Kraft pulp mill effluent treated by activated sludge and aerated lagoon genotoxicity assess

Claudia Regina Xavier (1), Elizabeth Oñate (1), María Angélica Mondaca (2) and Gladys Vidal (1).
(1) Environmental Science Center EULA- Chile. (2) Department of Microbiology, Faculty of Biological Sciences. University of Concepción. P. O. Box 160-C. Concepción- Chile. (e-mail: glvidal@udec.cl)

Organic compounds present in the bleached kraft pulp mill (BKPM) effluents may be reduced by biological treatment like: activated sludge (AS) or aerated lagoon (AL). However, after biological treatment, BKPM effluent still contains specific compounds. Some of them have been related with chronic effects (DNA damage, reproductive and physiological) observed on fishes exposed to BKPM effluents. The DNA damages or genotoxicity caused by BKPM effluent has been also studied using bacterial test. *Bacillus subtilis* mutant deficient in repair functions has been isolated. The comparison between the mutant bacteria and the wild-type serves to assess DNA- damaging agents. The aim of this work is to evaluate genotoxicity of kraft pulp mill effluent using *Bacillus subtilis* "rec" assay, before and after treatment by activated sludge (AS) and aerated lagoon (AL).
Effluent sample from a local BKPM, which process *Pinus radiata* was used. It was characterized regarding to Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD_5), phenolic compounds (UV_{215}), color (VIS_{440}), pH, lignins, resin acid contents. AL and AS reactors (0.9 L) were operated for more than 200 days. The organic load rate (OLR) increases from 0.2 to 0.6 g COD/L·d on three steps. Reactor's performance were verified by measuring COD, BOD_5, color (VIS_{440}), total phenolic compounds (UV_{215}) and lignin derived (UV_{272} and UV_{280}). The genotoxicity was assessed by using *Bacillus subtilis* PB1791 (rec-) and *Bacillus subtilis* PB 1652 (rec+), through a quantitative methods of the efficiency of plating (A). The "rec" assay using *Bacillus subtilis* was verified on influents and effluents. The COD and BOD_5 removal were around 65 % and 97 % in the AS and around 60% and 98 % in the AL. Color and total phenolic compounds are only partially removed. AS and AL reactors remove genotoxicity as well as specific chemical compounds removal during biological treatment.

Keywords: Activated sludge, aerated lagoon, *Bacillus subtilis*, genotoxicity, kraft pulp mill effluent.

INTRODUCTION

Bleached kraft pulp mill (BKPM) effluents contain mainly: suspended solids (10-40 kg/ton pulp), organic matter (100-140 kg COD/ton pulp), color and toxicity. Because of this and the high water use (60-90 m^3/ ton pulp), BKPM requires efficient systems to treat their effluents. In order to mitigating the environmental impacts of these effluents discharge, biological treatment like: activated sludge (AS) or aerated lagoon (AL) have been extensively used (Thompson et al. 2001, Pokhrel and Viraraghavan 2004).

Furthermore, acute toxicity is not detected in treated effluents, due to improvements done respect to bleaching processes on BKPM, and effluent biological treatment (Vidal 1999, Thompson et al. 2001, Diez et al. 2002). However chronic toxicity is still observed on it (Bailey and Young 1997). Wood extractive constituents (sterols, triglycerides, resin acids, phenols etc.) are released during the pulping process. They are expected to produce part of the chronic toxicity observed. Although, part of these compounds are burned or recovered in a separate process, some are dissolved in the effluent (Rod´ko et al. 1996, Thompson et al. 2001).

Effect on growth and reproduction of fishes exposed to BKPM treated effluent have been observed by many authors (Munkittrick et al. 1992, Lehtinen et al. 1999, Mattsson et al. 2001, Larsson et al. 2002). Also effects related to DNA damaging have been associated with fishes exposed to BKPM effluent in concentration of 0.78 to 12.5 % (Pacheco and Santos 1999, Gravato and Santos 2002b). The BKPM effluent genotoxicity are related with resin acid presence on it, mainly de dehydroabietic acid (0.004 - 0.8 mg/L) and abietic acid (0.283 - 0.8 mg/L) (Pacheco and Santos 1997, Pacheco and Santos 1999, Gravato and Santos 2002a). Resin acid contents on BKPM treated effluent are usually between 0.002 - 2.4 mg/L, where abietic and dihydroabietic acid represents more than 70 % of total resin acid concentration (Hewitt et al. 1996, Williams et al. 1996).

Genotoxicity could be reduced by aerobic treatment in one or more orders magnitude. It happens especially when there is no recirculation of the effluent during treatment process. However the genotoxicity removal observed is not the optimal, because genotoxic compounds are not completely removed, and also part of it could remain adsorbed on the sludge. Humic substances complex genotoxic compounds, and decreases their solubility in water. It also affects their bioavailability, and as consequence diminishes their biodegradation (Takigami et al. 2002, Kostamo and Kukkonen 2003).

As is showing in the Figure 1, the exposure of organisms to a genotoxic agent could generate DNA damage, mutation and tumor process. The DNA damaging could be similar to different kinds of animals, therefore its detection and understanding is essential to avoid mutations and tumor processes which demanding a long latent time (Segner and Braunbeck 1998, Shugart et al. 2002). The common methods used to assess genotoxicity are: (i) "rec" assay using *Bacillus subtilis*, (ii) test Ames, (iii) erythrocytic micronuclei (EMN), (iv) sister chromatic exchange (SCE) tests in "Chinese hamster ovary" (CHO) cells, and (iv) nuclear abnormalities (ENA).

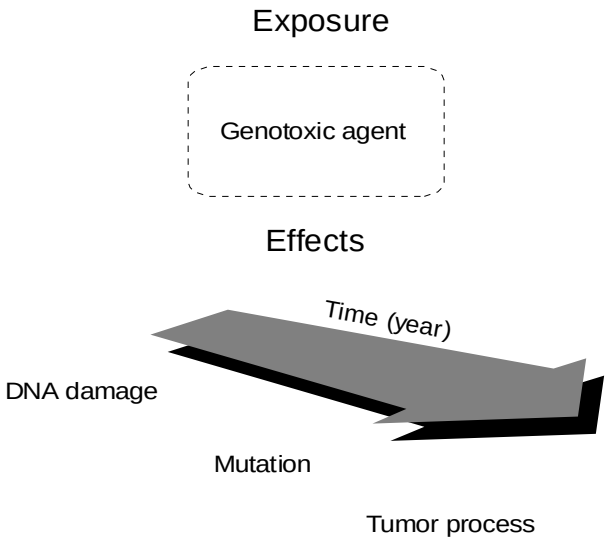

Figure 1. DNA damaging detection to avoid mutations and tumor processes.

The "rec" assay using *Bacillus subtilis* to determinate genotoxicity, is based on the comparison between *Bacillus subtilis* rec-, and the wild-type (rec+). This assay allows assessing DNA damages causes by genotoxic agent (Mazza 1982, Takigami *et al.* 2002). The *Bacillus subtilis* "rec" assay is a useful tool since it allows to detect: (i) wide spectra of DNA damages, (ii) mutagens at a much lower level concentration than the others bacterial strains, and (iii) genotoxicity together with cytotoxicity (Mazza 1982, Mondaca *et al*. 2000, Takigami *et al*. 2002). The aim of this work is to evaluate genotoxicity of kraft pulp mill effluent using *Bacillus subtilis* "rec" assay, before and after treatment by activated sludge (AS) and aerated lagoon (AL).

MATERIALS AND METHODS

BKPM effluent sample was obtained from a local plant, which employ *Pinus radiata* and an Elemental Chlorine Free (ECF) bleaching process. This effluent sample had been primary treated in a settler tank to reduce fiber and total solids. The sample was transported on ice in insulated cooler to the laboratory and stored in the dark at 4 ± 1 °C.

The BKPM effluent characteristics were verified by measuring Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD_5), color, total phenolic compounds, lignin derived and lignosulphonic and resin acid. An AS and an AL reactors of 0.9 L were used. The pH of BKPM effluent was adjusted to 7.0 ± 0.1, and it was supplemented with NH_4Cl and K_2HPO_4 as a nitrogen and phosphate nutrient (COD:N:P= 100:5:1). A consortium of aerobic bacteria (5 g/L volatile suspended solid (VSS)) was inoculated on each reactor. This consortium arises from an aerated lagoon system that treats BKPM effluent. The AS and AL reactors were operated continuously for more than 200 days. The operation strategy was to increase the OLR from 0.2 to 0.6 g COD/L·d, through three different steps. COD, BOD_5, color, total phenolic compounds and lignin derived were measured in the influent and effluent. The performance of the reactors was obtained by calculating the efficiency removal of each analyzed parameter.

The influent and effluent genotoxicity of aerobic treatments was assessed using *Bacillus subtilis* PB 1791 (rec-) and *Bacillus subtilis* PB 1652 (rec+). A quantitative method of efficiency of plate (A) was used. Fractional survival was measured in presence of increasing influent or effluent doses. Influent and effluent (pH=7.0) were previously filtrated on 0.45 μm membrane, and they were sterilized by using filter syringe (0.20 μm). All "rec" assays on tubes containing 2 mL of soft agar, maintained at 45 ^{0}C, on dry batch device were done in duplicated. They were supplemented with: (i) increasing concentration of influent or effluent (0.1, 0.3 and 0.5 mL), (ii) 0.1 mL of a 10^{-4} CFU/mL dilution of an overnight culture of tester strain in "nutrient broth (difco) with 0.5 % glucose added" (NBG). The contents of each tube were mixed in vortex and distributed onto the surface of agar plate. Plates were incubated at 37^{0}C for 24 h. After that, control survivalship and three concentrations samples were employed to make dose-response curves. Where N means the bacterial growth of rec+ or rec- in influent or effluent presence, and N_0 means bacterial growth of rec+ or rec- in influent/effluent absence. The survivalship (N/N_0) of *Bacillus subtilis* rec- and rec+ ratio provide the efficiency of plate (A) value, when A<1.0 effluent or influent shows genotoxicity. Statistix for Windows 2.2 software was used to evaluate sample variance analysis (ANOVA- Bonferroni test).

COD and BOD_5 were measured according to Standard Methods (APHA 1985). Total phenolic compounds concentration was measured by UV absorbance at 215 nm (UV_{215}). Total phenolic concentration was expressed as mg/L of phenol. Aromatic compounds at (UV_{254}) and lignin derived (UV_{272}), (UV_{280}), they were measured in a 1 cm quartz cell. Color and lignosulphonic acid were measured by VIS absorbance at 440 nm (VIS_{440}) and 346 nm (VIS_{346}), respectively. The analyses were measured in a 1 cm glass cell. The pH of all measurements was 9.1 (0.2 M KH_2PO_4 buffer) (Çeçen 2003). The measurements of COD, BOD_5, color, total phenolic compounds, lignin derived, lignosulphonic and resin acid were done after membrane (0.45 μm) filtration. Resin acid likes abietic acid concentration was determined by HPLC in a Shimadzu LC-10 ATVP Liquid Chromatograph with a Shimadzu Diode Array Detector SPD-M10 AVP. Sample pH was adjusted to 3.0 ± 0.1 before extraction with dichloromethane. After the extraction, the solvents were evaporated to dryness under vacuum and the dried extracts were redissolved in methanol:water (70:30). Sample was injected into a Lichrospher-60 column under a constant flow rate of 1mL/min at 20 ^{0}C (Li et al.1996, Latorre et al. 2003). Abietic acid (75 %, Sigma) was employed as standard.

RESULTS AND DISCUSSION

Table 1 shows the physico-chemical characteristics of BKPM effluent from a local plant. The COD/BOD_5 ratio (2.9) indicates that high concentrations of recalcitrant compounds are present on it. Compounds with high molecular weight (over 1000 Da), such as lignin, do not produce BOD_5; nevertheless COD and a dark color are found. Resin acid measured as abietic acid concentration (1.45 ± 0.93 mg/L) agrees with the values found into untreated BKPM effluent (0.28 -12.1 mg/L) (Strömberg et al. 1996, Williams et al. 1996).

Table 1. BKPM effluent characteristics

Parameter	Value
pH	3.4 ± 0.17
COD (mg/L)	881.5 ± 24.3
BOD_5 (mg/L)	300.5 ± 9.5
Total phenolic compounds (UV_{215}) (mg/L)	271.9 ± 14.2
Color (VIS_{440}) (1x1 cm)	0.41 ± 0.01
Aromatic compounds (UV_{254}) (1x1 cm)	6.69 ± 0.07
Lignin derived (UV_{272}) (1x1 cm)	5.90 ± 0.08
Lignin derived (UV_{280}) (1x1 cm)	5.37 ± 0.06
Lignosulphonic acid (VIS_{346}) (1x1 cm)	1.73 ± 0.04
Resin acid (abietic acid) (mg/L)	1.45 ± 0.93

Figure 2 shows the average removal of COD, BOD_5, color (VIS_{440}) and phenolic compounds (UV_{215}) for the three different OLR employed. COD removal was ranged between 63.3 ± 10.3 % to 64.2 ± 2.82 % to AS reactor. But it ranged from 57.1 ± 7.1 % to 58.3 ± 0.36 % during the AL operation. BOD_5 removal was similar in AS and AL reactors, the values were from 95.5 ± 3.32 % to 99.2 ± 0.16 % whereas OLR increased from 0.2 to 0.6 g COD/L·d. COD and BOD_5 removal agree to others authors (Videla and Diez 1997, Mattsson et al. 2001, Diez et al. 2002, Larsson et al. 2002, Pokhrel and Viraraghavan 2004). Conversely, an efficient removal of phenolic compounds and color in an AS system were reported by Chandra (2001). However, when the OLR was 0.6 g COD/L·d, the AS reactor of this study shows no total phenolic compounds (UV_{215}) removal (-28.1 ± 11.1 %) (c). The same trend was observed for color (VIS_{440}) removal at 0.6 g COD/L·d (d). The low total phenolic compounds removal is due to polymerization process, which occurs during AS treatment (Larrea et al. 1989). In spite of that, AL removes only about 7 % of total phenolic compounds (UV_{215}) and 15 % of color (VIS_{440}) (c-d). Because of compounds determinations, mainly lignin or chlorolignins, it is possible to conclude that these compounds are recalcitrant to aerobic treatment (Çeçen 1993).

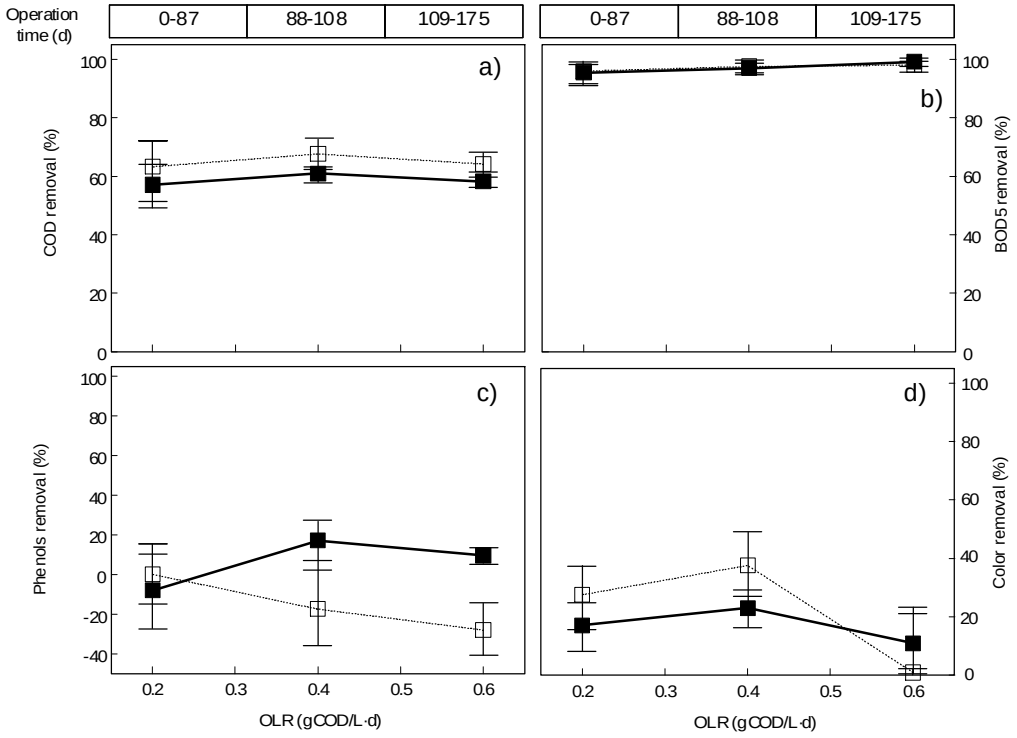

Figure 2. Performance of AS ( ), and AL ( ) reactors. a) COD, b) BOD_5, c) Total phenolic compounds (UV_{215}), d) Color (VIS_{440}) removal.

Lignins derived ($UV_{272,280}$), aromatic compounds (UV_{254}) and lignosulphonic acid (VIS_{346}) efficiency removal are shown on Figure 3. Whereas OLR increases from 0.2 to 0.6 g COD/L·d the removal of aromatic compounds (UV_{254}) (a), lignin derived (UV_{272} and UV_{280})(b-c) and lignosulphonic acid (VIS_{346}) (d) decreases. However, AS performance was slight better than AL performance. The presence of aromatic compounds (UV_{254}) on AS and AL effluents, and the low changes on lignin derived (UV_{272} and UV_{280})(b-c) after treatment, agree with previous results. Thus, low color removal (0.98 ± 21.1 %) for AS reactor (OLR: 0.6 g COD/L·d) was observed (Figure 2 d).

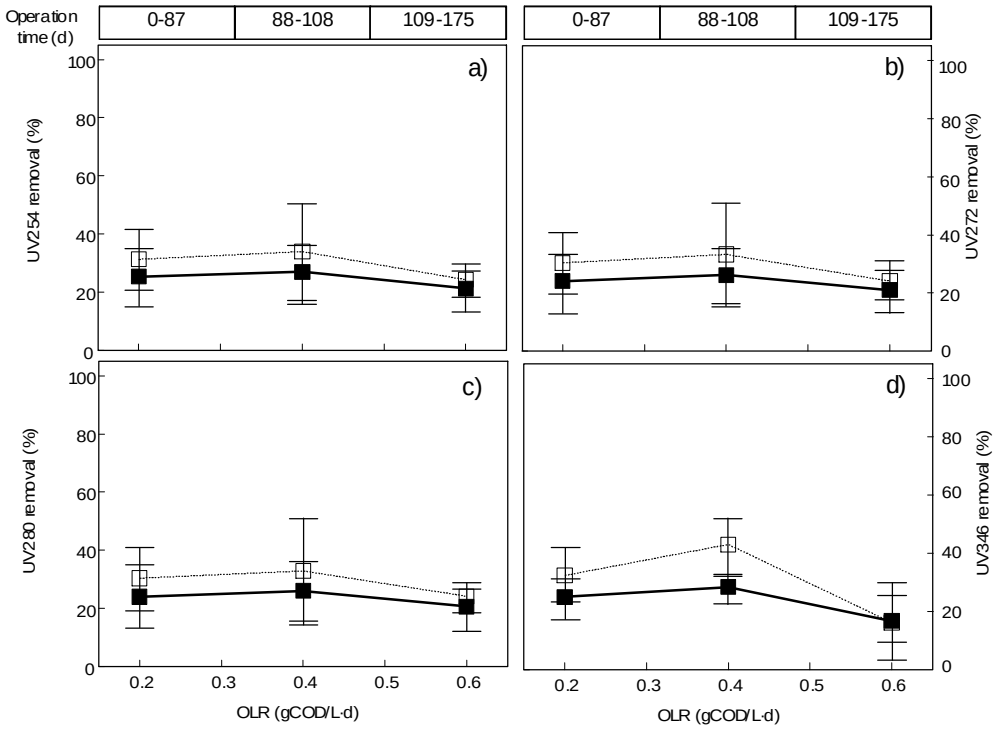

Figure 3. Performance of AS ( ), and AL ( ) reactors. a) Aromatic compounds (UV_{254}), b) Lignin derived (UV_{272}), c) Lignin derived (UV_{280}), d) Lignosulphonic acid (VIS_{346}).

The BKPM influent and effluent of AS and AL were assessing through "rec" assay. The average of the efficiency of plate was obtained thought the efficiency of plate (A); which was calculated for each dose (0.1, 0.3 and 0.5 mL) (Table 2). The "rec" assay was determined for OLR from 0.2 to 0.6 g COD/L·d. The average of the efficiency of plate (A) to 0.2 g COD/L·d, increases for AS and AL effluent. It means that genotoxicity removal was done by biological treatment. It seems to be higher for AS effluent. However, not significant difference was found between AS and AL genotoxicity removal ($p = 0.6186$).

On the other hand, when OLR was 0.6 g COD//L·d, the average of the efficiency of plate (A) for AS and AL effluents were different. Thus, while "A" of AS-effluent decreases, AL-effluent increases. However, statistically the differences of these values are not significant ($p = 0.1511$). To evaluate genotoxic effect using "rec" assay is necessary to make a dose- response curves. Figures 4 and 5 show the dose- response curves to OLR of 0.2 g COD//L·d and 0.6 g COD//L·d.

Table 2. Efficiency of plate

	Efficiency of Plate (A) Value	
OLR (g COD/L·d)	0.2	0.6
AS and AL influent	0.930 ± 0.100	0.879 ± 0.121
AS effluent	1.028 ± 0.140	0.715 ± 0.196
AL effluent	0.957 ± 0.066	0.991 ± 0.139

Figure 4 shows the dose- response curves for OLR 0.2 g COD/L·d. On the other hand, Figure 5 shows curves for OLR of 0.6 g COD/L·d. The results of AS and AL influent (a), AS effluent (b) and AL effluent (c) are presented. From 13.6 % to 8.7 % of genotoxicity was removed by AS and AL reactors respectively when OLR was 0.2 g COD/L·d (Figure 4 a-c). In this case, the influent genotoxic effect occurs mainly for low dose of it (0.1 mL), and it is because *Bacillus subtilis* rec+ grew while *Bacillus subtilis* rec- did not grow. Metabolic compounds and nutrients presents in reactors influent could be affecting the growing of *Bacillus subtilis* rec+ and rec-. Furthermore a cytotoxic effect is evidenced when rec- bacteria grew more than rec+ ones (Figure 4 b). Despite of influent genotoxicity, AS and AL effluent genotoxicity are more important to intermediate dose (0.3 mL) (Figure 4 b-c).

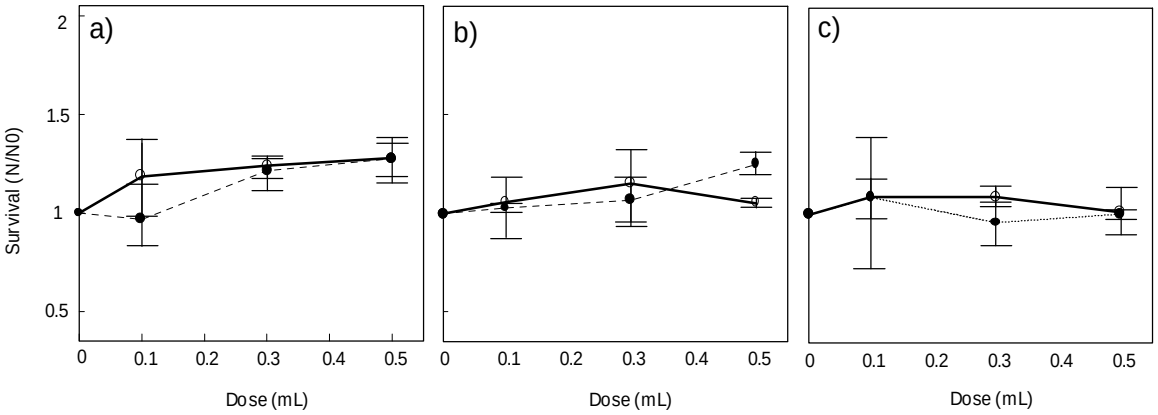

Figure 4. Dose response curves to *Bacillus subtilis* rec+ (¾) and rec- (·····) exposed to: a) AS influent, b) AL influent, c) AS effluent, d) AL effluent, during OLR of 0.2 g COD/L·d.

The genotoxic effect is better demonstrated by influent when was fed at OLR of 0.6 g COD/L·d (Figure 5 a). Because of the slope shows that rec- survival bacteria decrease when the dose increases. AS treatment do not remove genotoxicity. On the contrary, it increases genotoxicity even for low dose (0.1 mL) (Figure 5 b). In this case, genotoxic effect is due to the damages of rec- bacteria. On the other hand, AL effluent presents some genotoxicity (A= 0.64 to 0.3 mL dose), but it was specially related with rec+ bacteria growth effects (Figure 5 c).

Pérez- Alzola and Santos (1997) showed that a Chilean BKPM effluent was not mutagenic by test Ames, or erythrocytic micronuclei (EMN). However, they could contain genotoxicity, through the sister chromatic exchange (SCE) tests in “Chinese hamster ovary” (CHO) cells.

The evolution of resin acid removal was not determined during the AS or AL performance. The concentration of abietic acid on undiluted influent was range between 0.52 - 2.38 mg/L, which could contribute to genotoxic effects (Gravato and Santos 2002a). However, different authors had found that AS is able to remove resin acids totally, whereas AL could be able to remove from 96 to 98 % of resin acids (Strömberg et al. 1996, Williams et al. 1996, Kostamo and Kukkonen 2003, Kostamo et al. 2004). Despite of this, AS effluent shows genotoxicity.

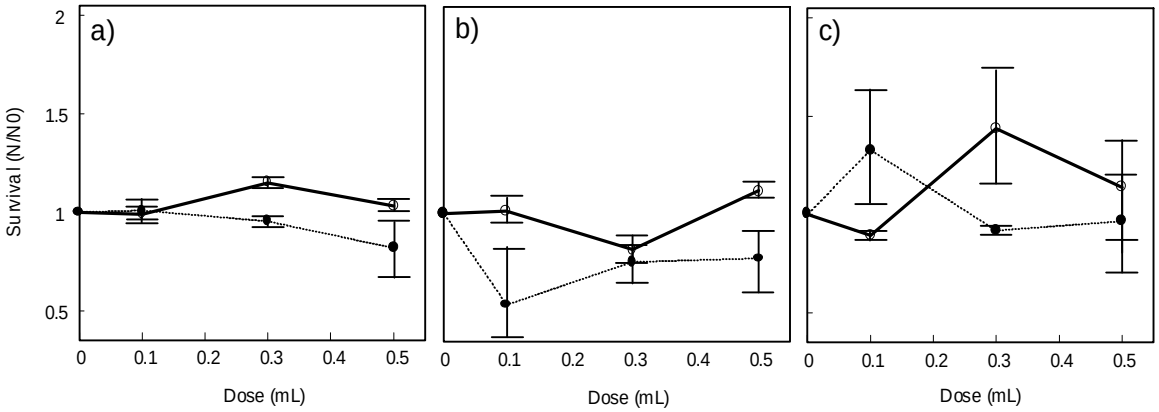

Figure 5. Dose response curves to *Bacillus subtilis* rec+ (¾) and rec- (⋯) exposed to: a) AS influent, b) AL influent, c) AS effluent, d) AL effluent, during OLR of 0.6 g COD/L·d.

AS and AL reactors remove genotoxicity as well as specific chemical compounds removal during biological treatment. At low OLR AS reactor had better performance than AL reactor. However, when OLR increase, AS reactor performance was affected. So, low removal of total phenolic compounds (-28.1 ± 11.1 %), color (0.98 ± 21.1 %) and specific compounds were observed. The low performance is expressed on genotoxicity of effluent AS values. Other assays like Ames test should be done to confirm the results obtained in this work.

CONCLUSION

Effluents from AS and AL reactors show a slight genotoxicity (A< 1.0) according to “rec” assay. AS and AL reactors remove the genotoxicity as well as specific chemical compounds removal during biological treatment. AS system shows better performance for removing genotoxicity at low OLR (0.2 g COD/L·d). However, AL was better to remove genotoxicity at high OLR (0.6 g COD/L·d).

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