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Preliminary assessment of terrestrial microalgae isolated from lichens as testing species for environmental monitoring: Lichen phycobionts present high sensitivity to environmental micropollutants

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ABSTRACT

Bioassays constitute a tool for pollution analysis providing a holistic approach and high-quality indication of the toxicity. Microbioassays allow evaluating the toxicity of many samples, implying lower costs and enabling routine monitoring and pollution control. But tests conducted so far are limited to the use of a small number of taxa. Lichens are excellent bioindicators of pollution with great ecological significance. Studies show that the phycobiont is more sensitive to pollutants than the mycobiont. Phycobiont have features such as adaptation to anhydrobiosis and relatively rapid growth *in vitro*, making them suitable for microbioassays. Our aim is to determine the sensitivity of phycobionts to the pharmaceutical micropollutants carbamazepine and diclofenac as a preliminary step for the development of a toxicity microbioassay based on phycobionts. Optical dispersion and chlorophyll autofluorescence were used as endpoints of toxicity on two algal species showing that suspensions present cyclic and taxon specific patterns of aggregation. *Trebouxia* TR9 suspensions present a very high grade of aggregation while *Asterochloris erici* cells do not. Both micropollutants alter optical properties of the suspensions of both species. No significant alteration of chlorophyll autofluorescence by carbamazepine is observed. *A. erici* chlorophyll autofluorescence is extremely sensitive to diclofenac but the effect is not dependent on the drug concentration or on the time of exposure. Differently, TR9 only shows punctual chlorophyll alterations. Fluctuations in optical dispersion may indicate changes in the population structure of the species, including reproductive strategy. *A. erici* seems more sensitive to micropollutants, is better characterized and is available from commercial collections.

1. Introduction

Chemical analysis has historically been the most used method to monitor and evaluate the environmental pollution (Wadhia and Thompson, 2007). These measurements can identify compounds in the environment, but do not give an indication of the bioavailability of toxic substances and of the joint effects these compounds may have on the biota (Hendriks et al., 1994, cited in Van der Griten et al., 2010). In this situation, the "bioassay" constitutes a complementary tool of analysis, capable of providing a holistic approach and an indication of the acute toxicity, genotoxicity or chronic effects that the organisms under study undergo (Wadhia and Thompson, 2007; Van der Griten et al., 2010).

When conducting bioassays routinely, there may be problems due to the high number of samples, and space and facilities (greenhouses, pools) required, usually reflected in increased economic costs. This has led to the development of bioassays with miniaturization or micro procedures, known as "microbioassays". They allow evaluating the toxicity of a high number of samples, implying lower costs and enabling structures for monitoring and pollution control (Wadhia & Thompson, 2007). But tests conducted so far are limited to the use of a small number of taxa, due to either practical or economic criteria, and not by ecological criteria (Catalá et al., 2009). It is therefore necessary to extend the range of action of these studies to more taxa, as usually the most unknown are the most threatened.

Microalgae rank among the most frequently used organisms because they provide features such as high sensitivity and a high reproducibility (Shitanda et al., 2009). The organisms referred to as algae are generally a collection of unrelated organisms that possess plastids (Del Campo et al., 2010) Green algae, or Chlorophyta, constitute a huge and extremely diverse phylum of eukaryotic organisms. These eukaryotes must not be confounded with prokaryotic cyanobacteria, also known as blue-green algae. At present, the

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toxicity bioassays use free-living aquatic algae as *Pseudokirchneriella subcapitata* or *Chlorella* sp. (Levy et al., 2009) but the possibility of using algae associated with lichens has never been studied.

Lichens are organisms very sensitive to environmental pollution, able to accumulate pollutants from the atmosphere or from soils. Therefore, these have been used as bioindicators of air or soil quality (Bačkor and Váczi, 2002; Bačkor et al., 2007). Besides being excellent bioindicators of pollution, lichens have great ecological significance, because they play a key role in soil formation, since in all cases, the first organisms to appear on the nude rocks are encrusting and foliose lichens (Cooper and Rudolph, 1953).

Lichens are complex organisms with a specific organization. Their thalli are composed of heterotrophic fungi (mycobiont) and photosynthetic algae or cyanobacteria, photobionts, or in the specific case of algae, phycobionts. Environmental studies with lichens show that, generally, the most sensitive element to pollutants is the phycobiont (Bačkor, Váczi (2002)). Over 50% of all lichen species are associated with algae of the genus *Trebouxia* (*sensu lato*, including *Asterochloris*) (Gasulla et al., 2010; Piovár et al., 2011). These algae have features such as the possibility to obtain large quantities at low economic costs and tolerance to a wide range of operating temperatures (Gasulla et al., 2010). In addition unlike free-living algae have high tolerance to desiccation (high tolerance to hydric stress) (Gasulla et al., 2009, 2013), qualities that free-living algae do not possess.

Symbiotic algae (phycobionts) belonging to *Astherochloris* and *Trebouxia* genera (class *Trebouxiophyceae*) can be isolated from lichens. Phycobionts are different to most algae used in toxicity bioassays belonging mostly to classes *Chlorophyceae* or *Chrysophyceae*. Apart from phylogenetic divergences, lichen phycobionts present dramatic ecophysiological differences:

1) Lichens are terrestrial organisms, and therefore, phycobiont (green microalgae) toxicity would be representative of terrestrial ecosystems vs. aquatic green algae currently used.
2) Linked to terrestrial dwelling, lichen phycobionts possess a high tolerance to desiccation (they are adapted to anhydrobiosis), a useful property that free-living algae do not possess, since they belong to aquatic ecosystems.
3) Phycobionts are symbiotic organisms. Recent works demonstrate remarkable similarities in certain biological traits among symbiotic organisms such as lichens, rhizobium or mycorrhizae (Meilhoc et al., 2011; Puppo et al., 2013). These symbioses are of utmost importance for both global nitrogen ecology and crop production. Phycobionts could serve as valuable testing organisms of how environmental pollution may disrupt symbiotic interaction.

These unique properties of phycobionts along with the need to increase the available taxa for use in toxicity bioassays, especially for terrestrial habitats, make them a good choice for the development of microbioassays of toxicity with low associated economic costs, increasing the number of taxa available. However, no study on the utility of lichen phycobionts as testing species in environmental monitoring or toxicology has been addressed yet. The first question to answer is whether they are sensitive to environmental pollutants. If this is the case, the next questions to solve are how to adapt their peculiar characteristics to a bioassay and what are the most appropriate endpoints to measure toxicity.

The revolutionary development of resources and new technologies has led to increased release of chemicals that may pose a threat to the environment and biodiversity (Bolong et al., 2009). Some of these compounds have been frequently detected in different environmental compartments (rivers, lakes or sediments) at low concentration (ng to μg L^{-1} and μg to mg $kg\ L^{-1}$ dry matter) and for this reason they are known as micropollutants (Delgadillo-Mirquez et al., 2011). These include pharmaceutical compounds and their metabolites (Fent et al., 2006). Pharmaceuticals may reach agricultural fields both through irrigation with micropolluted surface water and amendments with compost or biosolids derived from sewage sludges (Vazquez-Roig et al., 2011; Martin Ruel et al., 2012). Irrigation itself can generate water aerosols that directly reach lichens and soil tillage will produce contaminated soil aerosols. Terrestrial organisms can thus be exposed to these drugs by dry/wet deposition of aerosols generated in nearby agricultural fields (Fig. 1).

The antiepileptic carbamazepine is a Central Nervous System (CNS) drug that helps to quiet the abnormal firing of nerves. It blocks the sodium voltage-dependent channel of excitatory neurons. It is often used as an anticonvulsant and mood stabilizer (Van den Brandhof and Montforts, 2010). Diclofenac is a drug belonging to the family of Nonsteroidal Anti-inflammatory Drugs (NSAIDs). This is a phenylacetic acid derivative that inhibits cyclooxygenases, key enzymes catalyzing the biosynthesis of prostaglandin. Both drugs have been detected in water bodies at concentrations in the order of ng L^{-1} or even μg L^{-1} (Zhang et al., 2008; González Alonso et al., 2010; Valcárcel et al., 2010, 2011). Studies such as that conducted by Feito et al., (2012) highlight the potential threat that, these drugs can be for wild organisms, especially for terrestrial plants. Specifically, environmental concentrations of diclofenac can cause acute lethal and chronic sublethal toxicity in higher plant development (fern spore bioassay). Therefore, it is essential to monitor and evaluate the impact of drugs on different components of the ecosystems, especially if their presence is continued (Van der Griten et al., 2010).

Thus, the objective of this study is to determine the sensitivity of phycobionts to carbamazepine and diclofenac as a preliminary step for the development of a toxicity microbioassay based on isolated lichen phycobionts. Optical dispersion (O.D.) and chlorophyll autofluorescence were used as endpoints of toxicity.

2. Materials and method

2.1. Biological material

Two strains were used in this study, *Asterochloris erici* and *Trebouxia* sp. TR9. *A. erici* described by Skaloud and Peksa, 2010, (formerly known as *Trebouxia erici* Ahmadjian, 1960) is a phycobiont isolated from the lichen *Cladonia cristatella*. It has a growth temperature between 17 °C and 23 °C, with an optimum at 20 °C

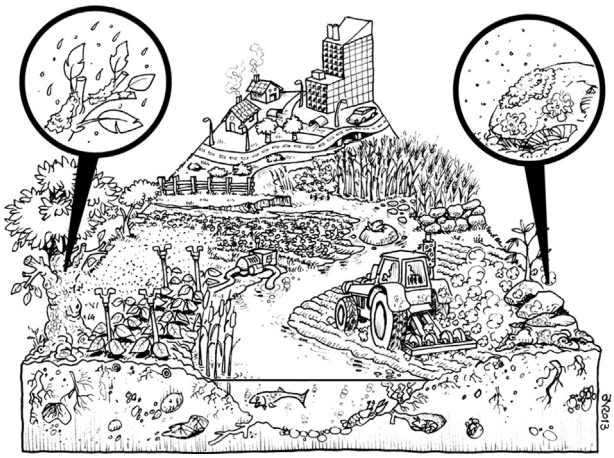

Fig. 1. Urban discharges and inefficacy of sewage treatment plants lead to pollution of surface waters with pharmaceuticals. Irrigation with these micropolluted waters can generate aerosols that may reach terrestrial organisms of the agro-ecosystem. Besides irrigation, pharmaceuticals can reach soils by the amendments done with compost or biosolids containing sewage sludge. Soil tillage also generates aerosols of contaminated dust that can deposit on adjacent ecosystems.

(Gasulla et al., 2010). *Trebouxia* sp. TR9 described by Casano et al., (2011), is a phycobiont isolated from the lichen *Ramalina farinacea*. It is characterized by optimal growth at 15 °C (Del Campo et al., 2011; Del Hoyo et al., 2011).

Both phycobionts were maintained in sterile conditions in semi-solid 3 N Bold's Basal Medium (3NBBM) plus 20 g glucose, 10 g of casein per liter and 15 g of agar per liter (Ahmadjian, 1960) in culture chambers under a photoperiod of 12 h of white light with an intensity of 30 μmol m^{-2} s^{-1} (Gasulla et al., 2010). *Asterochloris erici* cultures were maintained at a temperature of 20 °C and *Trebouxia* sp. TR9 cultures were maintained at a temperature of 15 °C (Gasulla et al., 2010).

2.2. Pharmaceuticals

We selected five different concentrations of carbamazepine (supplied by Sigma-Aldrich, Madrid, Spain) in a logarithmic range from 0.005 μg L^{-1} to 50 μg L^{-1} which covers relevant environmental concentrations (González Alonso et al., 2010; Valcárcel et al., 2010, 2011). Carbamazepine was previously dissolved in ethanol (Quinn et al., 2009) and later diluted in liquid 3NBBM.

We selected six different concentrations of diclofenac (supplied by Sigma-Aldrich, Madrid, Spain) in a logarithmic from 0.01 μg L^{-1} to 1000 μg L^{-1} covering relevant environmental concentrations (González Alonso et al., 2010; Valcárcel et al., 2010; Valcárcel et al., 2011). Diclofenac was dissolved directly in 3NBBM.

2.3. Treatments

Two different endpoints were used to evaluate pharmaceutical effects: optical dispersion (turbidity), and chlorophyll autofluorescence. Exposure times were 24, 48, 72 and 96 h, according to EPA PSEUDO Growth chronic-freshwater METHOD 1003.0.

Liquid cultures in 3NBBM were prepared 15 days before the bioassay from cells growing on agar (semi-solid cultures) and used at maximum of the growth curve of these phycobionts. The phycobiont suspension (500 μl, 10^6 cells ml^{-1}) were inoculated in 1.2 ml tubes (96 tubes rack). Then 500 μl of 3NBBM containing the pollutant and 0.2% ethanol was added. Eight replicates were performed per concentration of drug. Controls with 3NBBM and 0.2% ethanol were performed. Cell suspensions were maintained in a growth chamber at 20 °C in the case of *Asterochloris erici* and at 15 °C in the case of *Trebouxia sp.* TR9, under a photoperiod 12 h of white light with an intensity of 30 μmol m^{-2} s^{-1} for both strains. After the exposure time, 200 μl were taken for the measurement of optical dispersion and 200 μl were frozen for the determination of autofluorescence of chlorophyll.

2.4. Determination of cell aggregates number and size

Fuchs–Rosenthal cytometric chambers were used for the determination of aggregate number and size under an optical microscope (40× magnification). Samples were diluted ($F=10$) and the counting was repeated twice for each of the eights replicates prepared.

2.5. Optical dispersion (O.D.)

Two replicates of 100 μl were placed in transparent multiwell plates. Absorbance measurements were done in a multiwell plate reader (17–550 model Anthos 2010) at $\lambda_{abs}=650$ nm (Chung et al., 2007).

2.6. Chlorophyll autofluorescence

Two replicates of 100 μl were placed in black Greiner plates. Measurements were done a with Spectrafluor Plus plate reader (Tecan Group Ltd., Männedorf, Switzerland) with an excitation filter of $\lambda=485$ nm and an emision filter of $\lambda=635$ nm (Feito et al., 2012).

2.7. Toxicity data analysis

All data are expressed as the mean of the parameter studied for each concentration ± standard error of the mean, as a percentage of the control. Statistically significant differences between groups of data have been determined using ANOVA single-factor (analysis of variance), in combination with the LSD test (Fisher minor differences), using the statistical program Statgraphics. The presence of statistically significant differences are given with a confidence level of 95% ($p < 0.05$).

3. Results

The number of cell aggregates and the aggregate cell number for both species of phycobionts were determined. Then phycobionts were exposed to carbamazepine and diclofenac, pharmaceuticals in the conditions explained in Methods section. LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration) were noted for carbamazepine and diclofenac for each biomarker used, at the shortest time an effect was observed.

3.1. Number and size of cell aggregates

The number of cell aggregates and the number of cells per aggregate for both species of phycobionts were determined.Cell aggregates were classified into four categories: 1–2, 3–8, 9–16 or > 16 cells. Fig. 2 shows the relative proportion of aggregates of different size in *A. erici* (A) and TR9 (B) liquid cultures along time. *A. erici* suspensions contain mainly individual cells or small aggregates (2–8 cells). The proportion of medium size aggregates (9–16) is minority but remains constant along time. Large aggregates (> 16 cells) present cyclic variations in 14-day periods. TR9 suspensions are mainly composed of large aggregates (> 16 cells). Medium size aggregates (9–16) proportion strongly fluctuates along time. Small aggregates (3–8 cells) are only present in low proportion at some culture times. Single or two-cell aggregates are present at all times and their proportion fluctuates along time. Changes in cell size were also observed, smaller cells in the bigger aggregates.

3.2. Carbamazepine

Carbamazepine induces alterations in the optical properties of *A. erici* liquid cultures (Fig. 3A–D). The effects are biphasic and dose dependent. Overall, low doses induce decreases in O.D. while high doses induce increases. This effect is especially significant at 24 and 96 h time points (Fig. 3, A and D respectively). LOEC (24 h) is recorded for the lowest concentration assayed 0.005 μg L^{-1} (Fig. 3A). The sensitivity of the O.D. of TR9 suspensions to carbamazepine is lower, and only slight increases are observed mainly at the highest doses (Fig. 3a–d). In this case, there is not a clear dose response. LOEC (24 h) is recorded at 5 μg L^{-1} (Fig. 3a),

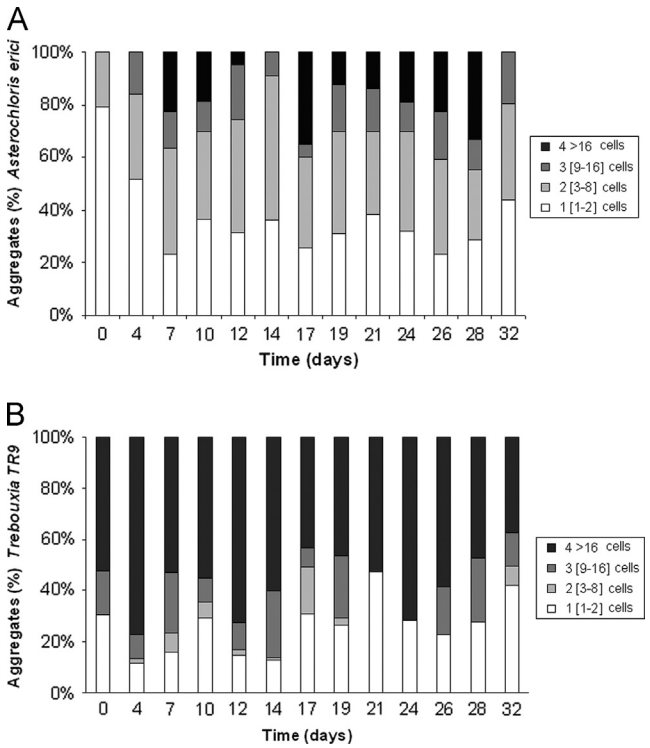

Fig. 2. Characterization of of *Asterochloris erici* (A) and *Trebouxia* sp. *TR9* (B) cell aggregates number and size.

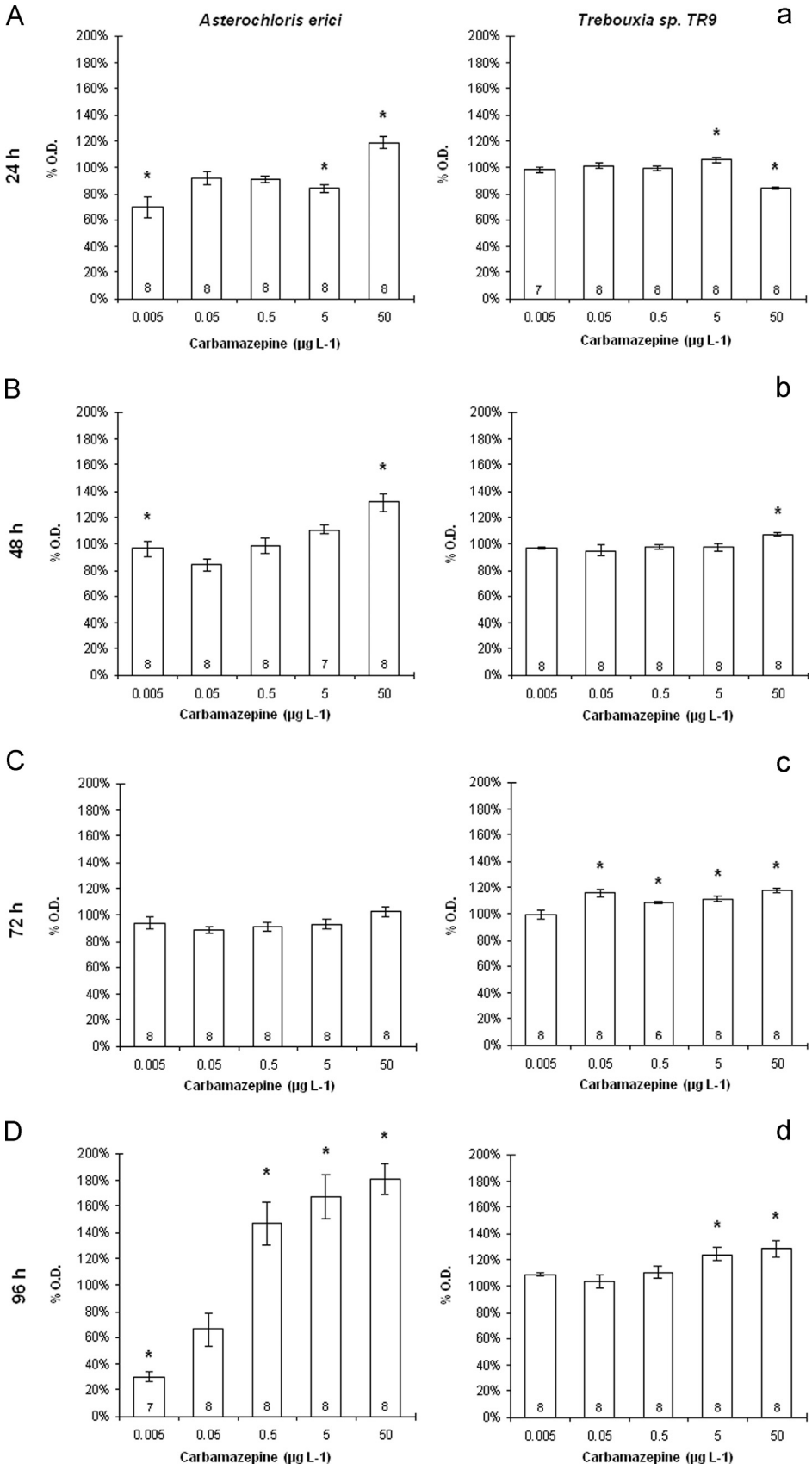

Fig. 3. Optical dispersion referred to controls at 24, 48, 72 and 96 h for five concentrations of carbamazepine in *Asterochloris erici* (A, B, C, D) and *Trebouxia* sp. *TR9* (a, b, c, d). The bars show the standard error of the mean. The base of each column shows the value of *n* at each point. *Indicates statistically significant difference versus control (ANOVA, $p < 0.05$).

although it decreases to 0.05 μg L^{-1} at 72 h (Fig. 3c), and returns again to 5 μg L^{-1} at 96 h (Fig. 3d).

Regarding chlorophyll autofluorescence, for *A. erici*, discarding an isolated small statistically significant decrease at 24 h, the LOEC can be set at 5 μg L^{-1} after 96 h of exposure (Fig. 4A). Chlorophyll autofluorescence alterations in TR9 suspensions consist of small increases (10–20%) at some concentrations and time points with no apparent trend. The LOEC (24 h) is 0.005 μg L^{-1} of carbamazepine.

3.3. Diclofenac

For *A. erici*, LOEC (24 h) is 0.01 μg L^{-1}, the lowest concentration assayed (Fig. 5A). No clear dose response effect is observable. Also for TR9 LOEC (24 h) coincides with the lowest concentration assayed, 0.01 μg L^{-1} (Fig. 5a). A dose-response effect on TR9 is observed at all time points except for 4 h where the O.D. increase is similar for all concentrations (Fig. 5b). At 24 h, O.D. decreases with the increase in diclofenac (Fig. 5a). Differently, O.D. increases with increasing diclofenac concentrations at 48 and 96 h (Fig. 5c and d respectively). In the longer exposure O.D. reaches values of 200% over controls at high concentrations.

The effects of diclofenac on phycobiont chlorophyll are of smaller magnitude than on O.D. and do not surpass 120% in any case. *A. erici* presents a generalized significant increase of chlorophyll autofluorescence at all times and doses studied (Fig. 6A–D). No dose–response effect can be observed and LOEC (24 h) is 0.01 μg L^{-1}, the lowest concentration studied. Only punctual significant increases (~110%) in TR9 chlorophyll autofluorescence have been recorded at 72 h. LOEC (72 h) is 0.01 μg L^{-1}, although the effects disappear at 96 h.

4. Discussion

The results presented above show that phycobiont suspensions may present different patterns of aggregation. This pattern is taxon specific and cyclic variations with time. *Trebouxia* TR9 suspension presents a very high grade of aggregation while *A. erici* cells present mainly individually or in 2-cell aggregates. There are also differences in the response of both species to the pharmaceutical micropollutants assayed. Carbamazepine seems to alter optical properties (O.D.) of the suspensions of both species, being *A. erici* more sensitive than TR9. The alteration of chlorophyll autofluorescence by carbamazepine is very subtle for both species and only punctual significant increases can be observed. Diclofenac also induces significant and generalized alterations in the optical properties of both phycobiont suspensions. The sign of the effect strongly depends on the species, while generalized O.D. decreases are observed for *A. erici*, important increases occur for TR9. *A. erici* chlorophyll autofluorescence is also extremely sensitive to diclofenac despite the effect observed is not dependent on the drug concentration or on the time of exposure. Differently, TR9 only shows punctual chlorophyll alterations.

The optical properties (O.D.) of the suspensions of both phycobionts are extremely sensitive to the presence of the pharmaceuticals showing LOECs (24 h) of 0.005 μg L^{-1} for carbamazepine and 0.01 μg L^{-1} for diclofenac. Optical density has traditionally been used as a surrogate measure of cell number in microbiology. However, O.D. largely depends on the size and density of the cell suspension under study. Throughout this work processes of aggregation and disaggregation of cells were observed, as well as changes in cell size for both phycobionts. Both mitotic and meiotic divisions as well as the production of different kinds of spores have been observed for the taxa chosen (Gasulla et al., 2010; Barreno, pers. comm.). Fluctuations in optical dispersion would indicate then, changes or variations in the population structure of the species, including reproductive strategy. Population level parameters are rarely studied in toxicology but are of utmost importance to predict the ecotoxicological effect of a pollutant. These processes are complex and an important amount of research and standardization remains to be done before their use in ecotoxicology. In any case, these variations of O.D. could potentially be developed as very sensitive indicators of water pollution.

Numerous algal bioassays use the autofluorescence of chlorophyll as a surrogate of cell number and biomarker of function. Overall, the phycobionts' chlorophyll autofluorescence remains quite constant irrespective of the species or time of exposure and hardly correlates with alterations in O.D. The variations observed are almost exclusively small increases (under 120%) but they become statistically significant thanks to the especially low dispersion of individual measurements. Only diclofenac causes a generalized increase in *A. erici* chlorophyll autofluorescence yielding a LOEC (24 h) of 0.01 μg L^{-1}. This effect could be due either to increases in cell number or to increases in the cellular ratio of fluorescent chlorophyll, which may well be related to the phenomenon known as hormesis, whereby moderate levels of toxins induce a compensatory response, thus promoting the activation of defense/repair mechanisms (Calabrese, 2008). The reason why an anti-inflammatory animal drug interferes with algal chlorophyll regulation remains to be elucidated but could be related to lipid metabolism.

Both species *A. erici* and *Trebouxia* sp. TR9 are sensitive to very low doses of the pharmaceuticals carbamazepine and diclofenac. Despite the similar results obtained for the LOECs of both species, the magnitude of variation seems larger for *A. erici* than for TR9 (especially in the case of diclofenac). On the other hand, *A. erici* shows a lower degree of aggregation in liquid culture, is better characterized than TR9 and available from commercial banks, for example the Culture Collection of Algae (SAG) at the University of Göttingen (Germany) or the Culture Collection of Algae at the University of Texas (Austin, TX, USA). These characteristics render *A. erici* a better candidate to initiate the development of a microbioassay using lichen phycobionts. Nonetheless, other phycobionts should be studied, especially those isolated from lichens characterized as excellent environmental quality bioindicators.

In order to achieve the greatest ecological relevance in ecotoxicological testing, representative or key organisms of each ecosystem potentially affected by a certain impact should be tested. The use of phycobionts in toxicity testing provides ecological relevance from a variety of points of view:

a) Terrestrial ecosystems are poorly represented in toxicity testing. Lichens have actually been able to colonize every terrestrial habitat, from the coldest tundra to the warmest rainforest, from the driest Deserts to the most humid marshlands. Therefore, phycobionts adapted to all these habitats can be potentially used.
b) Lichens are important members of biological soil crusts (BSC) which provide the first step for soil formation and protect soils from erosion, especially in arid and semi-arid environments (reviewed in Belnap, 2003, and Bowker et al., 2010).
c) Phycobionts are symbiotic organisms. As remarked above, the phyco-mycobiont associations show common biological traits in relation to other terrestrial symbiotic organisms. Symbioses, including legume—rhizobium or mycorrhizae, are key to plant–soil nutrient balance and plant biotic and abiotic stress tolerance (including toxics).

The absence of a dermal tissue, allows that water and the substances dissolved reach both symbiont partners. Despite being recognized as effective “early warning field bioindicators”, lichens present several drawbacks for a systematic use as testing species (i) as symbiotic multicellular organisms, thalli are intrinsically

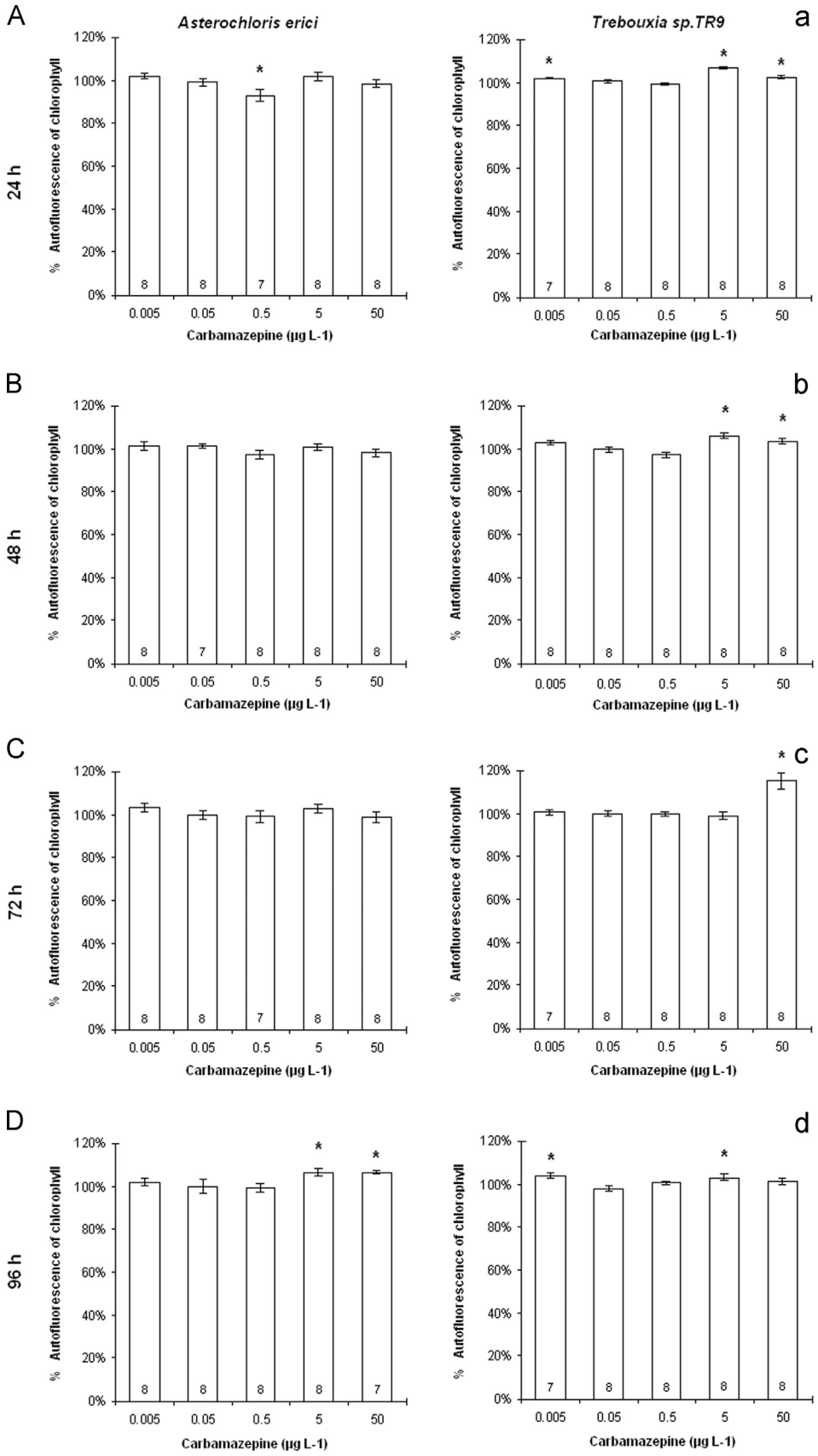

Fig. 4. Chlorophyll autofluorescence referred to controls at 24, 48, 72 and 96 h for five concentrations of carbamazepine in *Asterochloris erici* (A, B, C, D) and *Trebouxia* sp. *TR9* (a, b, c, d). The bars show the standard error of the mean. The base of each column shows the value of n at each point. *Indicates statistically significant difference versus control (ANOVA, $p < 0.05$).

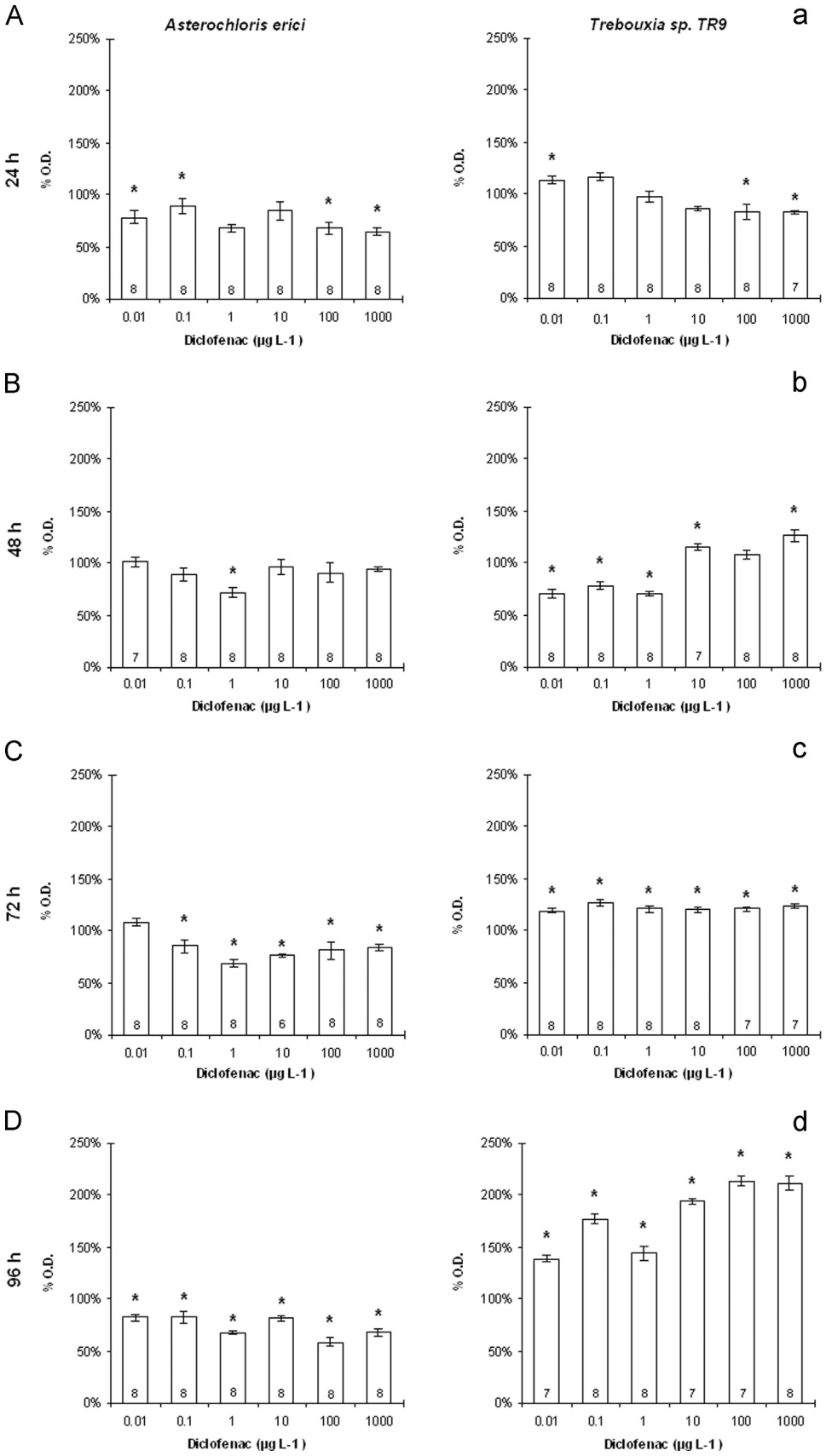

Fig. 5. Optical dispersion referred to controls at 24, 48, 72 and 96 hours for the 6 concentrations of diclofenac in *Asterochloris erici* (A, B, C, D) and *Trebouxia* sp. *TR9* (a, b, c, d). The bars show the standard error of the mean. The base of each column shows the value of *n* at each point .*Indicates statistically significant difference versus control (ANOVA, $p < 0.05$).

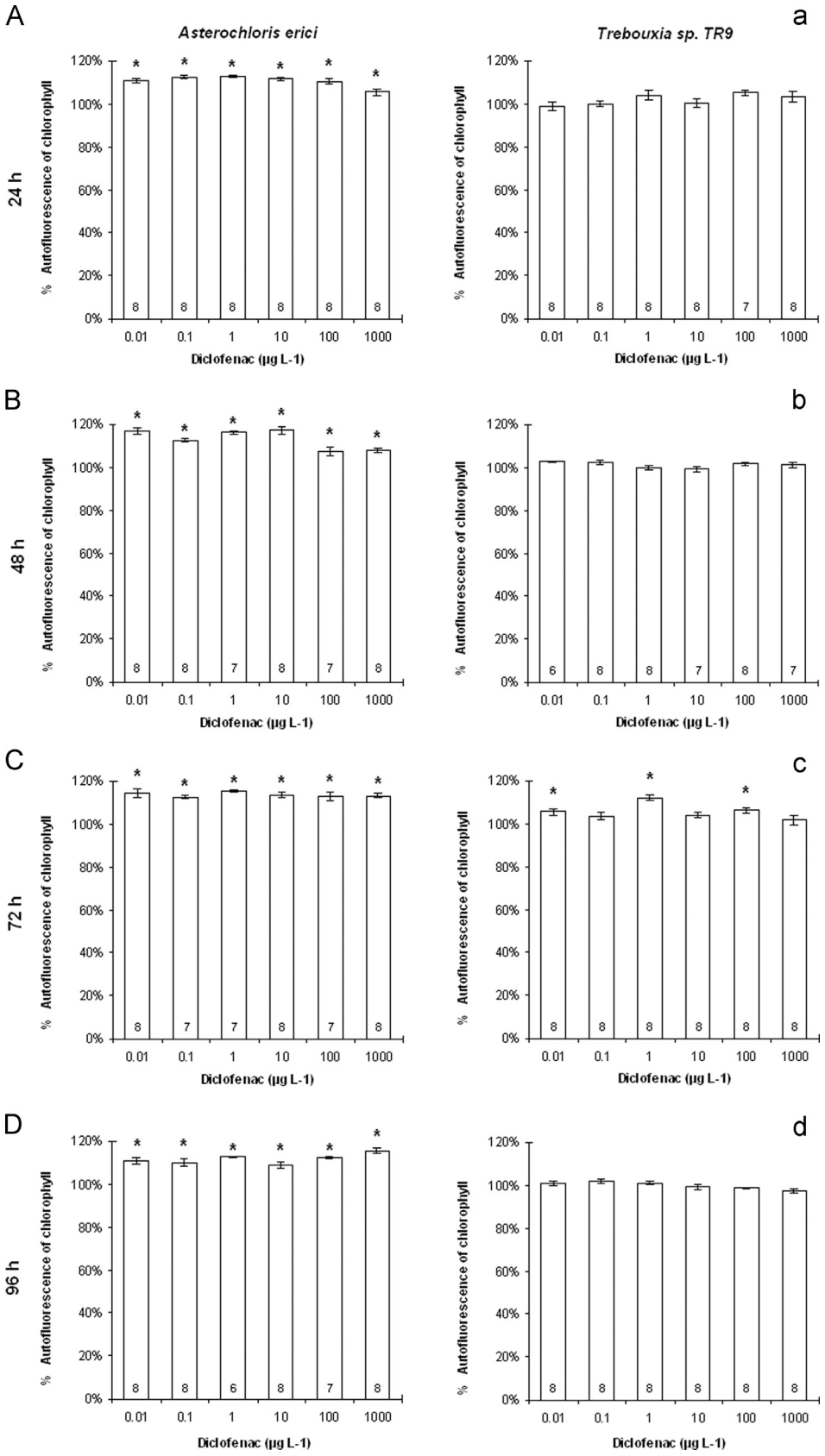

Fig. 6. Chlorophyll autofluorescence referred to control at 24, 48, 72 and 96 hours for the 6 concentrations of diclofenac in *Asterochloris erici* (A, B, C, D) and *Trebouxia* sp. *TR9* (a, b, c, d). The bars show the standard error of the mean. The base of each column shows the value of n at each point. *Indicates statistically significant difference versus control (ANOVA, $p < 0.05$).

heterogeneous, (ii) growth rates are very low and systematic growth has not been achieved yet. The use of phycobionts would be a step forward since the aforementioned difficulties are reduced.

Currently, available bioassays present problems such as the low number of taxa available, and the high costs of the routine use of continuous culture techniques used in conventional bioassays (Wadhia and Thompson, 2007; Catalá et al., 2009). The development of new microbioassays using lichen phycobionts has great environmental importance in order to possess a wide array of testing organisms endowed with biological and ecological relevance. Secondly, they also possess economic importance since these species may be available in sufficient quantities throughout the year, are easy to grow in a common laboratory at moderate temperatures (15–20 °C), can be stored alive at 4 °C or −20 °C long periods and are compatible with high throughput techniques (such as the 96-multiwell system used in the present work), which allows testing the toxicity of a high number of samples, implying lower costs and higher efficiency for monitoring and pollution control (Wadhia and Thompson, 2007).

The main limitation encountered in this study is the little knowledge about the phycobionts cell biology that hinders a comprehensive explanation of the mechanisms underlying the effects observed. The bioassays conducted to date use aquatic free-living algae that are characterized by single-celled organisms (Shitanda et al., 2009). The existence of aggregation and disaggregation processes as well as variations in cell size makes clear the important differences between free-living and lichenic algae. Thus, decreases in O.D. cannot be directly related with a decrease in cell number due to lethal toxicity. The triggering of different mechanism of proliferation by phycobionts including sexual reproduction in response to a pollutant might generate genetically distinct subpopulations within a culture especially in relative longer exposures. The toxic stress could provoke the selection of the most tolerant genetic lines within the population what could explain the fluctuations of LOEC at different exposure times. Even if lethal toxicity cannot be confirmed, these changes in the composition of phycobiont population could have negative effects in the ecosystem. On the other hand, most of the observed effects are not dose-dependent and O.D. results hardly correlate with chlorophyll autofluorescence.

5. Conclusions

We conclude that lichen phycobionts are very sensitive to both diclofenac and carbamazepine. The optical dispersion of phycobiont suspensions undergoes stronger and more frequent variations than chlorophyll autofluorescence by the effect of pharmaceuticals. Albeit none of the parameters used (optical dispersion and chlorophyll autofluorescence) can be directly associated with lethal toxicity, variations in optical density could indicate alterations in the population dynamics and structure with ecological implications. Although an important amount of research and standardization remains to be done before their use as testing species in ecotoxicology, they could help develop new microbioassays endowed with ecological relevance at lower costs. In this respect, *Asterochloris erici* seems the most suitable species. In any case, these variations of O.D. could potentially be developed as very sensitive indicators of water pollution.

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