Research article

Assessment of the ecotoxicity of phytotreatment substrate soil as landfill cover material for in-situ leachate management

Francesco Garbo[a], Alberto Pivato[b,*], Barbara Manachini[c], Carlo Giovanni Moretto[d], Maria Cristina Lavagnolo[b]

[a] DII - Department of Industrial Engineering, University of Padova, Via Marzolo 9, 35131, Padova, Italy
[b] DICEA - Department of Civil, Environmental and Architectural Engineering, University of Padova, Via Marzolo 9, 35131, Padova, Italy
[c] SAAF - Department of Agricultural, Food and Forest Sciences, University of Palermo, Viale delle Scienze 13, 90128, Palermo, Italy
[d] ARPAV - Inlad Water Observatory, via Rezzonico 41, 35131, Padova, Italy

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ABSTRACT

Phytotreatment capping in closed landfills is a promising, cost-effective, in situ option for sustainable leachate treatment and might be synergistically coupled with energy crops to produce renewable energy (e.g.: biodiesel or bioethanol). This study proposes to use 0.30 m of soil as growing substrate for plants cultivated on the temporary cover of closed landfills. Once the leachate phytotreatment process is no longer required, 0.70 m of the same soil would be added to attain the final top cover configuration. This solution would entail saving the costs of excavation and backfilling. However, worsening of the initial soil quality due to potential contaminant transfer from the liquid to the solid matrix must be avoided because EU legislation (such as that in Italy) fixes concentration limits for contaminants in soil. In this research, samples of soil used as substrate in a lab-scale leachate phytotreatment test with sunflowers were analysed to provide chemical characterization before, during, and at the end of the experiment. The results showed that the phytotreatment activity did not increase initial contaminant concentrations. These results are reinforced by those from ecotoxicological bioassays in which *Eisenia fetida* (earthworms), *Lepidium sativum* (cress), *Folsomia candida* (collembola), and *Caenorhabditis elegans* and *Steinernema carpocapsae* (nematodes) were used. It was observed that, by the end of the experiment, the substrate soil did not affect the earthworms, collembola and nematode behaviour, or the growth of cress.

1. Introduction

Landfilling is still considered the final element of most waste management strategies, so as to close the material usage loop. However, among others, the main problems linked to landfills are leachate management and the damage to the landscape that these waste masses can create (Cossu and Williams, 2015). In fact, one of the most onerous items of expenditure is the leachate management (Oloibiri et al., 2017), which is stored and then, most of times, treated ex situ often using highly sophisticated technologies such as reverse osmosis, evaporation systems and membrane bioreactors (Di Maria et al., 2018; Saleem et al., 2018). In addition, landfills are not typically accepted by citizens: following the "NIMBY" (Not In My Back Yard) principle (Ma and Hipel, 2016), they consider them dangerous accumulations of waste. These oppositions could be minimized by the utilization of energy crops growing on the top of closed landfills, not only for leachate phytotreatment purposes but also for renewable energy generation, offering a pleasant view of the site (Lavagnolo et al., 2016) and enhancing the process of environmental restoration (Pivato et al., 2018a) at the same time. The landfill leachate, which is collected and re-circulated to the top of the closed landfill, could be phytotreated on a portion of the surface area with little slope. Additionally, this would make it possible to save the huge amounts of water necessary to irrigate these types of plants (Garbo et al., 2017). Energy crops can be used effectively to treat landfill leachate because they are able to resist the organic and inorganic contaminants (Agostini et al., 2003; Brunetti et al., 2011; January et al., 2008; Marchiol et al., 2007; Schnoor et al., 1995; Tang et al., 2016). These plants were tested by several authors (Akinbile et al., 2012; Fraser et al., 2004; Hasselgren, 1992; Ma et al., 2016) who demonstrated their high efficiency in contaminant removal due to the synergic effects of the plants and the microorganisms living in the soil. The final objective of energy crop cultivation is the production of renewable energy: bioethanol from ligneous biomass, biodiesel from oleaginous crops and biogas from the biomass feedstock (Di Maria and

* Corresponding author.
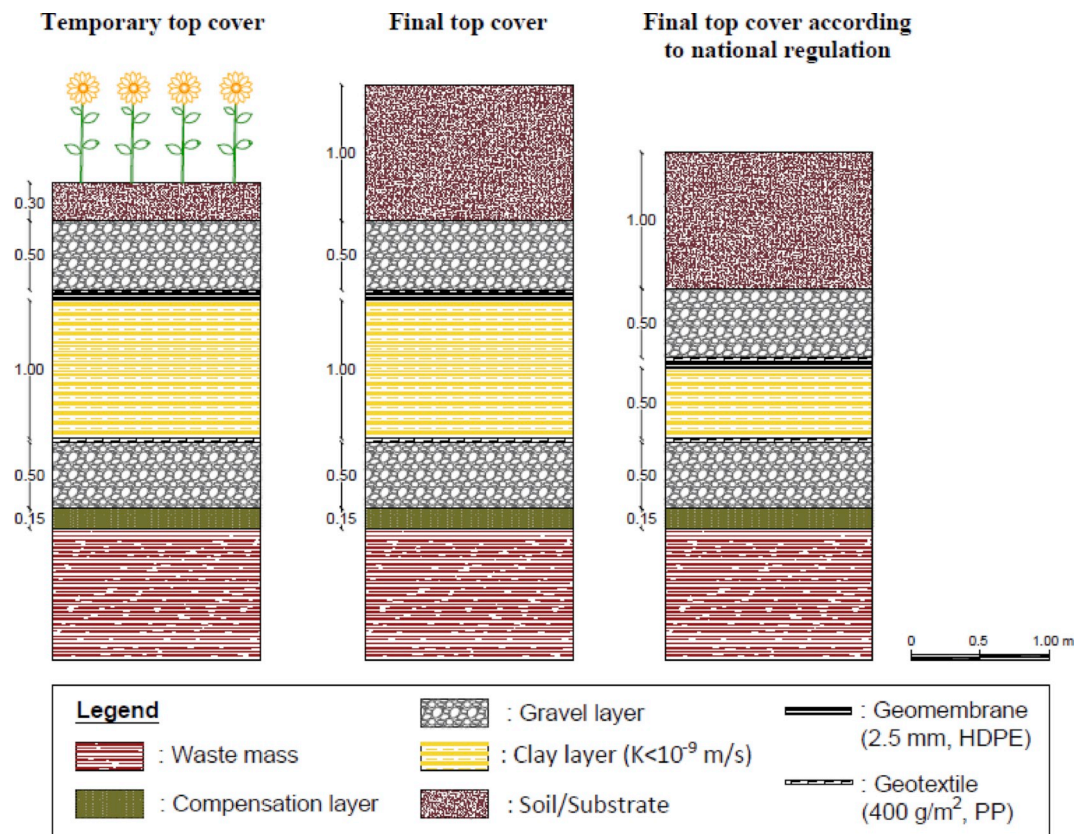 E-mail address: alberto.pivato@unipd.it (A. Pivato).

Fig. 1. Comparison between temporary and final top cover proposed in this article and final top cover prescribed by the current Italian legislation.

Sisani, 2017; Lavagnolo et al., 2017; Pandey et al., 2016). Garbo et al. (2017) and Lavagnolo et al. (2016) have already considered the use of oleaginous crops (e.g. sunflower, soybean, rapeseed) on the top of a landfill for leachate phytotreatment and biodiesel production. They reported good results, achieving efficiencies higher than 80% for Chemical Oxygen Demand (COD) reduction, and removal of more than 70% of total nitrogen (N) and more than 95% of total phosphorous (P_{tot}). Moreover, a significant fraction of the leachate volume was removed by natural evapo-transpiration (Garbo et al., 2017; Lavagnolo et al., 2016).

The EU Directive 1999/31/CE mandates the competent authority (region or province) to prescribe surface sealing of the landfill only if a potential hazard to the environment is recognized. On the other hand, the Italian transposition (D. Lgs. 36/2003) of the EU Directive and some regional regulations (e.g. DGR Lombardia n. X/2461/2014) prescribe a mandatory impermeable final top cover, aimed at minimizing the infiltration of liquids into the landfill body. Therefore, a phytotreatment basin built on the landfill final top cover is discouraged by the current Italian laws and regulations. To comply with the current national legislation, the following scenario was proposed (Fig. 1): the plants, irrigated with the leachate, are cultivated during the temporary cover period in 0.30 m of substrate soil, which is required for root development. At the end of the phytotreatment process, an additional layer of soil (0.70 m) is added to reach the final top cover configuration called for in D. Lgs. 36/2003 (at least 1.00 m of natural soil as superficial layer). In this manner, the costs of excavation and backfilling can be limited because the substrate soil used for phytotreatment is simply covered with the same type of soil. Moreover, to minimize leachate infiltration in the landfill body, an additional 0.50 m thickness of clay, for a total of 1.00 m, is also considered; in fact, the legislation (D. Lgs. 36/2003) requires a minimum thickness of 0.50 m. Therefore, the proposal for the final configuration is – from bottom to top – a 0.15 m compensation layer, 0.50 m of gravel to permit landfill gas drainage and collection, a 1.00 m layer of clay (instead of 0.50 m), with a

permeability k less than 10^{-9} m/s, a High-Density PolyEthylene (HDPE) geomembrane, a geotextile, 0.50 m of gravel to drain the water and 1.00 m of natural soil. Fig. 1 shows that the proposed final cover has the same configuration as the final cover now prescribed by law, except for the clay layer.

In this research, experiments were performed using the substrate soil on which sunflowers were cultivated. Sunflowers were irrigated with leachate to represent the scenario of leachate phytotreatment on the top of the landfill. One of the critical points of full-scale application could be the substrate soil quality at the end of phytotreatment period. Based on our literature review, there are no studies in which the wetlands growing medium has been chemically characterised and compared with the reference values set by the current legislations. However, a chemical analysis for a substance-based approach is not sufficient because the soil is a very complex living matrix including soil fauna along with microorganisms (EFSA, 2017; Manachini et al., 2009). These can absorb elements such as carbon and nitrogen, to degrade organic compounds and to amass stock substances in the form of humus (EFSA, 2017; Jacomini et al., 2000). Thus, it is necessary to consider also ecotoxicological analysis for a matrix-based approach (Pivato et al., 2017). Ecotoxicological testing involves the study of the effects of toxic compounds present in the soil on representative organisms (APAT, 2004; Hennebert, 2017).

In the past, some studies considered the use of earthworms, nematodes, and the germination of seeds as bio-indicators to determine the toxicity of a soil. For example, Dawson et al. (2007) considered earthworms and seed germination assays as indicators to assess the ecological health of soils from a former gas-works site undergoing various remediation treatments. Holmstrup et al. (2010) considered the effects of natural stresses during ecotoxicological analysis using earthworms and nematodes. Pivato et al. (2018b; 2016; 2014) utilized *Eisenia fetida* earthworms and *Folsomia candida* collembola to investigate the quality of compost and digestate for possible use in agriculture. There are no

references, however, reporting the ecotoxicological characterization of a substrate soil used for landfill leachate phytotreatment with energy crops.

In this work, chemical and ecotoxicological characterisations were conducted on the substrate soil before, during and after the leachate phytotreatment to determine if the substances contained in the leachate, or formed during the phytotreatment process, cause significant worsening of the soil quality. The concentrations of contaminants in the substrate soil were compared with reference values (screening values) for potentially contaminated sites defined in Table 1 of Annex 5 to Part IV of D. Lgs. 152/06 (soil for public, private and residential green areas in column A; soil for commercial and industrial activities in column B) to check if contamination occurred.

Chemical characterization was combined and reinforced by a series of ecotoxicological tests that were conducted using the following suitable vulnerable model species (EFSA, 2017): *Lepidium sativum* (cress), *Eisenia fetida* (earthworms), *Folsomia candida* (collembola) and the nematodes *Caenorhabditis elegans* and *Steinernema carpocapsae*, in which the potential toxicity of the substrate soil samples was assessed based on the growth and biological development of the organisms.

2. Materials and methods

2.1. Experimental design

The tested samples were collected from a lab-scale phytotreatment test, performed according to the experimental design described by Lavagnolo et al. (2016) and Garbo et al. (2017). Four 45 L polyethylene tanks, with a surface area of $0.16\,m^2$, were used. All tanks were placed in a controlled climatic chamber in which a 14 h photoperiod with $300\,\mu mol\,m^{-2}{\cdot}s^{-1}$ light intensity was imposed. The mean air temperature was maintained at 24 °C (MIN = 17 °C, MAX = 35 °C). To the four tanks were added – from the bottom to the top – 8 cm of gravel (20–30 mm diameter) for drainage, a small net to avoid clogging of the drainage system and 30 cm of substrate soil (the scheme of the tanks is reported in Supplementary Material - Fig. S1). Four sunflowers were planted in each experimental unit. Based on previous experiences (Garbo et al., 2017), the number of plants was considered to be sufficient. After an initial acclimation period, lasting for 14 days, in which tap water was used, sunflowers were irrigated with a mixture containing water and an increasing amount of landfill leachate, as reported in Table 1. The applied Hydraulic Loading Rate (HLR) was $4.5\,mm\,d^{-1}$. The irrigation was spread uniformly over the entire surface of each reactor. The leachate dose was increased gradually to adapt the plants to the increasing concentration of contaminants and to avoid sudden failure from potential phytotoxicity. The nitrogen concentration in the feed was used as a reference parameter in setting the irrigation timetable; previous studies had revealed that nitrogen exceeding 400 mg-N/ L could produce a negative effect on plants (Garbo et al., 2017;

Lavagnolo et al., 2016). Once a week, the tanks were drained through a valve at the bottom.

The substrate soil was the same in all the experimental units. The initial sample (initial substrate soil) was analysed before the start of the phytotreatment tests. After 35 days from the beginning, the substrate soil was excavated from two tanks, mixed, and analysed (intermediate substrate soil). The remaining two reactors were run until clear senescence of the sunflowers was reached (70 days from the beginning of the phytotreatment): then the plants were harvested, reactors were excavated, and the substrate soils were mixed and analysed (final substrate soil).

2.2. Leachate characterization

The leachate used in the experiment was collected from a sector of an operating landfill located in the North of Italy, in which residual waste from separate collection of Municipal Solid Waste (MSW) is disposed of. It was sampled once and analysed four times during the experiment to check whether the main parameters (e.g.: nitrogen) were changed over time. It was analysed according to the CNR-IRSA standard Italian analytical methods for liquid samples (CNR-IRSA, 29/2003). Its composition is reported in Supplementary Material - Table S1 and the results are consistent with the kind of waste landfilled.

2.3. Substrate soil characterization

2.3.1. Texture characterization

The substrate soil utilized for the lab-scale phytotreatment system was a locally available soil rich in sand. It was collected in the proximity of the research centre in which the experiments were performed, in the North-East of Italy. Long-term studies indicate that mixtures of soil and sand provide an optimal combination for phytotreatment systems (Lavagnolo et al., 2016; Stottmeister et al., 2003; Verakoon et al., 2013) because they provide sufficient air circulation, while at the same time guaranteeing proper root development. The texture was determined using the Bouyoucos Method (Bouyoucos, 1962) and, according to the soil taxonomy proposed by the USDA (USDA-NRCS, 1999), the substrate soil was classified as sandy loam (14% clay, 10% silt, and 76% sand).

2.3.2. Chemical characterization

The chemical characterization determined the presence of chemical compounds in the three substrate soil samples, which were analysed in triplicate. The compounds analysed were compared to the reference values (columns A and B) reported in Table 1 of Annex 5 to Part IV of D. Lgs. 152/06, already mentioned in the Introduction. The chemical analysis was performed according to the EPA Hazardous Waste Test Methods (SW-846).

2.3.3. Ecotoxicological characterization

2.3.3.1. Lepidium sativum (cress) tests. Soil quality can be evaluated using plants as bio-indicators. In this case, *Lepidium sativum* (cress) was used, according to the APAT guidelines (APAT, 2004), due to its ability to reveal quickly the potential toxicity of the soil. The tests were performed using Petri dishes (Ø = 9 cm). A mixture of 10 g of test-substrate soil (e.g. final substrate soil) and artificial soil (quartz sand with more than 50% of particles between 50 and 200 μm) was added to each dish. Increasing concentrations of the test-substrate soil were used: 0 (control), 2, 3, 5, 7, 10, 20, 30, 50, 70, and 100% (w/w referred to dry matter) to which deionized water was added to reach 100% of the Water Holding Capacity (WHC) of the mixture, plus 5 mL. As suggested by the USEPA (2005), the test concentrations were chosen to follow a geometrical series, with an average ratio of 1.5. Two controls were used: one with just 5 mL of deionized water (as prescribed by the APAT guidelines) and another with 10 g of artificial soil and 5 mL deionized water. The latter was used to be consistent with the testing procedure,

Table 1
Main characteristics of the irrigation water over the whole experimental period and the sampling timetable.

Week	HLR (mm·d⁻¹)	Leachate percentage	COD inlet (mg·L⁻¹)	P inlet (mg·L⁻¹)	TKN inlet (mg·L⁻¹)
Collection of initial substrate soil samples					
1	4.5	10%	49	0.23	50
2	4.5	20%	98	0.46	100
3	4.5	30%	147	0.69	150
4	4.5	40%	196	0.92	200
5	4.5	50%	245	1.15	250
Collection of intermediate substrate soil samples (35 days from the beginning of the test)					
6–10	4.5	60%	294	1.38	300
Collection of final substrate soil samples (70 days from the beginning of the test)					

which is based on the use of 10 g of material. Ten seeds were placed in each dish on a filter paper on top of the media, and the dishes were covered using parafilm. Seeds available in the market for bioassays were used. The tests were conducted under standardized conditions: 25 °C and complete darkness (0 lux). After 72 h, the elongation of the emerged roots was measured. As prescribed by the APAT guidelines, each concentration (including the controls) was tested using four replicas. The results were expressed as percentage Germination Index (GI%); each Germination Index (GI) was calculated by multiplying the number of germinated seeds with the mean root length of each plant, as follows:

$$GI = n.\ germinated\ seeds \cdot mean\ roots\ length \qquad (1)$$

The mean GI was calculated for each substrate soil sample ($\overline{GI}$) and control ($\bar{GI}_C$) and the percentage GI (GI%) was calculated as ratio between $\overline{GI}$ and $\bar{GI}_C$, as follows:

$$GI\% = \frac{\overline{GI}}{\bar{GI}_C} \cdot 100 \qquad (2)$$

2.3.3.2. Earthworms tests. The method adopted was a chronic test performed according to the OECD Guideline 222/2004. Ten *Eisenia fetida* adult earthworms were put in plastic containers (volume 1.2 L) filled with 500 ± 5 g of a mixture of artificial soil and test-substrate soils, at different concentrations (the same concentrations used for the cress tests). The artificial soil was composed of 70% sand, 20% clay and 10% peat (w/w), as prescribed by the OECD Guideline 222/2004. Its WHC was adjusted to 40%. The maximum WHC of the artificial soil was determined in accordance with the procedures described in Annex 2 of ISO 11274 (1998). The initial weight of the earthworms ranged from 0.3 to 0.9 g. Soil mixtures and earthworms were placed in the containers and closed with holed plastic lids to prevent the worms from escaping, to permit air passage and to limit evaporation. The earthworms were fed weekly with 5 g of dried cow manure. The test was performed in a thermostatic room with a monitored temperature of 20 ± 2 °C, light-dark cycles L:D 16:8 (L = 400–800 lux). After 28 days (Day 28), earthworms were counted and weighted. As prescribed by the OECD guidelines, each concentration was tested in triplicate. The results were expressed as percentage Relative Survival (RS%) and percentage Relative Growth (RG%). They were both defined as the average variation between the final and the initial earthworm conditions and were normalized using the values found in the controls (with 0% test-substrate soil), as follow:

$$RS = \frac{final\ n.\ of\ earthworms}{initial\ n.\ of\ earthworms} \qquad (3)$$

$$RG = \frac{final\ earthworms\ weight}{initial\ earthworms\ weight} \qquad (4)$$

The mean RS was calculated for each substrate soil sample ($\overline{RS}$) and control ($\overline{RS}_C$) and the percentage RS (RS%) was calculated as ratio between $\overline{RS}$ and $\bar{RS}_C$, as follows:

$$RS\% = \frac{\overline{RS}}{\overline{RS}_C} \cdot 100 \qquad (5)$$

The mean RG was calculated for each substrate soil sample ($\overline{RG}$) and control ($\overline{RG}_C$) and the percentage RG (RG%) was calculated as ratio between $\overline{RG}$ and $\bar{RG}_C$, as follows:

$$RG\% = \frac{\overline{RG}}{\overline{RG}_C} \cdot 100 \qquad (6)$$

2.3.3.3. Collembola tests. The collembola chronic bioassay was carried out using the common springtail (*Folsomia candida*) according to the ISO 17512-1 (2008) guideline. After preliminary bioassays that did not result in differences according to the concentrations, it was decided

(also for practical and economic reasons) to use 100% substrate soil concentration for all test samples (initial, intermediate, and final substrate soil). The test was carried out in glass containers with 10 g of test-substrate soil (dry weight). Ten specimens of *F. candida* were introduced into each container. At the beginning, deionized water and 10 mg of dried baker's yeast were added to each container. Test containers were closed with parafilm and incubated at 20 ± 2 °C, in the dark, for 28 days. At the end, exposure mortality of adults was determined. As prescribed by the ISO 17512-1 (2008) guideline, four replicates were used. The survival percentage (Su) at Day 28 was considered the endpoint.

2.3.3.4. Nematodes tests. The bacterial feeding nematode *Caenorhabditis elegans* was maintained as a stock of dauer larvae (juvenile stage that occurs with a lack of food) on nematode growth medium agar (Brenner, 1974), according to standard procedures (Lewis and Fleming, 1995; Sulston and Hodgkin, 1988). The nematode bioassay with *C. elegans* was carried out according to standard methods (ASTM guidelines E2172, 2014 and to the principles of ISO 10872, 2010). For the test, 0.5 g of each test-substrate (air-dry weight) was moistened with 0.35 mL of medium (containing Na_2HPO_4, KH_2PO_4, NaCl, and $MgSO_4$) in test wells and then mixed with *Escherichia coli* as the food supply. Ten first-stage juvenile nematodes were transferred to each test well (total of 160 nematodes). Their mean initial body length was 260 ± 38 μm. Four replicates were set up for each test-soil substrate (initial, intermediate, and final) and the control. Even in this case only the concentration of 100% test-substrate soil was considered. In fact, as for *F. candida*, preliminary bioassays indicated no difference in the lower concentrations, thus for practical and economic reasons it was decided to use only the highest soil concentration. After 96 h of incubation at 20 °C, the test was stopped by heat killing the nematodes at 50 °C, after checking the vitality of the specimens. The samples were then mixed with 0.5 mL of an aqueous solution of Rose Bengal to stain specimens for counting. Four different endpoints were considered: survival, growth, fertility, and reproduction. Survival percentage was considered also as the endpoint and was checked considering as alive the motile nematodes. Nematode growth was determined by measuring the body length at 100-fold magnification using a light microscope. Growth was calculated by subtracting the mean initial body length of the test organisms from the mean body length after incubation. Nematode fertility was quantified by calculating the percentage of gravid organisms. Nematode reproduction was quantified by counting the number of eggs under a dissecting microscope at 75-fold magnification.

The second nematode toxicity test examined the direct exposure of one of the entomopathogenic nematodes (EPN) most used in biological control, which is also one of the most common species living in agricultural soil, *Steinernema carpocapsae*. Monoxenic infective juveniles in a *S. carpocapsae* culture (Becker Underwood, Ltd) were used for the bioassay. For the test, 0.5 g of each test-substrate soil (air-dry weight) was moistened with 0.35 mL of medium (containing Na_2HPO_4, KH_2PO_4, NaCl, and $MgSO_4$). The toxicity test was carried out according to ASTM guidelines E2172, 2014 and ISO 10872, 2010. The results were expressed as Su at 24 h and at 48 h.

2.4. Statistical analysis

Statistical analysis was performed using Statgraphics® software. The responses to different substrate soil samples were compared by one-way analysis of variance. The F-test was used to assess whether there were significant differences amongst the means at the 95.0% confidence level ($p < 0.05$); pairwise comparisons were assessed with the Tukey's honestly significant difference (HSD) procedure.

Table 2
Chemical characterization of substrate soil samples. Comparison with reference values of Table 1 of Annex 5 to Part IV of D. Lgs. 152/2006.* denotes a statistically significant difference. Different apical characters indicate statistically significant differences among the samples.

	Reference values column A (mg/kgTS)	Reference values column B (mg/kgTS)	Initial substrate soil (mg/kgTS)	Intermediate substrate soil (mg/kgTS)	Final substrate soil (mg/kgTS)	p-value
Cadmium	2	15	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.296
Cobalt	20	250	8 ± 1	9 ± 2	11 ± 1	0.098
Total Chromium	150	800	20 ± 3^X	25 ± 2 ^{XY}	28 ± 2^Y	0.017*
Chromium VI	2	15	< 0.2	< 0.2	< 0.2	–
Mercury	1	5	< 0.05	< 0.05	< 0.05	–
Nickel	120	500	18 ± 3	21 ± 2	23 ± 2	0.105
Iron [a]	–	–	28884 ± 758	26718 ± 969	24903 ± 1352	0.072
Manganese [a]	–	–	176 ± 5	170 ± 7	163 ± 11	0.422
Lead	100	1000	19 ± 2^X	25 ± 3 ^{XY}	29 ± 3^Y	0.011*
Copper	120	600	27 ± 5^X	41 ± 5^Y	46 ± 3^Y	0.004*
Zinc	150	1500	65 ± 9^X	81 ± 9 ^{XY}	89 ± 7^Y	0.032*
Antimony	10	30	< 1	< 1	< 1	–
Arsenic	20	50	12 ± 3	13 ± 3	17 ± 2	0.113
Beryllium	2	10	0.7 ± 0.2	0.7 ± 0.1	0.8 ± 0.1	0.629
Selenium	**3**	**15**	**14 ± 2**	**14 ± 3**	**15 ± 2**	**0.842**
Thallium	1	10	< 0.2	< 0.2	< 0.2	–
Vanadium	90	250	31 ± 3	35 ± 3	37 ± 4	0.164
Cyanides	1	100	< 0.10	< 0.10	< 0.10	–
Fluorides	100	2000	< 10	< 10	< 10	–
Hydrocarbons C < 12	10	2000	< 0.05	< 0.05	< 0.05	–
Hydrocarbons C > 12	50	2000	< 10	< 10	< 10	–
Aromatic hydrocarbons	0.1–1	2–100	< 0.001	< 0.001	< 0.001	–
Aromatic polycyclic hydrocarbons	0.5–5	5–50	< 0.01	< 0.01	< 0.01	–
Aliphatic chlorinated carcinogenic hydrocarbons	0.01–1	0.1–20	< 0.001	< 0.001	< 0.001	–
Aliphatic chlorinated non-carcinogenic hydrocarbons	0.3–1	5–50	< 0.001	< 0.001	< 0.001	–
Aliphatic halogenated carcinogenic hydrocarbons	0.01–0.5	0.1–10	< 0.001	< 0.001	< 0.001	–
Nitrobenzene	0.1–0.5	10–30	< 0.01	< 0.01	< 0.01	–
Chlorobenzene	0.05–1	10–50	< 0.001	< 0.001	< 0.001	–
Phenol	1	60	0.039 ± 0.018	0.018 ± 0.012	0.017 ± 0.011	0.175
Methylphenol (o-, m-, p-)	0.1	25	0.0079 ± 0.001^X	0.0051 ± 0.001^Y	0.0059 ± 0.001 ^{XY}	0.034*
Chlorinated phenols	0.01–0.5	12–50	< 0.001	< 0.001	< 0.001	–
Aromatic amines	0.05–0.5	13–50	< 0.01	< 0.01	< 0.01	–
Esters of phthalic acid	10	2000	< 1.0	< 1.0	< 1.0	–

[a] Table 1 of Annex 5 to Part IV of D. Lgs. 152/2006 does not specify any reference value for Iron and Manganese.

3. Results and discussion

3.1. Substrate soil chemical characterization

The results of the chemical characterization are reported in Table 2 and were compared with the reference values from Italian legislation for soil contamination (Table 1 of Annex 5 to Part IV of D. Lgs. 152/2006). Statistical analysis revealed a statistically significant increase (from the initial substrate soil samples to the final ones) of the following chemical species: total chromium, lead, copper, zinc. However, treatment-related overall build-up of heavy metals spanned conditions from negligible to acceptable because concentrations remained well within the limits for residential soil. The concentration of each chemical element was always below the reference values, even in the final substrate soil, with the only exception being selenium. The concentration of this element exceeded the reference value of column A (screening values for public, private, and residential green areas), but remained below the corresponding reference value of column B (screening values for commercial and industrial activities). But it must be noted that selenium was above the reference value of column A even in the initial substrate soil and no change of concentration was recorded throughout the experiment. The initial substrate soil samples were collected before the beginning of the phytotreatment tests; therefore, the abnormal concentration of this element cannot be related to the leachate irrigation procedure, but rather to the characteristics of the locally available soil utilized in the experiment.

3.2. Substrate soil ecotoxicological characterization

3.2.1. Lepidium sativum bioassay

The GI% of *L. sativum* is shown in Fig. 2. Focusing on the results referred to deionized water as control (Fig. 2A,B,C), similar trends were detected for the three substrate soils for concentrations between 2 and 10%, characterised by peaks of the GI% up to 180% (Fig. 2B). For concentrations higher than 10%, the GI% of the initial substrate soil presented a slightly decreasing trend but remained always above 80% (Fig. 2A). The other samples presented some fluctuations, which were more marked for the intermediate substrate soil. For the intermediate sample, GI% ranged between 140 and 160% (Fig. 2B), while for the final sample, the GI% ranged between 100 and 120% (Fig. 2C). The trends of the GI% referred to controls in which artificial soil and deionized water were used are similar (Fig. 2D, E, F), especially for concentrations higher than 10%. In fact, the GI% decreased but remained above 50% for the initial soil (Fig. 2D), between 75 and 100% for the intermediate soil (Fig. 2E), and between 90 and 110% for the final substrate soil (Fig. 2F). Statistical analysis was performed on the results of the bioassays in which 100% test-soil substrate was used, in order to mimic the real scale conditions in which the substrate is not mixed with artificial soil (Supplementary material - Table S2). It revealed a statistically significant increase of the GI% between initial and intermediate substrate soil, and between initial and final substrate soil, respectively. The higher values of the GI% of intermediate and final samples could be due to an increased concentration of nutrients (especially nitrogen) in the substrate soil, compared to the initial
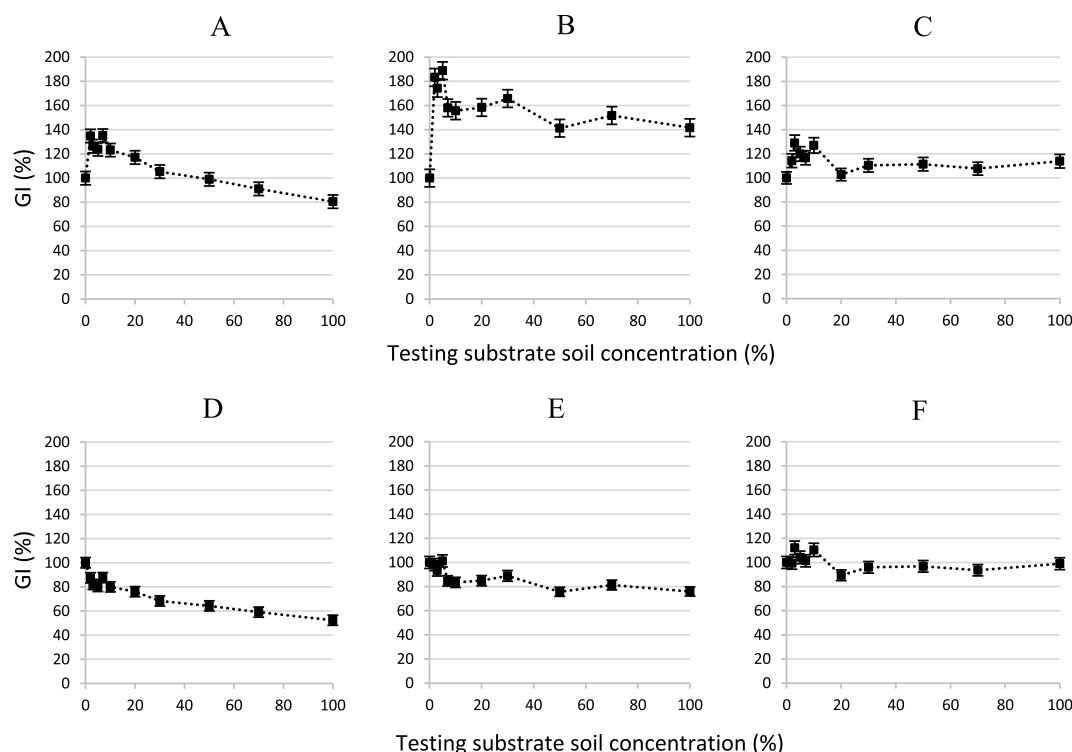
Fig. 2. Results of the percentage Germination Index (GI%) for initial (A), intermediate (B), and final (C) substrate soil with deionized water as control; results of the GI% for initial (D), intermediate (E), and final (F) substrate soil with artificial soil and deionized water as control. Deviation bars refer to the 95% confidence level.

sample. The increase of nitrogen and phosphorous content during the phytotreatment lab-scale tests is reported in Table 3. The maximum increase of the nitrogen content was 13% (Δ Final-Initial): it seemed to have a great influence on the cress development, although a statistically significant increase was not detected. The phosphorus concentration result was always below the detection limits. Summarizing, it is possible to affirm that the germination of *Lepidium sativum* did not present anomalies (e.g. phytotoxicity phenomena) induced by leachate application and the phytotreatment process.

3.2.2. Earthworm bioassay

Results of *E. fetida* earthworms percentage Relative Survival (RS%) and percentage Relative Growth (RG%) are reported in Fig. 3. Relative Survival close to 100% was detected for all three substrate soils, independent of the concentrations, meaning that almost all the earthworms remained alive in the initial, intermediate, and final substrate soils. Focusing on the lowest values, a minimum 90% of Relative Survival was observed with 5% of initial substrate soil (Fig. 3A), a minimum 85.7% of RS% with 2% of intermediate substrate soil (Fig. 3B) and a minimum 92.9% of RS% with 3% and 70% of final substrate soil (Fig. 3C). In the assays with the final substrate soil, some values exceeded 100%, indicating that survival of earthworms was even higher than in the controls, in which artificial soil, described in the OECD Guideline 222/2004 as optimal for the earthworms, was used.

Statistical analysis was applied to the results of the bioassays in which 100% test-soil substrate was used (Supplementary material - Table S3) and did not reveal any significant difference among the

different substrate soil samples (initial, intermediate, final).

The RG% increased with increasing concentrations of initial substrate soil, reaching a maximum value equal to approximately 200% (Fig. 3D) for concentrations of test samples with greater than 30% test-substrate soil. With regards to the intermediate and final substrate soils (Fig. 3E and F), after an initial increase, the trends of the RG% decreased with increasing concentration of the test substrate soils but were never below 100%, which is the value of the control. Statistical analysis was applied again to the results of the bioassays in which 100% test-soil substrate was used (Supplementary material - Table S3), revealing a statistically significant decrease of the RG% between initial and intermediate substrate soil, and between of initial and final substrate soil, respectively, clearly visible also in Fig. 3. However, RG% was always above 100%, the value of the control, in which artificial soil, specifically prepared the ensure optimal growing conditions, was used. Therefore, similarly to the *L. sativum* bioassays, the tests performed with *E. fetida* earthworms did not reveal anomalies which could be related to the applied process of phytotreatment.

3.2.3. Collembola bioassay

Endpoints results of toxicity tests on *F. candida,* expressed as Survival (Su), were compared with the corresponding control in which 100% Su was observed.

The average Su in the intermediate substrate soil (94.75%) was higher than the Su for the initial soil sample (92.50%); Su decreased to 90.50% in the final sample. These minimal variations of the Su were not statistically significant (Supplementary material - Table S4) and were

Table 3

Nitrogen and phosphorous content in the substrate soils (initial, intermediate, and final) used for the experiments.

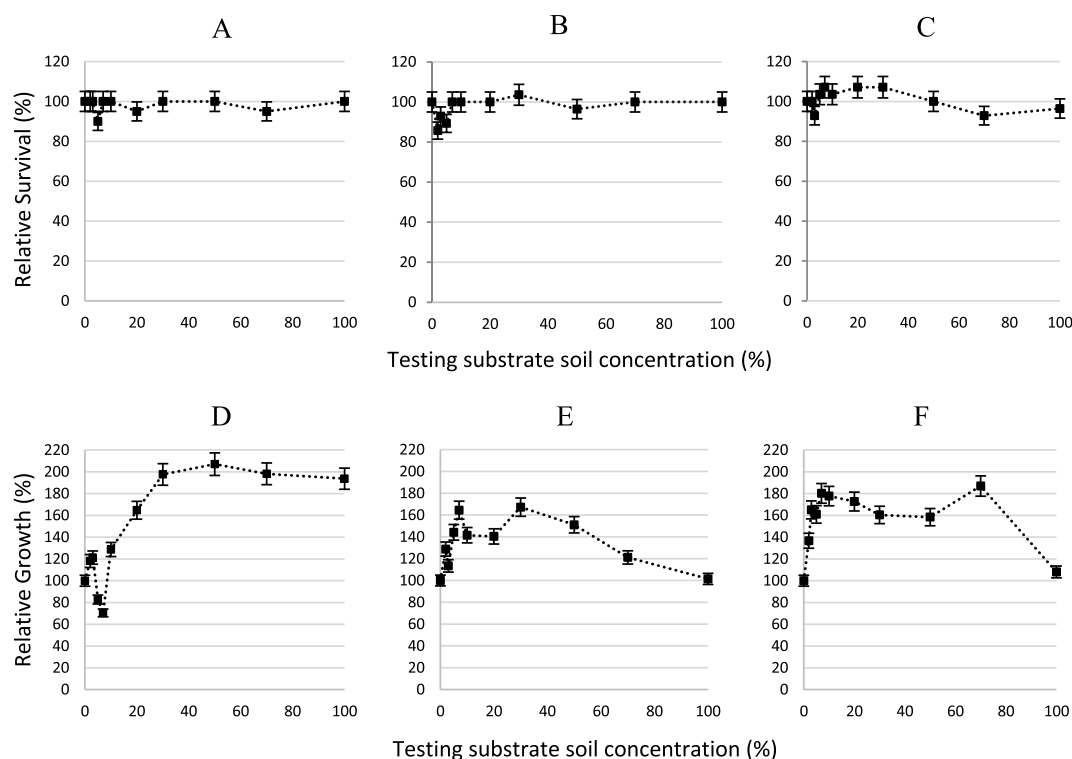|	Initial (%)	Intermediate (%)	Final (%)	p-value	Δ Intermediate-Initial (%)	Δ Final-Initial (%)
Nitrogen content	0.15 ± 0.1	0.16 ± 0.1	0.17 ± 0.1	0.1250	7	13
Phosphorous content	< 0.05	< 0.05	< 0.05	–	–	–

Fig. 3. Earthworm percentage Relative Survival (RS%) for initial (A), intermediate (B), and final (C) substrate soil and percentage Relative Growth (RG%) for initial (D), intermediate (E), and final (F) substrate soil. Deviation bars refer to the 95% confidence level.

not likely related to the applied phytotreatment process: the values of the intermediate and final samples are very close to the Su of the initial substrate soil, but the latter was sampled before the start of the test.

3.2.4. C. elegans and S. carpocapsae nematode bioassays

Results of the ecotoxicity tests on the nematodes *C. elegans* and *S. carpocapsae* are reported in Table 4. As already noticed for the *F. candida* assays, minimal variations (not statistically significant) were detected for all the endpoints considered (survival, growth, fertility, and reproduction) among the three substrate soils. Again, these minimal variations were not likely related to the applied phytotreatment process.

4. Conclusions

The aim of this study was to provide a contribution to the current Italian legislation regarding the properties of the substrate soil used for the leachate phytotreatment process on the top of closed landfills. The results of the chemical analyses were compared to the reference values for soil contamination. Almost all the parameters were below the reference values, except for selenium, which exceeded the reference even

in the initial sample. The tests on earthworms did not present any critical results; in fact, the survival percentages remained close to 100% and the growth results were equal or even higher than the control value, especially in intermediate and final substrate soil samples. The same consideration is valid for the bioassays in which *L. sativum* was used, which did not show significant variations in the Germination Index trend. The four endpoints of the nematode *C. elegans* (survival, growth, fertility, and reproduction) and the survival percentage results of the springtail *F. candida* and nematode *S. carpocapsae* also demonstrated that the three sample types did not affect the behaviour of these invertebrates.

The minimal quantity of contaminants detected in the substrate soil at the end of the test could be linked to phytotreatment activity by the sunflowers but further studies are required to understand the pathways of contaminants removal (e.g.: plants uptake, microbial degradation).

The results of this research indicate that phytotreatment on the top of closed landfills is a feasible option for in-situ leachate management. However, it is important to implement additional researches, for example by changing the quality of the leachate, the quality of the substrate soil, and by increasing the number of model and focal species in the ecotoxicological tests.

Table 4
C. elegans and *S. carpocapsae* nematode average endpoint results.

		Control	Initial substrate soil	Intermediate substrate soil	Final substrate soil	p-value
C. elegans	Survival (%)	100 ± 0	99.25 ± 0.9	99.75 ± 0.5	99.50 ± 1	0.716
	Growth (μm)	1325 ± 64	1275 ± 28	1313 ± 62	1350 ± 57	0.181
	Fertility (%)	100 ± 0	91.50 ± 5.9	96.75 ± 5.1	96.50 ± 5.1	0.250
	Reproduction (N° egg/female)	22.25 ± 1.7	19.00 ± 1.4	21.25 ± 1.7	20.75 ± 0.9	0.108
S. carpocapsae	Survival at 24 h (%)	100 ± 0	95.25 ± 3.3	94.00 ± 3.5	95.00 ± 3.6	0.900
	Survival at 48 h (%)	100 ± 0	91.00 ± 4.2	89.00 ± 3.5	92.00 ± 3.1	0.608

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2018.10.014.

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