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TITLE: IS DIGESTATE SAFE? A STUDY ON ITS ECOTOXICITY AND ENVIRONMENTAL RISK ON A PIG MANURE

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Abstract. Digestate represents a precious by-product in particular in agriculture, however its impact on the environment and human health is still unexplored. In this work, the toxicity of a pig slurry digestate was assessed through 7 ecotoxicity tests and considering 10 different endpoints. Besides, a synthetic index was applied to the outputs of the battery of tests for the environmental risk assessment, in order to evaluate the opportunity to use directly this kind of digestate in agriculture or to introduce an additional treatment. All the organisms were sensitive to digestate toxicity (EC_{50} ranged from 14.22% for *Cucumis sativum* to 0.77% for *Raphidocelis subcapitata*). The physical-chemical features at the base of this toxicity seem to be the high content of ammonium, salinity, COD, phosphate and colour. The synthetic index showed that the digestate was very toxic and associated to an extremely high environmental risk. Thus a pre-treatment is needed to reduce its toxicity and environmental impact, whatever could

be its exploitation.

Key Words: Anaerobic digestion, bioassays, bacteria, algae, phytotoxicity, crustaceans.

1. Introduction

Intensive livestock breeding produces a large quantity of biodegradable wastes that have to be managed adequately. EU Landfill Directive (1999/31/EC) has underlined the importance of waste reduction and management with sustainable methods such as recycling and composting. Since the anaerobic digestion of agriculture and zootechnical wastes is of great value both for livestock waste management and biogas production, the number of composting and anaerobic digestion plants increased in all the Europe Countries (Holm-Nielsen et al., 2009). Italy is the third country in the world for biogas production, after Germany and China, with approximately 1300 plants and 7400 Gwh produced in 2013 (Baronchelli, 2015). Benefits of anaerobic digestion basically consist in the production of biogas, and the reduction of both greenhouse gas emissions and water pollution (Möller and Stinner, 2010). On the other hand, anaerobic digestion produces the digestate, a residual material that is rich in recalcitrant organic molecules and nutrients, thus it has to be adequately managed and disposed (Provenzano et al., 2011).

In the light of Directive 2008/98/EC, which gives an adding value to wastes by means of their integrated management, digestate addition to soil is considered an appropriate option, with multiple benefits for agriculture and environment by reducing the use of mineral fertilisers (Zhang et al., 2015). However, applications of biogas digestates and their impacts on the environment and human health are still unexplored and the effectiveness of digestate as organic amendment and fertilizer is still under debate (Nkoa, 2014).

Ecotoxicity analyses of digestates before their exploitation in agriculture can predict their environmental impact and the necessity for additional treatments. Nevertheless, the few studies that have been done on this kind of samples used a limited number of bioassays and did not calculate a risk for the environment (Chen et al., 2014; Różyło et al., 2015). In ecotoxicity studies, indeed, the application of a battery of bioassays with organisms representing different positions in the food chain is essential, in order to obtain results that may realistically represent the impact on the environment. Moreover, the outputs of a battery should be summarised in a single datum, with the aim to give information about the environmental risk associated to the tested samples. This elaboration could allow to take decision for the digestate manage and use (Costan et al., 1992; Persoone et al., 2003; Canna-Michaelidou and Christodoulidou, 2008).

In the present study, the toxicity of a pig slurry digestate was assessed through 7 ecotoxicity tests and considering 10 different endpoints. Besides, the synthetic index developed by UNICHIM Water Quality Commission (UNICHIM, 2008) was used to for the environmental risk assessment, in order to evaluate the opportunity to use directly this kind of digestates in agriculture or to introduce an additional treatment.

2. Materials and Methods

2.1 Origin of samples and chemical analyses

Digestate was obtained from the effluent of an anaerobic digester, which treats pig slurry and corn, located in North West Italy. Samples of digestate liquid phase were stored at 4 °C after collection and analyzed periodically to check its stability for two months during which all the experiments were carried out. Parameters measured for the chemical characterization of the digestate were: ammonium, nitrate, total nitrogen, phosphate and COD. They were selected on account of their usual abundance and potential impact on the environment. All of them were spectrophotometrically estimated (LASA 100-HACH LANGE) according to APAT-IRSA CNR Standard Methods 2003 for nutrients and ISPRA Metodo 5135" – 2014 for COD. Moreover, pH and conductivity were measured by using the probe WTW Multi340i.

2.2 Ecotoxicity tests

Seven ecotoxicity test were selected on account of data in literature about their sensitivity to toxic substances and their low cost and easy availability also for a private company. Moreover, some of them were selected on account of their recommendation in the European legislations (i.e. Italian law Dlg 152/2006).

Vibrio fischeri strain NRRL B-11177 was bought at Ramcon A/S (Birkeroed, Denmark) and used for the test of luminescence inhibition (UNI EN ISO 11348-3) with Microtox® toxicity system (Microtox Model 500; Microbics Corp., USA) as described by Tigini et al. (2011). The luminescence intensity in all cuvettes was measured before the addition of the wastewaters and after 15 and 30 min exposition and automatic colour correction was performed. A computer program for Microtox Acute Toxicity Test (Azur Environmental Ltd., UK) was used for the data elaboration.

Raphidocelis subcapitata (Korshikov) Nygaard et al., originating from Agenzia Regionale per la Protezione dell'Ambiente (ARPA Piemonte, Grugliasco, TO), was used for the algal growth inhibition (UNI EN ISO 8692:2005). The tests were performed as described by Tigini et al. (2011), and data were elaborated using ToxCalc^{TM} 5.0.

The aquatic plant *Lemna minor* L. was used for the assessment inhibition of both biomass dry weight and frond number (ISO SO/WD 20079). The test was performed as described by Casieri et al. (2008).

Cucumis sativus L. and *Lepidium sativum* L. were used for phytotoxicity tests (UNICHIM N. 1651, 2003). Seeds were purchased from Blumen Group S.p.A. (Piacenza) and the test was performed as described by Tigini et al. (2011).

Daphnia magna Straus, cultured at ARPA Piemonte, was used for the immobilisation test (UNI EN ISO 6341:99). The tests were performed as described by Tigini et al. (2011), and immobile animals were counted after both 24 h and 48 h.

In the *Artemia franciscana* L. bioassay, after a preliminary test, 3 dilutions were chosen with 3 replicates each and 3 repetitions were used for the control. Three dilutions of 100 mg *A. franciscana* cysts were placed in a Petri dish (5 cm diameter) for hatching, containing 12 mL of saltwater and incubating for 48 h at 25 °C in the dark (changing saltwater after 24 h). After the incubation, 10 instar I and II nauplii were inoculated in 1 mL of sample, or saltwater for the control, for each replicate. Nauplii were incubated for 24 h at 25 °C in the dark, after that the nauplii mortality was assessed.

The sensitivity of the test organisms cultivated in directly in laboratory (*D. magna*, *L. minor*, *R. subcapitata*) was periodically assessed with a potassium dichromate solution ($K_2Cr_2O_7$).

Results of ecotoxicity tests were plotted on a dose-effect chart; the EC_{50} and its confidence limits ($p = 0.05$) and toxic units ($100/EC_{50}$) were estimated using standard procedures.

2.9 Synthetic index and ecotoxicological risk assessment

The synthetic index was developed by the Associazione per l'unificazione nel settore dell'industria chimica (UNICHIM) Commissione Qualità dell'Acqua, Gruppo di Lavoro Metodi Biologici, Sottogruppo Acque salate/salmastre e Sedimenti, Gruppo ad hoc Batterie, scale di tossicità e indici integrati. It is a modification of the model proposed by Hartwell (1997), and described by Baudo et al. (2011). This synthetic index allows to compare the results of ecotoxicity tests batteries through a toxicity score (BTS), that represents the mean of the relative toxicity of each test ($RT_{endpoint}$). This last parameter is calculated as follows:

$$RT_{endpoint} = 100 - 100 \cdot \frac{[\log (C \cdot EC_x) \cdot R \cdot S]_{max} - [\log (C \cdot EC_x)\, R \cdot S]_{endpoint}}{[\log (C \cdot EC_x)\, R \cdot S]_{max}} \qquad [1]$$

where C is a statistical corrective (C = 2 if the EC_x is higher than 100%; C = 1 if the EC_x and its 95% confidence limits are lower than 100%); S is a score depending on the considered endpoint (mortality = 8; bioluminescence = 7; development = 6; reproduction = 5; growth = 4; genotoxicity = 3; mutagenicity = 2, behaviour = 1); R is the rank of toxic concentrations and it is assigned from the lowest concentration to the highest one.

The synthetic index evaluates also the risk score of the battery (BRS) on the base of the consistence hat indicates the agreement rate among different endpoints, and is calculated with this formula:

consistence = $[(N/2)-X]^3$ [2]

where N is the number of the endpoints and X is the number of not significant (i.e. the EC_x is not calculable).

Thus, the higher the consistence the higher the risk score. Moreover, the total relevance is calculated as a percentage on the base of the severity of the kind of the considered endpoint, which affects the results with different weight.

Both the toxicity score and the risk score of the battery are converted in a scale based on the expert judgment (Baudo et al., 2011).

Eventually, the ECx (= $10^{(average(log(ECx))}$) and its confidence limits for the battery is calculated as a synthetic result.

3 Results

3.1 Chemical characterisation

Results of chemical analyses are reported in Table 1. Digestate was characterized by alkaline pH, a high conductivity, and high value of COD, phosphate and nitrogen. Most of the nitrogen was present as ammonium (61%).

Moreover the sample was characterised by a deep brown colour.

3.2 Sensitivity of bioassays

Results of ecotoxicity tests, expressed as inhibition percentage of each endpoint are reported in Figure 3. These data were also elaborated calculating the EC_{50} and its confidence limits (Table 2). The 10 endpoints, recorded by means of the 7 species representing different positions in the food chain, showed different sensitivity to the sample. The most sensitive organism was *R. subcapitata* with an EC_{50} of 0.77% and TU of 129.87. Then, *V. fischeri* (30') and *L. minor* (both fronds and biomass development) followed, with EC_{50} in the range of 1.02-1.77% and TU in the range of 58.82-98.03.

D. magna reported a result of all-or-nothing effect, and the threshold dose was ranging between 3.13% and 1.56%. Thus, these values were reported as the possible range of EC_{50}, and consequently TU of 32.2-62.5.

Another group of tests, *V. fischeri* (15'), *L. sativum* and *A. franciscana*, showed EC_{50} between 3.03% and 3.90%, and TU between 25.64 and 33.04.

Eventually, *C. sativus* was the lowest sensitive organism, with an EC_{50} of 14.22% and TU of 7.03.

3.3 Digestate toxicity and risk assessment

According to the synthetic index, all the 10 endpoints were significant; thus, they all contributed to the evaluation of the synthetic index. The EC_{50} of the battery was 2.33%, with the 95% CL_{low} of 1.88% and 95% CL_{up} 3.15%.

The relative toxicity of the digestate sample ranged from 10% to 100%, for *C. sativus*, *A. franciscana* and *V. fischeri* (30') (Figure 1).

The digestate showed a toxicity score (BTS) of 42.8% that corresponds to a very high toxicity value. Both the total relevance and the consistence of the battery were calculated, too. They were 64% and 100%, respectively. Consequently, the risk score (BRS) of the battery was 55.3% that corresponds to an extremely high risk (Figure 2).

4 Discussion

4.1 Chemical characterisation

Chemical characteristics of a digestate depend basically on the raw material directed towards the digestion process (Provenzano et al., 2011). The analysed sample can be considered representative of pig manure and energy crops digestate for the N content. Actually, it has values of total nitrogen and ammonia in the average of digestate from pig manure and energy crops, which are 2.5-4.5 kg t^{-1} of total nitrogen and 40-65% of ammonia (Rossi and Mantovi, 2012). On the contrary phosphate is higher than the average of this kind of digestate, which generally is 1-2.2 Kg t^{-1} (Rossi and Mantovi, 2012). Our digestate showed a slightly higher or comparable nutrient concentration even in comparison with pig slurry digestate in other international studies (De la Torre et al., 2000; Kumar et al., 2010; Park et al., 2010; Alburquerque et al., 2012).

On the contrary, with respect of cattle slurry and raw cheese whey digestate, the pig manure digestate analysed in this study presents a higher ammonium, nitrate, phosphate and TN, but a lower content of COD (Franchino et al., 2013). Digested dairy manure can have a lower phosphate, a higher COD and a nitrogen content comparable with our data (Wang et al., 2010), whereas digested poultry manure showed a lower N:P ratio (Cai et al., 2013; Yetilmezsoy and Sakar, 2008).

4.2 Bioassay sensitivity and possible cause of toxicity

The most sensitive organism for ecotoxicity assessment of digestate was the green alga *R. subcapitata*. At the best of our knowledge, this is the first time that this organism has been used as bioassay to test digestate toxicity, despite this organism is used as a routine test for ecotoxicity assessment (Tigini et al., 2011). Algae are also exploited for biofuel production from digestate (Uggetti et al., 2014). Thus, from an applicative point of view, this result is of particular interest: actually, if digestate induces an inhibition effect on alga growth, this implies the need of a pretreatment of the digestate or its dilution in order to achieve a good algal growth (Erkelens et al., 2014).

This alga has been indicated as the most sensitive organism also toward textile wastewaters (Novotný et al., 2006; Tigini et al., 2011, Bedoui et al., 2015). This kind of effluents have deep colour in common with digestate and this could be one of the reasons at the base of alga sensitivity. Actually, the sample colour has an inhibitory effect on photosynthetic organisms (Cleuvers and Ratte, 2002).

However, besides the colour, other physical-chemical aspects of the digestate must have a role in the inhibition of photosynthetic organisms, since also *L. minor* and *L. sativum* were sensitive towards the sample. Actually, in these tests the plants are not affected by the sample colour in their photosynthetic activity.

The high ammonium ion concentration could be a key factor in the toxicity of digestate. In landfill leachate with comparable ammonium concentration (2266 mg L^{-1}), it was likely the most important factor of toxicity towards *R. subcapitata*, *L. sativum* and also towards crustaceans (Tigini et al., 2014). Moreover, ammonium is the main cause of toxicity for the alga *Nephroselmis pyriformis* in industrial effluents (Källqvist and Svenson, 2003).

This fact could be at the base also of the high sensitivity of *D. magna*. This crustacean showed an all-or-nothing response, with a nonlinear relationship between dose and effect. *D. magna* showed the same behaviour also towards textile effluents (Bedoui et al., 2015). This indicate that this organism has a sort of resistance that acts as homeostatic compensation of the toxic effect of pollutant, up to a threshold limit that represents the extreme condition for its survival (Calow and Forbes, 2014).

Another factor of toxicity could be the salinity of the sample, which actually had high conductivity. In this case marine organism should be less sensitive with respect to fresh water and soil organisms. Nevertheless, *A. franciscana* and *V. fischeri* showed a high sensitivity towards the sample, too. In particular *V. fischeri* was very sensitive towards the digestate, and the bioluminescence inhibition was proportional to the exposition time. This species was already tested towards pig slurry digestate, showing an EC_{50} around 6.8% after 15' exposition (Chen et al., 2014), resulting less toxic than the actual pig digestate (EC_{50} 3.9%). Paying attention to the chemical features of the two samples, the actual digestate presents higher conductivity, COD and phosphate (27 mS cm^{-1} and 17600 mg L^{-1}, 319 mg L^{-1}, respectively), with respect to the digestate analysed by Chen and colleagues (10 mS cm^{-1} and 2667 mg L^{-1}, and 152 mg L^{-1} as TP, respectively). On the contrary, ammonium ion was lower (230 mg L^{-1} *vs* 845 mg L^{-1}, respectively). This suggests that ammonium is not the main cause of toxicity towards *V. fischeri*.

Eventually, the less sensitive test was that with *C. sativum*. However, the results indicate still high toxicity, since the EC_{50} was lower than 25% (Mekki et al., 2008). From literature, it is evident that high concentration of ammonium that characterises animal manure inhibits both the seed germination and the root elongation (Wong et al., 1983). Surprisingly, Gell et al. (2011) found no phytotoxicity in a pig manure digestate but they did not specify the ammonium concentration. The absence of digestate phytoxicity was recorded also by other authors, but the conditions were only partially comparable with the present work. Actually, some studies were performed in vivo (Różyło et al., 2015), other applied different exposition periods (Alvarenga et al., 2015) or other kinds of digestate (Massaccesi et al., 2013). In a most recent study, the use of proper concentrations of digestate as a biofertiliser is encouraged, since it causes biostimulation under 20% dose (Pivato et al., 2015).

4.3 Synthetic index and risk assessment for the evaluation of digestate exploitation

The applied synthetic index for toxicity and risk evaluation allows to describe a valid forecast of the environmental impact of the considered samples. This method attributes different weights to the endpoints on the base of an expert judgment. Thus the endpoints affect with different weight to the toxicity score attributed to the battery. On the basis of this elaboration, the impact of the tests with *L. sativum*, *C. sativus,* and *L. minor* increased, because the root development is characterised by high weight (= 4), soon after the bioluminescence (= 5), and before the algal reproduction (= 3), and the crustaceans behaviour (= 1). In the light of the possible use of digestates in agriculture, this evaluation of phytotoxicity tests acquires an additional importance.

The high toxicity and the extremely high risk associated to the sample indicate a serious danger for the environment associated to pig slurry digestate. This datum is alarming and confirms what underlined by Nkoa (2014) about the danger of digested animal manures. Actually, he observed that anaerobic digestates have a higher potential to harm the environment and human health than undigested animal manures and slurries, on account of their higher NH_3 emission potential.

Despite the N content in digestate, basically consisting of ammonium, can be easily up-taken by plants, its high toxicity effect can be explicated for many months (Różyło et al., 2015). Thus, the products of anaerobic digestion must satisfy certain level of stability and hygiene in order to fulfil sustainability criteria of reuse in agriculture, and this could be achieved by digestate dilution or pre-treatment (Alburquerque et al., 2012, Chen et al., 2014). This is the case of the actual digestate. An algal pretreatment aimed to biofuel production could represent a solution. Actually, besides allowing the achievement of the needed quality, it could lower the impact of the overall producing chain. However, on account of the high toxicity towards algae, a feasibility study should be performed. In alternative (or in addition) a pre-treatment with fungal biomasses could be a possible tool to reduce the toxicity of recalcitrant molecules or the colour, which are the two main cause of toxicity of digestate towards algae. Actually, fungal biomasses are able to decolourise very toxic wastewater coupling a decrease of the toxicity (Anastasi et al., 2011; Spina et al., 2014).

5. Conclusions

In this work, the toxicity of a pig slurry digestate was assessed through 7 ecotoxicity tests and considering 10 different endpoints. Besides, a synthetic index was used to summarise the obtained results for the environmental risk evaluation, in order to consider the opportunity to use directly this kind of digestates in agriculture or to introduce an additional treatment. All the organisms were sensitive to digestate toxicity. The chemical features at the base of this toxicity seem to be the high content of ammonium, salinity, COD, phosphate and colour. A more deep chemical characterisation (organic contaminants, metals, phenols) could give additional information about the cause of this high toxicity. According to the synthetic index the digestate was very toxic and was associated to an extremely high environmental risk. A digestate pretreatment is needed to reduce its toxicity and environmental impact.

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