Toxicity of the effluent from an anaerobic bioreactor treating cereal residues on *Lactuca sativa*

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ABSTRACT

Effluents generated during the process of anaerobic digestion should be treated before their disposal into the environment. The aim of this study was evaluating the effectiveness of the effluent treatment system from an anaerobic bioreactor, assessing the toxicity reduction with the *Lactuca sativa* seed germination and root elongation inhibition test. Three sampling points were selected along the effluent treatment system: inflow into the first treatment pond, outflow from the third pond and recirculated flow to the bioreactor. Effluent dilutions tested for each sampling point were 25% and 50% (v/v), undiluted sample and controls. The pH, conductivity, temperature, dissolved oxygen, BOD_5 and COD were measured. The decrease in the organic and inorganic loads was correlated with a reduction in the phytotoxicity. The use of the seed toxicity test allows evaluating the quality and effectiveness of the studied effluent treatment system.

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1. Introduction

The worldwide interest for the technological development of renewable energies has increased in the last decades (Flavin and Lenssen, 1994; Sawin, 2004). On the other hand, the organic waste generated by our society should be recycled in order to minimize the negative environmental impacts (Amaral, 2004; Khalid et al., 2011). There are various methods available for the treatment of organic waste, but anaerobic digestion appears to be the promising approach receiving worldwide attention (Lee et al., 2009; Karagiannidis and Perkoulidis, 2009). This technology has grown since it favors the greenhouse gases emission reduction (Mata-Alvarez et al., 2000). Therefore, the production of biogas has been proposed as a renewable energy source obtained by means of a fermentative process known as anaerobic digestion (Nallathambi Gunaseelan, 1997; Brändli, 2006; De Baere, 2006). A series of metabolic reactions such as hydrolysis, acidogenesis, acetogenesis and methanogenesis are involved in this decomposition process (Charles et al., 2009). Anaerobic digestion has been applied to various biosolids waste streams, including agricultural waste, industrial waste and municipal solid waste (Foster-Carneiro et al., 2007; Chen et al., 2008). Biogas is a mixture of gases composed by 55–70% of methane, 30–45% of carbon dioxide and fractions of water vapor (Harasimowicz et al., 2007; Deublein and Steinhauser, 2010). Typically biogas also contains < 1–17% of nitrogen, < 0.1% of oxygen, traces of hydrogen sulfide and other sulfur compounds, siloxanes, aromatic and halogenated compounds (Rasi et al., 2007). In this process of microbial transformation of organic matter, semi-liquid effluents or digestate, which must be treated before their application on soils (Kupper et al., 2008) or disposal into the environment, are also obtained (Lusk, 1998; Lindorfer et al., 2007). The digester effluents could provide the essential nutrients for plant growth when used as organic fertilizers (Zhang et al., 2007). These effluents can modify the fertility of the soil and positively influence the development and the growth of plants (Fuchs et al., 2008). Liedl et al. (2006) suggested that liquid and solid fractions of an effluent stream can be separated for using as crop fertilizers. However, elevated salt concentrations could also influence nutrient availability, competitive uptake, transport or partitioning in plants (Grattan and Grieve, 1999). Therefore, the high salinity associated with effluents used for irrigation water may pose a risk to the soil health and therefore decrease the production of pastures or crops (Ullman and Mukhtar, 2007).

Different test organisms are used in ecotoxicological monitoring or biomonitoring to assess biological effects induced by toxicants (Moriarty, 1999). The existence of sublethal effects in exposed organisms has been used as an advantage in monitoring strategies for early alert of toxicity (Kendall et al., 2001). In acute toxicity tests, organisms under a short and frequently intense exposure to a toxic compound or substance show adverse effects (Newman and Unger, 2003). Since effluents are complex mixtures of diverse substances, complexation or speciation of toxicants would be expected leading to enhancement or reduction of toxicity (Gómez et al., 2001). Biomonitoring of effluents allows evaluating their toxic loads by means of toxicity tests in addition with physicochemical parameters as an integrated approach for the assessment of impacts of discharges in the environment (Dorn and van Compernolle, 1995; Wang and Freemark, 1995). The presence of many complex chemicals in the sample makes it difficult to identify, which is the most significant factor causing damage during the testing (Tam and Tiquia, 1994). Particularly, toxicity tests with vascular plant species (EC, 2007) allow the assessment of adverse effects on seed germination and seedling development during the first days of growth (Dutka, 1989; Lewis, 1995). This type of tests allows assessing potential adverse effects on plants caused by the discharge of effluents over soil. Assessment of phytotoxicity has been used to characterize potential adverse impacts of diverse organic waste types (Pascual et al., 1997). Taking into account the previous considerations, the aim of this study was evaluating the effectiveness of the effluent treatment system from an anaerobic bioreactor, assessing the toxicity reduction with the *Lactuca sativa* seed germination and root elongation inhibition test.

2. Materials and methods

2.1. Anaerobic bioreactor and effluent treatment system

An anaerobic digestion pilot plant (Fig. 1) was designed and built in an experimental field of the Institute de Microbiology and Agricultural Zoology (IMYZA) of the National Institute of Agriculture and Cattle Technology (INTA), Castelar, Buenos Aires, Argentina (34°36′19.78″S 58°40′07.45″W). The anaerobic

bioreactor with a plug flow and 12 m^3 of working volume was loaded with an inoculum of active microorganisms extracted from a feedlot (7.26 pH, 0.65% total solids, 57% volatile solids and 0.36 volatile fatty acids/total acids ratio) as reported by Bres et al. (2009).

Aiming towards water reclaim, a treatment system allowing the reutilization of the effluent was designed. The feeding process was operated in a batch mode, with an estimated hydraulic retention time of 77 days. The anaerobic bioreactor was loaded daily with 35 kg of a mixture of fermented cereals and 125 L of treated effluents. The mixture was obtained from the residues composed of 20% of corn flour and 80% bran flour (44% total solids—loss on ignition at 105 °C, and 94% volatile solids—loss on ignition at 550 °C-), extracted from the housefly insectarium of the IMYZA, INTA Castelar. The follow-up and control of the physicochemical variables of the biodigestion process and biogas production were undertaken. The treatment system consisted of transferring the effluent from the biodigestion chamber (BC) into the discharge chamber (DC) used for solids sedimentation. The values of mean inflow into DC were 7.07 pH, 7.95 mS cm^{-1} of conductivity, 1.5% total solids and 44.3% volatile solids of the total solid fraction. The effluent was treated by physical methods in three 0.55-m^3 ponds in series (TP1, TP2 and TP3) and a 0.6-m^3 pond (SS), and then deposited in a buried tank (BT). Then, the treated effluent was elevated by pumping to a recirculation tank (RT) with 100-L capacity, where it was stored until its recirculation to the bioreactor via a feeding chamber (FC) during the feeding process.

Effluent samples were collected according to standardized specifications (APHA, 1992; IRAM, 2003) in the points marked with filled circles in Fig. 1. Sampling Point 1: inflow into the first pond (TP1); Sampling Point 2: outflow from the third pond (TP3); Sampling Point 3: recirculated flow to the bioreactor (outflow from RT). Samples were conserved at 4 °C.

2.2. Physicochemical characterization of the effluent

The physicochemical properties of the effluent were determined on the undiluted effluent. The pH, conductivity, temperature and dissolved oxygen (DO) were measured twice a week with a Thermo Orion 5 Star. The biochemical oxygen demand at 5 days (BOD$_5$) was also measured with a Hach BOD-Trak SL and the chemical oxygen demand (COD) with a Velp Eco 16 Thermoreactor using the method described by APHA (1992).

2.3. Toxicity tests

A non-chemically treated seed lot of *L. sativa* variety "divina" with 97% germination, certified by National Service of Health and Food Quality (SENASA) as an organic grown product, from the INTA, La Consulta, Mendoza, Argentina, was used for testing. The seeds were kept in a dry environment at 4 °C. Before toxicity testing, the samples were homogenized by gentle shaking (IRAM, 2003). Toxicity end points assessed were seed germination (effective concentration 50=EC50) and root elongation (relative growth index=RGI and germination index=GI). Observations of alterations in germination and normal development of seedlings were recorded. A seed was considered germinated when visible appearance of the radicle was detected. Quality controls were germination over 90% and a coefficient of variation for root elongation below 30% in control treatments (Sobrero and Ronco, 2004).

Tests were carried out in 90-mm diameter Petri dishes lined filter paper (Munktell AB Box 300, SE-790 20 GRYCKSBO, Sweden) with 10 seeds each, containing 4 mL of sample dilution or control water. Effluents tested concentrations were 25%, 50% and 100% (v/v), and deionized water was used as control. The samples were taken along the 14 weeks ($n=84$) time of process. Toxicity tests were carried out by duplicate once a week at 22 ± 2 °C in darkness for 120 h exposure, according to standardized protocols (EPA, 1989; Sobrero and Ronco, 2004).

The number of germinated seeds was used to calculate the EC50 of the effluent at each sampling point using probit analysis (Finney, 1971). Root elongation data was used to calculate the germination index (GI) according to Zucconi et al. (1981, 1985), and the relative growth index (RGI) according to Alvarenga et al. (2007) and Varnero et al. (2007). The calculation of these phytotoxicity indexes is shown in the following equations:

$$RGI = \frac{RLS}{RLC} \tag{1}$$

$$GI(\%) = \frac{RLS \times GSS \times 100}{RLC \times GSC} \tag{2}$$

where RLS is the radicle length of the sample, RLC is the radicle length of the control, GSS is the number of germinated seeds in the sample and GSC is the number of germinated seeds in the control.

The RGI values were differentiated into three categories according to the toxicity effects observed:

– Inhibition of the root elongation (I): $0 < x > 0.8$
– No significant effects (NSE): $0.8 \leq x \geq 1.2$
– Stimulation of the root elongation (S): $x > 1.2$
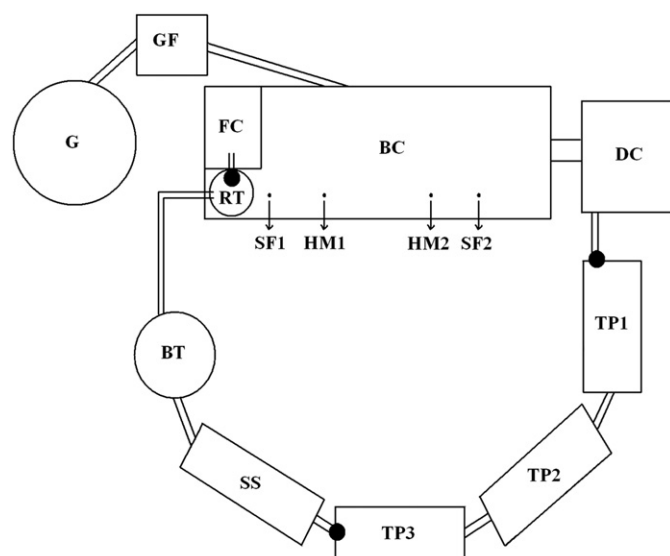
where x is the value obtained for RGI.

Fig. 1. Scheme of the anaerobic digestion experimental pilot plant with sampling points marked with filled circles (●), where BC=biodigestion chamber; DC=discharge chamber; HM1 and HM2=hand mixers; TP1=first treatment pond; TP2=second treatment pond; TP3=third treatment pond; SS=solids sedimentation pond; BT=buried tank; RT=recirculation tank; FC=feeding chamber; G=gasholder; GF=gas filter; SF1 and SF2=biodigestion chamber sampling faucet.

At the end of the exposure period, some of the seeds exhibiting a necrotic tegument were placed in deionized water and the remaining ones were transversally sectioned with a scalpel for microscopic examination. This corroborated an irreversible effect on seed germination.

2.4. Statistical analysis

Statistical analysis of data was done using a linear model for the analysis of variance (ANOVA). When the F values of the ANOVA were significant ($p < 0.05$), the means of the treatments were compared by Tukey's test. Regression analysis, principal component analysis (PCA) and correlation analysis between the toxicity indexes and the physicochemical variables of the non-diluted effluents were also carried out.

3. Results

3.1. Physicochemical characterization of the effluent

Table 1 shows the results of the physicochemical parameters analyzed at each sampling point. Both the organic and inorganic loads decreased significantly (COD by 83.41%, BOD_5 by 87.20% and conductivity by 68.35%; $p < 0.05$) from point 1 to point 3, whereas the concentration of dissolved oxygen increased by 50%.

3.2. Toxicity tests

The results of the toxicity tests were acceptable according to the criteria established by the quality controls. The coefficient of variation between the averages of radicle length in the controls was 18%, lower than that recommended in the test protocols. In Sampling Point 1, necrotic seeds were observed in all of the ones exposed to the non-diluted effluent, and an irreversible effect on germination was thus determined. The EC50 was $57.61 \pm 8.57\%$ in Sampling Point 1 and $87.79 \pm 6.33\%$ in Sampling Point 2. Besides, no seed germination inhibition was observed in the seeds exposed to the recirculated effluent to the bioreactor (Sampling Point 3).

Correlations between the toxicity indexes and the physicochemical variables were analyzed. The highest r^2 values from correlation matrix between all physicochemical variables with toxicity indexes were obtained for RGI. This indicated that RGI

was more sensitive than GI with respect to analyzed variables. In addition, a negative correlation of RGI with conductivity, BOD_5 and COD (r^2: 0.86, 0.8, and 0.72; $p < 0.05$) was observed. Table 2 shows the relationship between the categories of toxicity and the RGI values. The effluent in the Sampling Point 1 inhibited the root elongation in all treatments. However, along the process, the non-diluted sample from Point 3 did not induce significant adverse effects, showing effectiveness of treatment.

We observed an inflexion in the toxicity index trend for COD values higher than 2596 mg L^{-1} (Fig. 2A). The values above the inflexion point were not used in further correlations. Thus the
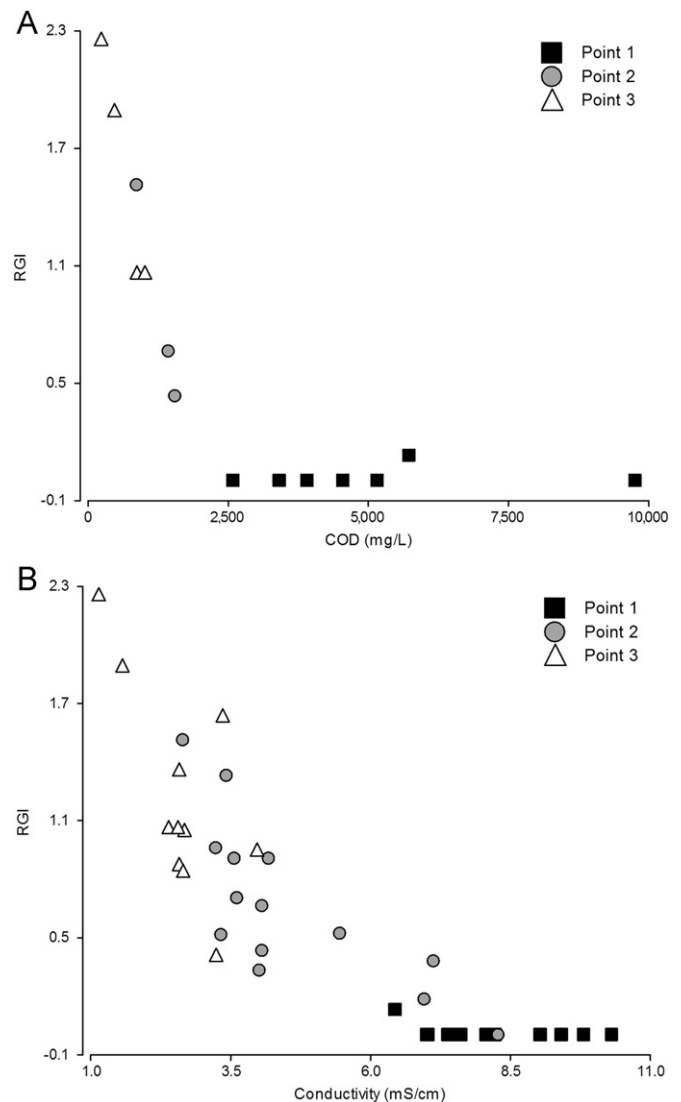
Table 1

Measured values for the physicochemical variables at each sampling point in the anaerobic digestion pilot plant.

Parameter	Point 1	Point 2	Point 3
pH	$7.6 \pm 0.2^{(a)}$	$8.1 \pm 0.1^{(c)}$	$7.9 \pm 0.1^{(b)}$
Conductivity (mS cm^{-1})	$7.9 \pm 1.5^{(c)}$	$4.7 \pm 1.8^{(b)}$	$2.5 \pm 0.8^{(a)}$
DO (mg L^{-1})	$1.0 \pm 0.9^{(a)}$	$1.5 \pm 1.5^{(ab)}$	$2.0 \pm 1.2^{(b)}$
Temperature (°C)	$22.8 \pm 3.2^{(a)}$	$21.9 \pm 3.5^{(a)}$	$23.7 \pm 2.7^{(a)}$
COD (mg L^{-1})	$4707 \pm 2059^{(b)}$	$1382 \pm 396^{(a)}$	$781 \pm 325^{(a)}$
BOD$_5$ (mg L^{-1})	$703 \pm 138^{(b)}$	$154^{(a)}$	$90 \pm 52^{(a)}$

Data are shown as mean $\pm$ S.D. Different letters (a, b, c) indicate significant differences ($p < 0.05$) among sampling points for each physicochemical parameter. Data with two letters (ab) indicate no statistical differences between this value and those marked with the same letter (a or b).

Fig. 2. Relative growth index variation with COD (A) or conductivity (B) for each sampling point of the anaerobic digestion system.

Table 2

Relative growth index (RGI) averages and toxicity categories in sampling points of process.

Treatment (tested effluent dilution % v/v)	Point 1	Point 2 (index values)	Point 3
25	0.65^a (0.41–0.89)^b I^c	1.77 (1.51–2.02) S	1.91 (1.16–2.16) S
50	0.10 (0.05–0.016) I	1.38 (1.12–1.64) S	1.73 (1.47–2.00) S
100	0.01 (0–0.03) I	0.65 (0.44–0.86) I	1.19 (0.91–1.47) NSE

^{a} Relative growth index (RGI) averages
^{b} Parentheses indicate the 95% confidence interval for the mean (lower limit–upper limit).
^{c} Toxicity categories I=inhibition; NSE=no significant effects; and S=stimulation.
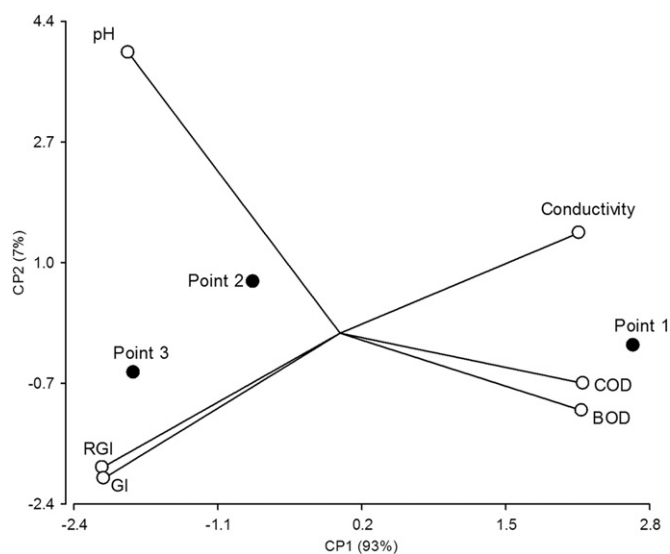
Fig. 3. Principal components analysis for physicochemical parameters, toxicity indexes and sampling points.

seed germination was inhibited in 99.29% of cases (139 necrotic seeds/140 total seeds). Therefore, the correlation between the RGI and the COD improved to $r^2 = 0.95$. However, the regression plot for this correlation (Eq. (3)) is linear between the following values of COD: 228 and 1554 mg L^{-1}. In addition, we observed an inflexion in the toxicity index trend for conductivity values higher than 7.4 mS cm^{-1} (Fig. 2B), also reaching 100% inhibition in seed germination.

$$y = -0.0013x + 2.4947 \tag{3}$$

where y being the RGI value and x the COD value.

Principal component analysis (Fig. 3) indicated that the phytotoxicity was reduced as the dissolved oxygen increased, and that this variation was associated with Sampling Point 3. The negative correlations of the toxicity indexes with conductivity, COD and BOD$_5$ associated with high values of Sampling Point 1 were also confirmed.

4. Discussion

Toxicity tests with vascular plants allow the detection of biological effects of toxicants contained in effluents, as indicated by Walsh et al. (1991). The results obtained in the present study are similar to those reported by other authors for wastewaters and raw effluents. Bazai and Achakzai (2006) also observed that the higher the concentrations of wastewaters, the lower the percentage of germinated seeds detected. However, results from this study and those obtained by the previous mentioned authors are different from those reported by Oberholster et al. (2008), who indicated a low sensitivity (5% inhibition in seed germination) of *L. sativa* seeds in effluent-receiving waters. This fact could be due to a higher content of DO and the "dilution effect" during the effluent discharge in receiving bodies of water.

In the present study, concentration of salts, BOD$_5$ and COD, were the major problems associated with effluents from the anaerobic bioreactor. Contrarily, Yetilmezsoy et al. (2009) reported no lethal effects using guppy fish (*Lebistes reticulatus*) in effluents of anaerobic bioreactors with 1:4 feed ratios, with similar values of COD and BOD$_5$ to those of Sampling Point 1 of the present study. Also, Zhang et al. (2008) recommend treatment of effluents from an anaerobic bioreactor with a COD equivalent to Sampling Point 1 (at which induction of necrosis in seeds was

detected in the present study). Finally, the inhibition of germination could be attributed to the concentration of organic matter as pointed out by Bohórquez-Echeverry and Campos-Pinilla (2007) and by the salinity of the effluents according to several reports (Tam and Tiquia, 1994; Marchiol et al., 1999; Komilis and Tziouvaras, 2009). In addition, negative correlations between conductivity and the root elongation have been also detected using seeds of lettuce (*L. sativa*), chinese cabbage (*Brassica parachinensis*) and tomato (*Lycopersicon esculentum*) by Tam and Tiquia (1994). Sánchez-Meza et al. (2007) also found negative correlations between COD and toxicity using *Daphnia pulex* and *L. sativa*. However, Filidei et al. (2003) reported positive correlations between conductivity and IG using seeds of *Lepidium sativum* exposed to olive oil mill effluents, mostly due to a lower conductivity of the treated effluents in their study. On the other hand, these authors suggested that the untreated olive oil mill effluent can be considered very phytotoxic. The phytotoxicity detected corresponds to levels of conductivity similar to those measured for Sampling Point 1 of the present study.

5. Conclusions

The present study demonstrates that the effluents from an anaerobic bioreactor may be monitored and treated before disposal into the environment or as soil amendment. The use of simple tool like the toxicity test seed inhibition of germination and root elongation with *L. sativa* seeds allowed assessing the quality and effectiveness of the effluent treatment system. This suggests that the toxicity of effluents from an anaerobic bioreactor could be reduced after treatment and potentially used for soil irrigation. In addition, treated effluents could also be used for feeding the anaerobic bioreactor, avoiding the consumption of drinking water or quality water supporting other uses. Future steps should include further studies in the adjustment of COD and conductivity guidelines, contributing to the standardization of the method, taking into account potential uses in technological applications.

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