Journal of Hazardous Materials 194 (2011) 435–439

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Short communication

Lignin and tannin toxicity to *Phaeodactylum tricornutum* (Bohlin)

Giovanni Libralato*, Francesco Avezzù, Annamaria Volpi Ghirardini

Department of Environmental Sciences, Informatics and Statistics, University Cà Foscari Venice, Campo della Celestia 2737/b, I-30122 Venice, Italy

ARTICLE INFO

Article history:
Received 24 May 2011
Received in revised form 3 July 2011
Accepted 27 July 2011
Available online 5 August 2011

Keywords:
Lignin
Tannin
Toxicity
Marine algae
Morphotype change

ABSTRACT

Lignin and tannin are widespread natural compounds traditionally used in tannery industries. Their presence is commonly detected in textile wastewater showing potential toxicity effects within various endpoints onto sea water organisms that generally represent the ultimate target of discharged effluents. Most data are available only as nominal concentrations or percentage volume of wastewater having an unknown lignin and tannin content. The aim of this study was to provide the ecotoxicological characterisation of both compounds considering as testing species the marine alga *Phaeodactylum tricornutum* (Bohlin). Lignin and tannin showed an E_rC_{50} of 113.84 (100.90–128.45) mg/L and 26.04 (20.10–33.95) mg/L, respectively. NOEC and LOEC values were together <0.1 mg/L and 0.1 mg/L, in that order. Moreover, it was observed a morphological change of the algae fusiform shape occurring only at tannin concentrations ≥75 mg/L and <185 mg/L.

1. Introduction

Recently, the use of natural compounds within wood preservation, the sustainable use of natural resources and agriculture is stressed in order to increase the economic trade off and reduce the environmental impact [1,2]. Traditionally, tannins are associated to leather tanning industries mainly as tannic acid [3], even though only developing countries are still making a widespread use of it [4]. The same occurs for lignin in the form of a sulphonate derivative [3]. Tannins in textile industries may be applied at various stages of the production process to increase the fixation rates and to enhance wet fastness onto the dyed fabric, whereas lignosulphonates are used as dispersing agents within various dyestuffs (i.e. reactive, disperse and metal complex) [3].

Nevertheless wastewater containing natural tannins with no pre-treatment stage showed to be more biodegradable than that having the synthetic one, various authors reported the presence of potential adverse effects towards the marine bacterium *Vibrio fischeri* [5], the marine diatoms *Phaeodactylum tricornutum* [6,7], *Dunaliella tertiolecta* [4,8] and *Nitzschia* sp. [9], and the sea urchins *Paracentrotus lividus* [4,8] and *Sphaerechinus granularis* [4]. Moreover, toxicity effects of tannin have also been reported regarding some freshwater organisms [9]. About lignin, in the form of lignin sulphonate, high acute toxicity was identified only onto *P. tricornutum* [3]. Actually, fresh and sea water communities could be potentially affected by discharges containing tannin and lignin, such as pulp and paper mill and tannery effluents, that also contain other natural organic compounds [10] that have already been partly classified as carcinogenic, mutagenic, clastogenic [11] and endocrinic [12]. A general overview of the available ecotoxicological information about lignin and tannin have been summarised in Table 1.

Although some toxicity data about lignin and tannin already exist, most of them are not based on real concentrations, but on nominal ones or wastewater percentage volume (v/v). The aim of this paper is to provide lignin and tannin concentration–response curves considering the growth inhibition of *P. tricornutum* highlighting the presence of unusual morphotypes within a certain range of tannin concentrations. Actually, this is a pennate diatom that can be found in coastal as well as in inland waters [13]. It may appear in three different morphotypes, i.e. the ovoid, fusiform and triradiate forms, even though their occurrence is not well understood being in dependence of strains, environmental conditions, light intensity and nutrient availability [14]. Usually, toxicity testing with *P. tricornutum* is carried on with the fusiform morphotype that is the most frequently form observed in liquid media [14] and the one standardised within the International Organization for Standardization [15]. This paper may potentially provide interesting tips whenever unusual *P. tricornutum* forms are observed.

2. Experimental

Tannin (tannic acid or gallotannin, Sigma–Aldrich) (CAS # 1401-55-4) and alkali-lignin (Sigma–Aldrich) (CAS # 8068-05-1) were

* Corresponding author. Tel.: +39 0412347737; fax: +39 0415281494.
E-mail address: giovanni.libralato@unive.it (G. Libralato).

0304-3894/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2011.07.103

Table 1
Overview of aquatic species toxicity data about lignin and tannin.

Testing organisms/community	FW/SW	End-point	Exposure type	Lignin (mg/L[a])	Tannin (mg/L[a])	Reference
Activated sludge	FW	Rate of respiration	Static		ED50 < 42% (v/v) (natural)	[6]
	FW	Rate of respiration	Static		ED50 < 45% (v/v) (synthetic)	[6]
Bacteria						
Vibrio fischeri	SW	Luminescence inhibition	Static		Luminescence inhibition at 40% (v/v) and 47% (v/v) of two raw tannery substreams	[5]
Algae						
Dunaliella tertiolecta	SW	IC50, growth rate	Static		Minimum growth was both observed at 0.1 mg/L and at 3 mg/L syntan water extracts	[9]
					Total growth inhibition at dilutions of raw wastewater < 3.1% and at 0.78%	[6]
Nitzschia sp.	SW	IC50, biomass	Renewal		0.1	[6]
Phaeodactylum tricornutum	SW	IC50, growth rate	Static	ED50 < 6–8% (v/v) (lignin sulphonate)	ED50 < 1% (v/v) (natural)	[4]
					ED50 < 1% (v/v) (synthetic)	[8]
Molluscs						
Dreissena polymorpha	FW	EC50, behaviour	Static		23.1–34.9	[9]
		LC50	Static		104–133	[9]
Crustaceans						
Daphnia magna	FW	LC50	Static		26	[9]
Echinoderms						
Paracentrotus lividus	SW	Embryotoxicity	Static		Adverse effects still present at 1% v/v for both endpoints: sperm cell and embryotoxicity hormetic effect at 0.1 and 0.3 mg/L of vegetable tannin and syntan water extracts was highly significant in *P. lividus* cultures with "low-quality" controls; 100% effect on embryos at 10 mg/L of vegetable tannin and 80% effect on embryos at 10 mg/L of syntan water extracts.	[8]
Sphaerechinus granularis	SW	Embryotoxicity	Static		56% effect on embryos at 10 mg/L of vegetable tannin and 80% effect on embryos at 10 mg/L of syntan water extracts	[4]
Fish						
Carassius auratus	FW	LC50	Static		10–100	[9]
Cyprinus carpio	FW	LC50	Renewal		10–33	[9]
Gambusia affinis	FW	LC50	Static		18–41[b]	[9]
Ictalurus punctatus	FW	LC50	Static		0.34–0.39	[9]
Labeo rohita	FW	LC50	Static		64.86–84.23	[9]
Lepomis macrochirus	FW	LC50	Static		0.14–0.16	[9]
Oncorhynchus kisutch	FW	LC50	Static		10	[9]
Oncorhynchus mykiss	FW	LC50	Static		0.34–10	[9]
Oncorhynchus tshawytscha	FW	LC50	Flow through		0.96–4.82	[9]
Pimephales promelas	FW	LC50	Static		500–1000	[9]
Ptychocheilus oregonensis	FW	LC50	Static		5–10	[9]
Salvelinus fontinalis	FW	LC50	Static		6.25	[9]
Tanichthys albonubes	FW	LC50			40–100	[9]
Amphibian						
Rana catesbeiana	FW	LC50	Static		500–1000	[9]

FS = fresh water. SW = salt water.
[a] Unless otherwise specified.
[b] Based on exposure time.

prepared in marine artificial seawater [15] considering various dilution scenarios. Chemicals were geometrically scaled with a factor of 2. Nevertheless, it was decided to introduce some other intermediate concentrations to strengthen the description of the concentration–response curves and increase the level of confidence of statistical analysis.

2.1. Tannin and lignin analysis

The concentration of tannin and lignin as pure substances in artificial seawater media was achieved considering the colorimetric tyrosine method (APHA standard 5550 method) [16] due to the absence of other hydroxylated aromatic compounds such as

Table 2
Lignin and tannin experimental design in addition to pH and redox potential values at zero time (t = 0 h) and after 72 h exposure (t = 72 h).

mg/L[a]	Lignin				mg/L	Tannin			
	t = 0 h		t = 72 h			t = 0 h		t = 72 h	
	pH	mV	pH	mV		pH	mV	pH	mV
0.1	7.89	−53.8	7.88	−54.0	**0.2**	7.88	−53.7	7.87	−53.1
0.3	7.88	−53.0	7.89	−53.3	**0.4**	7.92	−55.2	7.82	−55.3
0.8	7.88	−53.2	7.89	−53.3	**0.7**	7.86	−52.0	7.85	−52.1
1.7	7.90	−54.0	7.91	−53.8	**1.9**	7.85	−51.6	7.86	−51.4
3.6	7.91	−54.6	7.89	−54.3	**3.8**	7.82	−49.8	7.81	−49.7
7.3	7.90	−54.2	7.85	−54.1	**8.1**	7.77	−46.8	7.75	−46.5
40	7.90	−54.0	7.91	−54.0	**20**	7.57	−34.9	7.55	−34.3
50	7.90	−54.2	7.92	−54.5	**35**	7.48	−29.9	7.47	−29.7
70	7.93	−55.6	7.91	−54.9	**55**	7.33	−19.9	7.31	−19.6
80	7.89	−53.6	7.90	−54.0	75	7.14	−10.6	7.11	−10.4
110	7.88	−52.6	7.89	−52.9	90	7.01	−3.1	7.00	−2.8
140	7.91	−54.7	7.93	−54.6	120	7.06	−5.9	7.02	−4.0
200	7.91	−54.6	7.92	−54.3	185	6.89	3.6	6.88	3.4
300	7.95	−56.6	7.96	−56.2	295	6.83	7.2	6.85	7.3
400	7.97	−57.9	7.95	−57.7	400	6.72	14.4	6.69	14.6
500	8.00	−59.7	7.99	−59.4	440	6.68	16.0	6.69	16.2

[a] Concentrations used in E_rC_{xx} calculation are in bold.

phenol and cresol that could overestimate their concentrations [17]. Both lignin and tannin contain aromatic hydroxyl groups that react with Folin phenol reagent made of tungstophosphoric and molybdophosphoric acids, forming a blue colour (instrumental measurement at λ = 700 nm) that is proportional to the amount of these compounds present in the sample. The precision for both chemicals was set at 0.1 mg/L considering a 1-cm-path-length spectrophotometer (HACH DR 2800 Portable Spectrophotometer, Dr. Lange Company, Düsseldorf, Germany). A 2.0 mg/L and a 200 mg/L tannic acid standard solutions were considered to check the apparatus calibration. Lignin and tannin data have been normalised on the results of negative controls made of dilution artificial seawater.

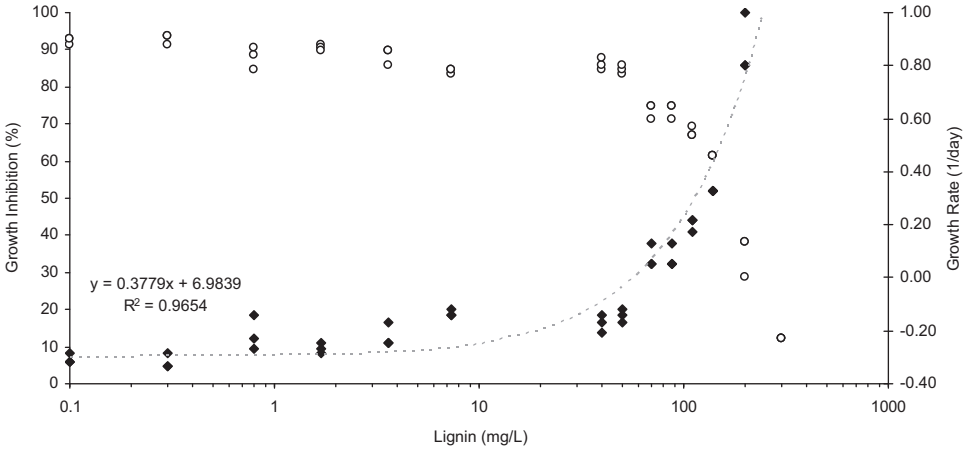

Fig. 1. Exposure to lignin: algae growth rate (1/day) (○) and growth inhibition rate (%) (♦).

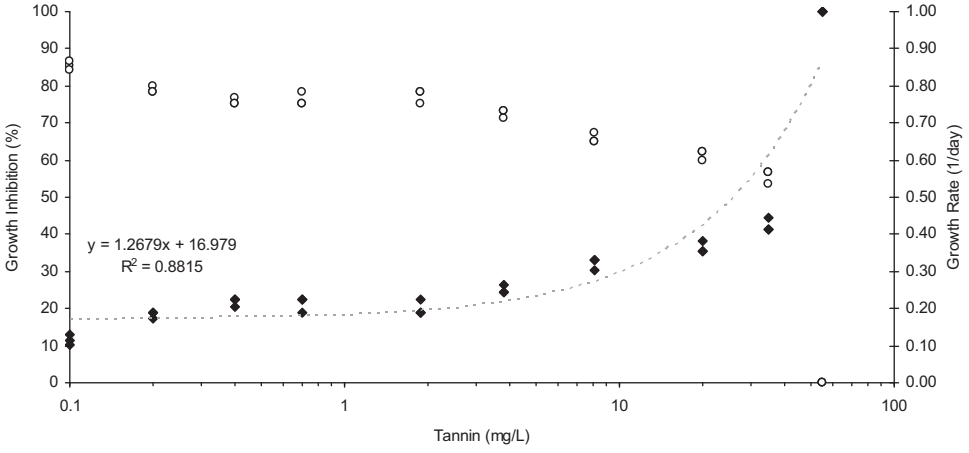

Fig. 2. Exposure to tannin: algae growth rate (1/day) (○) and growth inhibition rate (%) (♦).

Table 3
Ecotoxicological parameters.

E_rC_{xx}	Lignin (mg/L)	Tannin (mg/L)
E_rC_{10}	7.98 (1.08–15.77)	[a]
E_rC_{20}	34.45 (26.03–43.94)	2.38 (0.00–6.38)
E_rC_{50}	113.84 (100.90–128.45)	26.04 (20.10–33.95)

[a] Negative values.

A hand refractometer (Atago, Japan) was used to check salinity. pH was measured with an HI 9025 microprocessor-based pH meter (Hanna Instruments, Beverly, MA, USA) and dissolved oxygen with a WTW multi-parametric device (Nova Analytics, Weilheim, Germany) in order to verify that the values were in accordance with each relative toxicity test protocol.

2.2. Algal culturing and growth inhibition tests

The starting *P. tricornutum* inoculum was obtained from Micro-BioTests Inc. (Gent, Belgium) (PT190608) and subcultured in the laboratory. The culture media were in accordance with the UNI EN ISO standard protocol [15]. Weekly, cultures were visually inspected for contamination using a light microscope. Four days prior to the start of growth inhibition experiments, at least one new algal culture was prepared and allowed to grow at $20 \pm 2\,^{\circ}C$ in continuous light (6000–10,000 lx), obtaining a cellular density of more than 10^6 cells/mL.

The 72 h algal growth inhibition experiments were conducted in accordance with [15]. The initial algal density in the test was obtained by dilution of algal culture and ranged between 2×10^3 cells/mL and 10^4 cells/mL. *P. tricornutum* was exposed in triplicate to increasing concentrations of compounds for 72 ± 2 h at $20 \pm 2\,^{\circ}C$ and 6000–10,000 lx, with a light/dark photoperiod of 16/8 h. Negative (dilution artificial sea water) and positive ($K_2Cr_2O_7$ as reference toxicant) controls were included in each experiment. Cellular density was evaluated using a Bürker counting chamber. The point estimation and the calculation of the growth inhibition concentrations (E_rC_{xx}) and their relative 95% confidence limit values were carried on by a linear regression model after natural logarithm data transformation of the measured cell density (corrected for blank). The Lowest Observed Effect Concentration (LOEC) and No Observed Effect Concentration (NOEC) values were also calculated with the Dunnett's method.

3. Results and discussion

The experimental design reporting the concentrations of test solutions as well as their pH and redox potential was summarised in Table 2. Tested concentrations ranged between 0.1–500 mg/L for lignin and 0.2–440 mg/L for tannin. The value of pH and redox potential remained substantially constant after 72 h exposure for both lignin and tannin per each relative concentration. Anyhow, it has been evidenced a decrease of pH (from 7.88 at 0.2 mg/L to 6.68 at 440 mg/L) and an increase of redox potential (from −53.7 mV at 0.2 mg/L to 16.0 mV at 440 mg/L) at increasing tannin concentration due to its acid nature. Nevertheless, these variables did not negatively affect the determination of tannin ecotoxicological parameters that has been calculated on the basis of a reduced and unaffected set of data as shown in Table 2.

Negative (growth rate after 72 h > 0.9 1/day; coefficient of variation of the control specific growth rate not exceeding 7%; pH values did not increase more than 1.0 unit after 72 h exposure) and positive ($K_2Cr_2O_7$ as reference toxicant) (E_rC_{50} = 22.21 (19.32–24.51) mg/L) controls were in accordance with [15] standard protocol.

The concentration–response curves of lignin and tannin were presented in Figs. 1 and 2, respectively. Each figure summarised the trend of algae growth inhibition (%), counting the fusiform shaped algae, as well as its corresponding growth rate (1/day). Moreover, the equations resulting from linear regression of data were shown together with R^2 coefficients.

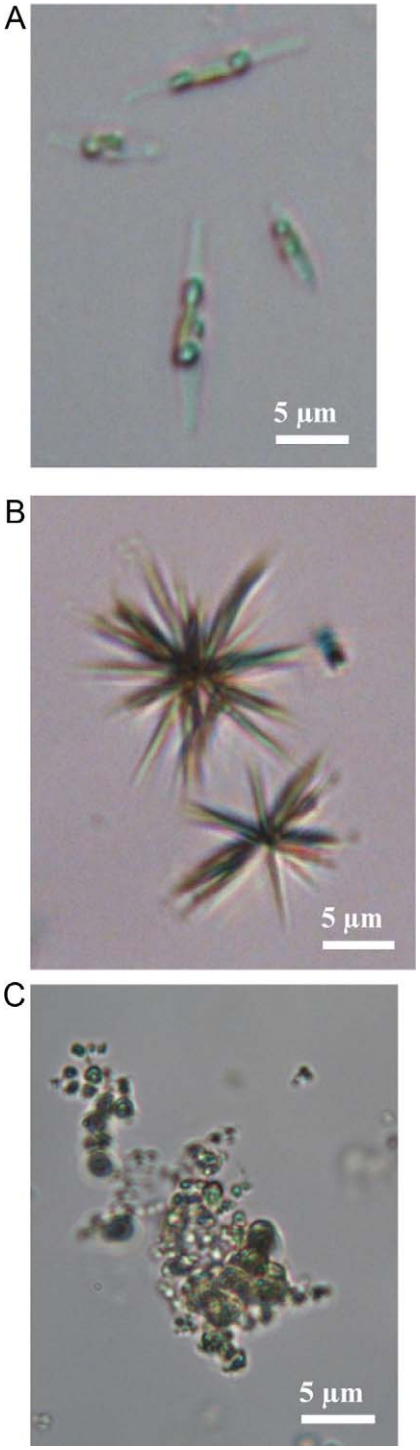

Fig. 3. (A) algae appear mainly as fusiform until 55 mg/L of tannin; (B) algae shape starting from 75 mg/L of tannin with a small number of fusiform organisms remaining to 185 mg/L (not included); (C) possible residues of the algal biomass presence at concentrations of tannin ≥185 mg/L and ≤295 mg/L.

The concentrations of lignin and tannin inhibiting the growth of algae populations by 10%, 20% and 50% were displayed in Table 3 on the basis of the aforementioned equations. NOEC and LOEC values for both compounds were determined as <0.1 mg/L and 0.1 mg/L, respectively, as a consequence of the limit of detection of the method selected to carry on the chemical analysis.

Although a traditional concentration–response curve can be defined from tannin toxicity data distribution, the experiments revealed that some morphological changes occurred in *P. tricornutum* on the basis of the exposure concentration range as shown in Fig. 3A–C. At exposure concentrations ≤55 mg/L algae assumed the expected fusiform shape (Fig. 3A), but for tannin concentrations ≥75 mg/L and <185 mg/L substantially prevailed the unidentified morphotype shown in Fig. 3B. For tannin ≥185 mg/L and ≤295 mg/L, it was not possible to detect any specific biological structure, but likely residues of the algal biomass as presented in Fig. 3C. At concentrations ≥400 mg/L and ≤440 mg/L, no biological residues were identified in the exposure solutions probably due to the combination of tannin levels and the consequent sub-acidic pH conditions (6.72 and 6.68, in that order).

4. Conclusions

It has been observed a lack of reliable data about the potential ecotoxicological effects to aquatic species of lignin and tannin. Thus the toxicity of these chemicals was investigated on the basis of the growth inhibition test with the alga *P. tricornutum*. The E_rC_{50} was set at 113.84 (100.90–128.45) mg/L for lignin and at 26.04 (20.10–33.95) mg/L for tannin. Moreover, it was observed that tannin is able to produce within a specific range of concentrations (75–185 mg/L) a change in the expected algae morphotype (i.e. fusiform). Higher tannin concentrations completely inhibited algal growth, probably, also due to the more slightly acidic conditions of the exposure solutions.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

[1] M.S. Jahan, D.A. Chowdhury, M.K. Isalm, Characterization and evaluation of golpata fronds as pulping raw materials, Biores. Technol. 97 (2006) 401–406.
[2] M.S. Jahan, D.A. Chowdhury, M.K. Isalm, S.M. Moeiz, Characterization of lignin isolated from some nonwood available in Bangladesh, Biores. Technol. 98 (2007) 465–469.
[3] D. Orhon, F. Germirli Babuna, O. Karahan, Industrial Wastewater Treatment by Activated Sludge, IWA Publishing, London, 2009.
[4] E. De Nicola, S. Meric, M. Gallo, M. Iaccarino, C. Della Rocca, G. Lofrano, T. Russo, G. Pagano, Vegetable and synthetic tannins induce hormesis/toxicity in sea urchin early development and in algal growth, Environ. Pollut. 146 (2007) 46–54.
[5] J.C. Jochimsen, M.R. Jeckel, Partial oxidation effects during the combined oxidative and biological treatment of separated streams of tannery wastewater, Water Sci. Technol. 35 (1997) 337–345.
[6] F. Germirli Babuna, Z. Yilmaz, O. Okay, I. Arslan Alaton, G. Iskender, Ozonation of synthetic versus natural tannin: inert COD and toxicity towards *Phaeodactylum tricornutum*, Water Sci. Technol. 55 (2007) 45–52.
[7] I. Arslan-Alaton, S. Koyunluoglu, Ozonation of two commercially important biocidal finishing agent, Ozone-Sci. Eng. 29 (2007) 335–342.
[8] S. Meriç, E. De Nicola, M. Iaccarino, M. Gallo, A. Di Gennaro, G. Morrone, M. Warnau, V. Belgiorno, G. Pagano, Toxicity of leather tanning wastewater effluents in sea urchin early development and in marine microalgae, Chemosphere 61 (2007) 208–217.
[9] S.E. Kegley, B.R. Hill, S. Orme, A.H. Choi, Pan Pesticide Database, Pesticide Action Network, North America (San Francisco, CA), 2010, http:www.pesticideinfor.org.
[10] A. Muna, T.R. Sreekrishnan, Aquatic toxicity from pulp and paper mill effluents: a review, Adv. Environ. Res. 5 (2001) 175–196.
[11] G. Ericson, A. Larsson, DNA adducts in Perch (*Perca fluviatilis*) living in coastal water polluted with bleached pulp mill effluents, Ecotoxicol. Environ. Safe. 46 (2000) 167–173.
[12] R. Orrego, J. Guchardi, R. Krause, D. Holdway, Estrogenic and anti-estrogenic effects of wood extractives present in pulp and paper mill effluents on rainbow trout, Aquat. Toxicol. 99 (2010) 160–167.
[13] S.R. Rushforth, J.R. Johansen, D.L. Sorensen, Occurrence of *Phaeodactylum tricornutum* in the great salt lake, Utah, USA, Great Basin Nat. 48 (1988) 324–326.
[14] G. Francius, B. Tesson, E. Dague, V. Martin-Jézéquel, Y.F. Dufrêne, Nanostructure and nanomechanics of live *Phaeodactylum tricornutum* morphotypes, Environ. Microbiol. 10 (2008) 1344–1356.
[15] UNI EN ISO 10253, Water quality – marine algal growth inhibition test with *Skeletonema costatum* and *Phaedactylum tricornutum*, 2006.
[16] SM, Standard Methods for the Examination of Water and Wastewater, APHA/AWWA/WEF, 21st ed., American Public Health Association, Washington, 2005.
[17] B. Karrasch, O. Parra, H. Cid, M. Mehrens, P. Pacheco, R. Urrutia, C. Valdovinos, C. Zaror, Effects of pulp and paper mill effluents on the microplankton and microbial self-purification capabilities of the Biobìo River, Chile, Sci. Total Environ. 359 (2006) 194–208.