Using an electrochemical assay to determine the biofilm elasticity change as a response to toxicant exposure

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Abstract: Elasticity is a trait of biofilm physiognomy which relates to cell clustering and can be measured by means of an electrochemical assay based on rotating disc electrode (RDE). This study aimed at testing the hypothesis according to which exposure of phototrophic biofilm to toxicant could reduce its elasticity. We compared biofilms developed for 21 days, in four sets of 6 replicated experimental units, in absence and presence of isoproturon at two concentrations of the inoculating suspension of biofilm, 103 and 104 diatom cell mL-1. Biofilm thickness and elasticity were measured based on RDE assay, bacterial and diatom density were measured by microscope-based numerations.Very thin biofilms (< 10 µm) were obtained as compared with a previous study. This might be linked with the way we selected the initial biofilm providing the suspension and the way we developed its growth. The biofilm elasticity mean values in the presence of isoproturon was quasi twice lower (60 ± 10 and 60 ± 41 µm rpm0.5) than the treatment without isoproturon (138 ± 93 and 115 ± 104 µm rpm0.5), for initial biofilm concentration of 103 and 104 respectively, but there was no significant difference between the mean values of each treatment. Nevertheless, the present preliminary study demonstrated the feasibility of an experiment dedicated to assessing biofilm elasticity changes as a response to toxicant exposure.

Keywords: electrochemical assay, biofilm elasticity, toxic

1. Introduction

Physiognomy is a term broadly used in plant ecology to describe the combination of the external appearance of vegetation, its vertical structure, and the growth forms of the dominant taxa e.g. coniferous forest or grassland [1]. Biofilm assembled microbial communities also have three dimensional architectures [2]. Biofilm thickness, porosity, viscoelasticity, size of cell clusters are some traits of biofilm physiognomy. Allowing the assessment of biofilm thickness and elasticity, the recent application of electrochemical method using Rotating Disc Electrode (RDE) provided the investigations in biofilm physiognomy with new insights [3].

Previous studies suggested the effect of toxicants on phototrophic biofilm physiognomy. One study found that the adpressed and stuck diatoms *Achnanthinidumminutissimum*, *Encyonemaminutum* and *Navicula minima* characterized the phototrophic biofilm in cadmium and zinc contaminated conditions[4]. Conversely, in uncontaminated conditions the phototrophic biofilm was characterized by the filamentous diatom *Melosiravarians*. Changes in the physiognomy of a *M. varians* dominated phototrophic biofilm were also observed after exposure to copper [5]. The authors reported no taxa replacement but shortening of the *M. varians* filaments to short tufts. Consistently, exposure to cerium oxide nanoparticles caused the flocculation in exopolymeric substances of a non-axenic strain of *Chlamydomonasrheinardti*[6]. Together the results of these studies suggest that cell clustering as a mean to attenuating individual cells exposure to a toxicant, could be an adaptive response of (some) microbial populations or communities to chemical stress.

In biofilms, cell clustering would be the reduction of the extracellular volume of the biofilm that could be measured by a loss of biofilm elasticity. We thus hypothesized that exposure to a toxicant would reduce phototrophic biofilm elasticity as a trait of biofilm physiognomy. To address this question we aimed to compare the biofilm thickness and elasticity of phototrophic biofilms exposed, or not, to a well-defined biocide. Thickness and elasticity were measured by means of the RDE electrochemical method on lentic phototrophic biofilms developed under controlled temperature, light and nutrient conditions in absence and presence of the phenylurea herbicide isoproturon (20 $\mu g\ L^{-1}$) at two concentrations of the inoculating biofilm suspension (10^3 and 10^4 diatom cell mL^{-1}).

2. Methodology

2.1. Experimental Design

Four sets of 6 replicated experimental units were prepared (Table 1). Isoproturon half live concentration is reached after 71 days of exposure[7]. Hence, during the present 21-day colonization experiment, isoproturon concentration was assumed to remain constant in the experimental units. Two different diatom concentrations of the inoculating suspension of biofilm, 10^3 and 10^4 cell mL^{-1}, were tested to ensure optimal biofilm colonization. The inoculating suspension of biofilm was prepared by scrapping and homogenizing two biofilm colonized glass slides (kindly provided by B. Chaumet and S. Morin, IRSTEA) in 150 mL of Dauta medium [8]modified by the addition of siliceous (10 mg L^{-1}), at pH ~ 7.7. In March 2016, clean glass slides had been immersed for 15 days in a pound (Gazinet-Cestas, France) then placed for 3 weeks, at 20°C, in a continuously illuminated aquaria filled with Dauta medium before use in this experiment.

Table **1. -** Experimental setup (RDE 1-24: identification number of the electrodes)

Isoproturon	Diatom cell concentration of the inoculating suspension of biofilm	
	10^3 cell mL^{-1}	10^4 cell mL^{-1}
0 $\mu g\ L^{-1}$	6 replicates RDE 1-6	6 replicates RDE 13-18
20 $\mu g\ L^{-1}$	6 replicates RDE 7-12	6 replicates RDE 19-24

The assay was conducted by developing phototrophic biofilm onto RDE as artificial substrate in a 100 mL propylene vials experimental unit. Identified RDE were immersed in 40 mL Dauta medium inoculated with 0.2 or 2 mL the biofilm suspension (10^5 cell mL^{-1}), with or without addition of 100 µL of a 50 $\mu g\ L^{-1}$isoproturon solution (stock solution: 50 mg L^{-1}). The 24 experimental units (4 treatments x 6 replicates) were incubated for 21 days, at 20°C, by moderate shacking (80 rpm), exposed to a 45 $\mu mol\ s^{-1}\ m^{-2}$ radiation (neon light) with a 12:12 photoperiod. The RDE was vertically suspended to the vial cap to prevent biofilm accrual by particle sedimentation onto the working surface of RDE. In such an assembly, however, the RDE working surface was not straight exposed to direct neon light radiation.

2.2. Biofilm thickness and elasticity measurements

The method is based on a basic experiment in electrochemistry, the RDE assay. A 3-electrode-system is immersed in an electrochemical cell filled with an electrolytic solution and connected to a potentiostat. The 3 electrodes are: (i) the working metallic electrode, RDE, on which biofilm develops;(ii) the counter electrode closing the electrical circuit and (iii) the reference electrode

monitoring the potential of the electrolyte. Intensity, I, of the steady-state diffusion current on the RDE working surface is controlled by the RDE rotation speed, □, and by the thickness of the biofilm colonising the RDE working surface (Figure 1).

For a given value of □□(rpm), the difference between the steady state current intensity measured on the naked RDE $I_{(t0)}$ and on the colonised RDE $I_{(t21)}$ permit to assess the biofilm thickness □ (µm) using Equation 1.

Equation 1: $\delta = n\ F\ D\ C^*\ S\ [i(t_{21j})^{-1} - i(t_0)^{-1}] \times 10{,}000$

Where n is the number of electrons, F the Faraday constant (96485 C mol^{-1}), D the diffusion coefficient in both water and biofilm set to 6.8 x 10-6 $cm^2\ s^{-1}$ at 20°C, C* the electroactive species concentration in the bulk solution (0.00001 mol cm^{-3}), and S the active RDE area (0.196 cm^2). The value measured at 300 rpm was retained as the estimate of biofilm thickness ($□_{300rpm}$).

The biofilm elasticity is determined by relating the biofilm thickness and rotation speed, considering the following law:

Equation 2**:** $\delta = 1 / (\delta_0^{-1} + K\Omega^{0,5})$

Where δ_0 (µm) is biofilm thickness at zero rpm, and K ($\mu m^{-1}\ rpm^{-0,5}$) the coefficient used to parametrise biofilm elasticity as 1/K ($\mu m\ rpm^{0,5}$).

The 24 identified RDE were 5-mm diameter platinum cylinder (electrical conductor) coated with a teflon® cylinder (electrical insulator)[3]. The reference electrode was a saturated calomel electrode (SCE) (REF 421, Radiometer Analytical, France). The counter electrode was a cylindrical grid of platinum immersed into the electrolyte solution that surrounded the working electrode. A 0.01M potassium ferrocyanide $[Fe(CN)_6]^{2-}$ and ferricyanide $[Fe(CN)_6]^{3-}$ solution was used as a electrolyte in 1M KCl. Ferrocyanide oxidation current intensity was measured at 0 V/SCE at which no water electrolysis and no oxygen reduction occur. Measurements were performed at 20°C.

In the laboratory, RDE was mounted on a motor axis plugged using mercury contacts and was rotated by a direct current motor system. The motor speed was controlled and measured using a tachymeter. Diffusion current was then measured at the potential 0V for each RDE rotation speed between 100 and 1200 by steps of 100 ppm. Rotation speed was limited to 1200 rpm to prevent biofilm erosion. Before t_0 measurements, every RDE were polished using sandpaper and cleaned with Milli-Q water. At the end of the incubation, RDE were sampled and their intensity were measured. Then, RDE working surface was immersed in 0.5 mL of Dauta media and 70 µL of neutralized formaldehyde and preserved for storage at 4°C until further analysis.

2.3. Numeration of diatom and bacteria colonizing the RDE working surface

The biofilm colonizing the working surface of the RDE was recovered by scrapping with a sterile plastic tool and sonication in an US bath at 37 kHz (30 s). After sonication the RDE was rinsed with 0.5 mL of Milli-Q water and removed. The resulting 1070 µL-biofilm suspension was vortexed (5 minutes) before storage at 4°C and prior to aliquoting for bacterial and diatom numerations within a few days.

For determination of bacterial density, 100 µL aliquot of the appropriate cell suspension dilution was stained with 200 µL 4'6-diamidino-2-phenylindole (DAPI, 0.01 mg mL^{-1}) and collected by filtration on 0.2 µm pore-size black polycarbonate filter. Counts were carried out on a OLYMPUS BH2 microscope fitted for epifluorescence at 1250x magnification and results were expressed as cell per cm^2 using equation 3.

Equation 3: Bacterial density (cell cm^{-2}) = NC x (SF/SC) x (VS/VF) x (1/SRDE)

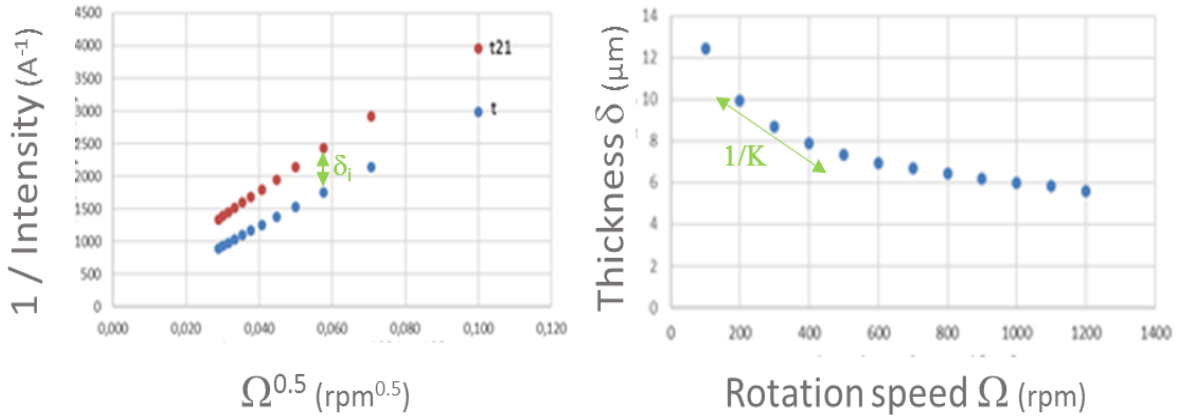

Figure 1. Inverse current intensity evolution with the electrode rotation speed measured on RDE #1 at t0 (blue symbols) and 21 days (red symbols). Thickness evolution with the electrode rotation speed measured on RDE #1 after 21 days of colonization.

Where NC (cell) is the mean number of bacteria on the calibrated counting grid, SF (mm^2) the filtration area on the filter, SC (mm^2) the area of the counting grid at the 1250x magnification, VS (mL) the total volume of biofilm suspension, VF (mL) the volume of filtered biofilm suspension and SRDE (cm^2) the RDE colonising surface.

Diatom density were determined directly or after 5-fold dilution. Counts were carried out on an OLYMPUS BH2 microscope at 200x magnification using a Nageotte counting chamber and results were expressed as cell per cm^2 using equation 4.

Equation 4**:** Diatom density (cell cm^{-2}) = (NC x VS) / (VN x SRDE)

Where NC (cell) is the mean number of diatom on the counting grid (5 to 10 fields counted, 1.25 µL each), VS (mL) the total volume of biofilm suspension, VN (mL) the Nageotte counting chamber volume, and SRDE (cm^2) the RDE colonising surface.

2. 4. Statistics

Biofilm elasticity was deduced by fitting experimental values to Equation 2 by means of non-linear least squares fits using Jkp Ads Excel add-in. Only significant fits (p-value ≤ 5%) were considered. At least, 3 out of 6 replicates were significant for each treatment. We thus assumed to consider only 3 replicates per treatment for further analyses.The non-parametric Mann-Whitney U-test (Merlin Excel Add-in) was used to check for differences in biofilm thickness and elasticity, diatom and bacterial cell numbers between the different treatments.

3. Results

Mean (± SD) biofilm thicknesses measured at 300 rpm were slightly lower in presence of isoproturon, 2 ± 1 and 3 ± 1 µm than in absence of isoproturon, 5 ± 3 and 4 ± 2 µm, for inoculating concentration of 10^3 and 10^4 diatom cell mL^{-1}, respectively (Table II and Figure 2.A). Differences between isoproturon treatments, however, were not significant (p-value > 0.05). The inoculating concentration of diatom has had no significant effect on biofilm thickness, either.

There were no significant differences between mean (± SD) values of diatom numbers in the biofilms developed in absence of isoproturon with an inoculum of 10^3 diatom cell mL^{-1} (1.1 ± 0.5 10^5 cell cm^{-2}) as compared to other treatments including biofilms developed in absence of isoproturon with an inoculum of 10^4 diatom cell mL^{-1} (2,2 ± 0.8 10^4 cell cm^{-2}) and those developed in presence of

isoproturon ($1.3 \pm 1.0\ 10^4$ and $1.0 \pm 0{,}3\ 10^4$ cell cm^{-2}, with inoculating concentration of biofilm suspension of 10^3 and 10^4 diatom cell mL^{-1}, respectively) (Table II and Figure 2.B).

Table 2. Raw data of replicate sets of measurements of biofilm thickness, elasticity, diatom and µbacteria densities of biofilms developed in May 2016 for 21 days in Dauta medium at 20°C in relation with different concentrations of inoculating biofilm suspension and/or isoproturon.

Inoculum concentration *cell ml^{-1}*	**Biocide treatment** *µg l^{-1}*	**RDE** *n°*	**Thickness** *µm*	**Elasticity** *1/K as µm rpm*	**Diatom numbers** *cell cm^{-2}*	**Bacteria numbers** *cell cm^2*
10^3 cell ml^{-1}	0	1	8,8	243	1,6 x 10^5	3,8 x 10^6
		2	4,8	93	1 x 10^5	4,2 x 10^6
		3	2,4	79	6,6 x 10^5	3,2 x 10^6
	20	1	2,7	55	2,4x 10^5	2,9 x 10^6
		2	1,7	55	9,6x 10^5	1,5 x 10^6
		3	3,3	72	4,8 x 10^5	1,8 x 10^6
10^4 cell ml^{-1}	0	1	4	68	3 x 10^5	3,7 x 10^6
		2	2,2	42	1,6 x 10^5	4,2 x 10^6
		3	6,4	235	1,9 x 10^5	6,3 x 10^6
	20	1	1,1	27	9,9 x 10^5	4,6 x 10^6
		2	3,8	105	1,2 x 10^5	5,6 x 10^6
		3	2,5	46	6,7 x 10^5	2,5 x 10^6

Among treatments, mean (± SD) bacterial densities in the biofilm were in the same order of magnitude ranging from 1.6 to 6.3 10^6 cell cm^{-2} (Table II). No significant differences in bacterial density means were found between biofilms developed with or without isoproturon and/or with inoculating concentration of biofilm suspension of 10^3 or 10^4 diatom cell mL^{-1} (Figure 2.C).

The mean (+/-SD) values of biofilm elasticity in the treatment without isoproturon were quasi twice higher (138 ± 93 and 115 ± 104 µm $rpm^{0.5}$, for inoculating concentration of biofilm suspension of 10^3 or 10^4 diatom cell mL^{-1} respectively) than values found for biofilms developed in presence of isoproturon (60 ± 10 and 60 ± 41 µm $rpm^{0.5}$, for inoculating concentration of biofilm suspension of 10^3 or 10^4 diatom cell mL^{-1} respectively) (Figure 2. D). Differences, however, were not significant (p-value > 5%).

4. Discussion

In the present study, diatom and bacteria densities onto the RDE working surface confirmed the occurrence of a biofilm colonising the RDE working surface after 21 days of incubation in controlled conditions. Measured thicknesses therefore may be related to biofilm occurrence on the RDE working surface. However, we found very thin biofilm (< 10 µm) as compared to values previously reported in the River Garonne for the same colonization duration (71 – 343 µm) [3]. In agreement, lower bacterial densities were found in the present study as compared to the River Garonne experiment. Diatom density, on the other hand, were not that low in the present experiment. This could be explained by the use of Dautamedium which is a culture medium dedicated to diatom culture. We thus hypothesize that conditions in the river such as current velocity or solar radiation were more likely to promote the development of complex and thick biofilm, except for the diatom community.

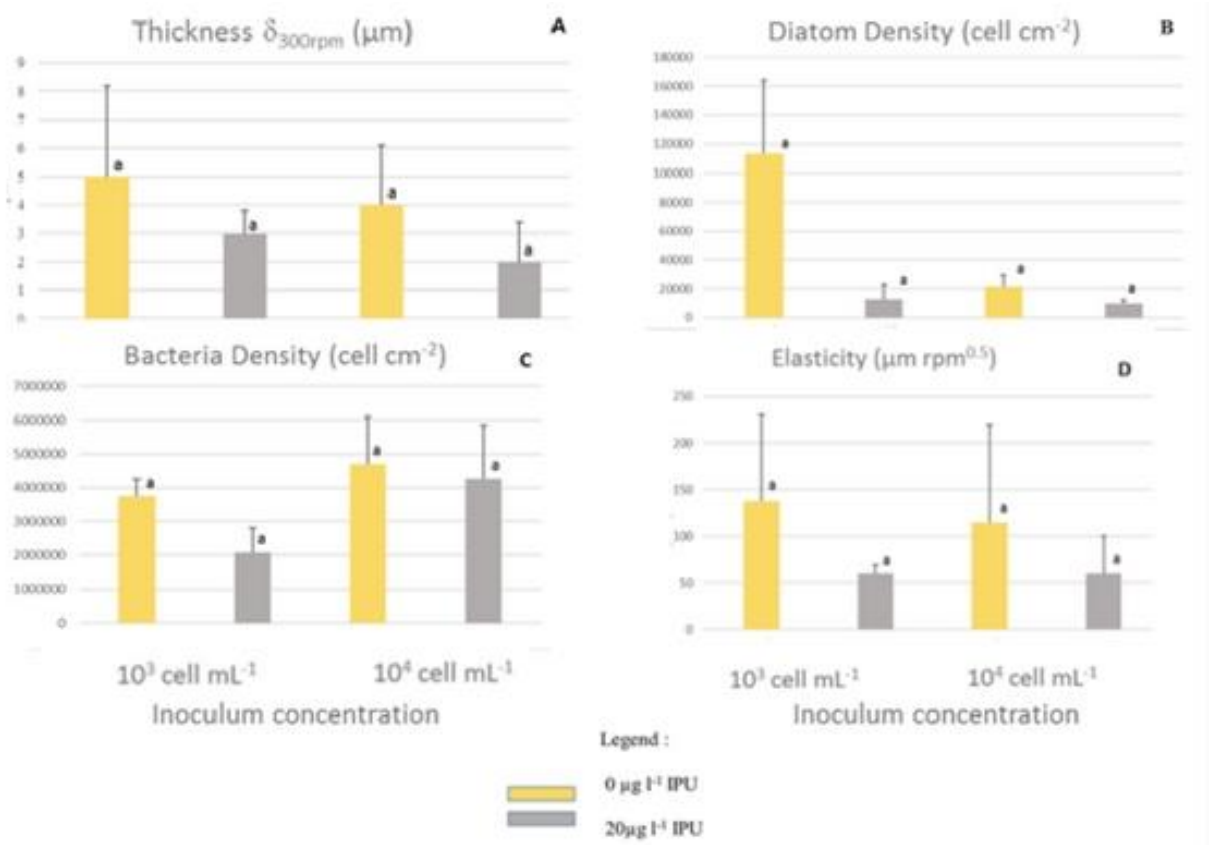

Figure 2. Biofilm thickness (a), elasticity (d), diatom (b) and bacteria (c) densities of biofilms developed for 21 days in Dauta medium at 20°C, in May 2016, with different concentrations of inoculating biofilm suspension and/or isoproturon. Bars represent the mean of 3 replicates and error bars represent the standard deviation. Distinct letters on bars indicate significantly different values at the 5% level.

Whether there were not significantly different values between the treatments, we obtained the lowest thickness values (ca. 2µm) when biofilms were developed in presence of isoproturon. An initial concentration of 5 µg L^{-1} of isoproturon was shown to reduce the rate of diatom colonization after 34 days of exposure [7]. The authors reported that small diatoms with high growth rate, *Naviculaparvulum* and *Sellaphoraseminulum,* dominated the isoproturon contaminated biofilms and could be indicators of isoproturon contamination. Diatom species replacement could explain biofilm development in spite of isoproturon addition. However, in the present experiment we did not perform diatom species identification to confirm it.

Biofilm elasticity ranged between 60 and 138 µm $rpm^{0.5}$. These values were not very important compared with the elasticity of River Garonne 21-day old biofilms (790 - 1300 µm $rpm^{0.5}$) [3]. This was nevertheless in agreement with the thickness data as elasticity is a function of the biofilm thickness. Again, there was a trend that isoproturon has a tendency to reduce by ca. two-fold the elasticity of biofilm. However, this result should not be considered as conclusive because (i) differences were not significant and (ii) the elasticity is a function of the biofilm thickness. In order to compare the elasticity, biofilm of similar thicknesses should be compared. To fulfill this condition, a different experiment designed with longer colonization duration in the higher contaminated treatment is needed to better characterize this contaminant effects on this physiognomic variables which are biofilms thickness and elasticity.

5. Conclusion

Biofilm colonisation was successfully measured in this study as diatoms and bacteria well developed on the rotating disc electrode, nevertheless there was no effect of initial inoculum concentration and/or isoproturon treatment on bacterial and diatom densities.As compared to previous experiment, developed biofilms were not very thick and this might be linked with the way we selected the initial biofilm providing the inoculating suspension and the way we developed its growth.Biofilm thickness and elasticity were lower in presence of isoproturon whereas differences were not significant.The present preliminary study demonstrated the feasibility of an experiment dedicated to assess biofilm elasticity changes as a response to toxicant exposure.

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7. References

[1] Whittaker, R. H. (1973). Editor. Ordination and Classification of Communities.Handbook of Vegetation Science 5. Junk, (The Hague, The Netherlands)
[2] Stoodley, P., Hall-Stoodley, L., Boyle, J. D., Jorgensen, F., Lappin-Scott, M. (20000. Environmental and genetic factors influencing biofilm structure. Community Structure and Co-operation in Biofilm. Cambridge University Press.
[3] Boulêtreau, S., Charchosset, J. Y., Gamby, J., Lyautey, E., Mastrorillo, S., Azémar, F., Moulin, F., Tribollet, B., Garabetian, F. (2011). Rotating disk electrodes to assess river biofilm thickness and elasticity. Water Research45, 1347 – 1357
[4] Feurtet-Mazel, A., Gold, C., Coste, M., Boudou, A. (2003). Study of periphytic diatoms communities exposed to metallic contamination through complementary field and laboratory experiments. Journal de Physique IV107, 467-470
[5] Barranguet, C., Plans, M., van der Grinten, E., Sinke, J.J., Admiraal, W. (2002). Development of photosynthetic biofilms affected by dissolved and sorbed copper in a eutrophic river. Environmental Toxicology and Chemistry 21 (9), 1955 – 1965
[6] Rohder, L. A., Brandt, T., Sig, L., Behra, R. (2014). Influence of agglomeration of cerium oxide nanoparticles and speciation of cerium (III) on short termeffects to green algae Chlamydomonasreinhardtii. Aquatic toxicology152, 121 – 130
[7] Pérès, F., Florin, D., Grollier, T., Feurtet-Mazel, A., Coste, M., Ribeyre, F., Ricard, M., Boudou, A. (1996). Effects of the phenylurea herbicide isoproturon on peryphytic diatom communities in freshwater indoor microcosms.Environmental Pollution94 (2), 141-152
[8] Dauta, A. (1982). Conditions de développement du phytoplancton.Annales de Limnologie18, 217- 268