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Ecotoxicological assessment of waste-derived organic fertilizers and long-term monitoring of fertilized soils using a multi-matrix and multi-species approach

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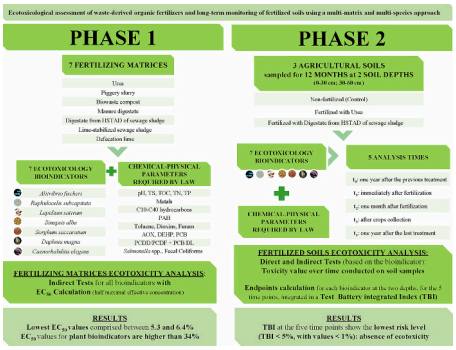
HIGHLIGHTS

- Ecotoxicological tests were based on 7 fertilizing matrixes and 7 bioindicators.
- Ecotoxicity of waste organic matrixes on soil is comparable to urea.
- 4-year application of sewage sludge digestate did not alter soil quality.
- Digestate ecotoxicity did not vary significantly with soil depth and sampling times.
- Ecotoxicity assessment of organic fertilizers should be based on real doses on soil.

GRAPHICAL ABSTRACT



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ABSTRACT

The present study investigates the ecotoxicity of 7 biofertilizers, including biowaste-derived organic matrices. Real-field tests were conducted to assess the impacts of soil fertilization with sewage sludge digestate from high-solid thermophilic anaerobic digestion (HSTAD) compared to those obtained on non-amended and urea-fertilized soils. The physical-chemical and ecotoxic impact of HSTAD digestate on soil was monitored for 12 months, at 5 time points and 2 soil depths, on a maize field divided in 3 portions (non-treated, fertilized with urea, amended with digestate). The chemical and physical characteristics of the soil were previously analyzed for 3 years to provide a long-term outlook of the impacts of biofertilizer application. Seven bioindicators were utilized for direct (on whole soil) and indirect (on soil elutriates) ecotoxicological tests on fertilizers and amended soils,

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including plant seeds (*Lepidium sativum*, *Sorghum saccharatum*, and *Sinapsis alba*), the aquatic organism *Daphnia magna*, the alga *Raphidocelis subcapitata*, the luminescent bacterium *Aliivibrio fischeri*, and the Nematode *Caenorhabditis elegans*. No serious negative effects on soil fertilized with HSTAD digestate were evidenced. Conversely, bioassays rather showed positive effects, encouraging the utilization of HSTAD digestate in agriculture, considering the proper concentrations of use. The obtained data were interpolated and a test battery integrated index was generated, confirming the absence of ecotoxicological risk for the soils amended with the applied fertilizers. The long-term evolution of the physical-chemical soil characteristics (including the concentrations of potential contaminants) was similar for both HSTAD digestate and urea application as well as for non-fertilized soil, indicating no negative effects due to digestate application on land. On the contrary, digestate application improved the content of stabilized organic matter and nutrients in soil. This study proposes a more correct approach to ecotoxicity assessment of fertilized soils for biofertilizer evaluation and demonstrates the long-term safe application of HSTAD digestate on agricultural soil.

1. Introduction

In the context of the modern paradigm of circular economy, the imperative of upcycling waste materials into new value-added resources has emerged. Organic wastes are produced from different industrial activities, including food production, animal farming, and wastewater treatment (European Commission, 2020). While waste materials are typically perceived as a burden, organic wastes represent an enormous pool of organic carbon and nutrients that can be reintroduced in the production and economic cycles in line with the principles of the Circular Economy Action Plan (European Commission, 2020). The easiest way to valorize these wastes and their carbon and nutrient content is their safe application in agriculture as fertilizers. In this way, the use of chemical fertilizers produced from the exploitation of dwindling mineral resources, e.g., phosphate rocks for phosphorus extraction, can be reduced and the economic and environmental burden of organic waste disposal via landfilling or incineration alleviated. This approach is in line with the Sustainable Development Goals set by the United Nations in the context of Agenda 2030, which assigns a key role to promoting sustainable agriculture (FAO, 2023).

Since 2019, approximately 180 million tonnes of digestate have been produced annually in the EU28: more than half (around 120 million tonnes) is derived from agricultural matrices, mainly energy crops; around 25 % is produced from the mechanical biological treatment of the organic fraction of municipal solid waste; about 7 million tonnes is produced from biowaste; less than 2 million tonnes are produced from sewage sludge and other industrial by-products (European Commission, 2019). In the past decades, the European legislation has encouraged the application of treated sewage sludge as fertilizer through the Sewage Sludge Directive 86/278/EEC. However, emerging concerns have risen recently about the correct use of sewage sludge and other waste materials in agriculture due to the possible presence of toxic compounds (e.g., xenobiotics and heavy metals) that may threaten the health of flora, fauna, and soil microbiomes as well as of humans as primary consumers. On the other hand, in the last decades, pollution prevention and the modernization of wastewater management in urban areas and industries have reduced the amount of toxic material entering municipal wastewater treatment plants, leading to an improvement of sewage sludge quality (Collivignarelli et al., 2020). Nevertheless, the manufacturing sector is continuously evolving, and new materials and substances are being introduced in the production cycles. Therefore, it is fundamental to assess the toxicity of the produced waste matrices, sewage sludge derivates, and their short- and long-term impacts on soil.

In this context, ecotoxicological tests are a useful tool to evaluate the environmental impacts on the soil biota of organic matrices, including domestic effluents (Gallego et al., 2021), sewage sludge (Tigini et al., 2016), biochar (Tomczyk et al., 2021), animal manure (Segat et al., 2015), and compost (Pivato et al., 2016). Several types of bioindicators, including earthworms (Cesar et al., 2012), bacteria (Godlewska et al., 2022), crustaceans (Picariello et al., 2021), and plants (Sommaggio et al., 2018) have been applied to evaluate the potential toxicity of waste-derived organic fertilizers such as sewage sludge and its derivates. Ecotoxicity has been often assessed directly on waste matrices (Tigini et al., 2016; Udebuani et al., 2021), but this approach does not allow to understand if toxicity is due to the presence of harmful molecules (Weissengruber et al., 2018) or to other adverse effects typical of the organic matrices (e.g., salinity, ammonia, volatile fatty acids accumulation) (Simplício et al., 2017; Tognetti et al., 2021) that assume temporary importance. Therefore, a more correct approach to evaluate the ecotoxicity of waste-derived fertilizers is to study their effect directly in soil by monitoring the evolution of soil toxicity over time through different bioindicators.

The ecotoxicological approach employs a battery of bioassays using diverse species, recognizing the absence of a universally sensitive species to all environmental contaminants (Grenni et al., 2018; Sforzini et al., 2016). This approach enhances ecological reliability and result interpretation, facilitating a comprehensive analysis of contaminant effects and the identification of specific mechanisms of action (Terekhova, 2022). Careful selection of appropriate test organisms is crucial for bioassay selection, and it is advantageous for these species to exhibit complementary sensitivity patterns, responding differently to contaminants, thereby enhancing the overall assessment of environmental risks (Manzo et al., 2014). In this way, a more reliable and useful approach for estimating the real impacts of applying waste-derived fertilizer on agricultural soil is provided.

This study aims to assess the ecotoxicity of sewage sludge digestate by comparing its toxicity with that of other chemical and organic matrices that can be applied in agriculture and analyzing soil amended with sewage sludge digestate as well as non-amended soil for 4 years. Ecotoxicity was assessed directly on both organic and mineral matrices and soil fertilized with sewage sludge digestate and urea. Data collected were used to set up a reliable and useful ecotoxicity index able to track the adverse effects of sludge used as fertilizer.

The ecotoxicological tests were carried out on fertilizers and fertilized soils using indirect (elutriate) and direct tests to account for differences in species sensitivity and exposure. Ecotoxicological tests were performed using seven target organisms from four different trophic levels (*Raphidocelis subcapitata*, *Daphnia magna*, *Aliivibrio fischeri*, *Lepidium sativum*, *Sinapsis alba*, *Sorghum saccharatum*, and *Caenorhabditis elegans*). Finally, the results of each individual test were combined in an ecotoxicological test battery integrated index to assess the environmental risk.

2. Materials and methods

2.1. Type and origin of the organic matrices

The organic matrices tested as fertilizers in this study (Fig. 1) were as follows: **i.** *Urea*: N-based organic compound typically applied in agriculture as fertilizer. Organic N is hydrolyzed by soil bacteria and transformed to NH_3 which is readily available for plant uptake; **ii.** *Piggery slurry*: slurry produced in pig farms composed of excrements and urine diluted into rinsing water used for cleaning livestock rooms; **iii.** *Biowaste compost:* fertilizer and/or soil enhancer produced from the bio-

oxidation and humification of source-segregated organic fraction of municipal solid waste; **iv.** *Manure digestate*: organic material produced from anaerobic digestion of zootechnical effluents; **v.** *Digestate from HSTAD of sewage sludge:* organic material composed of primary and secondary sludge with a high solid content (10–11 %) that has undergone to thermophilic anaerobic digestion; **vi.** *Lime-stabilized sewage sludge:* sewage sludge chemically treated with calcium oxide to drastically reduce fermentation activity and pathogen content to be used as soil fertilizer according to the Italian legislation (D. Lgs. 152/2006); **vii.** *Defecation lime:* sewage sludge hydrolysed to improve fertility efficiency by adding lime and sulfuric acid resulting in the subsequent precipitation of calcium sulphate to be used as fertilizers according to Italian fertilizers law D. Lgs. 75/2010. The complete analytical profile for each fertilizer matrix, including physical properties, and heavy metals, micropollutants and pathogens contents, is reported in Table 1.

All matrices originated from full-scale facilities located within the Lombardy region in northern Italy. Matrix selection was not only based on regional practices but tailored to make a thoughtful comparison among matrices being typically used as chemically synthesized (urea) or traditional (piggery slurry, biowaste compost and manure digestate) organic fertilizers and emerging biofertilizers (fertilizers originating from sewage sludge) whose application complies with the principles of material recycling in the context of circular economy.

2.2. Origin of soil samples

A maize field (*Zea mays* L.; hybrid Pioneer P1547, FAO 600) located in Lombardy region (Italy) was used for a comparative evaluation of the ecotoxicity of the sewage sludge digestate from HSTAD used in agriculture (injected at 15-cm depth) for 4 consecutively years in comparison with routine chemical fertilization. The choice of HSTAD digestate to be compared with a conventional chemical fertilizer (urea) was based on previous research highlighting the good fertilizing potential of this matrix and on the proximity of the full-scale digestion plant to the experimental field. The field was divided into 9 randomized parcels assigned to sewage sludge digestate (3), urea (3), and without fertilizer (control) (3). The field was located in the Po Valley (northern Italy) and had an extension of 0.7 ha. The fertilization procedure consisted in surface distribution of urea and digestate injection. Distribution and dosage of the applied fertilizers, as well as the chronological list of agronomic operations were described by Zilio et al. (2023). Overall, the experimental campaign lasted 4 years from 2019 to 2022. In the first three years, soil samples were sampled at the beginning and at the end of the experimental period from each parcel to compare the long-term impact of HSTAD fertilization on soil in comparison with mineral fertilization and control. Soils were sampled at a depth of 0–30 cm and were analyzed in terms of chemical-physical characteristics as well as heavy metal and micropollutant contents. During the fourth year, soils were sampled at two different depths, i.e., 0–30 cm and 30–60 cm, and five different periods (t_0 = before fertilization; t_1: immediately after fertilization, t_2: after one month from the fertilization, t_3: after the harvest, and t_4: one year after fertilization) to monitor the spatial (soil depth) and temporal (sampling time) impact of the tested matrices. The soil was analyzed for both physical and chemical characteristics and for heavy metal and nutrients content. Approximately 2 kg of composite soil sample was collected from each parcel for the subsequent analyses; samples were immediately transported to the laboratory, stored at 4 °C, and analyzed within short time. For chemical analysis, samples were air dried, sieved to 2 mm and then ground to 0.5 mm.

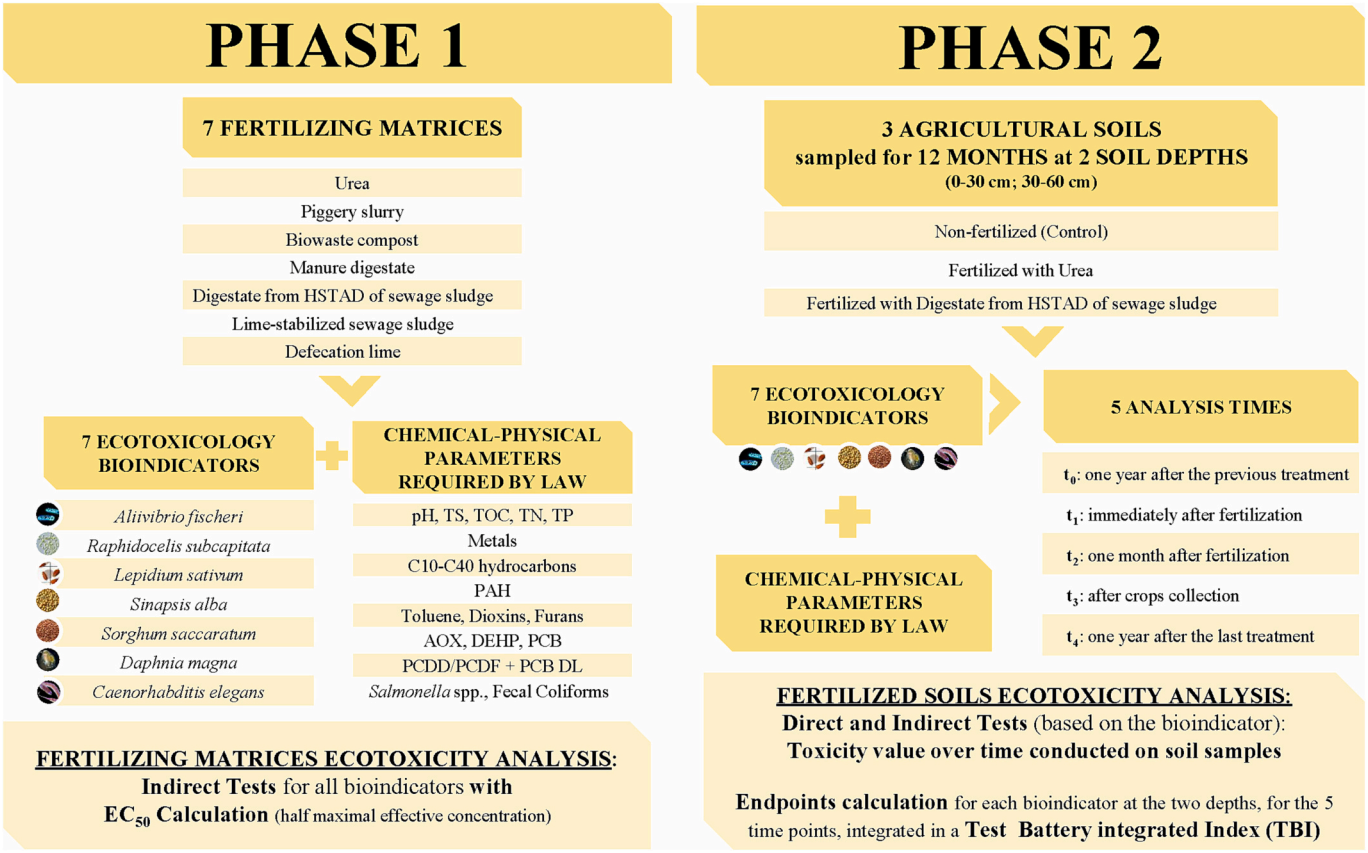

Fig. 1. Summary of the experimental protocol: following the analysis of the 7 fertilizing matrices, and the evaluation of EC_{50} for each bioindicator, the study focused on the evaluation of ecotoxicity and contaminant monitoring of soils amended with urea and digestate, sampling soils at different depth ranges (0–30 cm and 30–60 cm) and at five different times (t_0-t_4).

Table 1
Analytical profiles of the organic matrices tested as fertilizers in this study.

Matrix	Urea[a]	Piggery slurry[a]	Biowaste compost[a]	Manure digestate[a]	Sewage sludge digestate from HSTAD[b]	Lime-stabilized sewage sludge[c]	Defecation lime[c]
pH	8.3	7.0	7.1	8.2	8.5 ± 0.0	8.3 ± 0.1	11.4
TS (%)	98.5	2.98	80.9	4.49	10.4 ± 0.2	20.5 ± 1.7	32.3 ± 1.8
TOC (%TS)	18.6	33.7	33.6	34.2	33.3 ± 5.4	27.5	24.8
TN (%TS)	46	11	1.0	9.5	7.6 ± 0.2	1.7	1.5
TP (%TS)	<0.01	3.34	0.41	0.89	3.23 ± 0.26	2.78 ± 0.56	1.14 ± 0.23
Arsenic (mg/kg TS)	<2.05	5.5	<2.05	<2.05	8.4 ± 2.4	8.43 ± 1.69	3.55 ± 0.71
Beryllium (mg/kg TS)	<1	<1	<1	<1	<1	0.225 ± 0.045	0.118 ± 0.024
Cadmium (mg/kg TS)	<0.65	<0.65	0.65	<0.65	0.8 ± 0.2	0.197 ± 0.040	< 0.136
Chromium (mg/kg TS)	<9.5	10.3	10.5	9.9	78.7 ± 8.5	62.8 ± 12.6	–
Chromium (hexavalent) (mg/kg TS)	<0.5	<0.5	<0.5	<0.5	<0.5	n.d.	n.d.
Mercury (mg/kg TS)	<1.3	<1.3	<1.3	<1.3	<1.3	n.d.	n.d.
Nickel (mg/kg TS)	<8	<8	11	10.1	59.3 ± 7.1	46.1 ± 9.3	20.8 ± 4.2
Lead (mg/kg TS)	<7.5	<7.5	8.0	<7.5	64.6 ± 17.6	39.3 ± 7.9	16.3 ± 3.3
Potassium (g/kg TS)	1.88	73	12.3	81	5.5 ± 0.7	0.27 ± 0.05	4.14 ± 0.83
Selenium (mg/kg TS)	<1	8.24	<1	<1	2.0 ± 0.4	1.70 ± 0.34	n.d.
Copper (mg/kg TS)	<5	275	40	56	321 ± 32	319 ± 64	104 ± 21
Zinc (mg/kg TS)	<29	1184	93	244	992 ± 92	675 ± 136	272 ± 55
C10-C40 hydrocarbons (mg/kg)	<100	<100	580	<100	575 ± 187	1365 ± 285	–
PAH (mg/kg TS) [d]	<0.5	<0.5	<0.5	<0.5	<0.5	< 0.01	–
Toluene (mg/kg TS)	<0.1	<0.1	0.19	<0.1	2.64 ± 3.32	< 10	–
Dioxins and furans (μg/kg TEQ)	4.58	11.6	7.16	7.61	10.4 ± 1.6	–	–
AOX (mg/kg TS)	<0.6	<0.6	<0.6	<0.6	<0.6	< 35	–
DEHP (mg/kg TS)	0.31	0.52	8.0	0.18	28.9 ± 24.5	< 10	–
PCB (mg/kg TS)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	–
PCDD/PCDF + PCB DL (ng/kg TS)	5.26	13.4	8.23	8.77	9.30 ± 1.63	–	–
Salmonella spp. (MPN/g TS)	n.d.	>36,700	n.d.	n.d.	n.d.	n.d.	n.d.
Fecal coliforms (MPN/g TS)	<3	13,700	11	5100	29 ± 1	n.d.	<10–290

n.d. = not detected; - = not analyzed.
[a] Single values measured before the tests.
[b] Values are calculated based on 5-month monitoring (5 replicates) of matrix characteristics.
[c] Values are calculated based on three replicates of one sampling campaign.
[d] Calculated as indicated by the Italian law D.Lgs. 152/2006.

2.3. Ecotoxicological tests

In compliance with European regulations (Sewage Sludge Directive 86/278/EE C), Italian legislation (D. Lgs. 75/2010) and regional regulations (Lombardy Region – D.G.R. X/2031/2014, updated by D.G.R. X/7076/2017), it is mandatory to evaluate the ecotoxicity of biofertilizers using the germination index (GI) assay or phytotest.

For this study, three herbaceous species, namely *Lepidium sativum*, *Sinapsis alba*, and *Sorghum saccharatum*, were selected to conduct the GI assays. The regional legislation, i.e., D.G.R. X/7076/2017 and D.G.R. 16/04/2003 n. 7/12764 guidelines, were used as a reference. According to these guidelines, the GI should be higher than 60 % when considering a sample dilution of 30 %.

These regulatory requirements and standardized testing protocols ensure a comprehensive assessment of the potential ecotoxicological impacts of biofertilizers, guaranteeing compliance with safety and environmental standards. By utilizing specific plant species and established endpoints, the evaluation process becomes more rigorous and reliable, thereby contributing to the overall understanding of the ecological consequences associated with biofertilizer usage.

In this study, both direct and indirect tests were performed to evaluate the ecotoxicity of soils fertilized with sludge digestate. Direct tests involved four bioindicators: *Caenorhabditis elegans*, *Lepidium sativum*, *Sorghum saccharatum* and *Sinapsis alba*. The four bioindicators were directly exposed to the fertilized soils to assess their response to potential toxic effects.

On the other hand, indirect tests were conducted employing *Daphnia magna*, *Aliivibrio fischeri* and *Raphidocelis subcapitata*. These bioindicators were not directly exposed to the fertilized soils but instead to elutriates obtained by mixing the soils with distilled water. The elutriate samples represented the aqueous fraction containing dissolved or leached molecules from the fertilizers and soils. The threshold of 50 % effect was used to indicate toxicity in the study. This means that if the tested sample resulted in a 50 % or higher reduction in the measured parameter (e.g., germination rate, luminescence, immobilization), it was considered toxic. The use of the 50 % threshold provides an approach for determining the toxicity of the tested materials. It allows for easy comparison and interpretation of the results, indicating whether the samples have significant adverse effects on the tested organisms.

For the evaluation of organic fertilizer matrices, only indirect tests were conducted using all the above mentioned bioindicators. This means that the fertilizers themselves were not directly tested, but rather their effects on the bioindicators were assessed using elutriate samples. Conducting full-scale tests using undiluted fertilizers, as they naturally occur, would be impractical due to various reasons. To address this practical concern, the samples underwent to a dilution process during the analytical phase to facilitate testing. This dilution allowed us to obtain a more realistic representation of how fertilizers disperse and act in the environment, aiding in the understanding of the effective dose required to produce an effect and identifying a relevant threshold value. It also enabled us to evaluate fertilizer impacts across a range of concentrations, including lower levels that better reflect real-world application scenarios. Moreover, using direct tests to evaluate the ecotoxicity of fertilizing matrices can be misleading because adverse effects may be generated by other factors (e.g., non-optimal pH, ammonia content or salinity) rather than the presence of toxic substances.

2.3.1. Direct tests

Direct tests, i.e., the germination and mortality tests, pointed at the assessment of the effects of soils fertilized with the digestate on plant germination and nematode mortality. By doing so, tests were conducted by directly exposing organisms to fertilized soils that were moistened to achieve a water holding capacity of 80 %. For the germination tests, three plant species (*Lepidium sativum*, *Sinapsis alba*, and *Sorghum saccharatum)* were used (D.G.R. 16/04/2003 n. 7/12764 and ISO 11269-1:2012), and the seeds were supplied by Ecotox LDS (Milan, Italy). Ten seeds were placed in Petri dishes and incubated in darkness at a temperature of 25 ± 1 °C; all test were performed in triplicate. After 3 days, the number of germinated seeds and the length of the developed roots were measured. The nematode *Caenorhabditis elegans* - wild-type strain N2 variant Bristol was used in performing the mortality test. *C. elegans* was cultured on K-agar plates (2.36 g KCl, 3 g NaCl, 2.5 g Bacto-peptone, 17 g Bacto-agar, and 1 mL cholesterol (5 mg/mL)) and they were fed with the OP50 strain of *Escherichia coli*, which was seeded onto the plates. The mortality test was conducted following the ASTM E2172 Standard Method (2014), with some modifications. In each test, 20 age-synchronous adult nematodes were exposed to the soil samples for 24 h at a temperature of 20 °C. The tests were carried out in four replicates and the nematodes were not provided with food during the testing period. The standardized methods and replicates ensured the reliability and accuracy of the results obtained.

2.3.2. Indirect tests

Indirect tests were conducted by preparing elutriates, which were obtained by mixing fertilizers or the fertilized soils with distilled water at a 1:4 ratio (dry weight/volume of water). The mixture was stirred for 30 min and then allowed to settle for 1 h. The supernatant was then centrifuged for 20 min at 3000 rpm to minimize suspended solid particles. The resulting aqueous fraction, known as the elutriate sample, was used for the toxicity tests. First, elutriates from soils fertilized were tested at full concentration (100 %), whereas the elutriates derived from the fertilizers underwent dilution at various levels, resulting in final concentrations of 3.1 %, 6.2 %, 12.5 %, 25 %, 50 %, and 100 % (v/v). For the indirect tests on fertilized soils, three bioindicators were utilized, using standardized methods, i.e., *Raphidocelis subcapitata* for algal growth inhibition (ISO 8692:2012), *Aliivibrio fischeri* for bacteria luminescence inhibition (ISO 11348-3:2007), and *Daphnia magna* for crustacean immobilization (EN ISO 6341:2012). All tests were conducted in triplicate with constant illumination and temperature. The Daphnids were cultivated using ISO 6341 medium, while microalgae using ISO 8692 medium. Furthermore, Daphnids were fed daily, five days a week, with *R. subcapitata* ensuring a stable and controlled environment throughout the study. The lyophilized bacteria of *A. fischeri* were provided by Ecotox LDS (Milan, Italy). In the algal growth inhibition test, the density of *R. subcapitata* was measured using spectrophotometric analysis. The inhibition of cell growth was calculated by comparing the growth rate of the control with that of the sample (ISO 8692:2012). The bacteria luminescence inhibition test was performed using *A. fischeri*, according to ISO 11348-3:2007. The luminescence of the bacteria was measured using a Microtox® analyzer, and the light reading was recorded at specific time intervals. The tests were conducted in triplicate with constant illumination and temperature. For the crustacean immobilization test with *D. magna*, young daphnids were exposed to the samples, and immobilization was recorded after 24 h. The tests followed the guidelines outlined in EN ISO 6341:2012. In addition to these indirect tests, the toxicity assessment of organic fertilizer matrices was also performed using the germination and mortality assays described in Section 2.3.1. In this case, the tests were conducted using elutriates rather than the raw samples.

2.4. Analytical methods for chemical analyses

The list of chemical, physical, and microbiological parameters analyzed for each matrix is reported in Table 1, while the parameters monitored for the soils during the 4-year experimental campaign are listed in **Tables S1** and **S2**. The pH and concentrations of total solids (TS), total organic carbon (TOC), total nitrogen (TN), total phosphorus (TP), potassium (K), heavy metals (Cd, Cr, Ni, Pb, Cu, Zn), inorganic micropollutants (Hg, As, Be, Se), C10-C40 hydrocarbons, polycyclic aromatic hydrocarbons (PAH), halogenated organic compounds (AOX), polychlorinated biphenyls (PCB), polychlorinated dibenzo-p-dioxins and polychlorinated dibenzo-p-furans (PCDD/Fs), diethylhexyl

Table 2
EC_{50} values obtained with the selected test battery; values are in % (v/v). The lowest EC_{50} value obtained for each tested fertilizing matrix is highlighted in bold character. EC_{50} could not be evaluated for germination tests due to absence of toxicity.

Fertilizing matrix	EC_{50}[a] (%, v/v)			
	D. magna	*R. subcapitata*	*A. fischeri*	*C. elegans*
Urea	**6.4** [b]	29.4	31.2	7.5
	(5.5–7.4)	(27.00–31.9)	(26.1–37.6)	(6.4–8.8)
Piggery wastewater	9.7	38.5	**8.1**	10.3
	(8.6–10.9)	(34.9–41.4)	(7.2–9.1)	(9.1–12.7)
Biowaste compost	25.0	56.7	**9.3**	12.4
	(22.7–27.3)	(53.6–60.7)	(6.9–12.6)	(11.3–13.6)
Manure digestate	7.8	34.3	45.8	**6.4**
	(6.4–8.9)	(31.5–37.4)	(38.3–55.4)	(5.7–7.1)
Sewage sludge digestate from HSTAD	8.6	34.2	40.0	**5.3**
	(7.6–9.8)	(31.2–37.5)	(33.3–43.2)	(4.5–6.1)
Lime-stabilized	23.9	34.3	45.6	**17.7**
	(21.4–26.6)	(31.5–37.4)	(40.1–50.9)	(13.9–22.6)
Defecation-lime	11.5	30.1	**7.7**	10.3
	(9.7–13.5)	(26.8–33.7)	(6.3–9.3)	(8.6–12.2)

[a] EC_{50} are provided with ±95 % confidence limit values in brackets ($n = 3$).
[b] Values are in % (v/v).

phthalate (DEHP), fecal coliforms, and Salmonella were measured as described by Pigoli et al. (2021). Dioxins and furans, toluene, and nonylphenols concentrations were measured respectively according to UNI 11199:2007, UNI EN ISO 22155:2016, and ASTM D7485-16 methods. Soil granulometry and cationic exchange capacity (CEC) were measured as described by Zilio et al. (2023). Assimilable phosphorus (as P_2O_5) was measured according to Olsen et al. (1954).

2.5. Statistical analysis

2.5.1. Ecotoxicological tests

The results of the ecotoxicological tests were presented as the mean ± standard error. The inhibitory effect of *A. fischeri* was calculated using the following equation:

$$H_t = [(I_{ct} - I_t)/I_{ct}] \times 100 \quad (1)$$

where H_t represents the inhibitory effect, I_{ct} is the luminescence intensity of the control, and I_t is the luminescence intensity of the test sample after 30 min.

The equation for the immobility effect on *D. magna* and for mortality effect on *C. elegans*, where immobility/mortality was expressed as a percentage and was calculated by dividing the number of immobile/dead organisms by the total number of organisms.

The specific growth rate (μ) of *R. subcapitata* was calculated using the following equation:

$$\mu = \frac{lnN_n - ln\,N_0}{t_n - t_0} \quad (2)$$

where N_0 is the initial cell concentration, N_n is the final cell concentration after 72 h, t_0 is the start time, and t_n is the time of the last measurement (in hours from the start). Algal growth inhibition (%) was determined by comparing the sample's growth rate to the control.

As for plant seeds, the growth index was determined by multiplying the number of germinated seeds (G1) by the length of roots (L1). The GI was then utilized to calculate the effect, expressed as a percentage (%), in comparison to the control, using the following equation:

$$Germination\ Index\ (\%) = \frac{IGS}{IGC} \times 100 \quad (3)$$

where IGS and IGC represent the germination indices calculated for the samples and the control, respectively.

The statistical analysis for the ecotoxicological tests was performed using XLSTAT and GraphPad Prism software (Systat Software, San Jose, CA). The median effect concentrations (EC_{50}) for the tested fertilizing matrices were calculated using non-linear regression, and the mean values along with their relative 95 % confidence limits were determined. The EC_{50} represents the concentration at which a noticeable effect occurs in 50 % of the exposed population. To compare the effects of the treated soils, the data were normalized using Abbott's formula. This normalization involved calculating the effect values of the treated soils relative to the effect values of the non-fertilized control soils. The formula used was $((x_1\text{-}x_0)/(100\text{-}x_0)*100)$, where x_1 represents the treatment effect and x_0 represents the negative control effect (Finney, 1971). This normalization method allows for a more accurate comparison of the effects of the fertilizing matrices on the treated soils.

2.5.2. Chemical analyses

The statistical differences among the temporal profiles of soil characteristics were evaluated by one-way factor analysis of variance (ANOVA) using the Data Analysis Tool of Excel 365 (Microsoft Corporation, USA). The significant difference was set at 95 % ($p < 0.05$).

2.6. Toxicity test Battery integrated Index

The results of the tests within the ecotoxicological battery were integrated to assess the overall risk of the fertilized soil. According to Grenni et al. (2018), the integrated battery index was given by different "weight" to the different parameters available (severity and number of endpoints observed, kind of environmental matrix analyzed, level of agreement among test results).

The formulas utilized for generating and calculating the Toxicity Battery Index (TBI) adhere to the integrated approach recommended by the Italian National Institute for Environmental Protection and Research (ISPRA, 2013, 2011), which was further revised and modified by Manzo et al. (2014) for surface water and applied by Grenni et al. (2018) for assessing soil toxicity in the presence of foaming products. The values obtained for each analyzed sample were adjusted using the statistical correction criteria (referred to as CCS) outlined by Manzo et al. (2014) and Grenni et al. (2018). These criteria involve assessing the discrepancies between the sample and its corresponding control soil, considering factors such as the severity of each specific endpoint, the type of environmental matrix examined, the number of observed endpoints, and the level of agreement among test results.

In this study, a total of 7 endpoints were considered in the application of the battery index, as the algorithm requires a minimum of three endpoints. The TBI algorithm employed to generate an ecotoxicological risk scale yields two outputs: the integrated toxicity value (T%) and the risk battery (R%). These values were combined and categorized into five

main groups for the samples: (i) TBI ≤ 5 %, indicating an absent risk; 5 % < TBI ≤ 20 % (with the number of not statistically significant endpoints (C) ≤ 0), indicating low risk; 5 % < TBI ≤ 20 % (with C > 0), indicating medium risk; 20 % < TBI ≤ 50 %, indicating high risk; and TBI > 50 %, indicating very high risk (Grenni et al., 2018; ISPRA, 2013, 2011; Manzo et al., 2014) (see Supplementary Materials).

3. Results and discussion

3.1. Ecotoxicity tests performed on fertilizing matrices

The EC_{50} results obtained for each bioindicator using different fertilizing matrices are presented in Table 2. Analysis of the data reveals that the nematode *C. elegans*, followed by the crustacean *D. magna*, exhibited the highest sensitivity as bioindicators for assessing the environmental impact of fertilizers. This was evident from their generally lower EC_{50} values compared to *R. subcapitata* and *A. fischeri*. The lowest EC_{50} values overall were observed for *C. elegans*, measuring 5.3 % and 6.4 %, respectively. In contrast, the GI endpoint using *L. sativum*, *S. saccharatum*, and *S. alba* as bioindicators showed the least sensitivity. When seeds were used as bioindicators, it was not possible to calculate accurate EC_{50} values due to the absence of adverse effects exhibited by the fertilizer matrices even at a 25 % dilution of the elutriates. Therefore, with only three dilutions available, a precise determination of the

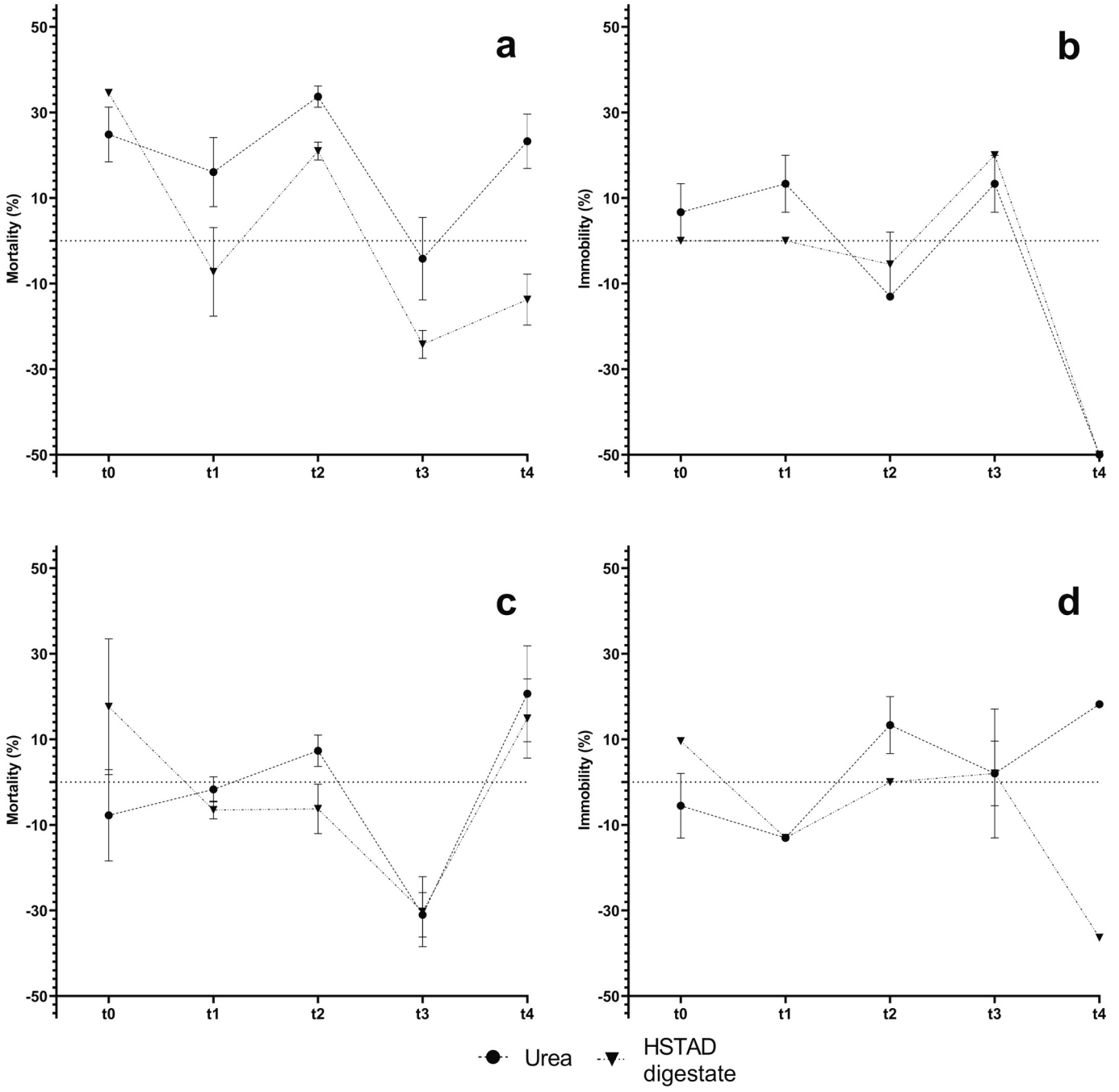

Fig. 2. Mortality (%) of *Caenorhabditis elegans* and Immobility (%) of *Daphnia magna*, following 24-hour exposure to soil and soil elutriate respectively, amended with urea and HSTAD digestate at different sampling times (t_0 = before fertilization; t_1 = immediately after fertilization; t_2 = one month after fertilization; t_3 = after the harvest; t_4 = one year after fertilization) and different soil depths (a) 0–30 cm and b) 30–60 cm for *Caenorhabditis elegans*; c) 0–30 cm and d) 30–60 cm for *Daphnia magna*. Results are presented as mean ± SD and are normalized with non-fertilized control soils.

EC_{50} value was not feasible.

Based on the obtained results, the relative sensitivity hierarchy of the specific bioindicators towards the seven evaluated fertilizers was as follows: *C. elegans* > *A. fischeri* > *D. magna* > *R. subcapitata* > *L. sativum, S. saccharatum*, and *S. alba*.

These findings align with a previous ecotoxicological study conducted by Pivato et al. (2016a) on digestate used as a biofertilizer. That study reported positive effects on the relative growth and GI of *Lepidium sativum* at low digestate doses (up to 15 % w/v), while higher concentrations (> 40 % w/v) showed inhibitory effects on the bioindicator, supporting the outcomes of this study. Additionally, the same authors observed higher toxicity in *D. magna* compared to *A. fischeri* and *Artemia* sp., (Pivato et al., 2016a), and a higher level of ecotoxicity in compost produced from aerobic digestion of food and green waste compared to digestate (Pivato et al., 2016), which aligns with our observations. Again, the analyses by Pivato et al. (2016a) on the worm *Eisenia fetida* also confirmed the results obtained for the nematode *C. elegans* in this study. In fact, they observed a hormesis trend in relative growth, indicating a positive effect on the bioindicator at lower digestate concentrations (up to 40 % w/v) and a toxic effect at concentrations higher than 50 % w/v. Furthermore, as emphasized by Pivato et al. (2016), the U.S. Environmental Protection Agency (U.S. EPA, 2017) advises the use of data points from at least five indicators representing different trophic levels for Species Sensitivity Distribution.

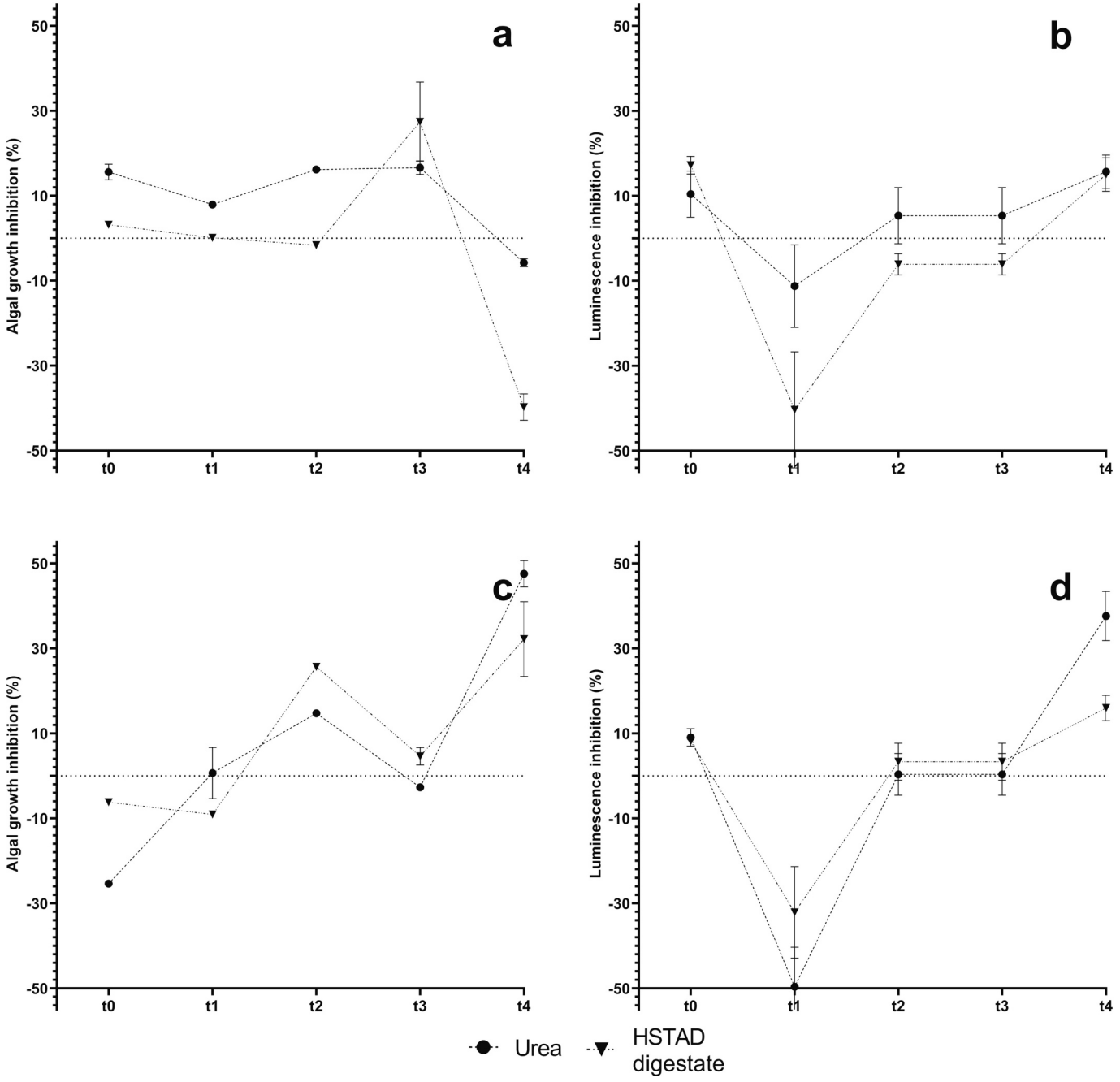

Fig. 3. Algal growth inhibition (%) of *Raphidocelis subcapitata* and Luminescence inhibition (%) of *Aliivibrio fischeri*, after exposure to soil elutriate amended with urea and HSTAD digestate at different sampling times (t0 = before fertilization; t1: immediately after fertilization; t2: after one month from fertilization; t3: after the harvest; t4: one year after fertilization), analyzing two soil depths: a) 0–30 cm and b) 30–60 cm for *Raphidocelis subcapitata*, and c) 0–30 cm and d) 30–60 cm for *Aliivibrio fischeri*. Results are presented as mean ± SD and normalized with non-fertilized control soils.

The results obtained in this study confirmed that the approach measuring ecotoxicity proposed in this work, enhances its significance, highlighting the higher sensitivity of terrestrial organisms compared to aquatic (Pivato et al., 2016). In fact, using aquatic organisms evaluating terrestrial environments may not provide useful data because of the different habitats and the need for indirect tests. Conversely, the simultaneous use of plants and earthworms, considering their respective physiology, may yield more specific results for soils environments, as they are more sensitive to any perturbations observed in soils (Pivato et al., 2016; U.S. EPA, 2017).

The comparable ecotoxicity observed for sewage sludge-based fertilizers vs. well-known and widely used in agriculture organic waste (compost, pig slurry and manure digestate), and chemical fertilizers (urea), suggested that the formers do not pose environmental concerns in terms of ecotoxicity.

3.2. Short- and long-term effects of fertilization with urea and HSTAD digestate at different soil depths

The lists of the contaminant profile of soil cultivated with urea and sewage sludge digestate at the end of the three-year monitoring period are reported in **Table S1**. No statistically significant differences ($p > 0.05$) were observed in terms of chemical-physical characteristics and pollutant contents among the soil treated with sludge digestate, soil fertilized with urea, and control. Similar results were obtained, also, for soils sampled during the fourth year of the experimental campaign (**Table S2**) at different sampling times and soil depths ($p > 0.05$). The results obtained in this study confirmed previous studies which indicated that sewage sludge from HSTAD digestate can act as a fertilizer and successfully substitute mineral fertilizers, as it presents an environmental profile similar to those of other organic matrices typically used in agriculture, such as agricultural digestates and green compost (Pigoli et al., 2021; Scaglia et al., 2018).

3.3. Ecotoxicity tests on soils fertilized with sewage sludge digestate and urea

The data obtained from the analysis of fertilized soils provided valuable insights into the endpoints of each bioindicator. Ecotoxicity was measured at two different soil depths (0–30 and 30–60 cm) and considering five specific time over the course of one year (Figs. 2–6).

The ecotoxicity values were normalized to the non-fertilized control soils by using the Abbott's formula to isolate the specific impact of the fertilizing matrix. This approach eliminated the direct contribution of individual matrices (soil) to the toxicity of each bioindicator, which are discussed in the following chapter.

3.3.1. Ecotoxicity analysis on Caenorhabditis elegans

The ecotoxicity assessment conducted on fertilized soils yielded intriguing results when considering the bioassays performed with the nematode *C. elegans* (Fig. 2), i.e., all soil samples showed an ecotoxicity level below the established threshold limit of 50 %. Furthermore, the findings obtained from the samples collected at t_1-t_3 revealed a decline in toxic effects for soils with higher relative toxicity compared to t_0, suggesting that contact time, i.e., the duration between fertilization and sampling, played an important role in determining toxicity levels. Higher mortality rates of *C. elegans* were observed in soil fertilized with urea, particularly in samples collected from the 0–30 cm depth range (Fig. 2a). The increase in mortality rates at t_2 and t_4 in the urea-fertilized soil may be attributed to several potential factors, including variations in nutrient availability, the presence of toxic substances resulting from the decomposition of the fertilizing material, alterations in environmental conditions like temperature and humidity, or the influence of other organisms impacting the survival of *C. elegans*. Prior to fertilization (t_0), soil fertilized with digestate displayed similar mortality rates to the urea-treated soil, while low or no toxicity was evidenced at t_4.

Moreover, soils amended with sludge digestate not only showed higher survival rates, but also exhibited increased replication percentages of *C. elegans* (Fig. 2c). These findings support existing scientific literature reporting that bio-fertilizers such as sewage sludge digestate can enhance not only the physical and chemical properties of soils, but also its biological functionality (Nkoa, 2014; Pigoli et al., 2021).

Previous studies on the ecotoxicity of biofertilizers, specifically focusing on earthworms under field conditions and long-term applications (4 years), are limited, so that results obtained represent in the present work are of particular importance. The few studies available in the literature (Butt and Putwain, 2017; Clements et al., 2012; Koblenz et al., 2015) outlined the presence of higher earthworm biomass in soils fertilized with solid or liquid digestate or undigested animal effluents, resulting in enhanced earthworm abundance ranging from +34 % to +120 % compared to non-fertilized soils (Butt and Putwain, 2017; Clements et al., 2012; Koblenz et al., 2015). Moinard et al. (2021) also described a positive effect on earthworm abundance (*L. terrestris* and *A. caliginosa*) in a long-term toxicity assessment of soils amended with mixed digestates, although negative effects were highlighted in the short-term.

3.3.2. Ecotoxicity analysis on Daphnia magna

None of the soil samples amended with urea and sewage sludge digestate from HSTAD showed an ecotoxic effect on *D. magna*. The assessment of toxicity was conducted by applying a threshold value of 50 % immobilization, beyond which samples were classified as toxic (Fig. 2b and Fig. 2d). The lowest absolute toxicity values were found in soil amended with digestate, at t_4, in soil samples collected at 0–30 cm and 30–60 cm soil depth (Fig. 2d). Soil fertilized with urea, also, registered low ecotoxic effects (immobility $\leq$20 %) for both the 0–30 cm and 30–60 cm soil depth (Fig. 2c). These results contrasted with those reported by Różyło et al. (2015), which evidenced an increase of toxicity on *D. magna* for soils amended with biogas digestate after 48 h contact time of soil with 3 % *v*/v digestate. In the study of Różyło et al. (2015), assays performed over a 12-month period comparing the treated soils with control soil, ecotoxicity showed fluctuation, with, in most cases, a marked decrease after 12 months.

3.3.3. Ecotoxicity analysis on Raphidocelis subcapitata

Data concerning the ecotoxicity on *R. subcapitata*, did not evidence toxicity after 72-h of exposition of the alga to the amended soils. Results on soil amended with sewage sludge digestate were different for the two soil depths considered (Fig. 3a and Fig. 3c): while no toxicity was evidenced at 30–60 cm soil depth at the time points considered, the 20 % algal growth inhibition was highlighted at t_3 in soil sampled at 0–30 cm depth (Fig. 3a). This result may depend on the lack of nutrients in the soil following the crop collection; however, in both cases, the toxicity never overcome 30 %, which proves that using such organic matrix did not cause ecotoxic effects (i.e., value below the 50 % threshold) on the algal indicator (Fig. 3c).

3.3.4. Ecotoxicity analysis on Aliivibrio fischeri

Test performed with *A. fischeri* did not show any toxic effect for soils fertilized with the two fertilizers **(**Fig. 3**)**. The lowest effects were registered for soil sampled at t_1, t_2, and t_3 (t_1: immediately after fertilization; t_2: after one month from the fertilization; t_3: after the harvest). At t_4, a slight increase of bioluminescence inhibition in soil amended with urea was evidenced for the 30–60 cm soil depth, but the effects were lower than the threshold limit previously indicated for the bioindicator. These findings were in line with Różyło et al. (2015) that, investigating the toxicity of soils amended with digestate and other biofertilizers, reported similar levels of luminescence inhibition compared to that of the control soil, i.e., the level of inhibition on the bioindicator was indeed the same detected at the beginning of the study. Despite the low sensitivity and the correlated low statistical significance registered for this bacterium as bioindicator, *A. fischeri* has been widely

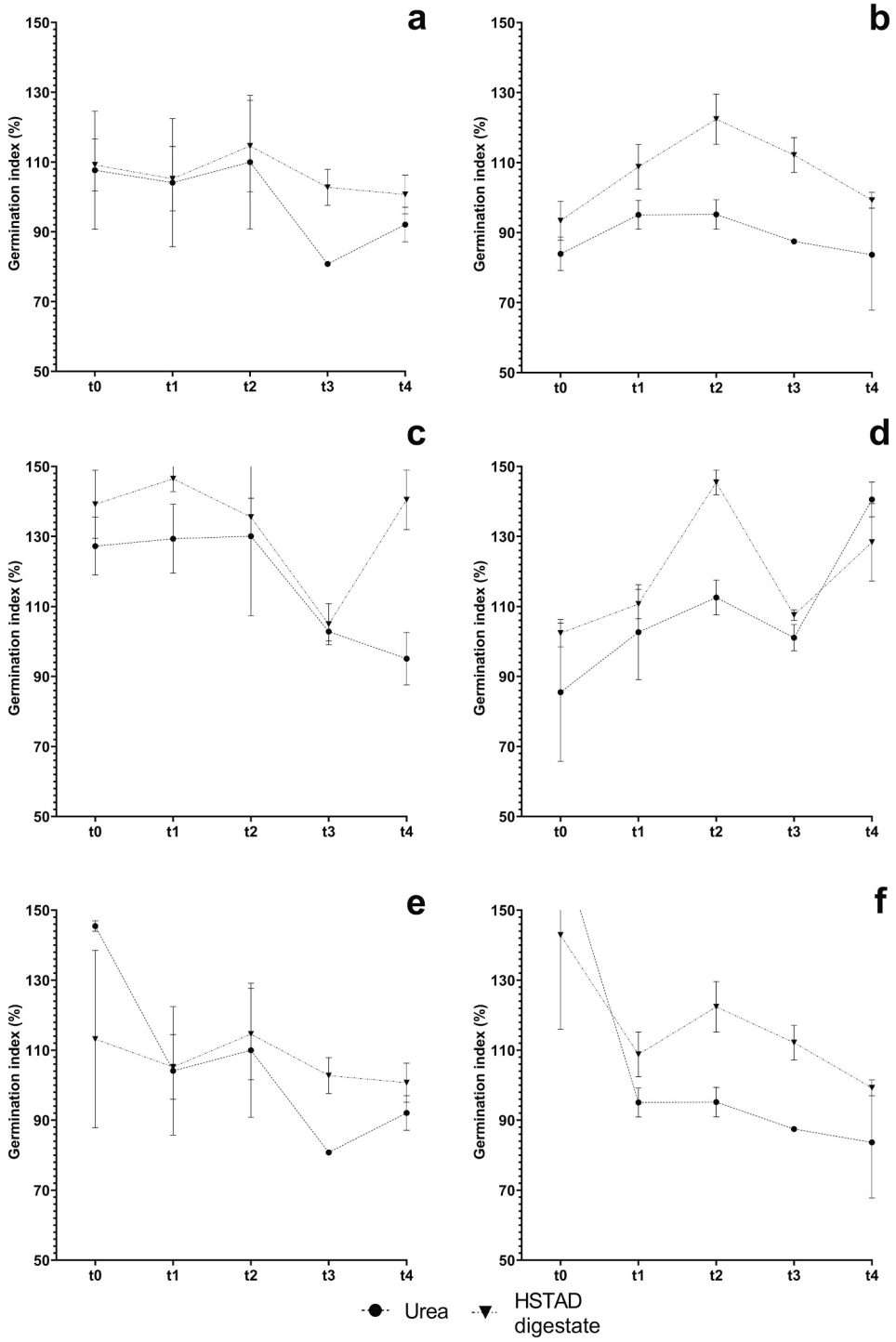

Fig. 4. Germination index (%) of *Lepidium sativum* (a-b), *Sinapis alba* (c-d) and *Sorghum saccharatum* (e-f) after 72 h of exposure to soils amended with urea and HSTAD digestate at five different sampling times (t_0 = before fertilization; t_1: immediately after fertilization; t_2: after one month from the fertilization; t_3: after the harvest; t_4: one year after fertilization), analyzing different soil depths: a) 0–30 cm b) 30–60 cm. Results are presented as mean ± SD and are normalized with non-fertilized control soils.

Table 3
Trend of the identified risk for each amended soil sample at the five diverse time points, employing the Toxicity test Battery integrated Index (TBI).

Soil depth	0–30 cm					30–60 cm				
Sampling times	t_0	t_1	t_2	t_3	t_4	t_0	t_1	t_2	t_3	t_4
TBI for urea	0.17	0.15	0.00	0.10	0.00	0.34	0.05	−0.02	0.07	0.05
TBI for sewage sludge digestate	0.21	0.39	0.10	0.04	0.00	0.11	0.00	0.02	0.02	0.13

employed in ecotoxic assays of environmental contamination considering diverse categories of chemical compounds (Hale et al., 2013; Malara and Oleszczuk, 2013). However, previous reports confirmed the low significancy of using *A. fischeri* as bioindicator for the assessment of the toxicity of contaminated soils or waste, registering an even less sensitivity in assays performed on soils amended with sewage sludge (Malara and Oleszczuk, 2013).

3.3.5. Ecotoxicity analysis on plants (phytotoxicity)

The phytotoxicity of soil samples was evaluated by exposing plant seeds of the bioindicators L. *sativum*, *S. saccharatum*, and *S. alba* to the fertilized soils. No toxic effects (GI comprised between 80 % and 120 %) were observed at the five time points, as the tests rather highlighted positive effects especially on soils amended with sludge digestate (Fig. 4). Różyło et al. (2015), who focused on the use of biosolids as fertilizers, reported a stimulating effect of such matrices on the growth of *L. sativum* roots; only when digestate concentration was employed over 3 %, the inhibition on the growth of *L. sativum* roots was observed.

The absence of toxicity in soils amended with biofertilizers depends on the positive effect that such matrices exert on the growth and development of plants, being rich in macro- and micronutrients (Malara and Oleszczuk, 2013; Pigoli et al., 2021; Różyło et al., 2015). In the present study, the stimulatory effect, rather than inhibitory effect, may be presumably linked to the degradation of the fertilizer itself. Despite, the decline of the stimulating action on L. *sativum* seeds (Fig. 4b) could be related to the decreased availability of nutrients, as much as transformations of the organic carbon sources exploitable from the biofertilizers (Różyło et al., 2015).

3.3.6. Ecotoxicity classification using the Test Battery integrated Index

In order to assess the ecotoxicity of soils amended with two fertilizing matrices and simplify the toxicity evaluation process, a synthetic index of toxicity was generated by integrating the overall results of the selected battery of bioindicators. This integration was achieved using Test Battery integrated Index (TBI), which employs a scoring system to calculate the index for each sample (see Supplementary Materials). The TBI model takes into account various factors, including the severity and degree of the effect, test variability, consistency between tests, and the number of measured endpoints. By combining and associating these parameters, the TBI provides a comprehensive methodology for evaluating ecotoxicity.

Table 3 presents the results of the TBI analysis, indicating the integrated ecotoxicity index values for the soils collected at different time points (t_0, t_1, t_2, t_3, and t_4). These index values were used to categorize the risk level associated with each soil sample.

Interestingly, all the analyzed soils were classified at the lowest risk level, with TBI values below 5 %. This suggested that the fertilizing matrices (urea and sludge digestate) did not induce significant ecotoxic effects on the bioindicators used in the study. Furthermore, some samples even displayed TBI values below 1 %, indicating an absence of ecotoxicity. These findings provided evidence that the fertilizing matrices, when applied to soils, did not pose a substantial risk to the tested organisms. Again, results obtained suggested that the integrated approach using TBI successfully captureed and evaluateed the overall ecotoxic potential of the soils amended with the fertilizing matrices.

4. Conclusions

Bio-fertilizers from organic waste materials represent a valid alternative to chemical fertilizers and may play a pivotal role in achieving the sustainable agriculture goal, contributing to the recovery of nutrients, thus embracing the principles of circular economy. Aiming at promoting sustainable agriculture, it is essential to perform a risk assessment of the effects that such biofertilizers exercise on the soil and on the autochthonous organisms. This research suggests specific insights concerning the long-term effects on soils of biofertilizers on a battery of seven bioindicators from different trophic levels. The addition to the soil of sewage sludge digestate from HSTAD held, in most cases, positive impacts on most tested organisms compared to non-treated control soils and urea-fertilized soils. Long-term monitoring of fertilized soils with urea and sewage sludge digestate at different depths revealed no significant alteration of the physical-chemical properties, including the concentrations of heavy metals and organic contaminants, due to fertilizer addition. Moreover, soil toxicity resulted lower when compared to the fertilizers itself, proving the higher significance of on-field long-term monitoring compared to the direct ecotoxicological evaluation of the fertilizing matrices. Data analysis of the fertilized soils highlighted the absence of toxicity for all the bioindicators considered. The utilization of bioindicators being more sensitive to ecotoxicity variations than the GI bioassays (demonstrated less sensitive) required by local, national, and international guidelines, may consent the detection of ecotoxic effects of specific fertilizers, thus enabling the application of risk mitigation strategies connected to the employment of safe fertilizers.

This study highlights that the determination of the ecotoxicity of fertilizers applied on soils is a reasonable approach that can be proposed for future methodological applications, as it allows the verification of the true onset of ecotoxicity, which could in some cases be transient and due to factors not related to the presence of toxic molecules (e.g., non-optimal pH, accumulation volatile fatty acids and/or ammonia, etc.). It should be pointed out that in this study soil ecotoxicity was evaluated based on the actual application methods of the tested fertilizing matrices. Indeed, the direct determination of ecotoxicity on complex matrices, such as the fertilizers analyzed in the present research (i.e., without considering their actual use and dosage in the soil), which is often adopted for evaluating the ecotoxicity of certain matrices, can be misleading as adverse effects may be generated by other factors rather than the presence of toxic molecules in the fertilizer matrix and does not consider the real fertilizer application and dosages on soil.

CRediT authorship contribution statement

Federica Carraturo: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Antonietta Siciliano:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Andrea Giordano:** Funding acquisition, Supervision, Writing – review & editing. **Francesco Di Capua:** Data curation, Writing – original draft, Writing – review & editing, Visualization. **Federica Barone:** Supervision. **Elisa Casaletta:** Funding acquisition, Supervision. **Flavia Cicotti:** Data curation, Formal analysis, Investigation, Methodology, Validation. **Marco Guida:** Conceptualization, Data curation, Methodology. **Fabrizio Adani:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Andrea Giordano reports a relationship with Acqua & Sole s.r.l. that includes: employment and funding grants. Elisa Casaletta reports a relationship with Agromatrici s.r.l. that includes: employment and funding grants.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.169341.

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