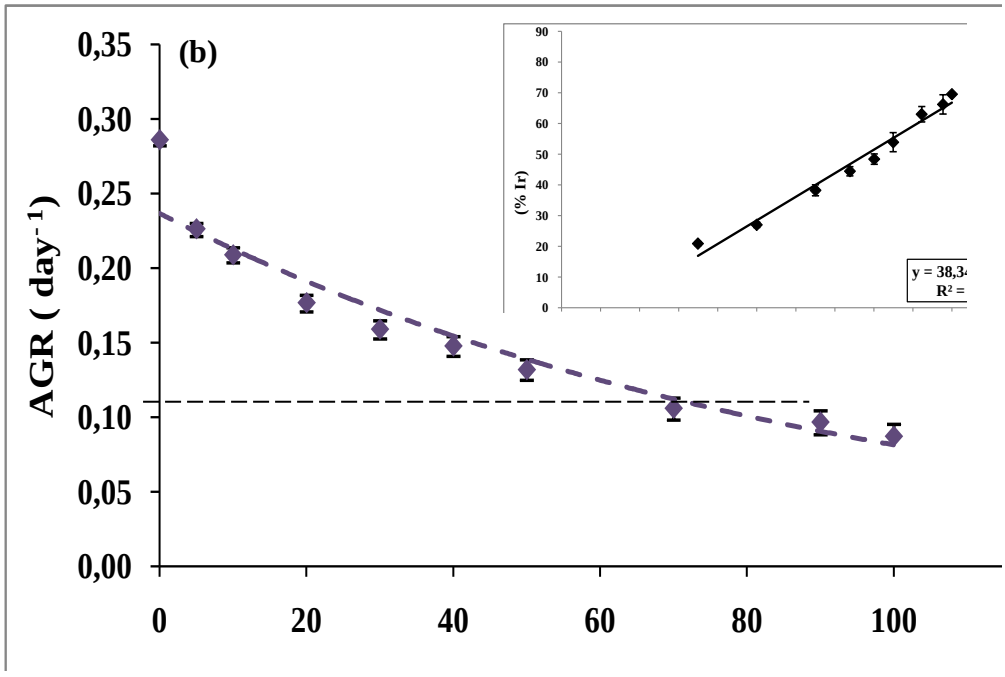
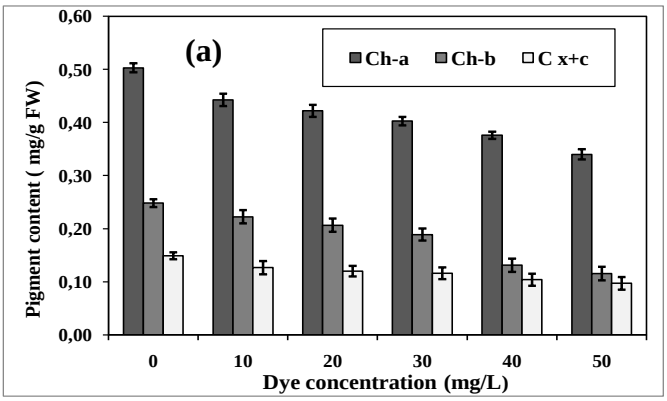


Fig. 3 Average growth rate of *L. gibba* with different concentrations of (a) DR-89 and (b) VB-20. Inserted curves showed percent inhibition of the growth rate versus the logarithmic dye concentration. Broken line indicate minimal growth rate. Vertical bars indicate standard deviation, n = 3



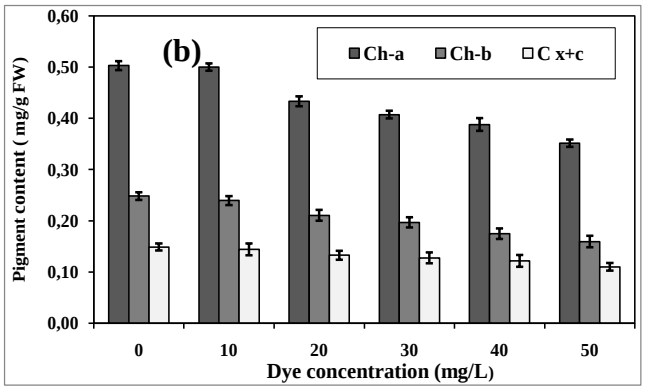


Fig. 4 Variation of chlorophyll and carotenoid pigments with increasing concentration of (a) DR-89 and (b) VB-20.Vertical bars indicate standard deviation, n = 3.