

# Activated Sludge-Assisted Phytoremediation For Dye Removal

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## Research Article

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# Abstract

We report the biodegradation of dye pollutants by a green process that combines the microbial activity of activated sludge with phytoremediation ability of the aquatic plant *L. gibba*. The obtained results showed that the combination of the two processes when the pollutant was present at concentration of 50 mg/L, lead to a dye removal of 95 and 70% for VB-20 and DR-89, respectively. The biodegradability index based on COD and BOD<sub>5</sub> measurement was equal to 3.1 for DR-89 and 2.0 for VB-20, confirming that DR-89 was removed by biosorption phenomena and only VB-20 was transformed into biodegradable compounds. UV-visible, FT-IR and LC/MS analysis were used as a tool for the monitoring of the biodegradation metabolite and the results showed that VB-20 biodegradation occurred by the cleavage of anthraquinone cycle and transformation of aromatic compounds to light hydrocarbon chain; this was further confirmed by the calculation of Fukui index using DFT method. This study highlighted the synergy between the phytoremediation and biodegradation process for organic dye removal.

## 1. Introduction

Water is altered by human activities as a result of domestic, industrial, artisanal, and agricultural use, resulting in a considerable amount of toxic organic pollutants such as additives, detergents and dyes that affect ecosystems by increasing biochemical oxygen demand (BOD) or chemical oxygen demand (COD) (Bisschops et al. 2003; Sun et al. 2015). Among these organic pollutants, textile dyes are considered as one of the most consumed chemicals which released into the environment worldwide (Ogugbue et al. 2011). The presence of these dyes in the environment have big issues such as eutrophication and non-aesthetic disturbance to aquatic life and therefore presents a potential danger of bioaccumulation which can affect humans through the food chain (Chowdhury et al. 2009). Therefore, this textile waste must be treated before being discharged into the natural receiving environments.

Some physicochemical treatments for eliminating pollutants are confronted with economic and technical obstacles, such as costs of treatment and the production of by-products even more dangerous than the basic pollutant (Robinson et al. 2001; Saratale et al. 2011). On the other hand, biological methods have received a significant awareness of the scientific community during the last decades, because of their many advantages, in terms of profitability, environmental benignity and simple operation (Sponza et al. 2005). These biological processes are based, on the processes of bioremediation, in which the main example of the most used wastewater treatment in the world is the activated sludge process (Du et al. 2020; Zeghioud et al. 2021). Microorganisms are used for subsequent treatment in an aerated bioreactor. In the activated sludge process, most of microorganisms and other solids are organized into discrete units called flocs. The flocs are kept in suspension in an aerated tank to ensure contact of the microorganisms with the dissolved organic matter and the nutrients available in the wastewater.

The organic pollutants are degraded and oxidized in the presence of oxygen by microbial metabolic activity for their survival and are transformed into harmless compounds such as carbon dioxide and water (El Defrawy et al. 2007; Ghosh et al. 2017; Tomei et al. 2016). This technology is considered to be a

sustainable solution due to the broad metabolic properties of mixed cultures, having great advantages over the use of pure algal or single strain microbial cultures. Also, they allow accumulating and degrading different dyes with a fairly complete mineralization due to the synergistic associations between the various microorganisms (Krishnan et al. 2016). However, elimination of pollutants by the activated sludge takes place after a long pretreatment time (several days) and in some cases the mineralization is not reached (Khellaf et al. 2017). In addition some pollutants such as azo dyes can only be degraded under anaerobic conditions (Aubert et al. 2004; Chang et al. 2000; Chang et al. 2001), or they will be biosorbed on the surface of the biological biomass and remains stable molecules thus reducing the sludge efficiency (Hitz et al. 1978). Furthermore, phytoremediation, a biological process that uses different plants having the capacity to eliminate organic pollutant, is considered as interesting method. The organic matter is either removed by degrading the pollutant molecule with different plant enzymes (Kaushal et al. 2021; Mahajan and Kaushal 2020; Mamirova et al. 2020), or by exploiting the accumulation capacities of these accumulating plants, where they absorb and concentrate the organic pollutants in their different parts (leaves, stems, etc.) (Ceschin et al. 2020; Kaushal et al. 2021; Saratale et al. 2011). *Lemna gibba* L. is an aquatic floating vascular plant belonging to the *Lemnaceae* family; it can be found all over the world on the surface of stationary or low-flow fresh and brackish waters, forming dense green carpets (Driever et al. 2005), due to its ability to adapt to climatic conditions (Sivakumar et al. 2014).

During wastewater treatment process, coupling the two biological processes (bioremediation and phytoremediation) should be very interesting from an economic and environmental point of view, because they can be reconciled by a mixed consortium of microorganisms (activated sludge) and/or of pure strains of microbes, algae, bacteria, and fungi (bioremediation) and even aquatic plants (phytoremediation) (Guo et al. 2020; Liang et al. 2014; Ito et al. 2016; Turgay et al. 2011). In order to contribute to the development of this axis, the present research concerns the coupling of two biological processes, for biodegradation of two textile dyes by activated sludge assisted by phytoremediation using the duckweed *L-gibba*. To highlight the efficiency of this process, two molecule dyes, Vat Blues 20 (VB-20) and Direct Red 89 (DR-89) were chosen as pollutants. The other objective was to reduce the treatment time relative to that achieved during processing without process coupling.

## 2. Materials And Methods

### Chemicals

The two textile dyes DR-89 and VB-20 were selected as organic pollutants; they were supplied by the Algerian textile industry located in the city of Constantine (eastern Algeria). All chemicals used were of the highest purity available and of analytical grades (HCl, NaOH and nutrients) and were purchased from Merck. All solutions were prepared using distilled water ( $\text{pH}=6.1\pm 0.01$ ,  $\lambda=6.0 \mu\text{S}/\text{cm}$ ).

### Activated sludge preparation

The activated sludge (AS) used in the biodegradation tests were taken from the aeration tank of the municipal WWTP located in the city of Annaba (East Algeria). This sludge in the form of a suspension

was transported in closed plastic containers to the laboratory and was suspended in a modified culture medium, necessary for the development of the microorganisms; it was then aerated under a moderate stirring, at ambient temperature  $T = 21 \pm 1 \text{ }^\circ\text{C}$  at  $\text{pH} = 6.1 \pm 0.1$  (Hocini et al. **2019**). The suspension of the activated sludge was placed in a 1 L graduated cylinder (test tube) to allow it to settle. After 30 minutes, the biomass was recovered and the latter was centrifuged at 4000 rpm for 20 min. Finally, the biomass was recovered at the end of the operation to be mixed with 20 ml of distilled water for subsequent use in various experiments.

### **Selection of the aquatic plant *Lemna gibba* L**

The aquatic plant *Lemna gibba* L used in this study was picked from a natural pond located in the town of El-Tarf (Northeastern Algeria). Healthy fronds with roots characterized by a green color were selected for the different experiments. They were then gently rinsed with tap water and distilled water to remove debris. After that, they were placed in a large aquarium containing the same nutrient medium as the AS microorganisms, at a temperature of  $21 \pm 1 \text{ }^\circ\text{C}$ , a pH of  $6.1 \pm 0.1$ , a photoperiod of 12 hours, and a continuous aeration system using air bubbling.

### **Analytical techniques**

Various analytical techniques were used in this study. Dye concentration was measured using a UV-Vis spectrophotometer (SECOMAM Prim Light V9B; S / N 2836) at maximum absorption wavelengths of each dye ( $\lambda_{\text{max}} = 495 \text{ nm}$  for DR-89) and ( $\lambda_{\text{max}} = 580 \text{ nm}$  for VB-20). Fourier transform infrared spectroscopy (FT-IR) was performed using a Perkin Elmer FT-IR spectrophotometer (Affinity-1 IR model: Perkin Elmer) in the ranges 4000 to  $450 \text{ cm}^{-1}$ . Liquid chromatography coupled with mass spectroscopy (LC/MS) was conducted using Agilent 6400 series (Agilent USA, technologies) equipped with columns (250 x 2.1 mm, 5 micron) and it was maintained at  $35 \text{ }^\circ\text{C}$ . The mobile phase was constituted with a mixture of pure water and methanol. The aliquots were taken every day and were injected using the apparatus auto sampler. Positive electrospray ionization ( $\text{ESI}^+$ ) was used for  $m/z$  identification in the range of 0-500 with capillary voltage of 3.0 kV.

The morphology of the aquatic plants was observed using scanning electron microscope (SEM) Quanta FEG-250 coupled with energy X-ray dispersion (EDX) for elemental analysis. Optic microscope was used to identify the microorganisms present in the activated sludge flocs and was carried out every week with an AMETEK type microscope at 100x magnifications. The chemical oxygen demand (COD) was measured by the normalized method NF T90-101 using a spectrophotometer (HACH DR2000) (AFNOR **1999**; APHA **1999**). To assess the biodegradability of our samples, the biochemical oxygen demand ( $\text{BOD}_5$ ) was also measured, according to the manometric respirometric method (APHA **1999**), using a BOD sensor (Hach Lange BOD Trak II type).

### **Microbial community development**

The activated sludge microbial community was monitoring using two parameters. The first parameter was the dry matter (DM) determined from the standard NFT 90-029 (AFNOR **1999**) and it was calculated from the following equation:

$$DM = \frac{(P_1 - P_2)}{V} \times 1000$$

Where  $P_1$  is the mass (g) of the cup and the activated sludge sample,  $P_2$  the mass (g) of the dry cup and  $V$  the volume of the activated sludge sample (50 mL).

The calculation of DM allows us to determine the microbial development percentage (MDP) and the activated sludge growth index (GI) using the following equations:

$$MDP (\%) = \frac{DM_t - DM_{initial}}{DM_t} \times 100$$

$$GI = \frac{DM_t}{DM_{initial}}$$

Where,  $DM_{initial}$  and  $DM_t$  are initial dry matter and the dry matter at time (t).

### DFT computational

DFT calculation was conducted on Gaussian 09 package (Scheiber et al. **2010**) to determine the Fukui index that used for the prediction of reactive sites of radical, electrophilic and nucleophilic attacks (Feng et al. **1997**). A full geometry optimization of pollutants molecules was first performed using B3LYP functional and 6-31G (d,p) as a basis set. Then, natural population analysis was calculated to determine the Fukui index using the following equation:

$$f_k^0 = (q_{N-1}^0 - q_{N+1}^0)$$

Where  $q_N^0$  is the atom charge population of atom K at corresponding state.

### Statistical analysis

Three independent assays were performed for each dye concentration. The errors given in the figures represent standard errors of the means. Results were analyzed using one-way analysis of variance (ANOVA). The comparison between the control and the treatments was analyzed statistically and the validity of the survey was expressed as a probability value of  $p < 0.05$ .

## Dye biodegradation experiments

Biodegradation of VB-20 and DR-89 by AS alone and by AS assisted by the phytoremediation with *L. gibba* and control tests (without pollutants), were carried out in three replications for 7 days in six stirred and aerated reactors with a capacity of 1 L (**Figure 1**), without and with the presence of glucose as co-substrate. The experiments were performed under the following experimental conditions: temperature =  $21 \pm 1$  °C, a pH of  $6.10 \pm 0.01$ , AS dosage of 20 mL/L. The plant dose was 100 and 200 fronds for VB-20 and DR-89 respectively, and the initial concentration of dyes was equal to 50 mg/L. A control test for AS without dyes was developed under the same conditions in order to monitor the development of microorganisms in the absence of pollutants.

Samples were taken at regular time intervals (for each day), centrifuged at 2000 rpm for 10 min then the separated liquid was analyzed to determine the dye concentration using a UV-Vis spectrophotometer and for COD and BOD<sub>5</sub> measurement. The removal percentage (RP%) and the degradation yield (DY%) were calculated according to the following relations :

$$RP (\%) = \frac{C_{\text{initial}} - C_{\text{final}}}{C_{\text{initial}}} \times 100$$

$$DY (\%) = \frac{COD_{\text{initial}} - COD_{\text{final}}}{COD_{\text{initial}}} \times 100$$

Where, C and DCO refer to the concentration of dyes and the chemical oxygen demand. The indices initial and final refer to the state of before and after treatment.

## 3. Results And Discussion

### Microbial development

According to our visual observations, the microbial development started on the third day for both control and biodegradation test. The result ported in Table 1 indicates that the growth index was ranging between 1.77 and 3.12 (VB-20) and 1.72 and 2.10 (DR-89). At this stage, we can assume that the chosen molecules were not toxic for the microbial community of the activated sludge, since in all cases (absence or presence of glucose as co-substrate), the presence of these compounds did not affect the growth of the biomass in the bioreactors. It is also noted that the growth of microorganisms in the activated sludge is much greater for the solutions enriched with glucose. This finding was confirmed by the measurement of microbial development percentage, where we noticed a MDP reaching 68 % (VB-20) and 52 % (DR-89) in the presence of glucose and dye. These percentages were higher than those obtained in the control solutions suggesting that the glucose substrate or the additional carbon promotes the development and growth of microorganisms in the activated sludge (Bibi et al. 2019; Nouren et al. 2017; Turgay et al.

2011). In the case of the presence only of dyes, the obtained percentage (44- 65 %) was always higher than that of control. The latter is explained by the fact that during an aerobic biological treatment, the microorganisms degrade the organic matter by oxidation, to obtain the necessary energy for their survival. Those results suggest that the organic dye molecule, whatever its nature, constitutes a source of nutrients for microorganisms in activated sludge (Vilaseca et al. 2010; Wang et al. 2019; Yan et al. 2021).

Among the two treated dyes, VB-20 was the best substrate for activated sludge with a growth index of 2.87 in the absence of glucose, showing higher uptake for this dye. These results are due to the fact that the dye molecules of small size can diffuse easily through the bacterial wall to be assimilated thereafter and constitute a source of nutrients, whereas those which are more complex as must be previously hydrolyzed by enzymatic systems before being assimilated (Dohanyos et al. 1978).

### **Microscopic observation of microbial communities**

Given that the microorganisms in activated sludge are the main contributors to the elimination of pollutants, the biological analysis of this activated sludge makes it possible to know the state of the active biomass and to adapt the control parameters in a biological treatment (Vilaseca 2001). In this case the identification of functional microbes is useful to improve the performance and treatment conditions of pollutants and to obtain efficient operation and good maintenance of the system (Al-Husseyen et al. 2014). The identification was conducted by microscopic observations at a magnification of 100 x, made it possible to carry out counts of various populations of the microfauna by a rapid enumeration procedure of the organisms visible.

From the microscopic observation of the AS consortium samples, which were taken, before and during biological treatment, it was found the presence of different genes of microorganisms, mainly protozoa (Short-stemmed *Vorticella*, *Euplotes sp*, *Epistylis sp*) and metazoa (*Rotifera*) (Figure 2). This identification was made on the basis of CURDS 1969 for *ciliates*, and VOIGT 1978 for *Rotifers* (Duchene et al. **1993**). In addition, we noted that there were no significant differences between the microorganisms found in the AS of control and the AS in treatments where the development and normal diversity of microorganisms were still observable. This indicates that the two pollutants contained in our solutions did not have a toxic effect on the microorganisms, and their evolution took place in a normal way. In the initial phase, there was abundance and development of a microfauna composed mainly of predatory metazoans such as *Rotifers* which were present in the sludge undergoing aerobic digestion. Their role as bacterial predators allows them to contribute to the renewal of the purifying biomass, stimulating the activity and decomposition of the microflora (algae); it can also help eliminate pathogenic bacteria (Duchene et al. **1993**; Sowinska and Makowska 2016). The abundance of *Rotifers* in our activated sludge reveals a good aeration conditions and indicates a low contaminant levels (Duchene et al. **1993**; Sowinska and Makowska **2017**), also to the stable functioning of biological treatment (Kocwa-Haluch and Wozniakiewicz **2011**).

The presence of ciliated protozoa such as *Vorticella*, *Epistylis sp* and *Euplotes sp*, play a main role in the clarification of the effluent by predation of free bacteria (Duchene et al. **1993**). The significant presence of

*Vorticella* goes hand in hand with a very complete carbon treatment. However, their abundances are always a sign of satisfactory oxygenation. On the other hand, the existence of *Epistylis sp* is correlated with periods or areas of anoxia with generally sufficient oxygen supply overall. Also *Euplotes sp* was only present in aqueous medium meaning that the elimination of carbon can be ensured and the elimination of nitrogen can be partial with very sufficient oxygenation (Duchene et al. 1993; Sowinska and Makowska 2016).

We also notice the presence of many species of filamentous bacteria flocculated in small quantities in our activated sludge. Three days after the start of treatment, the growth of the herds implied the presence of metazoa and protozoa of larger size, which indicated a good state of depuration and an index of good performance.

### **Effect of glucose on the elimination of dyes**

Glucose is considered to be the best source of carbon for the growth of microorganisms (Cervantes et al. 2006; Cui et al. 2020). In order to study the effect of glucose on the biodegradation of the two dyes by AS, experiments were carried out with and without glucose. The results reported in Figures 3a and 3b revealed that the microorganisms were responsible for the decrease in pollutant concentration, in the liquid phase. Overall, the reduction in the concentration of dyes increased more rapidly over time, especially in the presence of glucose, with a decrease that change from one dye to another. The discoloration of the solution loaded with VB-20 (Figure 3a) decreased rapidly during the first three days, and the elimination became slower at the end of the fourth day, reaching a stable level during seven days for both cases. However, an improvement of 20 % in the reduction was noted when glucose was added to the treated solution with a reduction percentage of 63 % against 43 % without glucose (Table 2).

In the case of DR-89 (Figure 3b), there was a decrease in the concentration during the first five days, then equilibrium was reached after seven days, with a reduction in the concentration which is less significant compared to VB-20. This may be due to the difference in weight and molecular structure of dyes (Dohanyos et al. 1978). However, with the addition of glucose, a greater reduction was obtained with a percentage of elimination of 44% against 33% (Table 2). These results are explained by the fact that the presence of an easily biodegradable organic product such as glucose, allowed a better bacterial growth and consequently a much greater biomass was obtained in the solution which favors the interaction of pollutants with the bacteria (Fu et al. 2001). This was confirmed by the results above of microbial development and the growth index of microorganisms grouped in Table 1. Other studies claimed that the addition of glucose as a source of organic carbon not only improves the cell growth of microorganisms but also biostimulates the production of enzymes for the co-metabolism of glucose and coloring compounds (Kanbouchi et al. 2013).

To better explore the phenomenon responsible for this reduction, measurements of the values of COD and BOD were carried out before and after the biological treatment with activated sludge; the coefficient of biodegradability K was then calculated. The results represented in Table 3 showed that in the case of the VB-20 dye, the initial biodegradability coefficient K is always lower than 3, meaning that the biological

treatment is favorable for this pollutant. However, the initial biodegradability ratio of DR-89 was higher than 3, indicating that the treatment of this pollutant by biological methods is not favorable due to their low biodegradability (Suschka and Ferreira 1986). In addition, the change in COD represented in Figure 3c, d showed a significant decrease due to the reduction in the concentration of pollutants in the liquid phase; this decrease is even greater in the presence of glucose. Furthermore, a fluctuation in the value of the COD inducing an increase in the latter after the seventh days of the two dyes was occurred for the solutions without glucose. This is probably due to the lysis of microorganisms that has occurred as a result of the absence of glucose from the nutrient medium, since this glucose substrate is essential for the survival of microorganisms. As a consequence, the death of microorganisms can increase the COD value.

The results in Table 2 showed that the degradation yields of the dyes calculated on the basis of COD reached a value of 63 and 47% for VB-20 and DR-89 respectively in presence of glucose. Compared to DR-89, a high biodegradation yield of VB-20 was noticed, which may indicate that the nature and type of bonds and the complexity of the chemical structure of the dyes played an important role in the dye's ability to degrade outside of experimental parameters such as pH and initial dye concentration (Dohanyos et al. 1978).

### **Biodegradation of dyes assisted by phytoremediation with *L. gibba***

The biodegradation of VB-20 and DR-89 with activated sludge and of the biodegradation assisted by the phytoremediation with *L. gibba* in presence of glucose substrate, were carried out under the same experimental conditions. In general, the pollutant concentration in the liquid phase decreased more rapidly over time, especially for solutions treated by coupling activated sludge and aquatic plants than for solutions treated with activated sludge alone (Figure 4a,b), which proved that the addition of the *L. gibba* improves the elimination of dyes. This elimination was carried out differently from one dye to another; for the solution loaded with VB-20 (Figure 4a), the decrease in dye concentration was rapid during the first three days, then it reached a stable value during the seventh day with a reduction percentage of 97% when duckweed was added to the solution (Table 4). In the case of DR-89, the results in Figure 4b showed a fair decrease in concentration during the first five days, and then equilibrium was reached after seven days with a reduction percentage of 72%. This was 28% higher than in solution treated with only activated sludge. The removal percentage for DR-89 was still less important compared to VB-20; this may be due to the type of bonds and the complexity of the chemical structure of the dye that play an important role in their removal (Dohanyos et al. 1978). The evolution of the COD over time for the combined treatment of the dyes is presented in Figure 4c, d, where we can notice that there was a significant decrease of COD over time. This reduction was carried out in a regular manner for both cases of treatments.

The degradation yields represented in Table 4 reached values greater than 96 and 69 % for VB-20 and DR-89 respectively, for the solutions treated by the combined process. The yield values were 63 (VB-20) and

47 % (DR-89) in the activated sludge treatment process. These results showed that the use of aquatic plants resulted in an increase of 33 and 22 % for VB-20 and DR-89, respectively.

According to Table 5, the measurements of the BOD values carried out after the treatment indicate a more remarkable reduction in carbon pollution by the combined treatment with a reduction in BOD of 95 and 69 % respectively for VB-20 and DR-89. In addition, the calculation of the coefficient of biodegradability K, for the biological treatment was more favorable to VB-20. However, the treatment of DR-89 by biological methods was not very favorable due to their low biodegradability.

From these results we can deduce that the combination of the aquatic plants *L. gibba* with the activated sludge for the treatment of textile dyes, improves the elimination of the selected pollutants. In addition, the combination process allowed minimizing the treatment time to seven days. This enhancement is probably related to the participation of the aquatic plants using phytoaccumulation mechanism allowing them to concentrate dyes on their different part such as roots or fronds (Hocini et al. 2020). This result supports the synergistic effect between the aquatic plants and activated sludge for the elimination of organic pollutant (Ambaye and Hagos 2020; Franciscon et al. 2015; Turgay et al. 2011).

### **Influence of aquatic plants on the development of Activated Sludge**

To demonstrate the synergistic effect existing between duckweed and activated sludge, we studied the influence of the plants on growth and microbial development of AS. The results obtained in Table 6 showed that the growth index of AS showed a slight reduction compared to that when AS was present alone in the culture medium. Indeed, it was ranging in intervals of 1.56-2.26 and 1.51-2.05 for VB-20 and DR-89, respectively. However, it was still higher than the control suggesting that the presence of *L. gibba* was not inhibiting the growth of activated sludge. Furthermore, a slight reduction in the MDP was observed for the control as well as for the treatment solutions, compared with MDP of the activated sludge found in Table 1. This is probably due to the competition of the aquatic plants in the nutrient components and on the organic carbon obtained after the biodegradation of the target molecules, given that the growth of plants require different components, namely macro and micro elements that are indispensable for plant metabolism and for the complete development of their vegetative cycle (Hocini et al. 2019; Kumar et al. 2021).

### **Biodegradation mechanism**

To evaluate the metabolites generated during the biodegradation of the two dye molecules, LC/MS, FT-IR and UV-visible spectroscopy were used as analysis tools. From Figure 5a-c we can notice that VB-20 and DR-89 have two peaks in UV region at 240 and 320 nm corresponding respectively to benzene and naphthalene cycle of the dye (Yu et al. 2005). The peaks appeared at 580 and 494 nm are related to anthraquinone cycle and the chromophore N=N for VB-20 and DR-89, respectively. In DR-89 spectrum, the dye exhibited a decrease in intensity without any peak change because most dyes, seven more azo dyes, are very resistant to microbial attack (Lodato et al. 2007). However, for VB-20, the peak obtained at 580 nm start to disappear on the third day, suggesting the cleavage of anthraquinone cycle by the AS

microbial community (Parmar et al. **2017**). In addition, a new peak appeared with higher absorbance intensity at a wavelength of 320 and 240 nm during the second day, indicating that benzene and naphthalene derivative concentration increased due to the biogenerated metabolites (Benabbas et al. 2020; Chowdhury, Bhattacharyya 2015). As long as the treatment continues, the absorbance for those peaks diminishes progressively suggesting the transformation of aromatic compounds to small organic molecules.

The transformation of dyes induced by activated sludge was also evaluated using FT-IR analysis and liquid aliquots were taken at different time intervals during the biodegradation process. The results represented in Figure 5e showed that for DR-89, the presence of peaks at 642, 1640, 2978  $\text{cm}^{-1}$  and a broad peaks between 3000-3500  $\text{cm}^{-1}$  are associated with the stretching vibration of C-H for benzene ring, N-H amine, C-H alkane and for O-H, respectively. During the dye removal, those peaks remains unchanged confirming the stability of azo dye during aerobic biodegradation process, because oxygen is a limiting factor in the biodegradation of xenobiotic and recalcitrant azo dyes, due to the competition for the NADH cofactor between respiration and azoreduction (Chang et al. 2001; Saratale et al. 2011; Sudarjanto et al. 2006). In addition, the azo dye DR-89 did not degrade in our operating conditions probably due to the lack of nitrifying microorganisms responsible for the degradation of azo bonds. Indeed, according to Le Bhian et al. (**2001**), other microorganisms such as protozoa and metazoan can cause a decrease in the growth rate of nitrifying bacteria by predation. Furthermore, the decrease in concentration of DR-89 was closely related to the biosorption phenomena rather than degradation process (Basibuyuk and Forster 2003; Porter and Snyder 1976; Sirianuntapiboon and Srisornsak 2007; Yang et al. 2013). In the case of VB-20, Figure 5d showed major peaks at 834, 1461, 1550, and 3500  $\text{cm}^{-1}$  which were attributed to the stretching of C=C and C-C of benzene ring, C-H alkane and C=O ketone function. Metabolites obtained during the biodegradation process showed that peaks relative to C=C and C-C practically disappeared and the peak relative to C=O becomes more intense during seven days confirming the cleavage of aromatic rings and anthraquinone cycle of the dye by the AS microbial community (Ayed et al. 2009). Also, a new peak appeared between 3000 and 3500  $\text{cm}^{-1}$  which corresponds to the stretching of O-H; as the treatment continued, the peak became more intense (6th and 7th day) suggesting that VB-20 was degraded into intermediate metabolites.

The obtained result was further supported by LC/MS analysis represented in Figure 6 which showed that during the biodegradation process, the anthraquinone cycle of the dye was cleaved, to produce 2-hydroxybenzoic acid and 2-(perylene-3-yl) benzoic acid at m/z values of 137 and 373, respectively. In the case of 2-hydroxybenzoic acid, the identified metabolite at m/z values of 131 and 61 were (Z)-pent-2-enedioic acid and acetic acid respectively that were transformed to low molecular carbon chain during the last day. The biodegradation of 2-(perylene-3-yl) benzoic acid produced Perylene and 2-hydroxybenzoic acid that was obtained at m/z values of 137 and 251 respectively. The Perylene metabolite was then transformed to carboxylic acid derivatives by firstly passing through 3-hydroxyphthalic acid (m/z = 183) as intermediate metabolite. This biodegradation occurred by two main steps; the first step is the transport of the substrates to the bacteria cell wall, this biosorption step is relatively rapid compared to that of

metabolization (Pandolfi and Pons 2004). After the biosorption phase, the microorganisms in the activated sludge oxidize the dye molecule aerobically to give other byproducts intoxicating for the environment, and produce either energy in the form of ATP, or synthesize new cellular cells. In addition, we visually saw that all of the bacterial strain granules retained their original color (dark brown) and were not deeply colored due to the biosorbed dye. This indicates that the color removal was mainly due to the biodegradation of the activated sludge (Chen al. **1999**). Several studies have shown that activated sludge (or bacteria isolated from activated sludge) successfully biodegrades organic dyes, including various types of dyes under aerobic conditions (Boonnorat et al. 2018; Kanagaraj et al. 2014).

Fukui index ( $f^-$ ) was used by many researchers as an important concept to explore the degradation of molecules (Esrafilii and Saeidi 2018; Huang et al. 2016; Maitarad et al. 2016; Lui et al. **2017**). This index was calculated using DFT theoretical calculation method. To further clarify the VB-20 molecule biodegradation, calculated Fukui index is represented in Figure 7. The higher Fukui index was obtained for atomic sites that were located in the anthraquinone cycle and are considered to be the most reactive sites for the biological degradation. This suggests that the biodegradation of the dye occurred firstly by destruction of anthraquinone cycles. In addition, the higher Fukui index for C19 (0.07464), C18 (0.03320), H46 (0.02502), H45 (0.02304), H27 (0.01942), C35 (0.01708), H28 (0.01072), C34 (0.0023), C17 (-0.008) and C30 (-0.0322) pointed out that phenanthrene cycle were also attacked by the activated sludge, then the oxidation continued to obtain benzoic acids that were then converted to low molecular acids.

These results demonstrated that the removal of VB-20 dye was occurring through biodegradation process by the microbial community of the activated sludge. The proposed biodegradation process of VB-20 is represented in Figure 8. The results confirmed that VB-20 was removed by the biodegradation process which firstly occurs by the attack of the anthraquinone cycle of the dye to obtain aromatic acids and low molecular compounds. Then as the biodegradation goes further the ring opening of the aromatic acids occurs to obtain short aliphatic acid chains. Those results are consistent with the biodegradation of anthraquinone dye found in the literature using different water treatment system (Alam et al. 2020; Chao et al. 2020; Fanchiang and Tseng 2009; Maitarad et al. 2016).

To investigate the mechanism responsible for the elimination of dyes, the morphology of *L. gibba* was observed under the SEM microscope before and after the combined biological treatments. From Figure 9a, it can be seen that the native biomass of *L. gibba* (before treatment) had a rough and porous surface with heterogeneous cavities and pores of various shapes and diameters, this porosity can be useful for dye fixation (Benabbas et al. 2021). The *Lemna* biomass was also observed after phytoremediation experiments using the CBS (centered backscattered electrons) mode which is sensitive to the differential phase mode. From Figure 9c, e we can observe that the two dyes are present on the surface of the white colored biomass suggesting that the plant biomass can accumulate organic dyes on the frond surface, revealing the significant affinity of duckweed for anionic dyes (Khaled et al. 2020). To support the above observation, the EDX results summarized in Table 7 showed that there was a major change in the composition of the biomass where the mass percentage of C, N and O increased after phytoremediation

experiments confirming that most of the dyes present in the solution have been eliminated by phytoaccumulation process (Hocini et al. 2020). The mass percentage of inorganic elements also varied when the biomass was exposed to DR-89; the amount of Na, Mg, K, Ca, P, S decreased which may be due to the toxicity of the dye which can inhibit the binding of these inorganic elements. These elements are reported to be important for plant growth, photosynthesis and are beneficial for plant survival under conditions of abiotic stress (Amtmann et al. 2008; Wang et al. 2013; Wang et al. 2014; Zhao et al. 2005).

## Conclusion

According to this study, the removal percentage and the degradation yield of VB-20 and DR-89 showed an important increase due to the coupling of the two biological processes. This efficiency was linked to the mixed consortium of microorganisms and aquatic plants that were responsible for the removal of dyes by biosorption and biodegradation mechanism on activated sludge and a phytoaccumulation process using aquatic plants. The growth and the development monitoring using different parameters showed excellent synergy between the two biological species even in the presence of dyes, especially when glucose was added to the solutions. The biodegradation mechanism showed that the removal of VB-20 was initiated by the cleavage of anthraquinone cycle and the degradation of the generated aromatic compounds to obtain low hydrocarbon chains that exhibited a low toxicity toward *L. gibba* during the toxicity assessment. This study showed that the combination of two biological processes could be interesting approach for the removal of anthraquinone dye.

## Declarations

### Ethical approval

Not applicable

### Consent to participate

Not applicable

### Consent to publish

Not applicable

### Contributions

All authors contributed to the study conception and design. Methodology, experiments and writing of the original draft: I. Hocini, K. Benabbas and N. Khellaf; supervision and review: N. Khellaf, H. Djelal and A. Amrane; all authors revised the manuscript and approved the final version.

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All authors declare no conflict of interest

### Availability of data and materials

The data are available from the corresponding author

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## Tables

**Table 1.** Dry matter, growth index and microbial development percentage of activated sludge measured in different mediums

Dyes	VB-20	DR-89
<sup>1</sup> AS DM-Control (g/L)	33.80	32.80
AS DM-without Glucose (g/L)	54.80	34.20
AS DM-with Glucose(g/L)	59.60	40.20
<sup>2</sup> AS GI-Control (-)	1.77	1.72
AS GI-without Glucose (-)	2.87	1.79
ASGI-with Glucose (-)	3.12	2.10
<sup>3</sup> AS MDP-Control (%)	43.43	41.71
AS MDP-without Glucose (%)	65.11	44.09
AS MDP-with Glucose (%)	67.92	52.44

<sup>1</sup>AS DM : Dry matter of activated sludge

<sup>2</sup>AS GI : Growth index of activated sludge

<sup>3</sup>AS MDP : Microbial development percentage of activated sludge

**Table 2.** Removal percentage and degradation yield of pollutants after biodegradation treatment with activated sludge

Dyes	RP (%)		DY (%)	
	Without glucose	With glucose	Without glucose	With glucose
VB-20	42.62	62.91	47.32	62.71
DR-89	33.14	43.89	38.78	47.29

**Table 3.** Estimation of COD and BOD<sub>5</sub> value and biodegradation coefficient during the biodegradation of the dyes using activated sludge

Dyes	Before biodegradation			
	COD (mgO <sub>2</sub> /L)	BOD <sub>5</sub> (mgO <sub>2</sub> /L)	K <sub>initial</sub>	BOD <sub>5</sub> reduction (%)
VB-20	212.4	78.0	2.7	0
DR-89	256.6	82.0	3.1	0
Dyes	After biodegradation			
	COD (mgO <sub>2</sub> /L)	BOD <sub>5</sub> (mgO <sub>2</sub> /L)	K <sub>final</sub>	BOD <sub>5</sub> reduction (%)
VB-20	79.2	38.9	2.0	50.1
DR-89	135.3	45.2	3.1	46.1

**Table 4.** Removal percentage and degradation yield of pollutants after biodegradation treatment with activated sludge assisted by phytoremediation

Dyes	RP (%)		DY (%)	
	Without plant	With plant	Without plant	With plant
VB-20	62.91	97.11	62.71	96.35
DR-89	43.89	71.83	47.29	68.65

**Table 5.** Estimation of COD, BOD<sub>5</sub> and biodegradation coefficient during the biodegradation of the dyes using the combined process

Dyes	Before biodegradation			
	COD (mgO <sub>2</sub> /L)	BOD <sub>5</sub> (mgO <sub>2</sub> /L)	K <sub>initial</sub>	BOD <sub>5</sub> reduction (%)
VB-20	212.4	78.0	2.7	0
DR-89	251.6	82.0	3.1	0
Dyes	After biodegradation			
	COD (mgO <sub>2</sub> /L)	BOD <sub>5</sub> (mgO <sub>2</sub> /L)	K <sub>final</sub>	BOD <sub>5</sub> reduction (%)
VB-20	7.8	3.8	2.0	95.1
DR-89	78.9	25.2	3.1	69.3

**Table 6.** Dry matter, growth index and microbial development percentage of activated sludge measured in different mediums

Dyes	VB-20	DR-89
DM Control (g/L)	29.80	28.80
<sup>1</sup> AS DM/LG without glucose (g/L)	37.60	33.40
AS DM/LG with glucose(g/L)	43.20	39.20
GI Control	1.56	1.51
<sup>2</sup> AS GI/LG without glucose	1.97	1.75
AS GI/LG with glucose	2.26	2.05
MPD control	38.84	33.61
<sup>3</sup> AS MDP/LG without glucose	49.15	42.75
AS MDP/LG without glucose	55.74	51.22

<sup>1</sup>AS DM/LG : Dry matter of activated sludge in the presence of *L. gibba*

<sup>2</sup>AS GI/LG : Growth index of activated sludge in the presence of *L. gibba*

<sup>3</sup>AS MDP/LG : Microbial development percentage of activated sludge in the presence of *L. gibba*

**Table 7. EDX analysis of *L. gibba* biomass before and after dye phytoremediation**

Elements	<i>L. gibba</i>	<i>L. gibba</i> + VB-20	<i>L. gibba</i> + DR-89
C	22.29	24.61	27.85
N	8.49	8.25	15.74
O	56.62	59.45	62.37
Na	3.83	3.86	2.59
Mg	2.24	1.46	1.26
P	2.37	1.05	1.21
S	0.27	0.35	1.29
K	2.59	10.00	9.22
Ca	1.30	0.97	0.93

# Figures

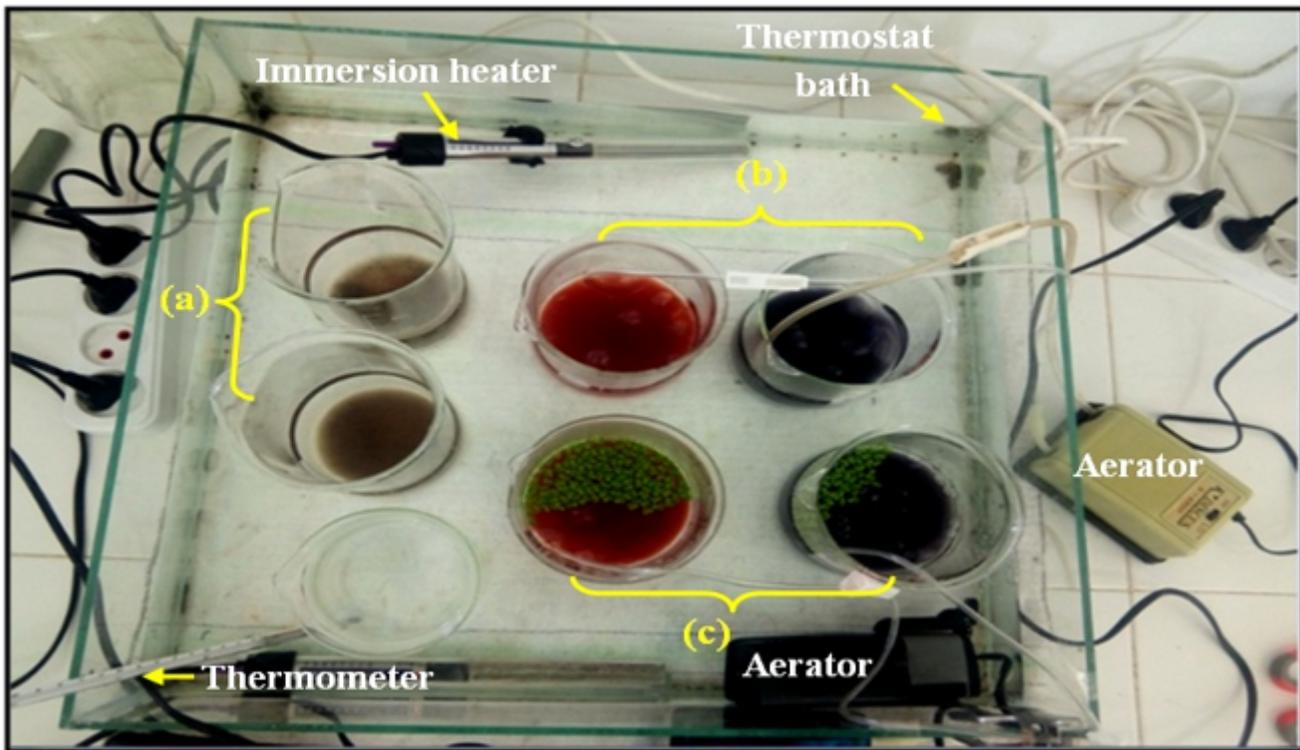
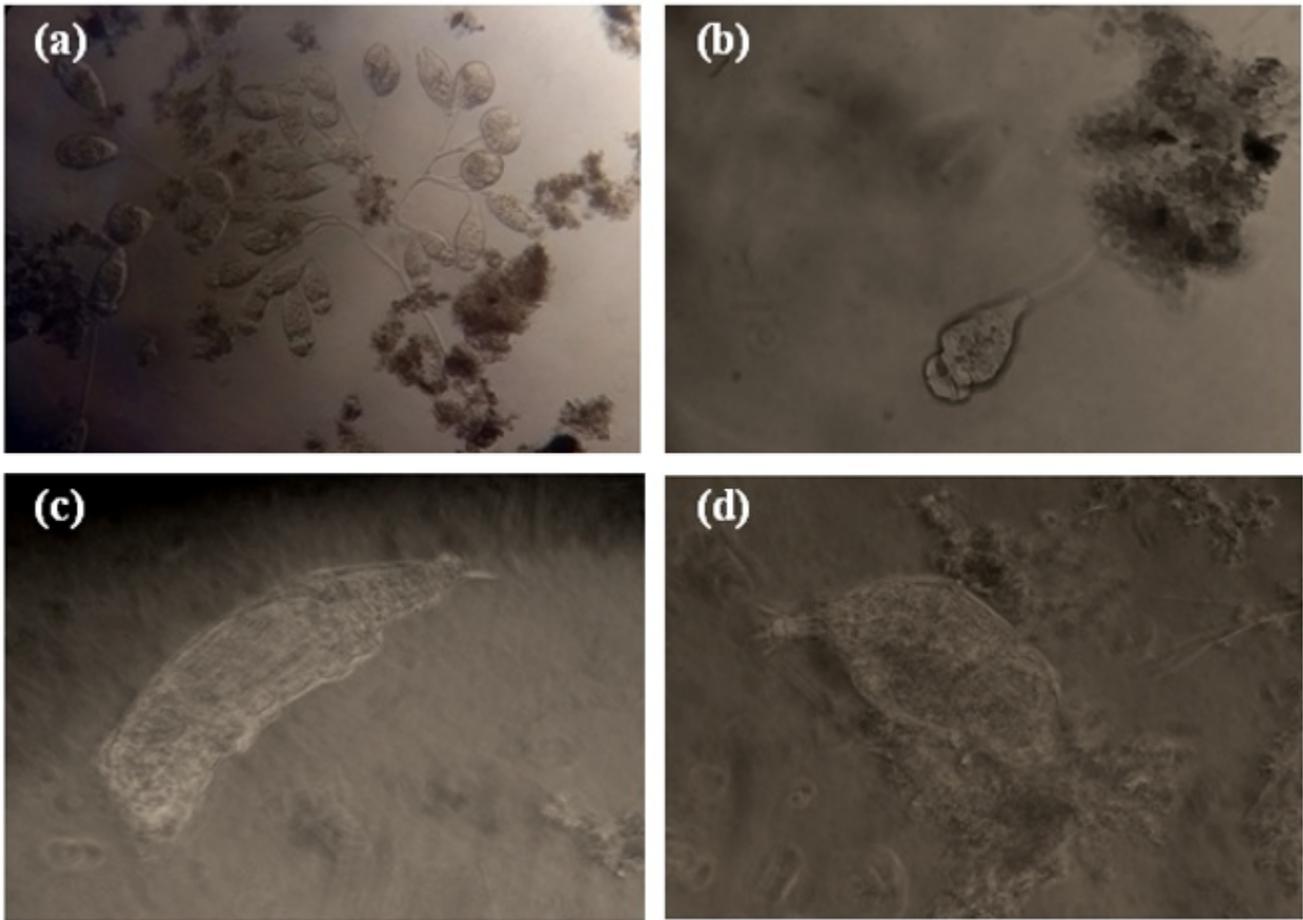


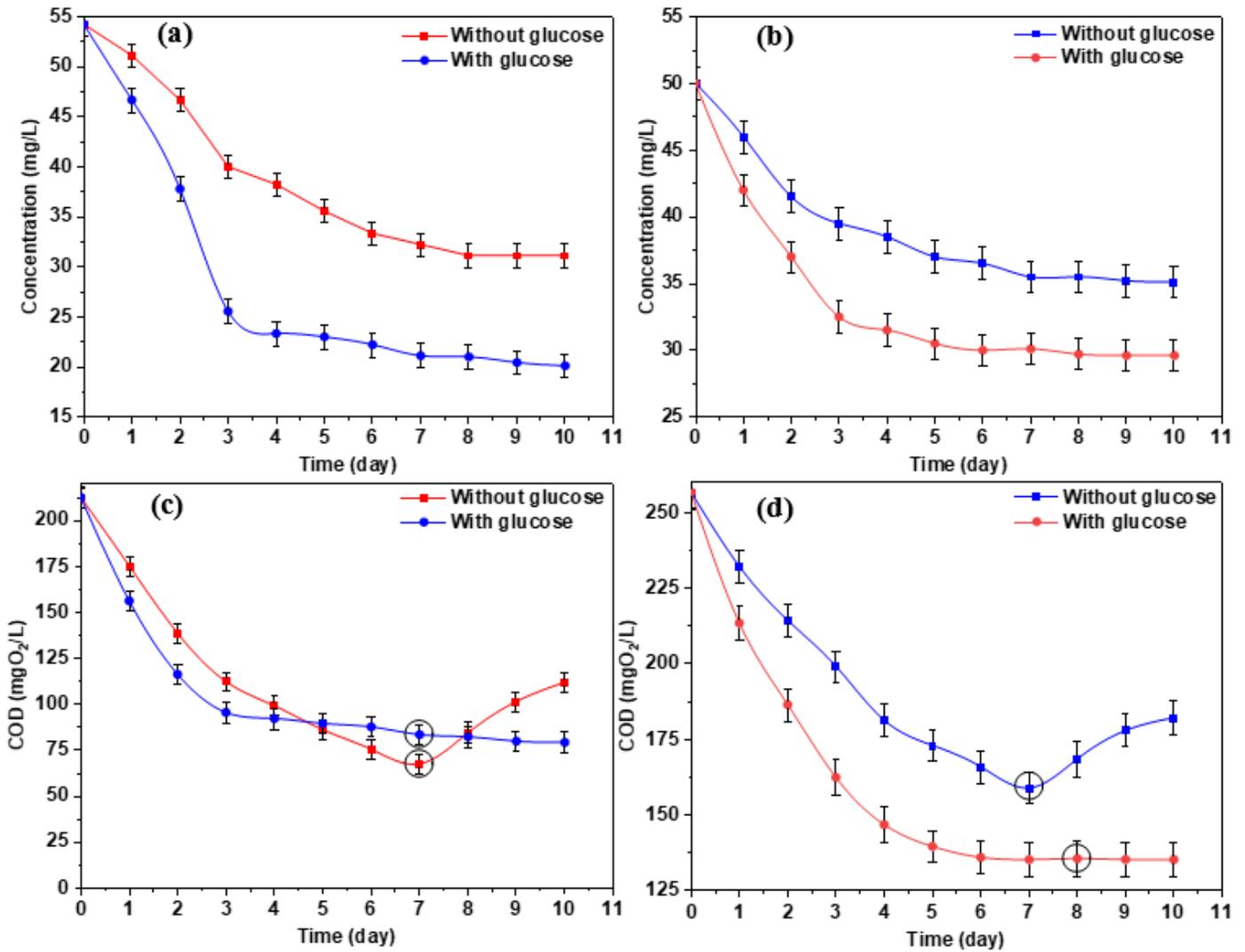
Figure 1

Experimental device for the treatment of dye pollutants: (a) Control tests, (b) Treatment with activated sludge, (c) Treatment with activated sludge/phytoremediation



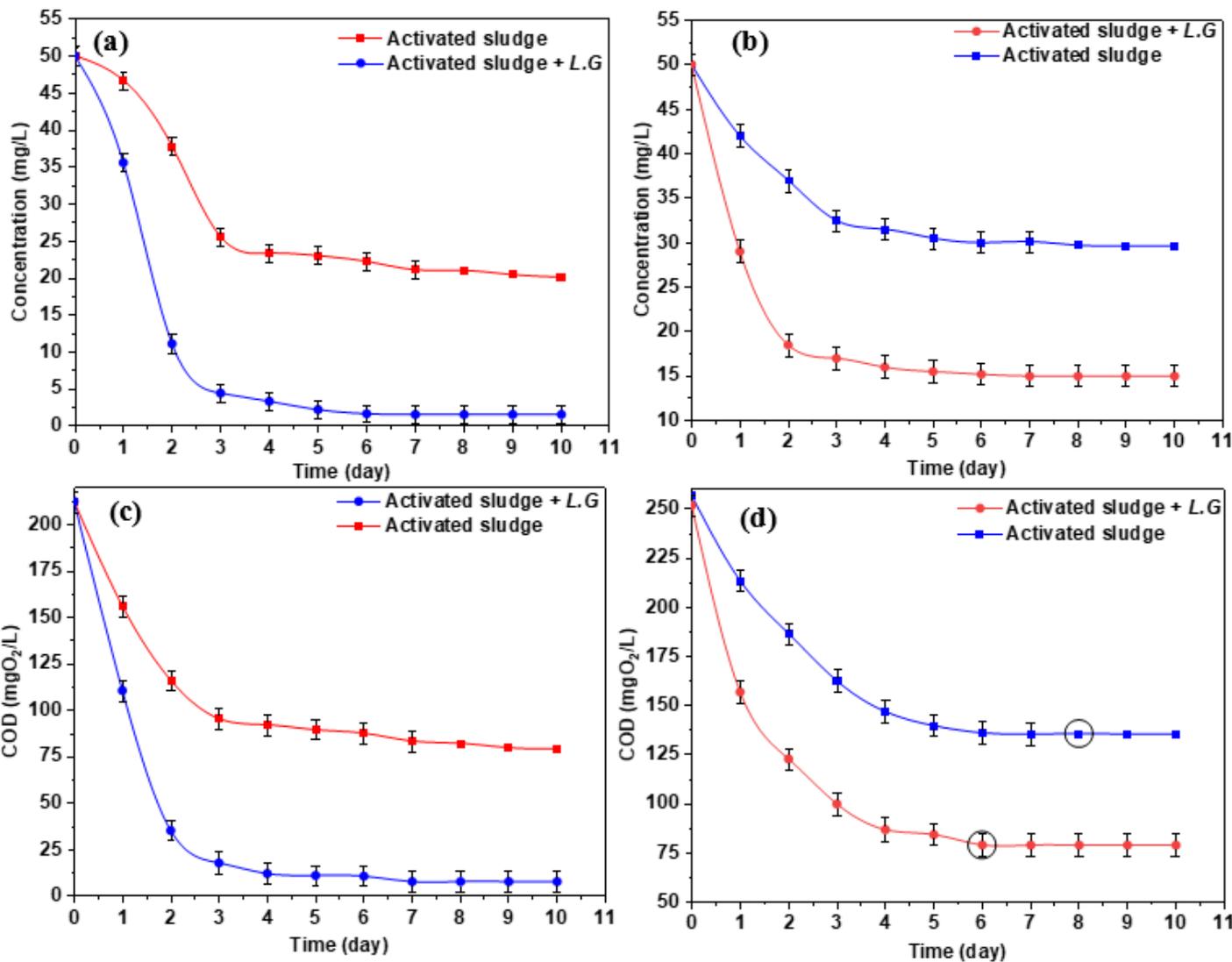
**Figure 2**

Microscopic observation of AS consortium, (a) *Epistylis sp.*, (b) *Vorticella*, (c) *Rotifera* and (d) *Euplotes sp.*



**Figure 3**

Effect of glucose on concentration and COD variation of (a, c) VB-20 and (b, d) DR-89 as a function of time during biodegradation with activated sludge



**Figure 4**

Concentration and COD variation of (a,c) VB-20 and (b,d) DR-89 over time during biodegradation using activated sludge assisted by phytoremediation with *L. gibba*

**Figure 5**

UV-visible and FTIR spectra of (a, b, d) VB-20 and (c,e) DR-89 during dye biodegradation using activated sludge

## Figure 6

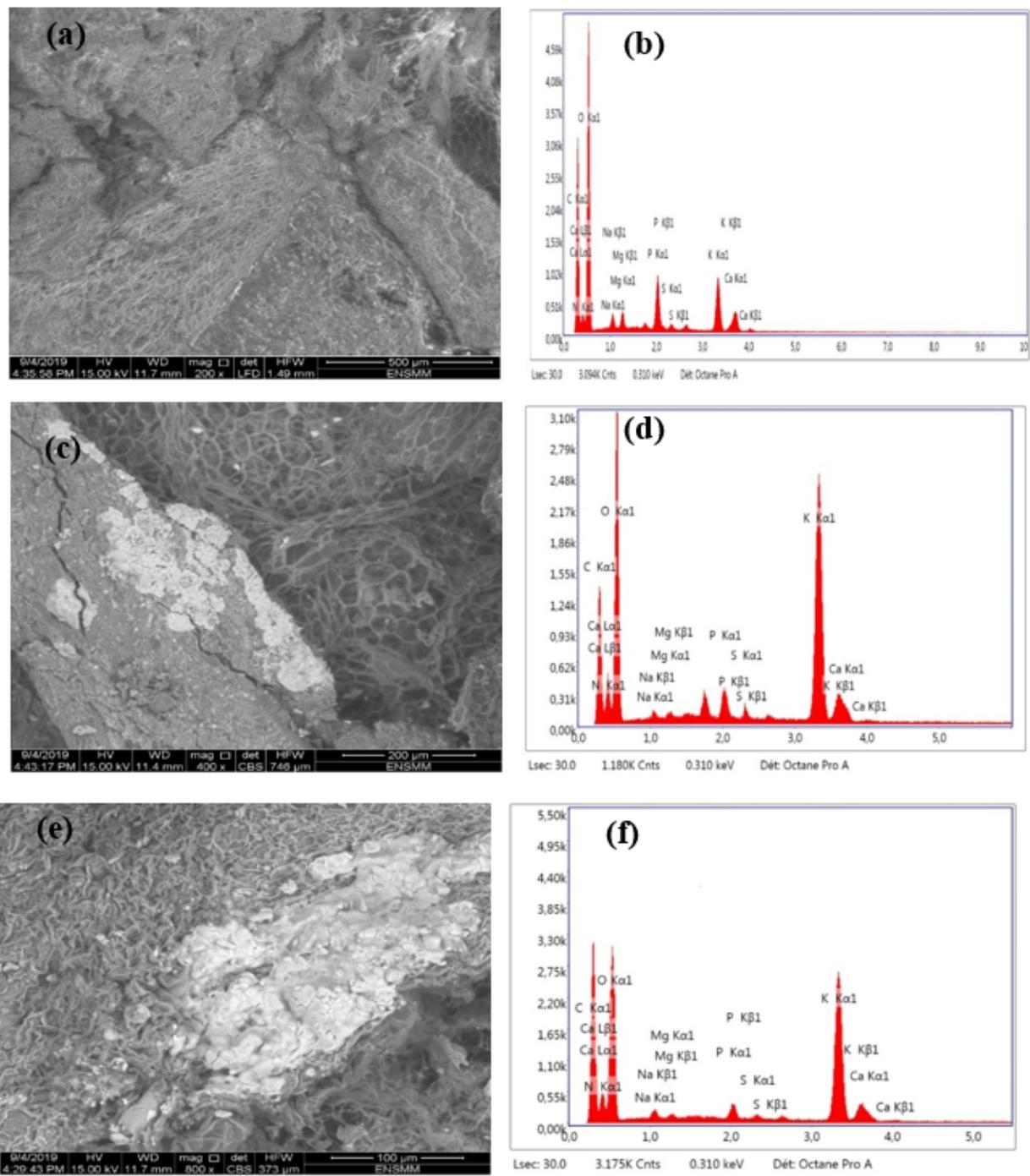
LC/MS spectra of VB-20 biodegradation metabolites at different time intervals

## Figure 7

(a) calculated Fukui index for VB-20, (b) optimal geometry of VB-20 molecules

## Figure 8

Proposed mechanism of VB-20 biodegradation



**Figure 9**

SEM images and EDX analysis of *L. gibba* biomass: (a, b) without dyes, (c, d) after DR-89 treatment, (e, f) after VB-20 treatment

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Graphicalabstract.doc](#)