Long-term amendment of Spanish soils with sewage sludge: Effects on soil functioning

Neus Roig [a,b], Jordi Sierra [a,c], Esther Martí [c], Martí Nadal [b,*], Marta Schuhmacher [a,b], José L. Domingo [b]

[a] Environmental Engineering Laboratory, Departament d'Enginyeria Química, Universitat Rovira i Virgili, Av. Països Catalans 26, 43007 Tarragona, Catalonia, Spain
[b] Laboratory of Toxicology and Environmental Health, School of Medicine, Institut d'Investigació Sanitària Pere Virgili (IISPV), Universitat Rovira i Virgili, Sant Llorenç 21, 43201 Reus, Catalonia, Spain
[c] Laboratori d'Edafologia, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Catalonia, Spain

A B S T R A C T

Wastewater treatment processes generate highly biodegradable solid wastes. As their fate is an environmental issue of great concern, public administrations promote a sustainable management of urban wastes. The objective of the present study was to analyze the systematic and periodical use, for 16 years, of anaerobically digested sewage sludge as an agricultural fertilizer by assessing the effects on some soil physical–chemical, functional, and ecotoxicological properties. The results showed that the input of sludge enhances soil properties proportionally to the application doses and/or frequency. The organic amendments increased the organic matter content (and its aromaticity), the soil nitrogen, and the microbial activity, improving carbon and nitrogen mineralization processes and some enzymatic functions. However, a maximum dose was identified (40 Mg ha^{-1} year^{-1}), beyond which soil properties do not improve, and may even worsen. Regarding environmental risks, although the bioluminescent bacteria test showed no toxicity on soil extracts, potential adverse effects such as some potentially toxic elements accumulation, phytotoxicity and the likelihood of groundwater pollution by nitrates or dissolved organic matter should be taken into account. The complementarity of studying soil functioning parameters and ecotoxicological effects, together with the analysis of pollutant content, must be enhanced. This assures a more realistic assessment of long-term effects of sewage sludge-amended soils.

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1. Introduction

The total quantity of sewage sludge production in the EU27 is currently estimated in more than 10 million tons (dry solids), of which nearly 40% is spread on land for agricultural use. Agriculture recycling of sewage sludge varies greatly among Member States (Milieu Ltd., 2008). In Spain, sewage sludge generation has significantly increased in recent years (64% in the period 1999–2005), 65% of sewage sludge is recycled through agricultural soils (MMA, 2008). Although this proportion may be similar across the EU, the situation is different in certain countries (Milieu Ltd., 2008) such as the Netherlands, where the development of stringent policies has actually resulted in an effective ban on using sewage sludge for agriculture. Alternatively, sewage sludge may be differently used. Some of these options are land reclamation, horticulture and landscaping, industrial processes, or energy recovery (Alabaster and Leblanc, 2008; Rovira et al., 2011).

Sewage sludges are composed mainly of organic matter, nutrients, pollutants, and micro- and mesofauna. Amending soil with these materials improves soil properties such as organic matter, nutrients content, soil porosity, bulk density, aggregate stability, and water holding capacity (Singh and Agrawal, 2008; Annabi et al., 2011). Sewage sludge materials, as organic amendments, cause initial enhancement in the soil microbial activity and biomass, as well as in the soil biochemical activity, due to higher organic matter and nutrients availability (Marschner et al., 2003). Mediterranean agricultural soils are poor in organic matter. Therefore, the use of this type of materials may be a good option for the management of these wastes. Among the beneficial aspects, the amelioration of soil physicochemical and nutritional properties has been reported (Singh and Agrawal, 2008). Moreover, the International Panel on Climate Change (IPCC) recognizes that over the past few centuries (especially the recent one), agricultural soils have lost more than one-half of their organic matter. Returning carbon in the form of sludge, animal manures, and composts could improve soil quality and crops while reducing carbon in the atmosphere (Soriano-Disla et al., 2010; Powlson et al., 2011).

There are some drawbacks derived from this practice (Achiba et al., 2009; Ippolito et al., 2009). The persistence and accumulation of persistent organic pollutants (POPs) and potentially toxic

* Corresponding author. Tel.: +34 977 758 390; fax: +34 977 759 322.
E-mail address: marti.nadal@urv.cat (M. Nadal).

elements (PTE) from sewage sludge may have notable consequences for the quality of the human food chain, plant health, and soil microbial processes (Smith, 2009; Passuello et al., 2010). Unfortunately, guidelines and legislation often refer to total contaminant burdens, while they do not consider information on what proportion of this total amount may be biologically available to organisms (Alvarenga et al., 2007; Barral and Paradelo, 2011). With regard to this, the application of complementary techniques, as toxicity bioassays (*i.e.*, germination tests), are recommended methods for the assessment of ecological risks in soils or other matrices (Alvarenga et al., 2007; Roig et al., 2011). As the disposal of sewage sludge on agricultural fields may result in an increase of the pollutants concentration in soil, the optimization in the dose and frequency of application to avoid an overload of contamination and the health and environmental risks derived (Passuello et al., 2012), is clearly desirable. Besides agronomic criteria, environmental restrictions play a role in the practical application of sewage sludge. Oleszczuk (2006) stated that the preferred method should be a one-year system, with a sludge application of ≤ 75 Mg ha^{-1}, allowing the degradation of polycyclic aromatic hydrocarbons (PAHs) contained in the sludge. For preventing water pollution by nitrates, a long-term rate application of 22 Mg per ha and year could be sustainable (Jin et al., 2011). Regarding possible ecotoxicological effects, Domene et al. (2008) estimated that the safe amendment rates of anaerobic sewage sludges should be between 2.7 and 13.9 Mg ha^{-1}, and Carbonell et al. (2009) observed different toxicity levels when applying sewage sludge, starting on 30 Mg ha^{-1} in a mesocosm study.

This study was aimed at assessing the effects of a repetitive application of sewage sludge for 16 years in a controlled field experiment. Physico-chemical characteristics, metals content, soil functioning properties related to carbon and nitrogen cycles, enzymatic activities, ecotoxicological tests and plant growth ratios were closely studied. Furthermore, the risk of aquifer contamination by nitrates or organic matter was also considered. Finally, the optimal dose and frequency of sewage sludge application was established in terms of lack of ecological risks.

2. Materials and methods

2.1. Experimental design

The field study was carried out in wheat experimental fields in Pamplona (N of Spain). Sewage sludge was produced in an urban wastewater treatment plant, with primary and secondary treatments. Sewage sludge was stabilized through anaerobic digestion and mechanical dewatering. Sewage sludge characteristics are summarized in Table 1. The soil used was clay-loam-textured (31% clay, 30% silt, 39% sand), rich in carbonates (24%), poor in organic matter (1% oxidizable carbon), and alkaline pH, corresponding to a Calcaric Fluvisol (FAO-UNESCO, 1998). The experimental field consisted of different plots, where sewage sludge had been continuously applied for 16 years (from 1993 to 2009) at different doses and frequency (eight treatments in total) (Table 2). Thirty-two composite soil samples (depth: 0–25 cm) were collected from 16 plots, each covering a surface a 35 m^2, in a completely randomized block design. Four different samples were taken from each different treatment. Control soils without sewage sludge or fertilizer, and conventional mineral amendment were included. Mineral fertilization was done by the application of 80 kg P ha^{-1}, before sowing, and 180 kg N ha^{-1}, divided into two applications along the wheat growing period. Soil sampling was carried out in July 2009, at least 6 months after applying sewage sludge.

2.2. Physico-chemical parameters of soil samples

Soil properties were determined by the usual soil characterization methods (Page et al., 1982). The pH in 1 N KCl (1:2.5) and pH and electrical conductivity (EC$_{25}$) in aqueous extracts (1:2.5) were measured. Oxidizable organic carbon was determined by the Walkley–Black method. Total Kjeldahl nitrogen (TKN) was determined by Kjeldahl digestion followed by ammonia distillation. Mineral nitrogen fractions were determined in 1 N KCl 1:10 extracts of the soils, and quantified by distillation (N-NH$_4^+$), and colorimetrically (NO$_3^-$), the latter according to US EPA method 352.1. The dissolved organic carbon (DOC) was analyzed in 0.01 N CaCl$_2$ soil 1:5 extracts, determining the total organic carbon (TOC) by means of a Multi N/C 3100 analyzer (with prior removal of inorganic carbon with HCl). In order to assess the organic matter quality (aromaticity degree), the specific absorbance of UV (SUVA$_{254}$) was measured by means of a UV–vis spectrophotometer according to US EPA (2005). Soluble phenolic compounds were analyzed in a 0.01 N CaCl$_2$ extract soil (ratio 1:5) and subsequently determined by the colorimetric method of Folin–Ciocalteau (Sierra et al., 2007). For potentially toxic elements analysis, 0.5 g dried soil samples were treated with HNO$_3$ (65% Suprapur, E. Merck, Darmstadt, Germany) in a Milestone Start D Microwave Digestion System (Milestone Srl, Bergamo, Italy) for 10 min until reaching 165 °C, and kept at this temperature for 20 min. After cooling, the extracts were made up to 25 mL with ultrapure water. The analytical determination was performed by means of inductively coupled plasma mass spectrometry (ICP-MS) for As, Cd, Cr, Cu, Hg and Pb, and inductively coupled plasma atomic emission spectroscopy (ICP-OES) for Mn, Ni and Zn. Blanks, control samples, and certified reference materials (CRM 052, loamy clay, Resource Technology Corporation US,) were used for quality control/quality assurance (Nadal et al., 2011). The recovery percentages of standards ranged from 62 to 91%.

2.3. Biochemical and ecotoxicological parameters

For the microbial activity assessment, soil samples (25 g) were incubated in manometric respirometers, which allow the determination of the sample oxygen consumption (Oxitop® OC 110, WTW GmbH, Weilheim, Germany). Samples were kept at 25 °C in the darkness, in an incubator equipped with a thermostat for 21 days. Oxygen consumption was periodically monitored. Cumulative respiration (CR) was determined by the cumulative oxygen consumption at the end of the incubation period. Once the incubation was completed, substrate induced respiration (SIR) was determined according to the OECD 216 carbon transformation test method (OECD, 2000a). Briefly, an aqueous solution equivalent to 4000 mg glucose per kg of soil was added to the incubated samples, and the oxygen consumed during the subsequent 12 h was determined. Basal respiration (BR) rate was estimated as the average hourly respiration rate over the last 5 days of incubation when the respiration was stable. The respiratory activation quotient (Q_R) was calculated, dividing BR by SIR (OECD, 2000b; ISO, 2002). Nitrogen mineralization potential was quantified by the OECD 217 method (OECD, 2000b). According to it, soil samples with added alfalfa meal and H$_2$O (equivalent to the 50% of the soil water holding capacity) were incubated at 25 °C for 28 days. Once completed, nitrates were determined in 0.1 N KCl extracts according to the US EPA method 352.1 (US EPA, 1971). Urease activity (UA) was quantified by the method of Kandeler and Gerber (1988), based on the determination of ammonia released during the incubation of soil at 37 °C for 2 h. Dehydrogenase activity (DH) was determined according to García et al. (2003). A germination elongation test was performed to assess the phytotoxicity of the amended soils. The test was done by using *Allium cepa* (onion) and *Raphanus sativus* (radish) seeds (monocotyledon and dicotyledon, respectively), using 5 seeds per

Table 1
Sewage sludge characteristics.

Physico-chemical characteristics		PTE concentrations (mg kg⁻¹)		Levels of organic pollutants	
Dry matter	20–25%	Zn	800–900	PCNs (mg kg⁻¹)	0.04–0.07
Organic matter	50–60% dm	Pb	100–150	PAHs (mg kg⁻¹)	1.41–5.63
C/N	7–10	Cu	200–300	PBDEs (mg kg⁻¹)	0.08–0.32
pH	7.5–8.0	Cr	100–150	PCBs (mg kg⁻¹)	0.26–1.35
N_t	3.0–3.5%	Ni	40–60	NPE (mg kg⁻¹)	4.27–321
P_2O_5	5.0–6.0%	Cd	<0.1	PFOS (μg kg⁻¹)	ND–10.5
K_2O	0.5–0.6%	Hg	<0.1	PFOA (μg kg⁻¹)	ND
MgO	0.7–0.8%				
CaO	10.0–12.0%				

ND: no detected; PTE: potentially toxic elements; N_t: total Kjeldahl nitrogen; PCN: polychlorinated naphthalenes; PAHs: polycyclic aromatic hydrocarbons; PBDEs: polybrominated diphenyl ethers; PCBs: polychlorinated biphenyls; NPE: nonylphenol ethoxylates; PFOS: perfluorooctane sulfonate; PFOA: perfluorooctanoic acid.

pot (soil amount: 15 g), in quadruplicates. After 14–28 days, the plants shoot lengths were measured according to OECD guideline 208 (OECD, 2006). Bacterial (*Vibrio fischeri*) bioluminescence inhibition acute bioassays (Microtox®) were conducted on soil NaCl 2% aqueous extracts (1:2) following the 90% basic test for aqueous extracts protocol (Azur Environmental, 1999).

2.4. Data treatment

Statistical analysis of the data was done by means of SPSS 13.0 (SPSS Inc., Chicago, USA). In order to determine the statistical significance of the differences between soil treatments and controls, an ANOVA followed by Duncan's *post hoc* test ($p < 0.05$) was executed on the results of the experiments. Complementarily, Pearson correlations were applied to determine the relationships between variables.

3. Results

Data from the physicochemical characterization of soil samples subjected to different treatments (sewage sludge amended and controls) are shown in Table 3. No significant differences were noted between the different treatments regarding pH-H_2O values. Despite that, a slight tendency to boost the acidification of soils was observed when sewage sludge was applied. The pH-KCl results showed a tendency to acidification of the soil exchange complex in the sewage sludge amended samples, contrasting with the mineral control. The amendment with sewage sludge produced an increase of the EC_{25} values. However, it did not imply the salinization of soil ($EC_{25} < 4000\,\mu S\,cm^{-1}$). The organic carbon concentrations and total nitrogen in the samples increased with the application of sewage sludge, whereas the mineral fertilization did not affect organic carbon concentrations of soil. No significant differences in N-NH_4^+ levels between the amended and control soils were found. Extractable nitrates concentrations were significantly higher in all sewage sludge treatments than in the control and mineral fertilizer plots. The concentrations of DOC and $SUVA_{254}$ values, as well as phenolic compounds concentrations, increased in the sewage sludge amended samples. Table 4 summarizes the concentrations of potentially toxic elements in soils. The levels of Hg, Mo, Zn, Cu, Cr and Pb were significantly higher in amended soils than in control plots. In turn, no differences were observed between both control sites (with or without mineral amendment). Furthermore, potentially toxic elements levels were well below the regulatory limits set by national and international guidelines.

The carbon mineralization process was, in general, enhanced by the long-term application of sewage sludge, especially regarding SIR parameters (Fig. 1). Nitrogen mineralization also increased significantly in all the amended samples (sewage sludge and mineral). The tested enzymatic activities showed that, although DHA tended to increase in most cases, UA was not significantly affected by the amendment (Fig. 2). Regarding the *A. cepa* germination-elongation test, the sewage sludge application produced a decrease in the germination-elongation rates, being this diminution only significant in the treatments 40/1 (40 Mg ha⁻¹ year⁻¹), 80/2 (80 Mg ha⁻¹ every two years) and 80/1 (80 Mg ha⁻¹ year⁻¹). In contrast, the sewage sludge amendment did not produce a clear effect on germination-elongation of *R. Sativus* (Fig. 3). Finally, no differences between amended samples and controls were observed in the Microtox® test results, as none of the treatments showed toxic levels ($EC_{50} > 1000\,mg\,soil\,mL^{-1}$ extract).

4. Discussion

When the correlations between parameters were analyzed (Electronic Supplementary Material), it was observed that the acidification of the exchange complex (pH-KCl) might be related to the organic matter mineralization (C and N mineralization) due to sewage sludge application. Significant correlations were noted ($p < 0.01$) between the decrease of pH-KCl and the organic matter mineralization (CR, SIR, N mineralization, DHA). Since the area of study was a carbonate rich soil capable to buffer the free acidity in the soil solution, the acidification could not be seen in the H_2O extracts. A pH decrease effect of long-term sewage sludge application was also observable in acidic soils with lower buffering ability, as previously reported (Enwall et al., 2007). The differences between pH-H_2O and pH-KCl showed a tendency to the salinization in the controls (base saturation of the soil exchange complex). This trend seemed to be compensated by the application of sewage sludge. The pH-KCl decrease showed a stronger correlation with the

Table 2
Details of the amending plan and treatment references.

Treatment	Sludge (Mg ha⁻¹ year⁻¹)	Frequency	Total applications	Total amount sewage sludge (Mg ha⁻¹)
C	0 (control)	–	–	–
CM	0 (control + mineral fertilizer)	Every year	–	–
40/4	40	Every 4 years	4	160
40/2	40	Every 2 years	8	320
40/1	40	Every year	16	640
80/4	80	Every 4 years	4	320
80/2	80	Every 2 years	8	640
80/1	80	Every year	16	1280

Table 3
Physicochemical characterization of the soil control and treated samples.

	C	MC	40/4	40/2	80/4	40/1	80/2	80/1
Total SS (Mg ha^{-1})	0	0	160	320	320	640	640	1280
pH$_{H_2O}$	7.67	7.69	7.66	7.67	7.62	7.52	7.59	7.56
pH$_{KCl}$	7.33[d]	7.32[cd]	7.16[ab]	7.14[ab]	7.21[bc]	7.22[bcd]	7.28[cd]	7.10[a]
EC$_{25}$ (μS cm^{-1})	214[a]	202[ab]	263[d]	227[bc]	240[c]	263[d]	280[d]	270[d]
Cox (%)	0.98[a]	0.98[a]	1.14[b]	1.08[b]	1.09[b]	1.12[b]	1.22[b]	1.28[c]
N$_t$ (% N)	0.07	0.07	0.08	0.08	0.08	0.08	0.08	0.09
C/N	14.1	14.2	14.8	13.7	13.9	15.2	14.1	14.6
NH$_4^+$-N (mg kg^{-1})	17.9	19.1	18.9	19.9	22.2	17.6	15.2	18.1
NO$_3^-$-N (mg kg^{-1})	8.60[a]	10.1[a]	15.3[b]	13.7[b]	19.4[c]	18.6[c]	22.9[c]	18.7[d]
DOC (mg kg^{-1})	59.8[ab]	57.2[a]	65.4[bc]	64.9[bc]	69.7[cd]	65.0[bc]	69.5[cd]	72.9[d]
SUVA (254 nm)	1.44[a]	1.47[ab]	1.68[d]	1.63[d]	1.59[cd]	1.65[d]	1.54[bc]	1.69[d]
Phenols (mg kg^{-1})	1.32[b]	1.20[ab]	1.53[bc]	1.53[bc]	1.70[c]	1.66[c]	1.45[bc]	1.74[d]

Different superscripts indicate significant differences at $p < 0.05$. SS: sewage sludge; EC: electrical conductivity; Cox: oxidizable carbon; N$_t$: total Kjeldahl nitrogen; DOC: dissolved organic carbon; SUVA: specific UV absorbance. Treatment references are defined in Table 2.

Table 4
Total potentially toxic elements concentrations (mg kg^{-1} dw) in the soil control and treated samples.

Ref.	C	MC	40/4	40/2	80/4	40/1	80/2	80/1	86/278/EC directive	EU[a] working document
Total SS (Mg ha^{-1})	0	0	160	320	320	640	640	1280		
Cd	0.243[a]	0.255[a]	0.267[a]	0.274[ab]	0.245[a]	0.317[b]	0.239[a]	0.279[ab]	40	2
Hg	0.057[a]	0.053[a]	0.126[cd]	0.093[b]	0.060[a]	0.106[bc]	0.093[b]	0.143[d]	25	2
Co	8.90[bc]	8.65[ab]	8.90[bc]	8.90[bc]	8.20[a]	8.95[c]	8.35[a]	8.45[ab]	–	–
Mo	0.11[a]	0.12[a]	0.15[b]	0.13[ab]	0.15[b]	0.15[b]	0.15[b]	0.18[c]	–	–
Ni	21.6[abc]	21.4[ab]	22.6[d]	22.3[cd]	21.2[ab]	22.8[d]	21.1[a]	22.1[bcd]	400	100
Pb	19.6[a]	19.7[a]	23.7[c]	22.8[bc]	19.8[a]	22.6[bc]	21.1[ab]	24.2[c]	1200	200
Cr	14.7[a]	14.8[a]	17.5[c]	16.6[b]	15.0[a]	17.4[c]	16.1[b]	18.4[d]	–	600
As	6.4[bc]	6.3[ab]	6.8[d]	6.7[cd]	6.6[bcd]	6.6[bcd]	6.0[a]	6.5[bcd]	–	–
Cu	18.3[a]	18.3[a]	23.6[d]	22.7[cd]	20.2[b]	22.8[cd]	21.8[c]	26.3[d]	1750	600
Zn	62.7[a]	60.7[a]	86.3[d]	79.4[c]	56.3[b]	80.9[cd]	79.1[c]	96.6[e]	4000	1500

Different superscripts indicate significant differences at $p < 0.05$. Treatment references are defined in Table 2.
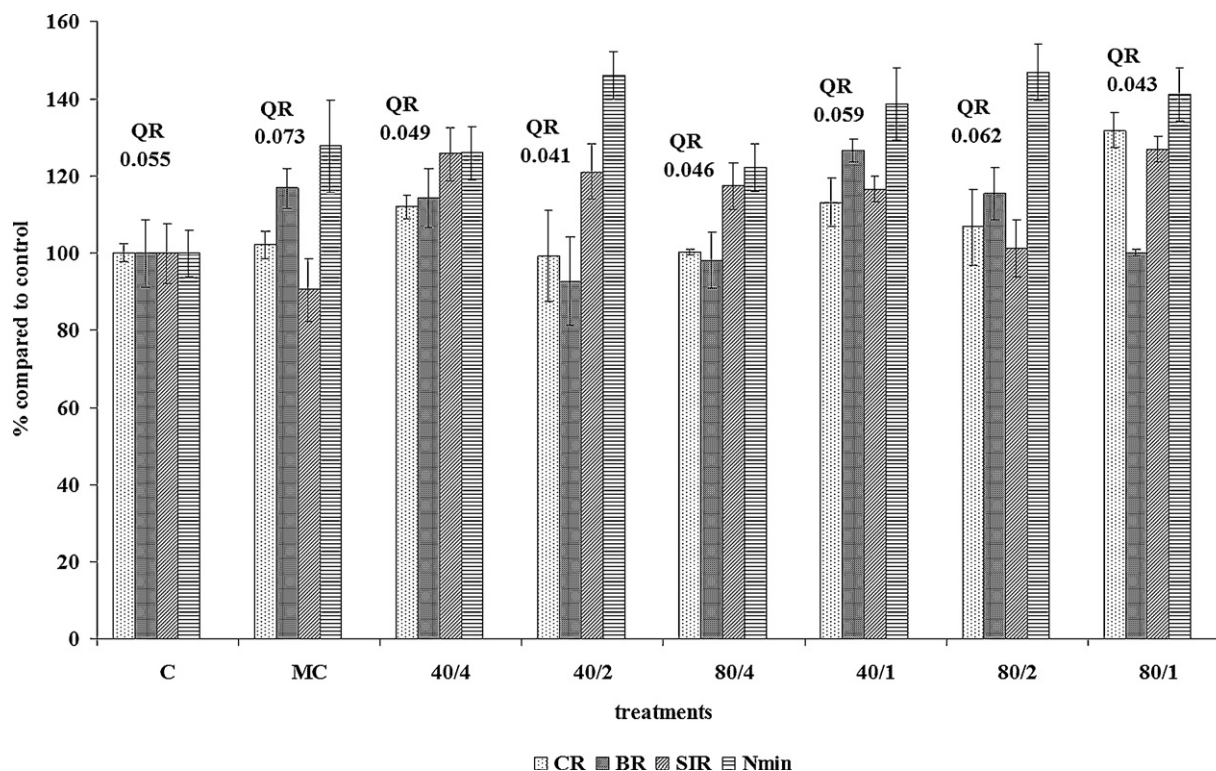
[a] EC (2000).

Fig. 1. Carbon and nitrogen mineralization. CR: cumulative respiration; BR basal respiration; SIR: substrate induced respiration: Nmin: nitrogen mineralization. Q_R: respiratory activation quotient. CR, BR, SIR and Nmin expressed as percentages related to the control soil. Different treatments are defined in Table 2.
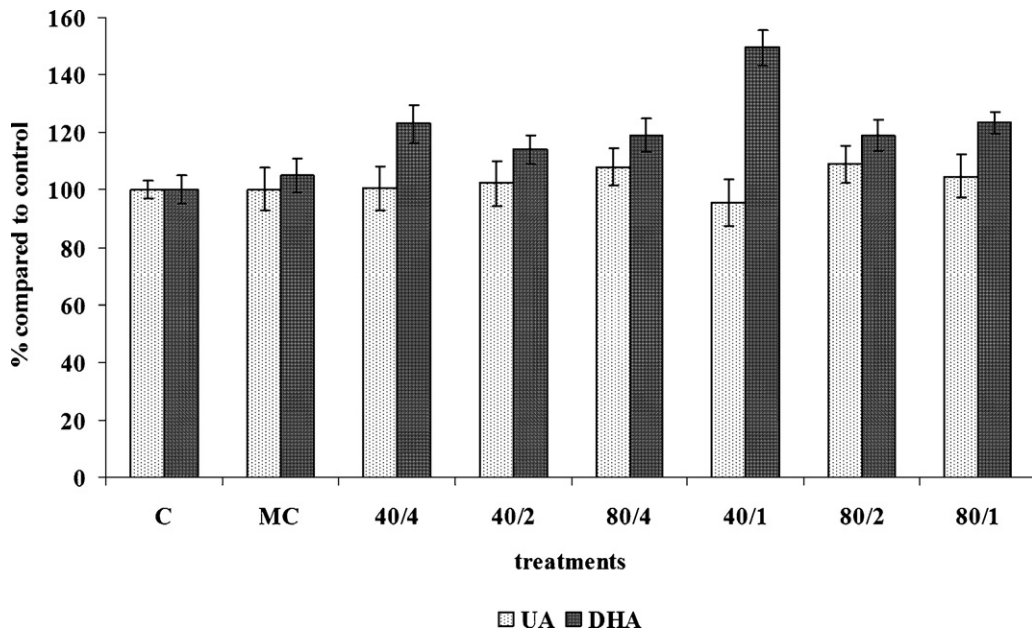
Fig. 2. Enzymatic activities of the control and treated samples expressed as percentages related to the control soil. UA: urease activity; DHA: dehydrogenase activity. Different treatments are defined in Table 2.

total amount of sewage sludge than with the dose or the number of applications. However, the increase in the EC_{25} was better correlated with the dose (0, 40 or 80 Mg ha^{-1}) than with the number of applications or total amount used.

In accordance with the results of previous experiments (Marschner et al., 2003), after the addition of organic material, most treatments showed an increase in the levels of organic matter, total nitrogen and nitrate, when compared to the respective controls. The dissolved organic matter (DOC) increased in the amended soil, in agreement with the results from recent investigations (Chiu and Tian, 2011; Sierra et al., 2012). The aromaticity (SUVA 254 values) of the soluble organic matter increases with its maturity degree (Surampalli and Tyagi, 2004), showing an improvement

in the quality of the DOC (Jaffrain et al., 2007). This leads to an increase of the soil ability to adsorb organic pollutants (De Paolis and Kukkonen, 1997). Among the aromatic constituents, free phenolic compounds play an important role in the synthesis of soil humic substances (Sanchez-Monedero et al., 1999) and also they allow some reactivity to the organic matter, which helps to face up an eventual pollution episode. Although these compounds may have a notable degree of phytotoxicity and antimicrobial activity (Souto et al., 2000), they did not hinder soil microbial activity in our experiment. In addition, this type of compounds tends to get biodegraded and/or incorporated to the soil organic matter in soils amended with high phenolic content organic wastes (Sierra et al., 2007). In the current investigation, soils presented a high degree
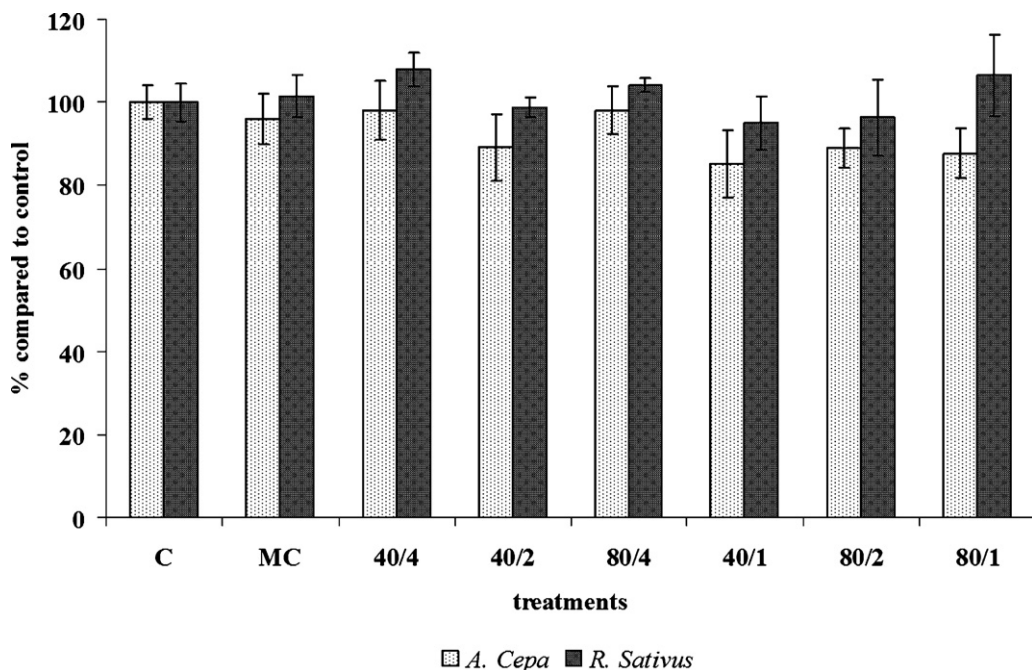

Fig. 3. Germination-elongation results expressed as percentages to the control for *Allium cepa* and *Raphanus sativus*. Different treatments are defined in Table 2.

of aromaticity, as it can be deduced from $SUVA_{254}$ results which also presented stronger correlations with the number of applications than with the total amount of sewage sludge deposited. In turn, phenolic compounds concentration showed a better correlation with the doses rather than with the number of applications. Moreover, whereas for the organic carbon increase the most influential factor seemed to be the total amount of sewage sludge used, the main factor influencing the DOC levels was the application dose. Nitrates increase was found to be especially correlated with the sewage sludge dose. Nitrate production is related to the N mineralization process, which is also enhanced by the addition of sewage sludge. In the present case-study, the nitrogen added in the applications 40/1 (40 Mg ha^{-1} year^{-1}), 80/2 (80 Mg ha^{-1} every two years) and 80/1 (80 Mg ha^{-1} year^{-1}) would be equivalent to 280, 280 and 560 kg N ha^{-1}, respectively. Between the 80/4 and 40/2, both bringing the same amount of N to the soil, nitrates were significantly higher when the nominal dose was also higher (Table 2). Consequently, organic amendments should be preferably applied at lower doses distributed over time in order to achieve a sustained mineral nitrogen supply and to minimize the nitrate losses, which may contaminate groundwater and misspend the nitrogen source.

With the only exception of As, the concentrations of all the elements were correlated with the number of applications and/or with the total amount of sludge applied. Although the levels of some elements were found to be significantly higher in the amended soils than in the controls, metal concentrations were still well below the threshold values established by different regulations such as those from Spain, the Netherlands and Canada (VROM, 2000; Ministerio de la Presidencia, 2005; CCME, 2007) for the protection of the environment and human health. Furthermore, according to the FOREGS geochemical database (Rodríguez Lado et al., 2008), the concentrations here found are near and even below the median of the corresponding geographical area, with only Hg exceeding that level, but remaining far below the Dutch target values (VROM, 2000).

Regarding the carbon mineralization experiments, no correlations were found between BR and sewage sludge application. BR is an indicator of the current biological activity, which remains stabilized once the labile carbon source is exhausted, and the microbiota is adapted to the conditions of the incubation. This depends on the microbiota and nutritional state of the soil. SIR has been found to be related to the active microbial biomass (Blagodatsky et al., 2000; Svensson and Friberg, 2007; Bradford et al., 2009; Hultgren et al., 2009), being often used as an indicator of both microbial biomass and toxicity in soils (Martí et al., 2007, 2011). The current results show a clear increase in the soil SIR due to the sewage sludge effect, which means that the active microbial biomass remains enhanced after relative long-term application of sewage sludge materials, showing a lack of toxicity in the amended soils, even considering the pollutants load and the time elapsed since the application. However, long-term potential effects associated to the increase of organic matter could appear and, therefore, they should be taken into account (Montserrat et al., 2006). SIR was especially favored by repeated applications of sewage sludge.

With respect to Q_R, it measures the relationship between the activity of microbiota and the number of active microorganisms, being an indicator of soil microbiota stress. Q_R values above 0.3 may indicate soil microbiota stress in polluted soils (ISO, 2002). Such scenario was not observed in the current experiment (Fig. 1), as Q_R was far below 0.3, with the highest value corresponding to the mineral amending. In previous studies, some relationship (negative correlations) was reported between BR/SIR ratios (Q_R) and pH (Enwall et al., 2007). It has been suggested that stress can be attributed to the acidity, as well as to the consequences of this acidity on the PTE availability. Although in the present study Q_R values correlated positively with pH ($r = 0.636$, $p < 0.01$), there were no observable differences compared to the control.

This might be due to the slightly alkaline pH that limits PTE bioavailability.

Regarding enzymatic activities, urease activity was not significantly affected by the amendment with sewage sludge, while dehydrogenase activity increased significantly with the number of sewage sludge applications (Fig. 2). In general, soil enzymatic activity is proportional to soil organic carbon content (Marschner et al., 2003), being in agreement with the current DHA results. The soil UA tends to increase with the organic matter application, becoming inhibited due to the presence of metals, as reported in some works (Tejada et al., 2011). In the present investigation, the UA was not significantly altered, so the expectable organic matter effect was not produced, or maybe it was counteracted by the presence of toxicity. In fact, if UA is expressed as UA to organic matter ratio, inhibitions compared to the control are highlighted, as they increased with the waste application. In relation to this, it must be noted that the correlation analysis shows that Co, Ni and As levels were negatively and significantly correlated with UA.

Finally, the amendment with sewage sludge did not derive in toxicity on R. sativus. In contrast, the growth of A. cepa was significantly lower ($p < 0.05$) in the highest application doses than in the control (Fig. 3). Additionally, the germination results with A. cepa correlated negatively with Cd, Hg, Pb, Cr, Cu and Zn concentrations, as well as the levels of free soluble phenols, which may exert phytotoxic properties. The inhibition of growth would be more related to the number of applications and total amount of sewage sludge than to the dose.

According to the current results, and despite the low profile of the differences between treatments, it seems that a biannual application of 40 Mg ha^{-1} could be recommended. This is based on the effects found on some parameters (differences between KCl and H$_2$O pH, C/N ratio, balance between ammonium/nitrate forms, soluble phenolic compounds, Q_R), but mainly on the fact that the number of applications (for the same total amount added) showed the highest correlations for some important parameters (i.e., potentially toxic elements concentrations and phytotoxicity). The accumulation of metals in soils due to the treatments, considering that the experiment lasted 16 years, still remained in a level similar to that of the background ones (Rodríguez Lado et al., 2008) and below the target values of some regulations as it has been stated above.

5. Conclusions

The application for 16 years of sewage sludge to a calcareous soil resulted in an increase in the acidity of the soil exchange complex, an improvement in the soil organic matter (C and N) and microbial activity and nitrogen mineralization potential, as well as in an increase in the soil organic matter aromaticity and the amount of soluble phenolic compounds. A varied range of increases in the PTE total concentrations was observed, although the levels reached did not exceed the statutory limits in any of the evaluated scenarios.

Some toxicity trends could be observed in the phytotoxicity test (A. cepa) related to Cd and in the urease activity (when expressed as relative to oxidizable carbon content) related to Co, Ni and As concentrations. Sewage sludge amendment at any dose (40 or 80 Mg ha^{-1} year^{-1}) was found not causing toxicity for C mineralization microorganisms. The results show that, for the same amount of sewage sludge used, the distribution along time of small doses is better than single applications because it increases soil fertility minimizing negative environmental impacts as ecotoxicity and soluble nitrate losses.

Overall, our findings highlight the importance of performing complementary studies, when evaluating the long-term effects derived from sewage sludge-amended soils. In order to interpret

a realistic scenario, the analysis of pollutant contents is not sufficient, as this must be applied in combination to studies of soil functioning parameters and/or ecotoxicological tests.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.agee.2012.05.016.

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