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Comparison of a test battery for assessing the toxicity of a bleached-kraft pulp mill effluent before and after secondary treatment implementation

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Abstract Pulp and paper mill effluents may cause harmful effects to the aquatic environment due to the combined influence of physical factors, toxic compounds, and nutrient enrichment. In the present study, the effectiveness of secondary treatment in reducing the toxicity of an elemental chlorine-free bleached-kraft pulp mill effluent was evaluated. To characterize the toxicity of the effluent, before and after the implementation of secondary treatment, a battery of tests with organisms bearing different functions at the ecosystem level was used, namely *Vibrio fischeri* (5-min luminescence), *Pseudokirchneriella subcapitata* (72-h growth), *Lemna minor* (7-day growth), *Daphnia magna* (21-day reproduction and 24-h postexposure feeding), *Chironomus riparius* (9-day growth), and *Danio rerio* (28-day growth). For the effluent sample collected before the implementation of secondary treatment, *P. subcapitata* was the most sensitive organism followed by *V. fischeri* and *D. magna*, and no toxic effects were observed toward the other organisms. For the effluent sample collected after the implementation of secondary treatment, the effluent caused no toxic effects on any of the tested species. The present results demonstrated not only that secondary treatment efficaciously reduced effluent toxicity toward the selected test organisms but also the usefulness of a battery of tests to characterize the toxicity of pulp mill effluents.

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Introduction

One of the major sources of environmental preoccupation posed by pulp and paper mills is the discharge of large volumes of effluent provoking considerable adverse impacts on aquatic ecosystems (Pokhrel and Viraraghavan 2004; Latorre

et al. 2005). These impacts are complex due to the combined action of: (1) physical aspects like odor, color, and turbidity; (2) potential toxicants consisting either of naturally occurring wood extractives or xenobiotic compounds added and formed during the pulp and paper making, including resin acids, fatty acids, lignin and its derivatives, sterols, chlorinated compounds, sulfur compounds, and metals; and (3) eutrophication, prompted mainly by elevated levels of phosphorus and nitrogen (Dubé and Culp 1996; Ali and Sreekrishnan 2001; Ferreira et al. 2002; Lacorte et al. 2003; Latorre et al. 2007). Moreover, the chemical composition of pulp and paper mill effluents is still not completely understood and characterized (many constituents remain unidentified; Latorre et al. 2005; Hewitt et al. 2006), varying with the type of wood used, the pulping process and the degree and type of effluent treatment procedures (Pokhrel and Viraraghavan 2004).

As a consequence, it is difficult to assess the degree of environmental risk from effluents discharged by these industries (O'Connor et al. 2003; Kovacs et al. 2007). To circumvent this difficulty, the evaluation of the potential effects of pulp and paper mill effluents toward the environment is frequently based on chemical sum parameters and not on determinations of individual compounds (Oanh and Bengtsson 1995; Tarkpea et al. 1999; Ferreira et al. 2002). In this regard and with the purpose of achieving a high level of protection of the environment, the European Commission issued a Council Directive on integrated pollution prevention and control, requiring the implementation of the best available techniques to achieve environmental improvements, and establishing maximum discharge limits for key chemical parameters, namely chemical oxygen demand (COD), biological oxygen demand (BOD), adsorbable organic halogens (AOX), total suspended solids (TSS), and total phosphorus and nitrogen (EC 1991). Yet, it should be kept in mind that equal levels of these parameters (mainly AOX) indicate neither identical composition of effluents nor equivalent toxicity to aquatic organisms (Hewitt et al. 2006).

Confronted with the environmental problems posed by pulp and paper mill effluents, large efforts were made in the last decade to reduce their toxicity by introducing technological changes along the production process and in the treatment of the effluent before discharge. These changes have been particularly concerned with effluents from bleaching processes (brightening of the pulp fibers) which may contain one class of compounds of major environmental concern: chlorinated organic compounds. Efforts have focused on the replacement of elemental chlorine by chlorine dioxide (elemental chlorine-free bleaching; ECF) or by oxygen-containing compounds, such as ozone and hydrogen peroxide (totally chlorine-free bleaching; TCF; Tarkpea et al. 1999; Latorre et al. 2005). Also, the installation or the improvement of secondary effluent treatment plants aims at reducing AOX values, together with the levels of other parameters like BOD, COD, and TSS (Hall et al. 1991; Kovacs et al. 1994; Kostamo et al. 2004).

Despite all the technological changes implemented to reduce toxicity, pulp and paper mill effluents continue often to present toxicity to various groups of aquatic organisms, namely bacteria (Tarkpea et al. 1999), algae (Kovacs et al. 1994), planktonic and benthic invertebrates (Dubé and Culp 1996; O'Connor et al. 2003), and fish (Kovacs et al. 2007). Moreover, a relationship between the levels of effluent parameters (e.g., AOX) and the degree of environmental impact is occasionally absent (Robinson et al. 1994; Hewitt et al. 2006; Latorre et al. 2007). Recent studies pointed out that further attention should be given to other compounds resulting from pulping operations, many of which are probably not yet identified (e.g., wood derivatives, additives, and their degradation products; Kostamo et al. 2004; Kovacs et al. 2006). Tests with a battery of organisms continue, therefore, to be a valuable tool to evaluate the toxicity of these effluents (Oanh and Bengtsson 1995; Bailey and Young 1997; Tarkpea et al. 1999).

The aim of the present study was to use a test battery with organisms representative of key functions at the ecosystem level to assess whether the implementation of secondary treatment reduced the toxicity of an effluent from an ECF bleached-kraft pulp mill. The mill uses *Eucalyptus globulus* (Tasmanian blue gum) as wood furnish and employs oxygen delignification as prebleaching treatment. Toxicity tests were performed with *Vibrio*

fischeri (bacteria; decomposer), *Pseudokirchneriella subcapitata* (planktonic microalgae; primary producer), *Lemna minor* (floating macrophyte; primary producer), *Daphnia magna* (planktonic cladoceran; primary consumer; filter feeder), *Chironomus riparius* (benthic midge larvae; deposit feeder), and *Danio rerio* (pelagic fish; secondary consumer).

Materials and methods

Effluent samples

"End-of-pipe" composite samples of an ECF bleached-kraft pulp mill effluent were collected into dark vessels, immediately transported to the laboratory and stored at 4°C until use in toxicity tests (within 1 month). Samples were taken at two occasions, before (S1) and after (S2) the implementation of secondary treatment. Chemical sum parameters of the ECF bleached-kraft pulp mill effluent monitored during 20 and 19 months prior to the collection of samples S1 and S2, respectively, are presented in Table 1; after the implementation of secondary treatment levels of BOD decreased by ninefold, of TSS, COD, and AOX by threefold, and those of total phosphorus by twofold. Values of pH (WTW 537 pH meter, Wissenschaftlich Technische Werkstätten, Weilheim, Germany), dissolved oxygen (WTW OXI 92 oxygen meter) and conductivity (WTW LF 92 conductivity meter), measured in both samples every time they were used for testing, ranged from 6.32 to 6.79, 9.0 to 9.5 mg/l, and 3.41 to 3.80 mS/cm, respectively, for sample S1, and from 7.61 to 8.04, 9.2 to 10.9 mg/l, and 3.72 to 3.85 mS/cm for sample S2.

Toxicity tests

The *V. fischeri* Lehmann and Neumann test was carried out according to the Microtox basic test protocol (www.azurenv.com/mtox.htm). The Microtox toxicity analyzer model 500 (Azur Environmental, Carlsbad, CA, USA) was used to measure the light emission of the luminescent marine bacteria *V. fischeri*. Sample S1 was subjected to a dilution gradient of 5.63%, 11.3%, 22.5%, and 45.0%, whereas a dilution gradient of 10.2%, 20.5%, 41.0%, and 81.9% was applied to sample S2. The luminescent activity of the two samples was measured after 5-min exposure.

The 72-h *P. subcapitata* (Koršhikov; formerly *Selenastrum capricornutum* Printz) growth test was carried out following OECD (1984) and EEC (1989) guidelines. *P. subcapitata* (strain Nr. WW 15-2521) was acquired from the Carolina Biological Supply Company (Burlington, NC, USA) and maintained in 100-ml nonaxenic batch cultures, with Woods Hole MBL growth medium (Stein 1973), at 19°C to 21°C under continuous cool-white fluorescent illumination (100 μE/m^2/s). Sample S1 was subjected to a dilution gradient of 2.81%, 5.63%, 11.3%, 22.5%, 45.0%, and 90.0%, whereas a dilution gradient of 6.25%, 12.5%, 25.0%, 50.0%, and 100% was applied to sample S2. The MBL medium was diluted 2.5 times to be

Table 1 Range of mean monthly values of waste water production, pH, total suspended solids (TSS), chemical oxygen demand (COD), biological oxygen demand (BOD), adsorbable organic halogens (AOX), total nitrogen, and total phosphorus of the ECF bleached-kraft pulp mill effluent before (B) and after (A) the implementation of secondary treatment

Parameter	B secondary treatment	A secondary treatment
Waste water (m^3/ton of pulp)	33–34	33–34
pH	6.5–8.0	7.5–8.0
TSS (mg/l)	70–100	10–40
COD (mg O_2/l)	450–800	160–260
BOD (mg O_2/l)	120–220	10–30
AOX (mg Cl/l)	2.0–3.9	0.4–1.3
Total nitrogen (mg N/l)	3.4–6.8	2.2–5.3
Total phosphorus (mg P/l)	3.9–8.1	2.4–4.6

Measurements were taken during 20 and 19 months for B and A, respectively

used as the control and dilution medium according to the required N/P ratios. Three 100-ml replicate cultures of each test dilution and six of the control were set up and inoculated with a 1-ml algal inoculum, so that the initial cell concentration was 10^4 cells/ml. The tests were carried out under the same conditions used for culturing. At the end of the 72-h exposure, the mean specific growth rate per day was estimated. Initial and final cell densities were counted on well-mixed aliquots of each replicate under a microscope (×400 magnification), using a Neubauer chamber (American Optical, Buffalo, NY, USA).

The 7-day *L. minor* Linnaeus growth test was carried out according to ISO (2005) guidelines. Uniformly sized fronds (mean ± standard deviation of 1.20 ± 0.138 cm^2) were obtained from the Institut für Biologische Testverfahren (Stuttgart, Germany). They were transferred from solid Jungnikel medium (ISO 2005) to liquid modified Steinberg medium (ISO 2005) for adaptation and sub-cultured weekly during 3 weeks before being used in the tests. Cultures were maintained under constant illumination (100 $\mu E/m^2/s$) at 24°C. Both samples were subjected to a dilution gradient of 6.25%, 12.5%, 25.0%, 50.0%, and 90.0%. A mixture of 10 ml of concentrated (10×) modified Steinberg medium and the required volume of distilled water was used as control and dilution medium. Each replicated culture consisted of a crystallizer filled with 100 ml of test solution inoculated with 10 to 15 fronds. Six control replicates and three replicates per test dilution were set up and incubated under the same conditions used for culturing. After the 7-day exposure, growth was estimated as the frond area using a digital image analysis system (Scanalyzer; Lemnatec, Wuerselen, Germany).

The 21-day *D. magna* Straus reproduction test was carried out according to OECD (1998a) guidelines. Organisms used for testing were third-brood 6- to 24-h-old neonates, obtained from cultures maintained at 20°C to 22°C under a 14-h/10-h light/dark photoperiod. Culture medium was a reconstituted hard water (ASTM 2002a) supplemented with vitamins (7.5 μg/l of B_1, 1 μg/l of B_{12}, and 0.75 μg/l of biotin) and Marinure extract (Glenside, Stirling, UK; 7.5 ml/l of a suspension with an absorbance of 620 units at 400 nm). Cultures (25 and 15 daphnids/l up to the first brood and from there onwards, respectively) were fed daily with *P. subcapitata* (3×10^5 cells/ml), and the medium was renewed every other day. Sample S1 was subjected to a dilution gradient of 25.0%, 33.8%, 45.6%, 61.5%, and 83.0% (because preliminary trials showed 25% to have no toxic effects on reproduction and 100% to be acutely toxic a multiplication factor of 1.35 was used), whereas a dilution gradient of 33.8%, 45.6%, 61.5%, 83.0%, and 100% was applied to sample S2 (as it was anticipated to be less toxic). The culture medium was used as the control and dilution medium. Ten replicates were set up for each treatment, with 50 ml of the test solution and one organism each. During testing, the feeding regime, medium renewal frequency, and incubation conditions were similar to those used for culturing. After the 21-day exposure, fecundity was determined as the total number of neonates released per female.

The *D. magna* postexposure feeding test was based on the methodology developed by McWilliam and Baird (2002). This test was adopted in the present study because pulp mill effluents contain suspended solids and particulates that may influence the feeding rate. Test organisms were 4-day-old juveniles obtained from cultures maintained under the conditions described above. Only sample S1 was tested because it was found to be nontoxic (see "Results" section) and the TSS of sample S2 was markedly lower than that of sample S1; a dilution gradient of 33.8%, 45.6%, 61.5%, and 83.0% of S1 was used. Aside from the test with plain effluent, an additional test was performed with a centrifuged effluent sample (for 10 minutes at 3,370×*g*) to investigate weather the presence of suspended solids in the effluent contributed to toxicity. Five replicates were used per treatment, and each one consisted of a glass vessel filled with 120 ml of test solution and five organisms. The tests were carried out at 20°C to 22°C, under a 14-h/10-h light/dark photoperiod, and no food was provided. After a 24-h exposure, organisms were immediately transferred into glass vessels containing 120 ml of control medium and fed 3×10^5 cells/ml of *P. subcapitata* during a 4-h postexposure in darkness. A blank treatment consisting of three replicates without daphnids was set up to account for the occurrence of algal growth.

Before and after the feeding period, algae counts were performed as previously described and feeding rates were expressed as the number of algal cells/organism/h.

The 10-day *C. riparius* Meigen growth test was carried out following the OECD (2004) and ASTM (2002b) guidelines. For testing, second- to third-instar larvae were used, with a mean ($n = 12$; ± standard deviation) dry weight (24 h at 60°C) of 0.214 (± 0.071) and 0.243 (± 0.040) mg for sample S1 and S2, respectively. Cultures were maintained in a transparent plastic cage (40 × 60 × 120 cm) sufficiently large to allow swarming and copulation of adults (OECD 2004), at 20°C to 22°C, under a 14-h/10-h light/dark photoperiod with 90-min dawn and dusk periods. Cultures consisted of crystallizing dishes containing 185 g of quartz sea sand (0.1–0.4 mm particle size; Merck, Darmstadt, Germany) and 300 ml of reconstituted hard water (ASTM 2002a) continuously aerated and were fed a suspension of ground Tetramin (Tetrawerk, Melle, Germany) every other day (0.1 g/dish, with 30 and 15 larvae/dish up to day 7 and from there onwards, respectively; for further details see Castro et al. 2003). The samples dilution gradient was 40.2%, 48.2%, 57.8%, 69.4%, 83.3%, and 100% for S1 and 48.2%, 57.8%, 69.4%, 83.3%, and 100% for S2. The culture medium was used as the control and dilution medium. Four replicates were set up for each test dilution and control, each with 50 g of sediment and 120 ml of medium. Vials were prepared 12 h prior to the beginning of the test and left with continuous aeration. Three larvae were added per replicate, and after 30 min, aeration was restarted. During testing, the organisms were fed daily with 1 mg of Tetramin per larvae. The tests were incubated at 20°C to 22°C under a 14-h/10-h light/dark photoperiod. After a 9-day exposure (instead of 10 days because larvae started to pupate), the growth of each larva was estimated as the daily increase in dry weight (gram per day) and the percentage of pupation was also determined.

The 28-days *D. rerio* Hamilton growth test followed the OECD (2000) guidelines. Nonspawning fish with a mean (± standard deviation; *n*) wet weight of 0.219 (± 0.015; 8) and 0.214 (± 0.030; 12) g for the test with sample S1 and S2, respectively, were obtained from a commercial supplier and acclimated for at least 3 weeks before tests. During acclimation, fish were maintained at 19°C to 21°C under a 8-h/16-h light/dark photoperiod, in an aquarium filled with 50 l of dechlorinated tap water continuously aerated and fed daily, ad libitum, with Tetramin. Sample S1 was subjected to a dilution gradient of 9.49%, 13.3%, 18.6%, 26.0%, 36.4%, 51.0%, 71.4%, and 100%, whereas a dilution gradient of 18.6%, 26.0%, 36.4%, 51.0%, 71.4%, and 100% was applied to sample S2. The culture medium was used as the control and dilution medium. Three replicates for each test dilution and four for the control were set up, each with 400 ml of test solution and two fish. During testing, constant aeration was provided, the medium suffered a renewal of 50% on Mondays and 35% on Wednesdays and Fridays, and organisms were fed daily with Tetramin in a quantity equivalent to 3% of their wet weight. Tests were incubated at 20°C to 22°C, under a 14-h/10-h light/dark photoperiod. At the end of the 28-day exposure growth was estimated as the daily increase in wet weight (microgram per day).

For the *V. fischeri* and *L. minor* tests, pH and conductivity were measured at test initiation, whereas for the *P. subcapitata* tests pH and conductivity were measured at the beginning and the end of each test. For the *D. magna* reproduction and *D. rerio* tests pH, conductivity and dissolved oxygen were measured in old and fresh medium at all medium renewals, whereas for the *C. riparius* and *D. magna* postexposure feeding tests, the same parameters were measured at the beginning and end of the tests. Values measured in the controls were within the limits established in the guidelines.

Data analysis

For all tests, except for the *V. fischeri* test, the organism responses were examined for significant differences between the control and tested effluent dilutions using one-way analysis of variance (ANOVA) or nested ANOVA. Although a nested design was used in the *C. riparius* test, a one-way ANOVA was applied to the growth and pupation data due to the elevated occurrence of

pupae. Pupation proportions were arcsine square root transformed to fulfill the assumptions of normality and homoscedasticy. When significant differences were found, the Dunnett's test was performed to determine not only the no-observed-effect dilution (NOEC) and the lowest-observed-effect dilution (LOEC; inhibition relatively to the control) but also the possible occurrence of stimulatory effects. Although the Microtox software did not provide replicate data to perform ANOVA, the effluent dilutions causing a luminescence inhibition lower and higher than 10% were considered as the NOEC and LOEC values, respectively.

For the *V. fischeri* and *L. minor* tests, the median effective dilutions (EC50) and respective 95% confidence limits (CL) were calculated using Microtox Omni Software 1.18 (Azur Environmental) and Biostat 2000 Software (Lemnatec), respectively. For all remaining tests, EC50 values and respective 95% CL were obtained by fitting organism responses to a logistic model using the least squares method (OECD 1998b).

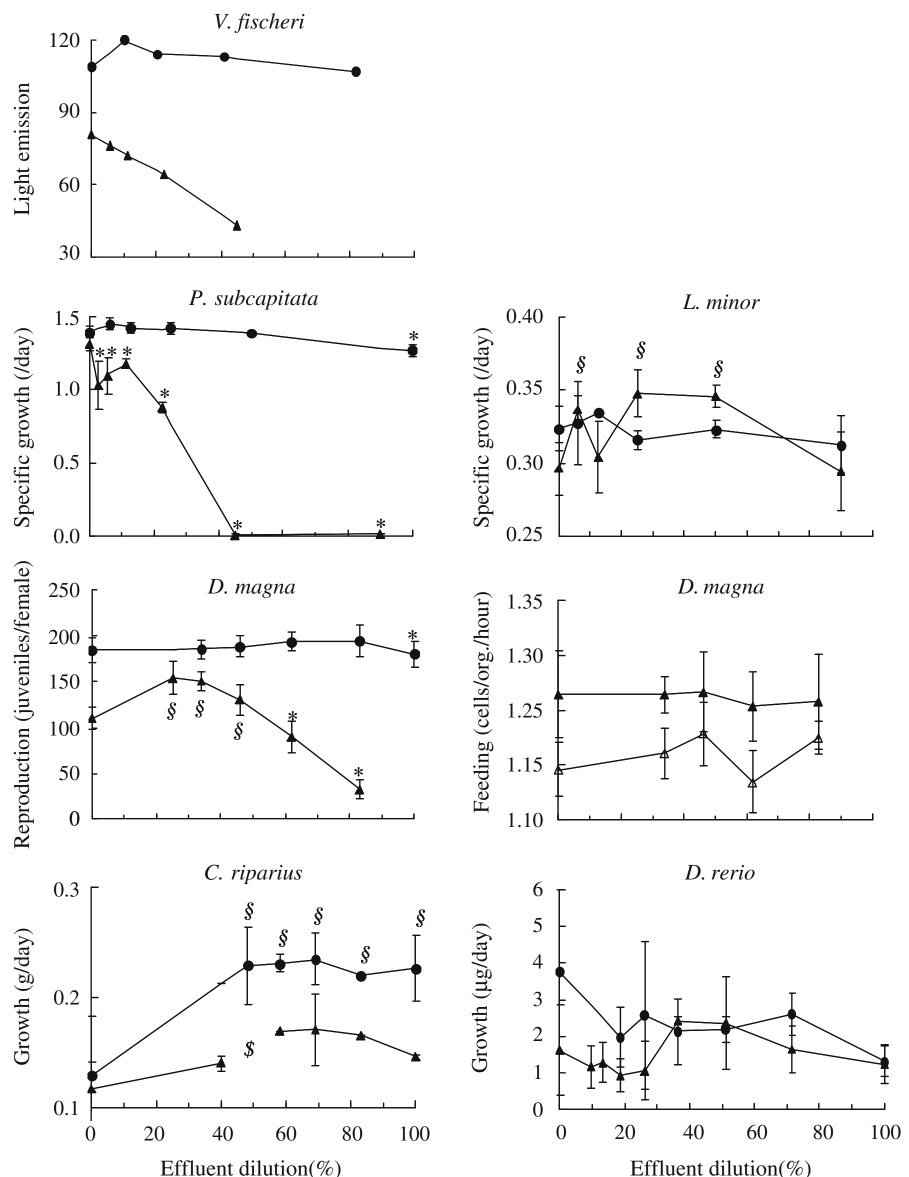

Fig. 1 Sublethal effects of an elemental chlorine-free bleached-kraft pulp mill effluent before (sample S1—*filled triangle*) and after (sample S2—*filled circle*) the implementation of secondary treatment, on *Vibrio fischeri* (5-min luminescence; no statistical testing performed since the test design does not include replication), *Pseudokirchneriella subcapitata* (72-h growth), *Lemna minor* (7-day growth), *Daphnia magna* (21-day reproduction and 24-h postexposure feeding; *closed symbols* for plain S1 and *open symbols* for centrifuged S1), *Chironomus riparius* (9-day growth; dry weight), and *Danio rerio* (28-day growth; wet weight). *Error bars* indicate ±1 standard deviation; *asterisk* and *section sign* denote, within each tested sample, means significantly lower and higher than control, respectively, by Dunnett's test; *dollar sign* indicates dilution not considered (48% of S1) due to excessive pupation

Results

All tests fulfilled the validity criteria for control performance required in the respective guidelines. Although the OECD (2000) guidelines for fish growth tests do not specify the recommended weight increase of control fish for *D. rerio* tests, the mean weight increase of the control fish observed in the present study both for sample S1 (28%) and S2 (51%) was within the range of previously reported values (Smolders et al. 2002). Figure 1 and Table 2 present the toxicity results of all tests performed. The *V. fischeri* test revealed toxicity for sample S1, with an EC50 value of 49%, being the NOEC (9.2% inhibition) and LOEC (22.7% inhibition) values 11.3% and 22.5%, respectively. For sample S2 no toxicity was observed. Significant differences in *P. subcapitata* growth between the control and the effluent dilutions were observed both for samples S1 (one-way ANOVA: $F_{6,16} = 161$, $P < 0.001$) and S2 (one-way ANOVA: $F_{5,15} = 10.3$, $P < 0.001$). Yet, a LOEC value was found only for sample S1 (21% inhibition at the 2.81% dilution), while for S2 growth was inhibited by merely 9%, only for 100% of effluent. For *L. minor*, significant differences in growth between the control and test dilutions were observed for sample S1 (one-way ANOVA: $F_{5,15} = 6.23$, $P < 0.01$) but not for S2 (one-way ANOVA: $F_{5,15} = 0.769$, $P = 0.59$). Yet, growth was not inhibited by sample S1. On the contrary, growth stimulation (from 13% to 17%) was observed at 6.25%, 25%, and 50% of S1.

Although sample S1 exerted a toxic effect on the reproduction of *D. magna* (one-way ANOVA: $F_{5,53} = 92.6$, $P < 0.001$), with a NOEC, LOEC, and EC50 of 46%, 62%, and 55%, respectively, reproduction was stimulated by up to 41% relatively to the control at the lowest percentages of effluent (from 25% up to 46%). For S2, no effects on reproduction were observed (one-way ANOVA: $F_{5,54} = 1.89$, $P = 0.11$). With regard to the postexposure feeding test, significant differences in feeding rate between the control and test dilutions were observed for the centrifuged effluent (one-way ANOVA: $F_{4,20} = 2.88$, $P = 0.049$) but not for the plain effluent (one-way ANOVA: $F_{4,20} = 0.124$, $P = 0.97$). Yet, NOEC and LOEC values could not be detected for the centrifuged effluent, probably because the observed significant differences were marginal, with an inhibition of only 0.9% at the 62% dilution and a stimulation ranging from 1% to 3% at the remaining dilutions.

When *C. riparius* was exposed to sample S1, significant differences between the control and test dilutions were observed for the percentage of pupation (one-way ANOVA: $F_{6,21} = 4.42$,

Table 2 Values of no-observed-effect dilutions (NOEC, in %), lowest-observed-effect dilutions (LOEC, in %), and median effective dilutions (EC50, in %) with 95% confidence limits inside brackets, for all tests performed with an elemental chlorine-free bleached-kraft pulp mill effluent before (sample S1) and after (sample S2) the implementation of secondary treatment

Test	S1			S2		
	EC50	NOEC	LOEC	EC50	NOEC	LOEC
V. fischeri	48.9 (43.2–55.3)	11.3	22.5	ne	ne	ne
P. subcapitata	25.4 (16.1–34.8)	< 2.81	2.81	ne	ne	ne
L. minor	ne	ne	ne	ne	ne	ne
D. magna	55.3 (50.9–59.7)	45.6	61.5	ne	ne	ne
D. magna (PEF)[a]	ne	ne	ne	nt	nt	nt
D. magna (PEF)[b]	ne	ne	ne	nt	nt	nt
C. riparius	ne	ne	ne	ne	ne	ne
D. rerio	ne	ne	ne	ne	ne	ne

Vibrio fischeri (Microtox, 5-min luminescence), *Pseudokirchneriella subcapitata* (72-h growth), *Lemna minor* (7-day growth), *Daphnia magna* (21-day reproduction and 24-h PEF), *Chironomus riparius* (9-day growth), and *Danio rerio* (28-day growth)
PEF postexposure feeding, *ne* no effect, *nt* not tested
[a]Test with plain sample
[b]Test with centrifuged sample

Table 3 Range of pH, conductivity (Cond.) and dissolved oxygen (DO) measurements, from lowest to highest percentage of effluent, measured during the tests performed with an elemental chlorine-free bleached-kraft pulp mill effluent before (sample S1) and after (sample S2) the implementation of secondary treatment

Test	S1			S2		
	pH	Cond. (μS/cm)	DO (mg/l)	pH	Cond. (μS/cm)	DO (mg/l)
V. fischeri[a]	6.6	3,842	7.2	7.5	3,100	8.1
P. subcapitata	5.8–7.6 (6.3)	308–3,410 (310)	nm	7.4–8.8	456–3,770	nm
L. minor	6.6	3,842	7.2	7.5	3,100	8.1
D. magna	7.2–7.1 (7.0)	1,392–3,370 (2,615)	9.0–6.3 (7.8)	7.8–8.0	1,680–3,720	9.5–9.2
D. magna (PEF)[b]	7.4–6.9	1,560–3,170	7.9–6.9	nt	nt	nt
D. magna (PEF)[c]	7.7–7.4	2,030–3,350	9.5–8.4	nt	nt	nt
C. riparius	8.0–7.9	1,864–3,720	9.6–9.5	8.2–8.4	2,120–3,730	9.6–9.5
D. rerio	6.5–6.7	446–6,590	7.2–7.6	7.3–7.9	833–3,760	8.4–9.5

Vibrio fischeri (Microtox, 5-min luminescence), *Pseudokirchneriella subcapitata* (72-h growth), *Lemna minor* (7-day growth), *Daphnia magna* (21-day reproduction and 24-h PEF), *Chironomus riparius* (9-day growth), and *Danio rerio* (28-day growth). Values inside brackets correspond to the measurements taken at the lowest-observed-effect dilution

nm not measured, *nt* not tested, *PEF* postexposure feeding

[a]Single value corresponding to the highest effluent percentage, as the small test volumes during testing did not allow to take measurements

[b]Test with plain sample

[c]Test with centrifuged sample

$P < 0.01$) but not for growth (one-way ANOVA: $F_{5,9} = 2.67$, $P = 0.094$). Yet, the former was due to an increase in pupation rate at all S1 dilutions (>83%) compared with the control (25%), and, thus, no toxicity was found for S1. For sample S2, significant differences were found for larval growth (one-way ANOVA: $F_{5,15} = 5.85$, $P < 0.01$) but not for pupation (one-way ANOVA: $F_{5,18} = 0.571$, $P = 0.72$; an overall mean of 70%). As for S1, a stimulatory effect was observed at all S2 dilutions, though this time on growth. Toxic effects on the growth of *D. rerio* were observed neither with sample S1 (nested ANOVA: $F_{8,19} = 1.23$, $P = 0.33$) nor with S2 (nested ANOVA: $F_{6,15} = 1.10$, $P = 0.41$). Table 3 presents the range of pH, conductivity, and dissolved oxygen measurements during testing and also the exact measurements taken for the LOEC values, when applicable.

Discussion

In this study, a battery of tests with organisms bearing different key functions at the ecosystem level was used to evaluate the effectiveness of secondary treatment in managing the toxicity of a hardwood ECF bleached-kraft pulp mill effluent. Overall, the implementation of secondary treatment resulted in the elimination of the toxicity of the effluent; toxic effects were observed in none of the six tested species. Reductions in the toxicity of pulp and paper mill effluents through the introduction of secondary treatment have been observed in previous studies (Tarkpea et al. 1999; Environment Canada 2003; Hewitt et al. 2006; Parrott et al. 2006). Such toxicity reductions are mainly due to decreases in the levels of critical chemical sum parameters, as was observed in the present study with regard to the TSS, COD, BOD, and AOX values, as well as to declines in the concentrations of chemical constituents (e.g., fatty and resin acids; Hall et al. 1991; Kovacs et al. 1994). After the implementation of secondary treatment, the levels of these parameters were within ranges observed in other secondary treated effluents, for which no toxic effects were observed toward microalgae, cladocerans, and fish (Kovacs et al. 1994; O'Connor et al. 2003).

Although results from different studies may not be readily comparable due to differences in the type of wood used, the pulping process, and the degree and type of effluent treatments, some considerations can be made regarding the observed differences in the toxic responses of the various species to the ECF bleached-kraft pulp

mill effluent prior to and after the implementation of secondary treatment (samples S1 and S2, respectively). The *V. fischeri* test was sensitive to effluent sample S1, with a 5-min EC50 value of 49%, whereas effluent sample S2 was not toxic to *V. fischeri*. This result is in agreement with other studies showing the 5- and 15-min EC50 values for *V. fischeri* of untreated elemental chlorine bleached effluents to vary from 36% to 58% (Oanh and Bengtsson 1995). The tendency for a higher toxicity observed in the latter study compared to the present study was probably due to the differences in the type of bleaching process used, since elemental chlorine bleaching leads to the formation of higher amounts of toxic chlorinated compounds than ECF bleaching, and also to the type of wood used. In a more recent study, Tarkpea et al. (1999) found a 15-min EC50 for *V. fischeri* exposed to ECF-bleached effluents of 31% and 61% before and after secondary treatment, respectively. Contrary to what was observed in the present study, the implementation of secondary treatment did not efficiently remove the toxicity of the effluent in the latter study. Since both these effluents resulted from ECF bleaching, were subjected to secondary treatment and had similar AOX levels, differences in natural wood constituents were probably at origin of the observed differences in effluent toxicity (Kovacs et al. 2006).

The overall effect of pulp and paper mill effluents on primary producers results from the interaction of different impact factors, namely the attenuation of light (due to turbidity and color), phytotoxicity, or eutrophication (due to nutrient level increases of nitrogen, phosphorus, and organic carbon; Amblard et al. 1990). The results from the algal growth tests showed that *P. subcapitata* was extremely sensitive to the effluent sample taken before the implementation of secondary treatment, with LOEC and 72-h EC50 values of 3% and 25%, respectively. Conversely, the secondary-treated effluent sample was not toxic to the algae; 100% of effluent sample S2 inhibited algal growth by 9%. The observed sensitivity of *P. subcapitata* to effluent sample S1 was most likely due to the presence of chlorate, a constituent of pulp mill effluents known to be toxic to algae that is usually produced when chlorine dioxide is incompletely reduced to chloride during pulp bleaching (van Wijk and Hutchinson 1995), as well as other toxic compounds (e.g., resin acids; Landner et al. 1994). Thus, the absence of toxicity in sample S2 was probably ascribed to the fact that secondary treatment reduced the discharges of such toxic compounds. The present result is consistent with results of other studies observing that secondary treatment considerably reduced the toxicity of pulp mill effluents toward phytoplankton (Kovacs et al. 1994; Tarkpea et al. 1999). Also, Hall et al. (1991) have found that a secondary-treated elemental chlorine-bleached effluent affected periphyton growth, causing stimulation at low and inhibition at high concentrations. Yet, they concluded that stimulation was due to nutrient addition from the effluent, and inhibition was due to the color effect reducing light availability. With regard to the 7-day growth test with *L. minor*, no toxicity was found toward the macrophyte, either prior to or after the implementation of secondary treatment. Macrophytes usually develop well in environments rich in nutrients and are many times used for their removal (Mohan and Hosetti 1999). This fact could explain the *L. minor* growth stimulation observed in the test with effluent sample S1 and not in sample S2, since a decrease in nitrogen and particularly phosphorus was observed after secondary treatment. The lack of toxic effects of pulp mill effluents toward macrophytes, and even the occurrence of growth stimulation, has also been reported in previous studies characterizing the toxicity of untreated and secondary-treated effluents (Oanh and Bengtsson 1995; Yen et al. 1996; Tarkpea et al. 1999).

In the 21-day *D. magna* reproduction test with sample S1, a significant increase in the number of neonates released was observed at the lowest effluent dilutions ($\leq$46%), whereas the opposite was found at the highest dilutions ($\geq$61%). It is possible that the fecundity was stimulated due to increases in food supply corresponding to the nutrient loading of the effluent, which increased the nutritive value of the diet (Robinson et al. 1994; Sobral et al. 1998). Yet, it appeared that after a certain threshold (between 46% and 61% of effluent), the effect of nutrient loading was overridden by the toxic effect of the effluent. As

expected, effluent sample S2 was found to be nontoxic toward *D. magna*, since secondary treatment reduces effluent toxicity (Tarkpea et al. 1999; Environment Canada 2003; Hewitt et al. 2006; Parrott et al. 2006). In agreement with the present results, Robinson et al. (1994) observed that the 7-day reproduction of *Ceriodaphnia dubia* Richard was significantly lower in samples from untreated than in those from secondary-treated effluents, i.e., the effects on reproduction were consistent with the degree of effluent treatment. Also, Kovacs et al. (2002) in their review study concluded that secondary treatment had significant beneficial effects on the 7-day reproductive performance of *C. dubia*. With regard to the influence of the effluent (sample S1) on the postexposure feeding rate of *D. magna*, no toxic effects were observed either with plain or with the centrifuged samples. The short exposure period of 24 h of this test could be the reason for the observed lack of toxicity. Also, the possible occurrence of the physiological recovery of the organisms during the postexposure feeding period should be considered. Pulp mill effluents are composed of several substances among which polycyclic aromatic hydrocarbons (McLeay 1987). In a study on the applicability of a postexposure feeding test with *D. magna* for toxicity assessments, McWilliam and Baird (2002) found that the polycyclic aromatic hydrocarbon fluoranthene only induced feeding depression during exposure and not during the postexposure feeding period.

No toxic effects were observed in the 9-day *C. riparius* growth test either prior to or after the implementation of secondary treatment. The growth stimulation observed with sample S2 could be the result of a reduction in the toxicant levels due to secondary treatment along with the presence of nutrients from the effluent (secondary treatment reduces but does not eliminate the organic load; Munkittrick et al. 1992). Chironomids are considered as opportunistic organisms, tolerant to contamination and with a high adaptative capacity (Groenendijk et al. 1998; de Haas et al. 2002), and, thus, enhanced growth due to exposure to biologically treated bleached-kraft pulp mill effluents has been observed before (Lowell et al. 1995; Dubé and Culp 1996). The observed increases in growth have been attributed to increases in food availability resulting from nutrients present in the effluent, to increases in the nutritive value of the food and of its palatability, and also to direct growth stimulation via hormonal responses to (plant) compounds present in the effluent that can interfere with the growth and development of insects (Lowell et al. 1995; Dubé and Culp 1996). Also, in the present study, no effects on growth were detected in the 28-day test with *D. rerio* for both effluent samples. Regarding effects of secondary-treated bleached-kraft pulp mill effluents on fish growth, stimulatory (Hall et al. 1991; Soimasuo et al. 1995), inhibitory (Kovacs et al. 2007), and no effects have been reported (Robinson et al. 1994). Although no effects were here observed on *D. rerio* growth, it should be stated that most of the detrimental effects of pulp mill effluents on fish have been found at the level of reproductive indicators, namely increased age to maturation, reduced fecundity, and reduced gonadal development or levels of active reproductive steroid hormones (Environment Canada 2003; Kovacs et al. 2006; McMaster et al. 2003). In particular, Kovacs et al. (2007) observed a significant reduction in egg production and in the number of spawning events in *D. rerio* after exposure to secondary-treated effluent, though the responsible agent(s) still have to be identified.

Overall, the use of a battery of tests to evaluate the toxicity of an ECF bleached-kraft pulp mill effluent before the implementation of secondary treatment (sample S1) revealed differences in the sensitivity of the six tests. The growth test with the microalga *P. subcapitata* was the most sensitive among all tests. The luminescent test with the bacterium *V. fischeri* and the reproduction test with the crustacean *D. magna* were less sensitive but were still useful to characterize the effluent; though the EC50 values showed these two tests as equally sensitive the LOEC values suggested the *V. fischeri* test to be more sensitive than the *D. magna* test. None of the remaining four tests was sensitive to effluent sample S1. This rank of sensitivities is in agreement with that observed in previous studies showing that tests with microalgae were always the most sensitive immediately followed by the *V. fischeri* test and that the macrophytes and fish usually rank among the most tolerant (Oanh and Bengtsson 1995; Yen et al.

1996; Tarkpea et al. 1999). The fact that algae are considered to be more sensitive to chlorate than invertebrates and fish (van Wijk and Hutchinson 1995) could explain their general higher sensitivity, though short-term tests measuring survival and growth are usually not able to detect effects of pulp mill effluents on fish (Parrott et al. 2006). The present results also demonstrated that the implementation of secondary treatment and consequent decrease in the levels of critical chemical sum parameters clearly reduced the toxicity of the ECF bleached-kraft pulp mill effluent, as no toxicity was found for any of the tested species. Therefore, the present study, along with previous studies (Kovacs et al. 1994; Bailey and Young 1997; Tarkpea et al. 1999; Sponza 2003), supports the usefulness of a battery of tests with organisms of different sensitivities that bear different ecosystem functions to fully characterize and compare the toxicity of pulp mill effluents.

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