Manuscript Number: ECOLIND-12441R1

Title: Phytotoxicity assessment of conventional and biodegradable plastic bags using seed germination test

Article Type: Research paper

Keywords: Compostable; Leachate; Additives; Lepidium sativum; Phytotoxicity

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First Author: Elena Balestri, Ph.D.

Order of Authors: Elena Balestri, Ph.D.; Virginia Menicagli; Viviana Ligorini; Sara Fulignati; Anna Maria Raspolli Galletti; Claudio Lardicci

Abstract: A large fraction of plastic litter found in natural environments is constituted by conventional not biodegradable plastic bags, and their adverse effects via ingestion or entanglement on terrestrial and marine organisms are largely documented. Biodegradable and compostable shoppers have been recently developed as alternative to traditional ones. These bags are specifically designed to degrade in composting facilities and generate a product devoid of toxicity to soils and crops. However, very little is known on the effects of bag leaching, i.e. the transfer of chemicals from plastic into natural environments, on vegetation. Some plant species are highly sensitive to a variety of chemicals, and seedling growth is generally the most affected life history stage. In this study we assessed the potential effects of conventional (high-density polyethylene, HDPE) and compostable (Mater-bi®, MB) bags, when left in natural environments, on water quality and plant development. To this end, seeds of Lepidium sativum L., a terrestrial plant commonly used in phytotoxicity standard tests, were exposed to leachates obtained from different amount of HDPE and MB bags, simulating various pollution degrees occurring in nature, for 72 hours. Both not-exposed (or virgin) bags and natural weathering exposed bags were used. Variations of chemical-physical characteristics of extracts were used as indicative of water quality deterioration, while alterations of seed gemination and seedling radicle and hypocotyl length were considered as indicative of phytotoxicity. A chemical qualitative screening of the leachates was also performed to identify the compounds with potential phytotoxicity. Both types of bags affected water characteristics (pH, salinity and total dissolved solids) relevant to plants, and released into water intentionally added chemicals, such as the noxious bisphenol A, and other phytotoxic substances probably generated during bag manufacturing. Leachates from both bag types did not affect seed germination. But, a significant number of seedlings showed developmental abnormalities or reduced seedling growth. The hypocotyl was the most sensible seedling organ to HDPE bag leachates while the radicle was the most vulnerable to MB ones. These findings indicate that plastic bags, including those that meet biodegradability

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***Highlights (for review)**

- Conventional and compostable bags are a consistent fraction of plastic litter.
- Harmful chemicals readily leached out of bags and affected water quality.
- Leachates altered seedling development in the test plant *Lepidium sativum*.
- Compostable bags if discarded in natural habitats may adversely affect plants.
- Standard phytotoxicity tests could be useful in developing more eco-friendly bags.

Phytotoxicity assessment of conventional and biodegradable plastic bags using seed germination test

Elena Balestri[a,*], Virginia Menicagli[a], Viviana Ligorini[a], Sara Fulignati[b], Anna Maria Raspolli Galletti[b], Claudio Lardicci [a]

[a] *Department of Biology, University of Pisa, via Derna 1, 56126, Pisa, Italy*

[b] *Department of Chemistry and Industrial Chemistry, University of Pisa, via Moruzzi,13, 6124 Pisa, Italy*

ABSTRACT

A large fraction of plastic litter found in natural environments is constituted by conventional not biodegradable plastic bags, and their adverse effects via ingestion or entanglement on terrestrial and marine organisms are largely documented. Biodegradable and compostable shoppers have been recently developed as alternative to traditional ones. These bags are specifically designed to degrade in composting facilities and generate a product devoid of toxicity to soils and crops. However, very little is known on the effects of bag leaching, i.e. the transfer of chemicals from plastic into natural environments, on vegetation. Some plant species are highly sensitive to a variety of chemicals, and seedling growth is generally the most affected life history stage. In this study we assessed the potential effects of conventional (high-density polyethylene, HDPE) and compostable (Mater-bi®, MB) bags, when left in natural environments, on water quality and plant development. To this end, seeds of *Lepidium sativum* L., a terrestrial plant commonly used in phytotoxicity standard tests, were exposed to leachates obtained from different amount of HDPE and MB bags, simulating various pollution degrees occurring in nature, for 72 hours. Both not-exposed (or virgin) bags and natural weathering exposed bags were used. Variations of chemical-physical characteristics of extracts were used as indicative of water quality deterioration, while alterations of seed gemination and seedling radicle and hypocotyl length were considered as indicative of phytotoxicity. A chemical qualitative screening of the leachates was also performed to identify the compounds with potential phytotoxicity. Both types of bags affected water characteristics (pH, salinity and total dissolved solids) relevant to plants, and released into water intentionally added chemicals, such as the noxious bisphenol A, and other phytotoxic substances probably generated during bag manufacturing. Leachates from both bag types did not affect seed germination. But, a significant number of seedlings showed developmental abnormalities or reduced seedling growth. The hypocotyl was the most sensible seedling organ to HDPE bag leachates while the radicle was the most vulnerable to MB ones. These findings indicate that plastic bags, including those that meet biodegradability and compostability standards, represent a potential threat to plants, if left in natural environments. Therefore, people and managers should be adequately informed about the potential environmental impact of an incorrect bag disposal. Simple, rapid standard phytotoxicity tests, such as the *L. sativum* bioassay, applied to bag leachates could be used in the future to select not noxious additives so to develop more eco-friendly bags.

Keywords.

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1. Introduction

Pollution by plastics is a serious environmental problem affecting the whole world. Plastic bags manufactured with conventional, virtually non-biodegradable polymers, such as polyethylene (PE), polypropylene (PP) and high-density polyethylene (HDPE), are a consistent fraction (17-23%) of the total amount of plastic litter found in terrestrial and marine habitats (Moore et al., 2001; Barnes et al., 2009; Li et al., 2016; Pasternak et al., 2017; Schmuck et al., 2017). The adverse effects of bag litter on a variety of animal organisms via entanglement or ingestion are largely documented (Muller et al., 2012; Besseling et al., 2014; Galloway et al., 2017). Yet relatively few studies have examined the potential effects of bags on organisms via leaching, i.e., the transfer of chemicals from bags to the surrounding environment (Bejgarn et al., 2015), and none of these studies has focused on vegetation. Plants play a key role in the development and maintenance of soil structure, microbial communities and ecosystem functions (Beare et al., 1995; Kuzyakov and Blagodatskaya, 2015). Some plant species are known to be sensitive to a variety of pollutants, and the effects of pollutant exposure are often more detrimental at the early life history stages, seed germination and seedling growth (Li et al., 2005; Macoustra et al., 2015).

All plastic materials, including bags, contain plasticizers, pigments and organometallic compounds (hereafter referred to as additives) that are encapsulated with the polymeric matrix during the manufacturing process to improve item properties (Oehlmann et al., 2009; Bejgarn et al., 2015; Avio et al., 2017; Alam et al., 2018). Plastics can also adsorb harmful chemicals from the environment, for example persistent organic pollutants, polycyclic aromatic hydrocarbons and metals (Avio et al., 2017; Ceccarini et al., 2018). When plastic bags are exposed to natural environmental conditions, water-soluble additives and adsorbed chemicals can leach out (Bejgarn et al., 2015; Alam et al., 2018) and migrate into soils where they may imbibe seeds or be taken up by seedling roots (Wu et al., 2013; Martin-Closas et al., 2014; Rani et al., 2015; Zhang et al., 2017). The consequences of such phenomenon on plant development could be relevant. Indeed, studies on the individual effects of some chemicals frequently added to traditional plastic bags, such as bisphenol A (BPA), nonylphenol polyethoxylates and phthalic acid esters, indicate that such substances can inhibit seed germination and reduce seedling growth in some crop- and not crop-species (Domene et al., 2009; Staples et al., 2010; Ma et al., 2013; Pan et al., 2013; Li et al., 2018), in addition to be noxious to animals and human health (Vandenberg et al., 2007; Talsness et al., 2009).

Recently, several countries have banned traditional plastic bags (UNEP, 2018) and replaced them with a new generation of bags labelled as biodegradable and compostable to mitigate the social-ecological impact associated with plastic disposal and littering. Many of the compostable shoppers currently marked in Europe are manufactured with environmental-friendly polymers such as starch-based polymers (Shah et al., 2008). These new bags have specifically been developed to be disposed at the end of their life in industrial or "home" composting systems under specific conditions of temperature, moisture, and pH (Song et al., 2009) to totally biodegrade and generate carbon- and nutrient-rich compost for agriculture applications. According to international standards, the final compost has to meet fundamental requirements including safety, and it is expected to have no impact or any ecotoxicity effects on the soil environment and plants (OECD, 2006; ISO, 2012a; ISO, 2012b). However, studies have shown that also biodegradable/compostable bags are entered into natural environments, and their possible impact on the receiving ecosystems is of increasing concern (Balestri et al., 2017; Sharma and Chatterjee, 2017; Harrison et al., 2018). In fact, once left in natural habitats these items can require over than six months to biodegrade (Accinelli et al., 2012; Muller et al., 2012; UNEP 2015; Balestri et al., 2017), causing alterations of chemical-physical characteristics of the substrate and water (for example pH, temperature and redox potential) that may be relevant to organisms (Carson et al., 2011; Green, et al., 2015), including plants (Balestri et al., 2017). Unfortunately, the exact chemical composition of biodegradable bags is often secret, but there is evidence that during degradation these bags can release some additives potentially toxic to organisms (Bejgarn et al., 2015). However, current biodegradability test methods do not take into account the potentially adverse ecological impacts of plastic bags, additives and intermediate polymer degradation products that can leach out of these items. Since the market of biodegradable bags is expected to greatly increase in the next decades (European Bioplastic, 2017), assessing the effects of new generation of bags not only on animals but also on plants should be extremely useful.

The aim of this study was to evaluate the impact of plastic bags via leaching on terrestrial higher plants through a rapid and cost-effective standard phytotoxicity test. We hypothesized that (i) when exposed to rainwater both types of bags would affect water quality by releasing processing compounds and that (ii) bag leachates would influence early life plant stages. To test these hypotheses, we examined the effects of bags on water chemical/physical properties that regulate the availability of nutrients to plants (Hinsinger, 2001; Shrivastava and Kumar, 2015), and we identified potentially phytotoxic compounds released by bags in leachates. Then, the phytotoxicity of bag leachates was assessed by using a laboratory seed germination and root elongation test. The application of standard phytotoxicity tests to plastic bag leachates could be critical for developing alternative more eco-sustainable materials.

2. Materials and methods

To assess the effects of different types of plastic bags, conventional and compostable bags, on water quality, leachates from bags were prepared in laboratory and chemical/physical analyses of the leachates were performed. Both virgin bags and bags previously exposed to natural environmental conditions were used to discriminate between the effects of plastic chemical content and bag interaction with abiotic/biotic factors. A qualitative screening of the leachates from virgin bags was also carried out by using gas chromatography-mass spectrometry to detect the processing compounds leached out from bags. Then, the phytotoxicity of the leachates was evaluated in a separate seed gemination and seedling growth experiment by using *Lepidium sativum* L. (garden cress, Brassicaceae) as test species.

2.1. Leachate preparation, chemical/physical analysis and qualitative additive screening

To prepare bag leachates for chemical/physical analyses and qualitative screening, two different types of commercially available shopper bags, a high-density polyethylene (HDPE) bag and a compostable and biodegradable bag purchased from local supermarkets in Italy, were used. This latter type of bag was made of Mater-bi®(MB), a biopolymer based on starch and vinyl-alcohol copolymers (Sforzini et al., 2016), and it is certified for industrial composting (EN13432) and “home” composting scheme (OK Compost Home).

A group of bags (hereafter referred to as virgin or not-exposed bags, NE) was left in laboratory at a temperature of 22 ± 1 °C. Another group of bags (hereafter referred to as exposed bags, E) was placed outdoor (average daily temperature of 13 ± 2 °C, total amount of precipitations of 57 mm and mean daylength of 10 hours) in a back-dune area at Rosignano Solvay (43°23’N 10°26’ E, Italy) over a period of 10 days for natural weathering. The duration of the exposure period was similar to that of a previous study on the toxicity of bag leachates to a marine organism (Bejgarn et al., 2015). To avoid their dispersion, the bags were individually placed on the soil substrate and fixed with pebbles. At the end of exposition period, the bags were collected and transported to the laboratory.

All plastic bags were then cut into pieces of approximately 1 cm^2. Different amounts of pieces obtained from each type of bag were placed into glass flasks containing sterilized deionized water to obtain liquid (water)- to- solid (plastic) ratios of 100, 10 and 5, corresponding respectively to approximately 4.1 x 10^{-4}, 4.1 x 10^{-3} and 8.3 x 10^{-3} bag/mL, hereafter referred to as low (L), medium (M) and high (H) pollution degrees. These ratios were chosen to mimic various degrees of bag pollution, in terms of number of bags per m^2, occurring in natural environments (Munari et al., 2016; Alshawafi et al., 2017; Pasternak et al., 2017; Schmuck et al., 2017). For each bag type, sterile deionized water with no bag material was used as control (no pollution). Each treatment was performed in triplicate. Flasks were wrapped in aluminum foil and placed in a culture chamber on a rotary shaker (95 rotations per minute) for 72 h (Bejgarn et al., 2015) at a temperature of 24 ± 1 °C. Thus, the experiment was a full factorial design consisting of three factors, plastic type (fixed, two levels: HDPE and MB), exposure (fixed, two levels: not-exposed or virgin bags and exposed) and pollution degree (fixed, four levels: control or no pollution, low, medium and high). After the incubation period, plastic pieces were separated from the liquid phase by filtration using nylon mesh filter (200 µm) and the filtered aqueous phase obtained from each treatment were used for water quality analysis, qualitative additive screening and seed germination test.

To examine the effect of bag leaching on water quality, a sample (30 mL) of the filtered aqueous solution obtained from each replicate of the MB and HDPE treatments was collected, and water chemical/physical indicators such as pH, salinity and total dissolved solids (TDS) were measured by a multiparameter meter (HI98194, Hanna Instruments). To detect the volatile organic compounds, present in leachates, samples of the filtered aqueous phases obtained from HDPE and MB virgin bags at the highest concentration were collected. About 30 mL of aqueous solutions were extracted for three times with 6 mL of an organic solvent in a separation funnel. Chloroform and n-hexane were employed for the extraction of solution deriving from HDPE bags, whilst methyl isobutylketone (MIBK) was used for the extraction of leachates from MB bag leachates. The aqueous and organic phases were separated, and the organic extracts were concentrated under reduced pressure and analyzed through gas chromatography-mass spectrometry (GC-MS, Agilent 7890B-5977A) equipped with HP-5MS capillary column (30 m×0.25 mm×0.25 µm) (5%-phenyl)-methylpolysiloxane. The carrier gas was helium with a flow of 1 mL/min. The injector and detector temperatures were maintained at 250°C and 280°C, respectively, and the following temperature program was adopted for the chromatographic run: 60 °C isothermal for 2 min; 10 °C/min up to 260 °C; 260 °C isothermal for 10 min. This non-polar column has an excellent inertness and low bleed characteristics and has been successfully adopted under similar chromatographic conditions for the analysis of different types of plasticizers and of a wide range of organic contaminants recovered by extraction from the aqueous phase (Hardesty et al., 2015). Organic compounds were identified on the basis of the NIST 2.0 Mass Spectral Database and by the comparison with literature data. Identification was restricted to the most abundant and representative organic constituents of the leachates.

2.2. Lepidium sativum seed germination test

Lepidium sativum was used as indicator of bag leachate phytotoxicity. This species is considered as a biological indicator for toxicity of wastes, soils and water contaminated with chemicals, including heavy metals, petrochemical compounds and polycyclic aromatic hydrocarbons (Gong et al., 1999). The fast growth and root sensitivity of the species to different compounds enable its use in standard ecotoxicity tests on environmental matrices (UNICHIM, 2003; OECD, 2006; ISO, 2012a; ISO, 2012b). In the presence of cytotoxic compounds, inhibition of the dividing processes of the root meristematic cells causes a reduction of roots length relative to control (Gong et al., 1999). Commercially available seeds of *L. sativum* (Italsementi s.n.c, Italy) were rinsed with sterile deionized water and placed in Petri dishes (12 seeds per dish) containing a layer of cellulose filter paper (Whatman N°1), previously moistened with 1.5 mL of one of the obtained filtered leachates, following the same design of the leaching experiment described above. Petri dishes were sealed with parafilm to prevent water evaporation and randomly placed in a culture chamber for 72 hours at 24 ± 1 °C in darkness, as recommended in standard phytotoxicity tests (UNICHIM, 2003). For each treatment there were five replicate dishes. At the end of the incubation period, the number of germinated seeds in each dish was counted and the percentage of germination was calculated. A seed was considered to have germinated when the length of the radicle had reached at least 2 mm (Luo et al., 2017). A visual evaluation of developmental abnormalities in seedlings (ISTA, 2003; Chandler, 2008) was also carried out, and the percentage of abnormal seedlings was calculated. Then, a sample of the remaining normal seedlings (n = 5) was collected at random from each Petri dish, and the seedlings carefully placed on squared paper and photographed for morphological measurements. The length of the radicle and the hypocotyl of each seedling was measured with an image analysis software (ImageJ 2, Rueden et al., 2017). The radicle to hypocotyl length ratio was also computed as it is considered an indicator of relative allocation of biomass to belowground and aboveground organs (Poorter et al., 2011). Before the germination test, seeds were examined under a stereomicroscope (Wild M3C, Leica) and pressure tested with forceps. Moldy or empty seeds, and those that did not resist the pressure were considered non-viable and thus discarded. A sample of seeds was tested using distilled water for rapid and homogeneous germination under the assay conditions. This test was considered valid as the end of the incubation period seed germination was higher than 90%.

2.3. Data analysis

Non-metric multidimensional scaling (MDS) based on the Euclidean distance was conducted on chemical-physical variables of leachates (pH, salinity and total dissolved solids) from each bag type separately (HDPE or MB) to visualize differences among samples. To reduce the leachate variables and determine the relationship among them, principal component analysis (PCA) was performed for leachates from each bag type. Three-way permutational multivariate analysis of variance (PERMANOVA) was also performed on chemical-physical data to examine the overall effect of treatments (plastic type, bag exposure and pollution). Then, univariate three-way PERMANOVAs were conducted on each individual chemical-physical variable.

For each bag type, separate MDS graphical representations based on the Euclidean distance were conducted on *L. sativum* data (percentage of germination, percentage of abnormal seedlings, hypocotyl and radicle length, and radicle to hypocotyl ratio) to visualize differences among samples. A three-way multivariate PERMANOVA was also conducted on all plant variables to compare the effects of the two different types of plastic bags (HDPE and MB), exposure and pollution degree on *L. sativum* performance, followed by separate univariate PERMANOVAs. Prior to the analyses, data were appropriately transformed and normalized when necessary. In PERMANOVAs, dissimilarities were calculated as Euclidean distances from 9999 permutations of the residuals under a reduced model, and when significant effects were detected posteriori pair-wise comparisons using 9999 random permutations were performed. Statistically significant terms were checked for differences in multivariate group dispersion through permutational analysis of multivariate dispersion (PERMDISP). All analyses were performed using PERMANOVA + add on to PRIMER 6 statistical software (Clarke and Warwick, 2006; Anderson et al., 2008).

3. Results and discussion

3.1. Leachate chemical/physical analysis and qualitative additive screening

MDSs performed on chemical-physical variables of water extracts (pH, salinity and TDS) obtained from each type of bag (MB or HDPE) showed an overlapping of samples belonging to controls (no pollution) and a clear segregation of these samples from those of the pollution treatments (Fig. 1). These findings suggest that the presence of the tested bags altered the quality of water. For MB bag leachates, a segregation between samples from virgin and exposed bags was also observed (Fig. 1), indicating that the examined characteristics were influenced by abiotic/biotic factors.

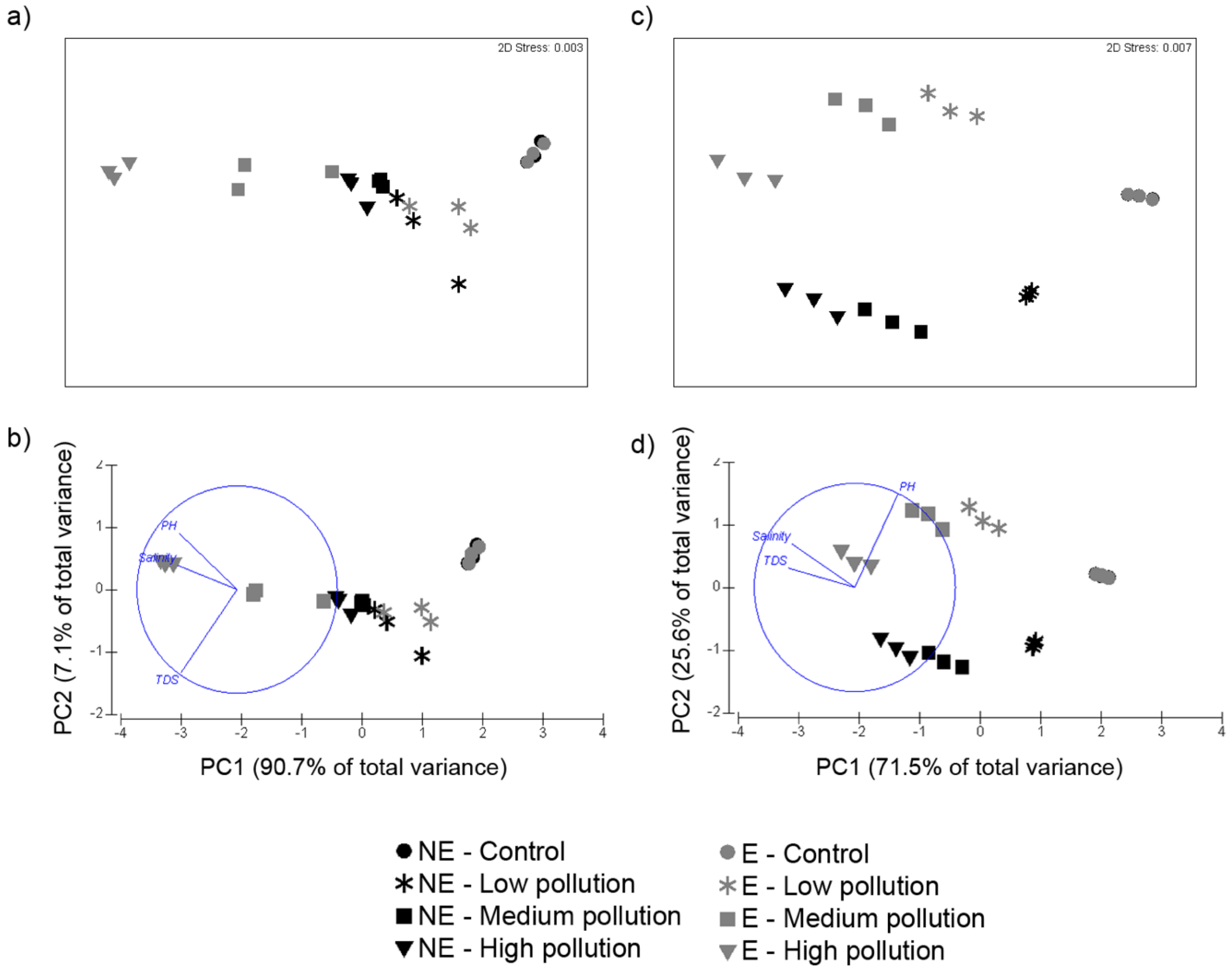


Fig. 1. Non-metric multidimensional scaling ordination (MDS) and principal component analysis biplots of chemical-physical variables of leachates from different concentrations of not-exposed (NE) and exposed (E) HDPE (a, b) and MB (c, d) plastic bags.

2-column fitting image, color image in online version and black-and-white image in printed version

Similar graphical representations were observed in principal component analyses (Fig. 1). The first (PC1) and the second (PC2) principal components accounted for 97.8% and 97.1% of the total variance for HDPE and MB leachates, respectively (Table 1). For both the materials, all the examined variables contributed to the construction of PC1 (Table 1), and thus they were consistently responsible for the observed differences in leachate quality among treatments.

Table 1

Results of principal component analysis performed on chemical/physical data of leachates from high-density polyethylene and Mater-bi® bags. Explained (a) and cumulative (b) variance, eigenvalues and eigenvectors of leachates variables are reported.

Principal components	High-density polyethylene			Mater-bi®		
	1	2	3	1	2	3
a) Variance explained						
Eigenvalues	2.720	0.213	0.065	2.140	0.768	0.087
% of variance	90.7	7.1	2.2	71.5	25.6	2.9
b) Cumulative variance %	90.7	97.8	100	71.5	97.1	100
Eigenvectors						
pH	-0.579	0.544	0.607	0.425	0.891	-0.158
Salinity	-0.591	0.233	-0.773	-0.622	0.414	0.664
TDS	-0.562	-0.806	0.187	-0.658	0.184	-0.730

Results of multivariate PERMANOVA analyses revealed a significant effect of all investigated factors (plastic type, exposure and pollution degree), as well as of their interaction, on the quality of leachate (Table 2). A consistent effect of the interaction among all factors was also detected by separate univariate PERMANOVAs for all examined variables (Table 2).

Table 2

Results of multivariate (a) and univariate (b) PERMANOVA analysis on pH, total dissolved solids (TDS) and salinity of leachates. Significant results are in bold, and pair-wise comparisons are reported. NE: not-exposed bag, E: exposed bag, H: high pollution, M: medium pollution, L: low pollution, C: control, no pollution, HDPE: high-density polyethylene bag, MB: Mater-bi$^{®}$ bag.

a) Multivariate PERMANOVA analysis

Source	df	Pseudo-F	P
Plastic (Pl)	1	223.11	**<0.001**
Exposure (E)	1	187.80	**<0.001**
Pollution (Po)	3	308.46	**<0.001**
Pl x E	1	45.57	**<0.001**
Pl x Po	3	45.27	**<0.001**
E x Po	3	34.38	**<0.001**
Pl x E x Po	3	26.02	**<0.001**
Residual	32		
Transformation		Log (x+1), normalized	

b) Univariate PERMANOVA analysis

		pH		Salinity		TDS	
Source	df	Pseudo-F	P	Pseudo-F	P	Pseudo-F	P
Plastic (Pl)	1	6215.3	**<0.001**	3.33	0.075	2.97	0.09
Exposure (E)	1	1606.1	**<0.001**	112.13	**<0.001**	248.9	**<0.001**
Pollution (Po)	3	556.4	**<0.001**	136.09	**<0.001**	1343.6	**<0.001**
Pl x E	1	997.85	**<0.001**	0.53	0.464	10.61	**<0.001**
Pl x Po	3	1218.2	**<0.001**	1.82	0.160	1.13	0.34
E x Po	3	214.42	**<0.001**	26.36	**<0.001**	36.91	**<0.001**
Pl x E x Po	3	205.24	**<0.001**	13.96	**<0.001**	55.48	**<0.001**
Residual	32						
Transformation		Normalized		Normalized		Log (x+1), normalized	
Pair-wise comparisons		MB ≠ C: NE, E		HDPE ≠ C: E		HDPE ≠ C: NE, E	
		HDPE ≠ C: E (H, M)		MB ≠ C: NE, E (H, M)		MB ≠ C: NE, E	
		MB ≠ HDPE: E (H, M), NE		MB ≠ HDPE: NE, E (H)		MB ≠ HDPE: NE (H), E	
		NE ≠ E: HDPE (H, M), MB		NE ≠ E: HDPE (H, M), MB		NE ≠ E: HDPE (H, M); MB	
		MB (NE, E): H ≠ M ≠ L		HDPE (E): H ≠ M ≠ L		HDPE: H ≠ M ≠ L	
		HDPE (E): H, M ≠ L				MB: H ≠ M ≠ L	

The pH of MB leachates was significantly lower that of control (pH 7.29 -7.31), regardless of exposure and pollution degree, while that of HDPE leachates was slightly higher than that of control but only with exposed bags and at medium and high pollution degree (Fig. 2). For MB leachates, the pH decreased with increasing pollution degree regardless of exposure, while that of HDPE leachates increased with increasing pollution but only with exposed bags (Fig. 2; Table 2). The pH of MB leachates was lower than that of HDPE irrespectively of pollution for virgin bags, and only at high and medium pollution degree for exposed bags. Leachates from virgin bags had significantly lower pH values than those from exposed bags, except that with HDPE bags at the low pollution degree (Fig. 2; Table 2). These findings are in accordance with results of a study by Bejgarn et al. (2015) on the leachate produced from plastic bags, showing an increase of pH in leachates from HDPE bags and a decrease of pH with compostable/biodegradable bags. However, Bejgarn et al. (2015) found a lower pH in the leachates obtained from compostable bags previously exposed to artificial weathering (exposure to UV radiation) than that measured in virgin bag leachates, and thus the opposite of that observed in the present study with MB bags. This discrepancy could be due to the different composition of the employed biodegradable bags. In addition, in our study exposed bags were subjected to real natural conditions for 10 days, and during this period abiotic factors (solar UV-radiation, rain and temperature) and microorganisms (Andrady, 2015) might have promoted plastic decomposition, causing the release of acid cations from plastics.

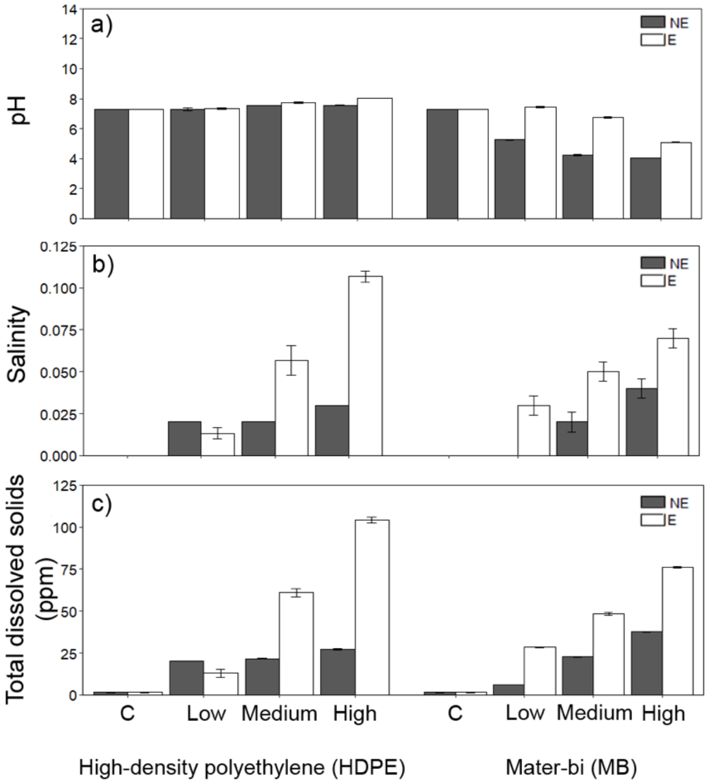


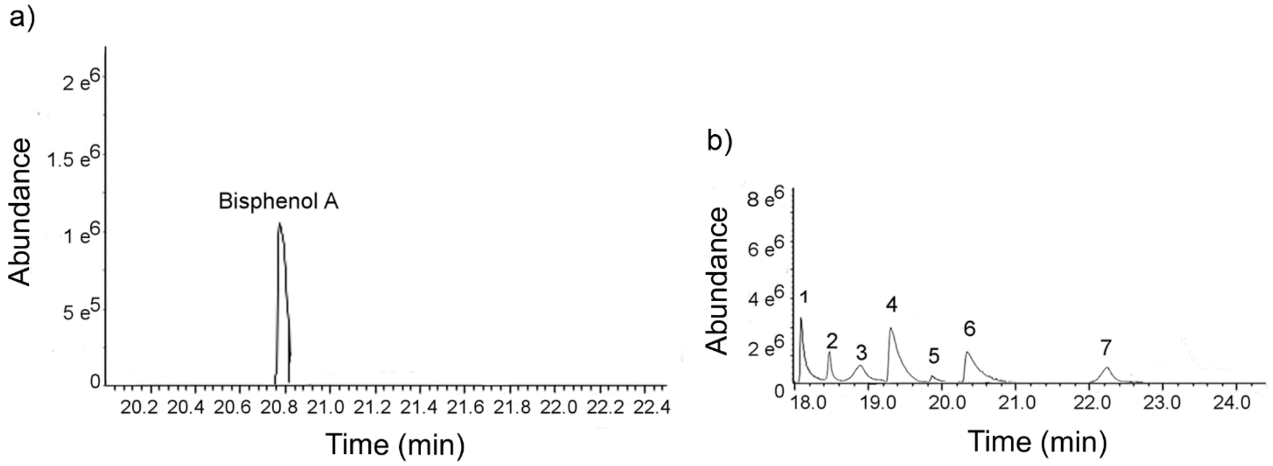
Fig. 2. Values of pH (a), salinity (b) and total dissolved solids (c) of leachates from different concentrations of not-exposed (NE) and exposed (E) HDPE and MB plastic bags. Data are mean ± SE, n = 3.

1.5- column fitting image

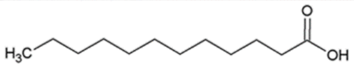
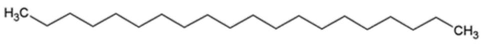
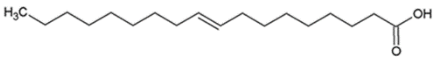
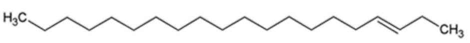
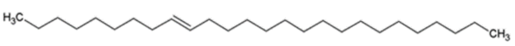
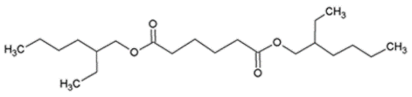
The salinity of HDPE leachates was significantly higher compared to that of control treatments but only with exposed bags, while that of MB leachates was higher than controls with virgin bags regardless of pollution degree, and only at high and medium pollution levels with exposed HDPE bags. For both types of bag, salinity increased with increasing pollution, and HDPE leachate salinity was higher than that of MB regardless of exposure at the high pollution degree. Leachates from exposed bags had significantly higher salinity than those from virgin ones irrespectively of pollution for MB bags, and at high and medium pollution degrees for HDPE bags (Fig. 2; Table 2). This finding suggests that MB bags contained or might have released more salts in water than HDPE ones, and that both bag types might have adsorbed salts deposited with salt spray from the adjacent coastal habitat during the exposure period.

The values of TDS in plastic leachates were significantly higher than controls and increased with increasing pollution degree irrespectively of exposure. The amount of TDS of MB leachates was significantly higher than that of HDPE ones at the highest pollution degree for virgin material, while was lower at the medium and high pollution degree for exposed materials (Fig. 2; Table 2). Significantly higher TDS amounts were found in leachates from exposed than virgin bags, except that for HDPE at low pollution level, indicating that weathering might have increased plastic embrittlement, and that MB bags were more prone to fragmentation than HDPE ones due to their greater susceptibility towards biodegradation.

Identifying potential toxic compounds in plastic bags is difficult, since their additive content can vary widely, even for the same type of item, depending on the manufacturer and the process of production (Groh et al., 2018; Hahladakis et al., 2018). In most previous studies, the chemical screening of plastic leachates has failed to identify chemical structures (Lithner et al., 2012; Bejgarn et al., 2015; Li et al., 2016). Here, in total twelve compounds have been identified in virgin plastic bag leachates. Mass spectra interpreted by NIST library were well matched and a qualitative analysis of the extracts has been performed. The largely prevailing compound in the chromatogram of HDPE chloroform extract (Fig. 3) was identified as BPA, an antioxidant, flame retardant commonly added to plastics. On the other hand, the extraction of HDPE leachate with n-hexane allowed to reveal the presence of seven different organic compounds with a long alkyl chain, soluble in this alkane (Fig. 3). Linear long-chain alkanes and alkenes resulted the prevailing components. These products can derive from the addition of a wax, by-product of polyethylene manufacture, which gives to the polymer more pronounced plasticization (AlMaadeed et al., 2015).



c)

Peak	Organic compound	
1	n-Dodecanoic acid	
2	1-Nonadecene	
3	Eicosane	
4	Oleic acid	
5	3-Eicosene	
6	9-Hexacosene	
7	di-(2-ethylhexyl)adipate	

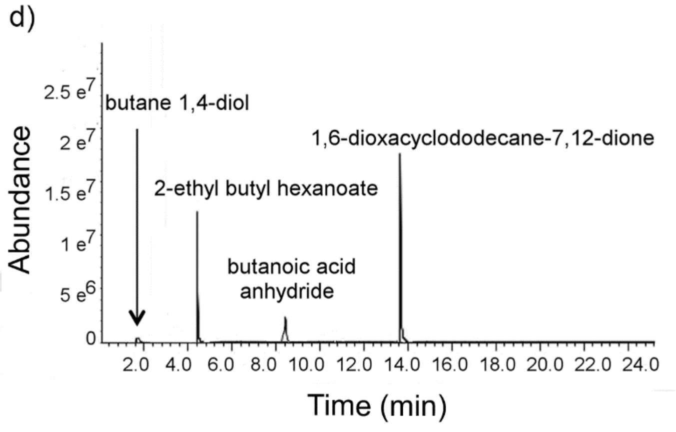


Fig. 3. Chromatograms of the chloroform extract (a) and of the hexane extract (b) from HDPE virgin bag leachate, and organic molecules present in the chromatogram of n-hexane extract (c). Chromatogram of the methyl isobutylketone extract (d) from the MB virgin bag leachate.

1.5- column fitting image

Dodecanoic and oleic acids have been also ascertained, probably deriving from ester plasticizers used as co-processing additives for the polyolefin (Mantese Sander et al., 2012). In fact, also di-(2-ethylhexyl)adipate, also known as DEHA, a largely used plasticizer, was identified. Instead, the four organic compounds extracted from MB bag leachate with MIBK were identified as traces of butane1,4-diol, 2-ethylbutyl hexanoate, 1,6-dioxacyclododecane-7,12-dione and traces of butanoic acid anhydride (Fig. 3). The first compound, in very low amount is the co-monomer of adipic acid in the polyester poly(butylene adipate), employed with corn starch for the preparation of Mater-bi®. The second compound is a hydrolytically stable ester used as volatile plasticizer or coalescing agent for coatings (Patent EP0026982A1, 1979). The third and largely prevailing compound could be a non-intentionally added substance or it could derive from decomposition of the initial components, or because of chemical interactions between them (Watanabe et al., 2007). It has been indeed found as new formed molecule from the reaction between butane 1,4-diol and adipic acid, the monomers of the polyester poly(butylene adipate) present in Mater-bi® (Canellas et al., 2015). Thus, the significant presence of 1,6-dioxacyclododecane-7,12-dione, as well of low amounts of free butane 1,4-diol, can suggest the depolymerization of the polyester with release of the free monomers, that could be probably responsible for the observed higher acidity and salinity of MB leachates (Rychter et al., 2010). Finally, traces of butanoic acid anhydride can be a residue of the starch esterification reaction. Butyrated corn starch is often present in Mater-bi® because this modified starch is able to increase the hydrophobicity and the flexibility of the biopolymer (Rahim et al., 2012).

3.2 Lepidium sativum seed germination test

After three days of incubation, total percent of germination of *L. sativum* seeds treated with plastic leachates was similar to that of the controls (Fig. 4). However, a significant number of germinated seeds treated with plastic leachates produced deformed seedlings (Fig. 5) and inhibitory growth effects were detected in many normal seedlings. MDS plots performed on all plant variables showed a segregation between samples belonging to plastic treatments and controls, more evident with MB bag samples than with HDPE ones (Fig. 6). Results of multivariate PERMANOVA revealed a significant effect of the interaction among plastic type, exposure and pollution, and this effect was effectively ascribable to the investigated factors and not to heterogeneity in multivariate dispersion (Table 3). Results of univariate PERMANOVA for all examined plant variables are reported in Table 4.

Table 3

Results of multivariate PERMANOVA analysis performed on *L. sativum* variables (percentage of germination, percentage of abnormal seedlings, hypocotyl length, radicle length and radicle to hypocotyl ratio). The transformation applied to data is also reported. Significant results are in bold.

Source	df	Pseudo-F	P
Plastic (Pl)	1	4.43	**0.003**
Exposure (E)	1	3.44	**0.015**
Pollution (Po)	3	22.33	**<0.001**
Pol x E	1	6.25	**<0.001**
Pl x Po	3	1.79	0.063
E x Po	3	1.94	**0.044**
Pl x E x Po	3	4.07	**<0.001**
Residual	64		
Transformation		Log (x+1), normalized	

Table 4

Results of univariate PERMANOVA analysis performed on *L. sativum* variables (percentage of germination, percentage of abnormal seedlings, radicle length, hypocotyl length and radicle to hypocotyl ratio). Significant results are in bold. Transformation applied to data and pair-wise comparisons are reported. NE: not-exposed bag, E: exposed bag, H: high pollution, M: medium pollution, L: low pollution, C: control or no pollution, HDPE: high-density polyethylene bag, MB: Mater-bi® bag.

		Germination (%)		Abnormalities (%)	
Source	d.f.	Pseudo-F	P	Pseudo-F	P
Plastic (Pl)	1	0.13	0.713	13.99	**<0.001**
Exposure (E)	1	0.54	0.461	8.44	**0.004**
Pollution (Po)	3	1.08	0.359	30.98	**<0.001**
Pl x E	1	0.14	0.724	0.53	0.465
Pl x Po	3	0.86	0.471	1.93	0.125
E x Po	3	1.99	0.122	1.14	0.342
Pl x E x Po	3	0.86	0.466	1.19	0.322
Residual	64				
Transformation		None		Fourth root	
Pair-wise comparisons				MB ≠ HDPE ≠ C ; NE ≠ E	

		Radicle length (cm)		Hypocotyl length (cm)		Radicle to hypocotyl ratio	
Source	d.f.	Pseudo-F	P	Pseudo-F	P	Pseudo-F	P
Plastic (Pl)	1	10.83	**0.001**	16.85	**<0.001**	4.37E-2	0.830
Exposure (E)	1	0.80	0.374	12.32	**<0.001**	4.83	**0.032**
Pollution (Po)	3	96.15	<**0.001**	138.4	**<0.001**	1.58	0.201
Pl x E	1	2.26	0.130	7.30	**0.009**	22.84	**<0.001**
Pl x Po	3	6.19	<**0.001**	8.43	**<0.001**	0.23	0.870
E x Po	3	3.99	**0.013**	5.82	**0.001**	0.92	0.444
Pl x E x Po	3	2.23	0.092	10.43	**<0.001**	9.85	<**0.001**
Residual	64						
Transformation		Log (x+1)		Square root		None	
Pair-wise comparisons		MB, HDPE ≠ C MB ≠ HDPE: H HDPE, MB: H ≠ M, L		HDPE ≠ C: NE, E (M, L) MB ≠ C MB ≠ HDPE: E (H) NE ≠ E: HDPE (H) MB (NE): M ≠ L		HDPE ≠ C: E (H) MB ≠ HDPE: NE, E (H) NE ≠ E: HDPE, MB (H)	

There was no consistent effect of plastic leachate on percent seed germination, probably because the germination stage is independent from substrate and the radicle does still not contribute to pollutant-uptake (Milberg and Lamont, 1997).

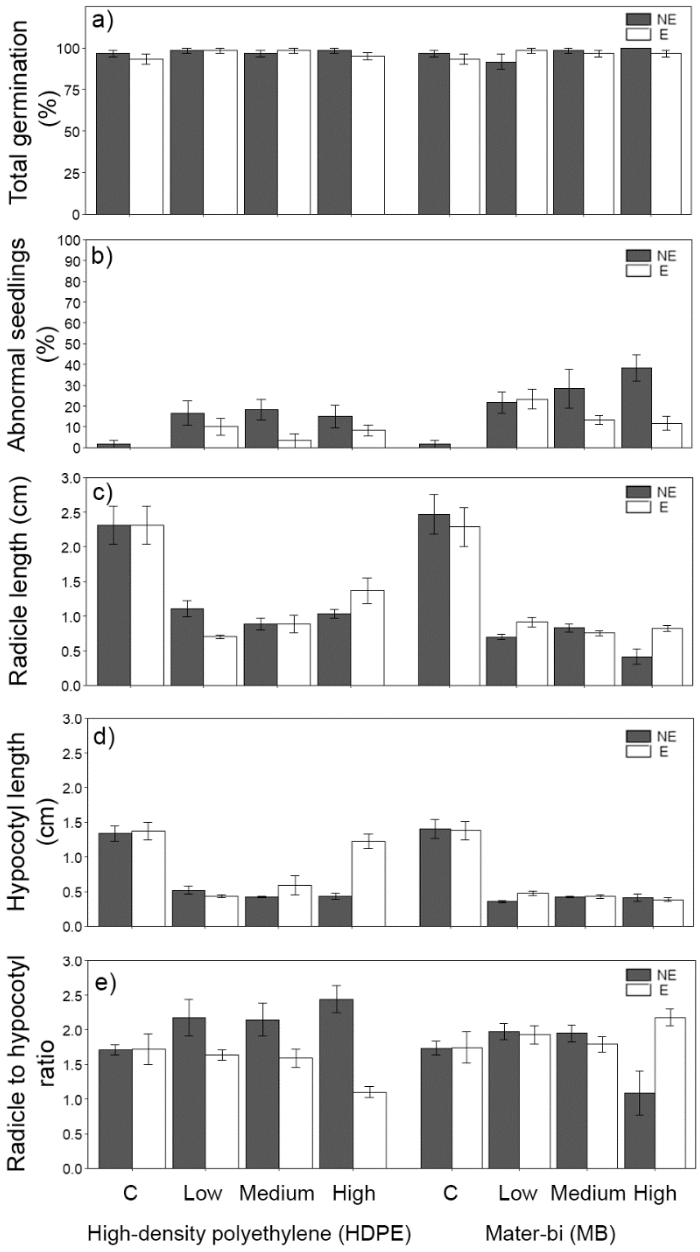


Fig. 4. Percentages of seed germination (a) and abnormal seedlings (b), radicle length (c), hypocotyl length (d), and radicle to hypocotyl ratio (e) of *L. sativum* seedlings treated with different concentrations of leachates from not-exposed (NE) and exposed (E) HDPE and MB plastic bags. Data are mean ± SE, n = 5.

1.5-column fitting image

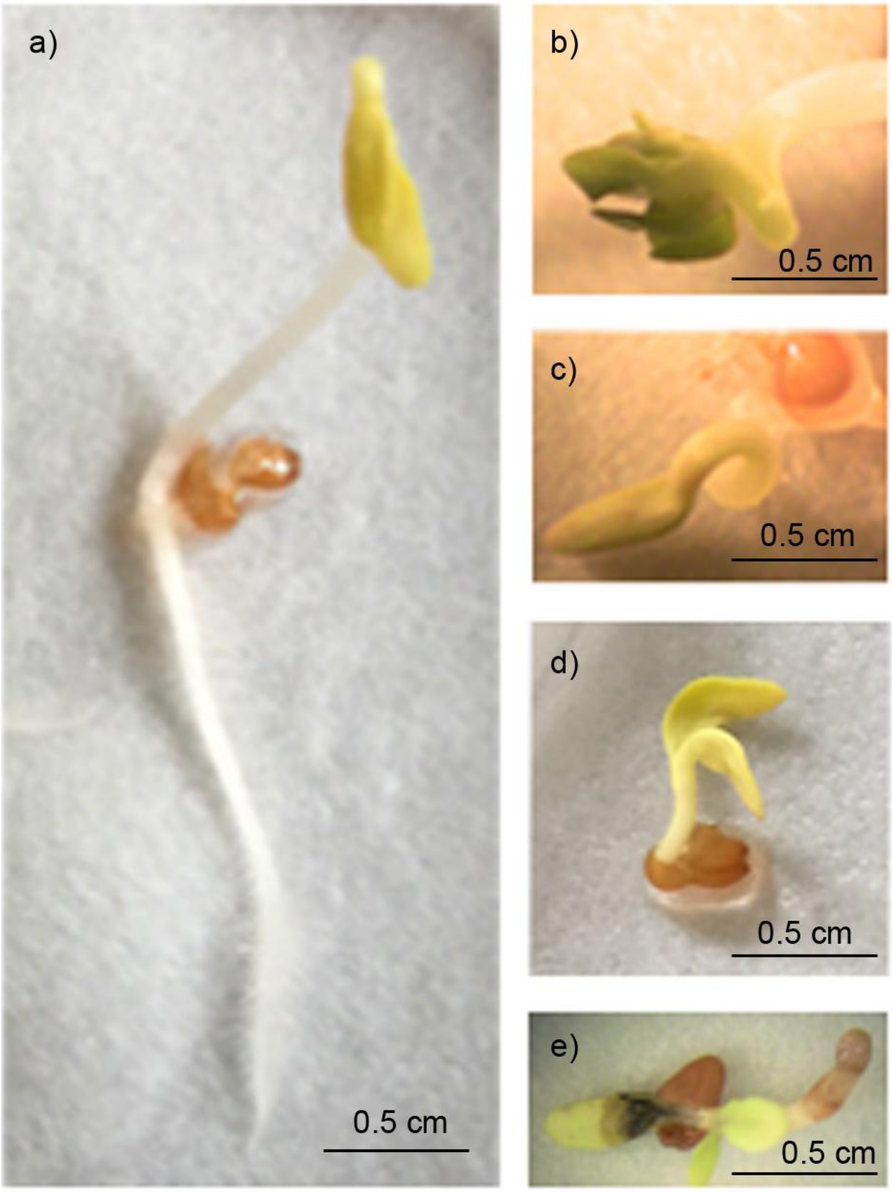


Fig. 5. *Lepidium sativum* seedlings. A normal seedling (a), and abnormal seedlings grown with bag leachates showing deformed cotyledons (b), a twisted hypocotyl (c), missing radicle (d) and deformity of the whole seedling (e).

1.5-column fitting image

Color image in online version and black-and-white image in printed version

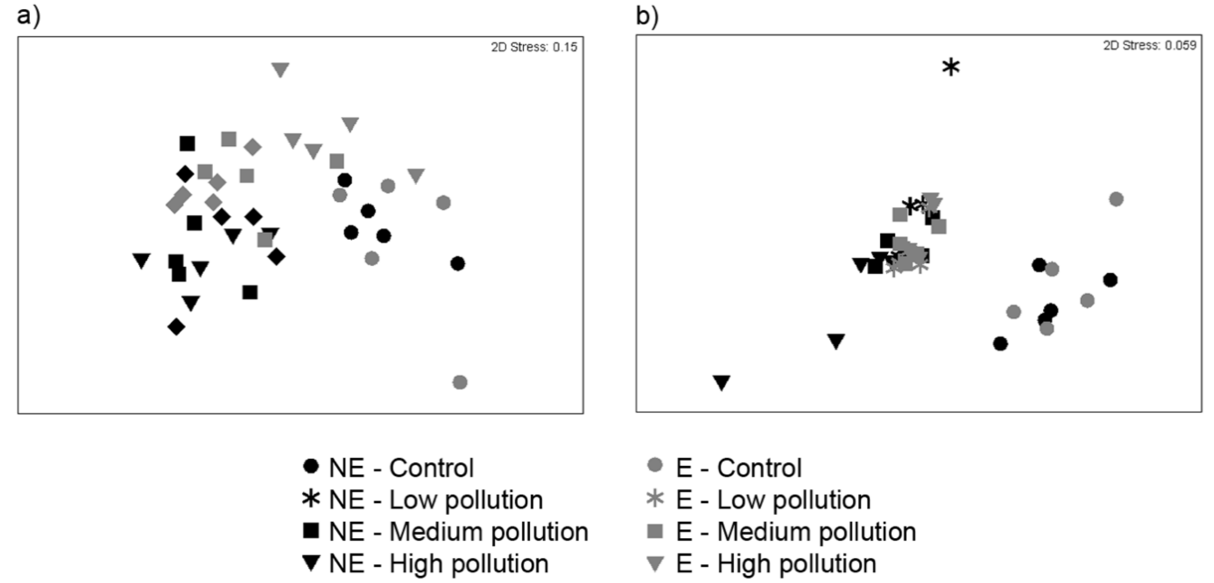


Fig. 6. Non-metric multidimensional scaling ordination of *L. sativum* seed germination and seedling growth data recorded with different pollution degrees of leachates from not-exposed (NE) and exposed (E) HDPE (a) and MB (b) bags.

2-column fitting image

However, up to 40% of the seeds germinated in the presence of plastic leachates showed developmental abnormalities, such as a short or stubby radicle, twisted hypocotyl and malformed and/or supernumerary cotyledons (Fig. 5; Table 5), indicating leachate phytotoxic effects (ISTA, 2003; Chandler, 2008; De Barro, 2008).

Table 5

Number of normal and abnormal *L. sativum* seedlings observed after incubation of seeds with leachates obtained from not-exposed and exposed high-density polyethylene bags (a) and Mater-bi$^{®}$ bags (b) at the different pollution degrees, low, medium and high.

		Total number of seeds	Total number of abnormal seedlings		
			Short/ stubby radicle	Twisted hypocotyl	Malformed supernumerary/ cotyledon
a) High-density polyethylene					
Not-exposed	High pollution	60	5	2	1
	Medium pollution	60	5	2	4
	Low pollution	60	3	2	3
Exposed	High pollution	60	3	1	0
	Medium pollution	60	0	1	0
	Low pollution	60	3	1	1
b) Mater-bi$^{®}$					
Not-exposed	High pollution	60	18	3	2
	Medium pollution	60	9	4	4
	Low pollution	60	8	3	2
Exposed	High pollution	60	4	0	1
	Medium pollution	60	4	2	2
	Low pollution	60	5	3	5

Indeed, similar abnormalities have been observed in crops species and are considered as characteristics of seedlings exposed to toxic chemicals (Mitchell et al., 1988; De Barro, 2008). Overall, a higher number of abnormal seedlings, as well as of normal seedlings with reduced growth, was detected with leachates from not-exposed bags compared to exposed ones (Fig. 4; Table 4). These findings demonstrate that the chemicals responsible for such detrimental effects were present in virgin material, and were not absorbed by bags from the environment. They also indicate that a fraction of these chemicals was migrated into the environment during the exposure period. Available data on the effects of additives used in the manufacturing of traditional bags on plants indicate that BPA can have a clastogenic activity and induce morphological alterations of roots and shoots through the inhibition of both cell elongation and cell division, as well as alteration of gene expression and hormone function (Ferrara et al., 2006; Chandler, 2008; Weizbauer et al., 2011; Gupta et al., 2012). Instead, DEHA was ascertained as toxic at high concentration to some aquatic organisms (Lambert et al., 2010). Therefore, the developmental abnormalities observed in the present study in seedlings grown with HDPE bag leachates could be mainly related to the interference of BPA released from bags with plant hormonal metabolism and signaling. However, also the other identified compounds with phytotoxicity effects, such as for example oleic acid (Jyothi et al., 2014), might have contributed. Instead, the abnormalities detected in seedlings treated with MB leachates could mainly be attributed to the presence of 1,6-dioxacyclododecane-7,12-dione. Its presence, as well as that of traces of butane 1,4-diol, suggests a certain extent of depolymerization of the polyester poly(butylene adipate) with the release of butane 1,4-diol and adipic acid. This latter monomer is known to be toxic to aquatic organisms, including algae (Kennedy, 2002). Clearly, further studies are needed to confirm our hypotheses about the effects of these compounds on higher plants.

As concerning early seedling growth, the radicle of seedlings treated with leachates was significantly shorter than that of control groups, regardless of bag type, exposure and pollution degree (Fig. 4; Table 4). At the highest pollution level, the radicle of seedlings treated with MB leachates was reduced compared to that of seedlings grown with HDPE leachates, regardless of exposure condition (Fig. 4; Table 4). Also, the hypocotyl of seedlings treated with plastic leachates was shorter than that of controls, except that of those exposed to HDPE bags at the highest pollution degree. Leachates from exposed MB bags were more effective in reducing hypocotyl elongation than those from HDPE bags, even if only at the highest pollution degree (Fig. 4; Table 4). Here, seedlings treated with virgin MB bag leachates showed lower radicle to hypocotyl ratio than those grown with HDPE at the high pollution degree, while the reverse occurred in seedlings treated with leachates from exposed materials (Fig. 4; Table 4). This could be due to differential effects of the two type of bags on biomass allocation to belowground and aboveground organs (Poorter, 2011). With HDPE leachates, significantly lower radicle to hypocotyl ratios were detected with exposed materials compared to virgin ones, suggesting that the chemicals migrated from bags to the environment before the leaching experiment could have greater growth inhibitory effect on the hypocotyl than on the radicle (Fig. 4; Table 4). Instead, with MB leachates higher radicle to hypocotyl ratios were observed with exposed materials compared to virgin ones, thus the compounds released in the environment could have larger adverse effects on radicle growth (Fig. 4; Table 4). Previous studies have shown that the growth of *L. sativum* seedlings is not influenced by variations of substrate pH between 4.4 and 8.8, while the presence of relatively high salt concentrations (about NaCl 50 mM) can inhibit the growth of the radicle and the plumule (El-Darier and Youssef, 2000; Bonanomi et al., 2006). In our study, the pH of leachates was lower than 4.4 only for MB virgin bags at the highest pollution level, and the highest values of salinity measured in leachates (about 2 mM) was lower than that reported to affect *L. sativum.* Therefore, the inhibitory effects on radicle growth observed with MB bag leachates could be explained by both water acidification and presence of released compounds. Instead, the suppression of hypocotyl growth observed with HDPE leachates could be mainly related to the presence of BPA. Overall, the results of this study show that leachates of traditional and compostable bags are toxic to the test species *L. sativum*, and provide new experimental evidence that leaching can occur in natural habitats causing contamination of soils and water available to plants.

4. Conclusions

The substitution of conventional plastic bags with biodegradable ones is a widely accepted strategy to reduce the environmental impact of plastic litter. Results of the present study demonstrate, however, that both types of bags can release processing compounds when incorrectly discarded in natural terrestrial environments due to precipitations, and hence can contaminate soils and waters. The *Lepidium sativum* seed germination test reveals that bag leachates can adversely affect seedling growth, and they could be thus potentially toxic to other higher plants. These findings are of particular ecological and managerial relevance. They indicate that international standards currently used to certify the compostability of bags, although more stringent than those developed for testing their biodegradability, cannot exclude the occurrence of adverse environmental effects of bags when abandoned on natural habitats. This is because in composting facilities phytotoxic water-soluble compounds, intentionally or non-intentionally added to plastics, can be gradually eliminated from litter as the degradation proceeds. Instead, in natural environments, these chemicals can quickly migrate out of plastic and be absorbed by roots affecting plant development. Thus, people and managers need to be adequately informed about the potential environmental impact of an incorrect disposal of bags.

The presence of additives used to manufacture plastic items, such as for example BPA, in natural environments is of great concern, due to their notorious adverse effects on human health and on marine and terrestrial organisms (Vandenberg et al., 2007; Talsness et al., 2009; Bejgarn et al., 2015). Many efforts have recently been made to develop eco-friendly, biodegradable substances (including bio-based compounds) as alternative to traditional additives. Our results suggest that in screening new plastic bag additives, special attention should be also paid in the future to their eventual transformation during the manufacturing process in products toxic to animals and plants. In this context, simple, rapid standard phytotoxicity tests performed on bag leachates, based on higher plants such as the *Lepidium sativum* seed germination and radicle elongation assay, could be useful tools.

Acknowledgements

We sincerely thank Flavia Vallerini for her support in the laboratory experiment. This work is part of the PhD research project of Virginia Menicagli funded by the University of Pisa (PRA and FA).

References

Accinelli, C., Saccà, M.L., Mencarelli, M., Vicari, A., 2012. Deterioration of bioplastic carrier bags in the environment and assessment of a new recycling alternative. Chemosphere. 89, 136-143. https://doi.org/10.1016/j.chemosphere.2012.05.028.

Alam, O., Billah, M., Yajie, D., 2018. Characteristics of plastic bags and their potential environmental hazards. Resour Conserv Recycl. 132, 121-129. https://doi.org/10.1016/j.resconrec.2018.01.037

AlMaadeed, M.A., Labidi, S., Krupa, I., Ouederni, M., 2015. Effect of waste wax and chain structure on the mechanical and physical properties of polyethylene. Arab J Chem. 8, 388-399. http://dx.doi.org/10.1016/j.arabjc.2014.01.006

Alshawafi, A., Analla, M., Alwashali, E., Aksissou, M., 2017. Assessment of marine debris on the coastal wetland of Martil in the North-East of Morocco. Mar Pollut Bull. 117, 302-310. https://doi.org/10.1016/j.marpolbul.2017.01.079

Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. PERMANOVA+ for PRIMER: Guide to software and statistical methods. PRIMER-E: Plymouth, UK.

Andrady A.L., 2015. Persistence of plastic litter in the oceans. In Bergmann M., Gutow L., Klages M. (Eds.), Marine Anthropogenic Litter, Berlin: Springer.

Avio, C.G., Gorbi, S., Regoli, F., 2017. Plastics and microplastics in the oceans: from emerging pollutants to emerged threat. Mar Environ Res. 128, 2-11. https://doi.org/10.1016/j.marenvres.2016.05.012

Balestri¸ E., Menicagli, V., Vallerini, F., Lardicci C., 2017. Biodegradable plastic bags on the seafloor: a future threat for seagrass meadows? Sci Total Environ. 605-606, 755-763. https://doi.org/10.1016/j.scitotenv.2017.06.249

Barnes, D.K.A, Galgani, F., Thompson, R.C, Barlaz, M., 2009. Accumulation and fragmentation of plastic debris in global environments. Philos Trans R Soc London B Biol Sci. 364, 1985-1998. https://doi.org/10.1098/rstb.2008.0205

Beare, M.H., Coleman, D.C., Crossley, D.A., Hendrix, P.F., Odum, E.P., 1995. A hierarchical approach to evaluating the significance of soil biodiversity to biogeochemical cycling. Plant Soil. 170, 5-22. https://doi.org/10.1007/BF02183051

Besseling, E., Wang, B., Lürling, M., Koelmans, A.A., 2014. Nanoplastic affects growth of *S. obliquus* and reproduction of *D. magna*. Environ Sci Technol. 48, 12336-12243. https://doi.org/10.1021/es503001d

Bejgarn, S., MacLeod, M., Bogdal, C., Breitholtz, M., 2015. Toxicity of leachate from weathering plastics: An exploratory screening study with *Nitocra spinipes*. Chemosphere. 132, 114-119. https://doi.org/10.1016/j.chemosphere.2015.03.010

Bonanomi, G., Sicurezza, M.G., Caporaso, S., Esposito, A., Mazzoleni, S., 2006. Phytotoxicity dynamics of decaying plant materials. New Phytol. 169, 571-578. https://doi.org/10.1111/j.1469-8137.2005.01611.x

Canellas, E., Vera, P., Nerin, C., 2015. UPLC-ESI-Q-TOF-MS(E) and GC-MS identification and quantification of non-intentionally added substances coming from biodegradable food packaging. Anal Bioanal Chem. 407, 6781-6790. https://doi.org/10.1007/s00216-015-8848-2

Carson, H.S, Colbert, S.L., Kaylor, M., McDermid, K.J., 2011. Small plastic debris changes water movement and heat transfer through beach sediments. Mar Pollut Bull. 62, 1708-1713. https://doi.org/10.1016/j.marpolbul.2011.05.032

Ceccarini, A., Corti, A., Erba, F., Modugno, F., La Nasa, J., Bianchi, S., Castelvetro, V, 2018. The hidden microplastics: new insights and figures from the thorough separation and characterization of microplastics and of their degradation by-products in coastal sediments. Environ Sci Technol. 52, 5634-5643. https://doi.org/10.1021/acs.est.8b01487

Chandler, J.W., 2008. Cotyledon organogenesis. J Exp Bot. 59, 2917-2931. https://doi.org/10.1093/jxb/ern167

Clarke, K.R., Gorley, R.N., 2006. *PRIMER* v6: User manual/tutorial. PRIMER-E: Plymouth.

De Barro, J., 2008. Understanding and managing the causes of abnormal seedlings in Lucerne. Rural Industries Research and Development Corporation.

Domene, X., Ramirez, W., Solà, L., Alcañiz, J.M., Andrés, P., 2009. Soil pollution by nonylphenol and nonylphenol ethoxylates and their effects to plants and invertebrates. J Soils Sediments. 9, 555-567. https://doi.org/10.1007/s11368-009-0117-6

El-Darier, S.M., Youssef, R.S., 2000. Effect of soil type, salinity, and allelochemicals on germination and seedling growth of a medicinal plant *Lepidium sativum* L.. Ann Appl Biol. 156, 273-279. https://doi.org/10.1111/j.1744-7348.2000.tb00035.x

European Bioplastic, 2017. Bioplastic market data 2017.

Ferrara, G., Loffredo, E., Senesi, N., 2006. Phytotoxic, clastogenic and bioaccumulation effects of the environmental endocrine disruptor bisphenol A in various crops grown hydroponically. Planta. 223, 910-916. https://doi.org/10.1007/s00425-005-0147-2

Galloway, T. S., Cole, M., Lewis, C., 2017. Interactions of microplastic debris throughout the marine ecosystem. Nat Ecol Evol. http://dx.doi.org/10.1038/s41559-017-0116

Gong, P., Wilke, B.-M., Fleischmann, S., 1999. Soil-based phytotoxicity of 2,4,6-trinitrotoluene (TNT) to terrestrial higher plants. Arch. Environ. Contam. Toxicol. 36, 152–157. https://doi.org/10.1007/s002449900455

Green, D.S., Boots, B., Blockley, D.J., Rocha, C., Thompson, R.C., 2015. Impacts of discarded plastic bags on marine assemblages and ecosystem functioning. Environ Sci Technol. 49, 5380-5389. https://doi.org/10.1021/acs.est.5b00277.

Groh, K.J., Backhaus, T., Carney-Almroth, B., Geueke, B., Inostroza, P.A., Lennquist, A., Leslie, H.A., Maffini, M., Slunge, D., Trasande, L., Warhust, A.M., Muncke, J., 2018. Overview of known plastic packaging-associated chemicals and their hazards. Sci Total Environ. 651, 3253-3268. https://doi.org/10.1016/j.scitotenv.2018.10.015

Gupta, A., Singh, M., Jones, A.M., Laxmi, A., 2012. Hypocotyl directional growth in Arabidopsis: A complex trait. Plant Physiol. 159, 1463-1476. https://doi.org/10.1104/pp.112.195776

Hahladakis, J.N., Velis, C.A., Weber, R., Iacovidou, E., Purnell, P., 2018. An overview of chemical additives present in plastics: migration, release, fate and environmental impact during their use, disposal and recycling. J Hazard Mater. 334, 179-199. https://doi.org/10.1016/j.jhazmat.2017.10.014

Hardesty, B.D., Holdsworth, D., Revilland, A.T., Wilcox C., 2015. A biochemical approach for identifying plastics exposure in live wildlife. Methods Ecol Evol 6, 92–98. https://doi.org/10.1111/2041-210X.12277

Harrison, J.P., Boardman, C., O' Callaghan, K., Delort, A.-M., Song, J., 2017. Biodegradability standards for carrier bags and plastic films in aquatic environments: a critical review. R Soc open sci. 5, 171792. http://dx.doi.org/10.1098/rsos.171792

Hinsinger, P., 2001. Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. Plant Soil. 237, 173-195. https://doi.org/10.1023/A:1013351617532

ISO 11269-1: 2012a. Soil quality — Determination of the effects of pollutants on soil flora — Part 1: Method for the measurement of inhibition of root growth. International Organization for Standardization.

ISO 11269-2: 2012b. Soil quality — Determination of the effects of pollutants on soil flora — Part 2: Effects of contaminated soil on the emergence and early growth of higher plants. International Organization for Standardization.

ISTA 2003. International rules for seed testing. Edition 2003, International seed testing association, Zurich, Switzerland.

Jyothi, G., Reddy, K.R.K., Reddy, K.R.N., 2014. Oleic acid: a mycoherbicide from *Cochliobolus* lunatus for management of *Echinochloa crusgalli* in paddy. JEBAS. 2, 405-409.

Kennedy, G.L. Jr., 2002. Toxicity of adipic acid. Drug Chem Toxicol. 25, 191-202.

Kuzyakov, Y., Blagodatskaya, E., 2015. Microbial hotspots and hot moments in soil: concept & review. Soil Biol Biochem. 83, 184-199. http://dx.doi.org/10.1016/j.soilbio.2015.01.025

Lambert, N., Rostock, C., Hetland, S., 2010. Environmental risks associated to plasticizers and sweeteners in the Nordic countries. Bergfald miljørådgivere, kongens gate 3 0153 Oslo.

Li, W., Khan, M.A., Yamaguchi, S., Kamiya, Y., 2005. Effects of heavy metals on seed germination and early seedling growth of *Arabidopsis thaliana.* Plant Growth Regul. 46, 46-50. https://doi.org/10.1007/s10725-005-6324-2

Li, H.X., Getzinger, G.J., Lee Ferguson, P., Orihuela, B., Zhu, M., Rittschof, D., 2016. Effects of toxic leachate from commercial plastics on larval survival and settlement of the barnacle *Amphibalanus amphitrite*. Environ Sci Technol. 50, 924-931. 10.1021/acs.est.5b02781.

Li, W.C., Tse, H.F., Fok, L., 2016. Plastic waste in the marine environment: a review of sources, occurrence and effects. Sci Total Environ. 566–567, 333-349. https://doi.org/10.1016/j.scitotenv.2016.05.084

Li, X., Wang, L., Wang, S., Yang, Q., Zhou, Q., Huang, X., 2018. A preliminary analysis of the effects of bisphenol A on the plant root growth via changes in endogenous plant hormones. Ecotoxicol Environ Saf. 150, 152-158. https://doi.org/10.1016/j.ecoenv.2017.12.031

Lithner, D., Nordensvan, I., Dave, G., 2012. Comparative acute toxicity of leachates from plastic products made of polypropylene, polyethylene, PVC, acrylonitrile–butadiene–styrene, and epoxy to *Daphnia magna.* Environ Sci Pollut Res. 19, 1763-1772. https://doi.org/10.1007/s11356-011-0663-5

Luo, Y., Liang, J., Zeng, G., Chen, M., Mo, D., Li, G., Zhang, D., 2018. Seed germination test for toxicity evaluation of compost: its roles, problems and prospects. Waste Manag. 71, 109-114. https://doi.org/10.1016/j.wasman.2017.09.023

Ma, T., Christie, P., Teng, Y., Luo, Y., 2013. Rape (*Brassica chinensis* L.) seed germination, seedling growth, and physiology in soil polluted with di-*n*-butyl phthalate and bis(2-ethylhexyl) phthalate. Environ Sci Pollut Res. 20, 5289-5298. https://doi.org/10.1007/s11356-013-1520-5

Macoustra, G.K., King, C.K., Wasley, J., Robinson, S.A., Jolley, D.F., 2015. Impact of hydrocarbons from a diesel fuel on the germination and early growth of subantarctic plants. Environ Sci Process Impacts, 17, 1238-1248. http://dx.doi.org/10.1039/C4EM00680A

Mantese Sander, M., Nicolau, A., Guzatto, R., Samios, D., 2012. Plasticiser effect of oleic acid polyester on polyethylene and polypropylene. Polym Test. 31, 1077-1082. http://dx.doi.org/10.1016/j.polymertesting.2012.08.006

Martin-Closas, L., Botet, R., Pelacho, A.M., 2014. An in vitro crop plant ecotoxicity test for agricultural bioplastic constituents. Polym Degrad Stab. 108, 250-256. http://dx.doi.org/10.1016/j.polymdegradstab.2014.03.037

Milberg, P., Lamont, B.B., 1997. Seed/cotyledon size and nutrient content play a major role in early performance of species on nutrient- poor soils. New Phytol. 137, 665-672. https://doi.org/10.1046/j.1469-8137.1997.00870.x

Mitchell, R.L., Burchett, M.D., Pulkownik, A., McCluskey, L., 1988. Effects of environmentally hazardous chemicals on the emergence and early growth of selected Australian plants. Plant Soil. 112, 195-199. https://doi.org/10.1007/BF02139995

Moore, S.L., Gregorio, D., Carreon, M., Weisberg, S.B., Leecaster, M.K., 2001. Composition and distribution of beach debris in orange county, California. Mar Pollut Bull. 42, 241-245. https://doi.org/10.1016/S0025-326X(00)00148-X

Müller, C., Townsend, K., Matschullat, J., 2012. Experimental degradation of polymer shopping bags (standard and degradable plastic, and biodegradable) in the gastrointestinal fluids of sea turtles. Sci Total Environ. 416, 464–467. https://doi.org/10.1016/j.scitotenv.2011.10.069

Munari, C., Corbau, C., Simeoni, U., Mistri, M., 2015. Marine litter on Mediterranean shores: analysis of composition, spatial distribution and sources in north-western Adriatic beaches. Waste Manag. 49, 483-490. https://doi.org/10.1016/j.wasman.2015.12.010

OECD 2006. Test No. 208: Terrestrial plant test: seedling emergence and seedling growth test, OECD Guidelines for the testing of chemicals, section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264070066-en

Oehlmann, J., Schulte-Oehlmann, U., Kloas, W., Jagnytsch, O., Lutz, I., Kusk, K.O., Wollenberger, L., Santos, E.M., Paull, G.C., van Look, K.J.W., Tyler, C.R., 2009. A critical analysis of the biological impacts of plasticizers on wildlife. Philos Trans R Soc Lond B Biol Sci. 364, 2047-2062. 10.1098/rstb.2008.0242

Pan, W.J., Xiong, C., Wu, Q.P., Liu, J.X., Liao, H.M., Chen, W., Liu, Y.S., Zheng, L., 2013. Effect of BPA on the germination, root development, seedling growth and leaf differentiation under different light conditions in *Arabidopsis thaliana*. Chemosphere. 93, 2585-2592. https://doi.org/10.1016/j.chemosphere.2013.09.081

Pasternak, G., Zviely, D., Ribic, C.A., Ariel, A., Spanier, E., 2017. Sources, composition and spatial distribution of marine debris along the Mediterranean coast of Israel. Mar Pollut Bull. 114, 1036-1045. https://doi.org/10.1016/j.marpolbul.2016.11.023

Poorter, H., Niklas, K.J., Reich, P.B., Oleksyn, J., Poot, P., Mommer, L., 2011. Biomass allocation to leaves, stems and roots: meta-analyses of interspecific variation and environmental control. New Phytol. 193, 30-50. https://doi.org/10.1111/j.1469-8137.2011.03952.x

Rahim, A., Haryadi, Cahyanto, M.N., Pranoto, Y., 2012. Characteristics of butyrylated arenga starch prepared at different reaction time and butyric anhydride concentration. Int Food Res J. 19, 1655-1660.

Rani, M., Shim, W.J., Han, G.M., Jang, M., Al-Odaini, N.A., Song, Y.K., Hong, S.H., 2015. Qualitative analysis of additives in plastic marine debris and its new products. Arc Environ Contam Toxicol. 69, 352-366. https://doi.org/10.1007/s00244-015-0224-x

Rychter, P., Kawalec, M., Sobota, M., Kurcok, P., Kowalczuk, M., 2010. Study of aliphatic-aromatic copolyester degradation in sandy soil and its ecotoxicological impact. Biomacromolecules. 11, 839-847. https://doi.org/10.1021/bm901331t

Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., Eliceiri, K.W., 2017. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics 18:529, https://doi.org/10.1186/s12859-017-1934-z

Schmuck, A.M., Lavers, J.L., Stuckenbrock, S., Sharp, P.B., Bond, A.L., 2017. Geophysical features influence the accumulation of beach debris on Caribbean islands. Mar Pollut Bull. 121, 45-51. https://doi.org/10.1016/j.marpolbul.2017.05.043

Sforzini, S., Oliveri, L., Chinaglia, S., Viarengo, A., 2016. Application of biotest for the determination of soil ecotoxicity after exposure to biodegradable plastics. Front Env Sci. 4: 68. https://doi.org/10.3389/fenvs.2016.00068

Shah, A.A., Hasan, F., Hameed, A., Ahmed, S., 2008. Biological degradation of plastics: a comprehensive review. Biotechnol Adv. 26, 246-265. https://doi.org/10.1016/j.biotechadv.2007.12.005

Sharma, S., Chatterjee, S., 2017. Microplastic pollution, a threat to marine ecosystem and human health: a short review. Environ Sci Pollut Res. 24, 21530-21547. https://doi.org/10.1007/s11356-017-9910-8

Shrivastava, P., Kumar, R., 2015. Soil salinity: a serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. Saudi J Biol Sci. 22, 123-131. http://dx.doi.org/10.1016/j.sjbs.2014.12.001

Song, J.H., Murphy, R.J., Narayan, R., Davies, G.B.H., 2009. Biodegradable and compostable alternatives to conventional plastics. Philos Trans R Soc Lond B Biol Sci. 364, 2127-2139. https://doi.org/10.1098/rstb.2008.0289

Staples, C., Friederich, U., Hall, T., Klečka, G., Mihaich, E., Ortego, L., Caspers, N., Hentges, S., 2010. Estimating potential risks to terrestrial invertebrates and plants exposed to bisphenol A in soil amended with activated sludge biosolids. Environ Toxicol Chem. 29, 467-475. https://doi.org/10.1002/etc.49

Talsness, C.E., Andrade, A.J.M., Kuriyama, S.N., Taylor, J.A., vom Saal, F. S., 2009. Components of plastic: experimental studies in animals and relevance for human health. Phylos Trans R Soc B. 364, 2079-2096. https://doi.org/10.1098/rstb.2008.0281

Vandenberg, L.N., Hauser, R., Marcus, M., Olea, N., Welshons, W.V., 2007. Human exposure to bisphenol A (BPA). Reproductive Toxicol. 24, 139-177. https://doi.org/10.1016/j.reprotox.2007.07.010

UNEP, 2015. Biodegradable plastics and marine litter: misconceptions, concerns and impacts on marine environments. United Nations Environment Programme, Nairobi, 2015.

UNEP, 2018. Single-use plastics: A Roadmap for Sustainability. United Nations Environment Programme, Nairobi, 2018.

UNICHIM 1651: 2003. Qualità dell’acqua - Determinazione dell’inibizione della germinazione e allungamento radicale in *Cucumis sativus* L. (Cetriolo), *Lepidium sativum* L. (Crescione), *Sorghum saccharatum* Moench (Sorgo) – (saggio di tossicità cronica breve). UNICHIM.

Watanabe, M., Nakata, C., Wu, W., Kawamoto, K., Noma, Y., 2007. Characterization of semi-volatile organic compounds emitted during heating of nitrogen-containing plastics at low temperature. Chemosphere. 68, 2063-2072. https://doi.org/10.1016/j.chemosphere.2007.02.022

Weizbauer, R., Peters, W.S., Schulz, B., 2011. Geometric constraints and the anatomical interpretation of twisted plant organ phenotypes. Front Plant Sci. 2, 62. https://doi.org/10.3389/fpls.2011.00062

Wu, Z., Zhang, X., Wu, X., Shen, G., Du, Q., Mo, C., 2013. Uptake of di(2-ethylhexyl) phthalate (DEHP) by plant *Benincasa hispida* and its use for lowering DEHP content of intercropped vegetables. J Agric Food Chem. 61, 5220-5225. https://dx.doi.org/10.1021/jf401378u

Zhang, C., Feng, Y., Liu, Y., Chang, H., Li, Z., Xue, J., 2017. Uptake and translocation of organic pollutants in plants: a review. J Integr Agric. 16, 1659-1668. https://doi.org/10.1016/S2095-3119(16)61590-3

Phytotoxicity assessment of conventional and biodegradable plastic bags using seed germination test

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ABSTRACT

A large fraction of plastic litter found in natural environments is constituted by conventional not biodegradable plastic bags, and their adverse effects via ingestion or entanglement on terrestrial and marine organisms are largely documented. Biodegradable and compostable shoppers have been recently developed as alternative to traditional ones. These bags are specifically designed to degrade in composting facilities and generate a product devoid of toxicity to soils and crops. However, very little is known on the effects of bag leaching, i.e. the transfer of chemicals from plastic into natural environments, on vegetation. Some plant species are highly sensitive to a variety of chemicals, and seedling growth is generally the most affected life history stage. In this study we assessed the potential effects of conventional (high-density polyethylene, HDPE) and compostable (Mater-bi®, MB) bags, when left in natural environments, on water quality and plant development. To this end, seeds of *Lepidium sativum* L., a terrestrial plant commonly used in phytotoxicity standard tests, were exposed to leachates obtained from different amount of HDPE and MB bags, simulating various pollution degrees occurring in nature, for 72 hours. Both not-exposed (or virgin) bags and natural weathering exposed bags were used. Variations of chemical-physical characteristics of extracts were used as indicative of water quality deterioration, while alterations of seed gemination and seedling radicle and hypocotyl length were considered as indicative of phytotoxicity. A chemical qualitative screening of the leachates was also performed to identify the compounds with potential phytotoxicity. Both types of bags affected water characteristics (pH, salinity and total dissolved solids) relevant to plants, and released into water intentionally added chemicals, such as the noxious bisphenol A, and other phytotoxic substances probably generated during bag manufacturing. Leachates from both bag types did not affect seed germination. But, a significant number of seedlings showed developmental abnormalities or reduced seedling growth. The hypocotyl was the most sensible seedling organ to HDPE bag leachates while the radicle was the most vulnerable to MB ones. These findings indicate that plastic bags, including those that meet biodegradability and compostability standards, represent a potential threat to plants, if left in natural environments. Therefore, people and managers should be adequately informed about the potential environmental impact of an incorrect bag disposal. Simple, rapid standard phytotoxicity tests, such as the *L. sativum* bioassay, applied to bag leachates could be used in the future to select not noxious additives so to develop more eco-friendly bags.

Keywords.

Compostable; Leachate; Additives; *Lepidium sativum*; Phytotoxicity

1. Introduction

Pollution by plastics is a serious environmental problem affecting the whole world. Plastic bags manufactured with conventional, virtually non-biodegradable polymers, such as polyethylene (PE), polypropylene (PP) and high-density polyethylene (HDPE), are a consistent fraction (17-23%) of the total amount of plastic litter found in terrestrial and marine habitats (Moore et al., 2001; Barnes et al., 2009; Li et al., 2016; Pasternak et al., 2017; Schmuck et al., 2017). The adverse effects of bag litter on a variety of animal organisms via entanglement or ingestion are largely documented (Muller et al., 2012; Besseling et al., 2014; Galloway et al., 2017). Yet relatively few studies have examined the potential effects of bags on organisms via leaching, i.e., the transfer of chemicals from bags to the surrounding environment (Bejgarn et al., 2015), and none of these studies has focused on vegetation. Plants play a key role in the development and maintenance of soil structure, microbial communities and ecosystem functions (Beare et al., 1995; Kuzyakov and Blagodatskaya, 2015). Some plant species are known to be sensitive to a variety of pollutants, and the effects of pollutant exposure are often more detrimental at the early life history stages, seed germination and seedling growth (Li et al., 2005; Macoustra et al., 2015).

All plastic materials, including bags, contain plasticizers, pigments and organometallic compounds (hereafter referred to as additives) that are encapsulated with the polymeric matrix during the manufacturing process to improve item properties (Oehlmann et al., 2009; Bejgarn et al., 2015; Avio et al., 2017; Alam et al., 2018). Plastics can also adsorb harmful chemicals from the environment, for example persistent organic pollutants, polycyclic aromatic hydrocarbons and metals (Avio et al., 2017; Ceccarini et al., 2018). When plastic bags are exposed to natural environmental conditions, water-soluble additives and adsorbed chemicals can leach out (Bejgarn et al., 2015; Alam et al., 2018) and migrate into soils where they may imbibe seeds or be taken up by seedling roots (Wu et al., 2013; Martin-Closas et al., 2014; Rani et al., 2015; Zhang et al., 2017). The consequences of such phenomenon on plant development could be relevant. Indeed, studies on the individual effects of some chemicals frequently added to traditional plastic bags, such as bisphenol A (BPA), nonylphenol polyethoxylates and phthalic acid esters, indicate that such substances can inhibit seed germination and reduce seedling growth in some crop- and not crop-species (Domene et al., 2009; Staples et al., 2010; Ma et al., 2013; Pan et al., 2013; Li et al., 2018), in addition to be noxious to animals and human health (Vandenberg et al., 2007; Talsness et al., 2009).

Recently, several countries have banned traditional plastic bags (UNEP, 2018) and replaced them with a new generation of bags labelled as biodegradable and compostable to mitigate the social-ecological impact associated with plastic disposal and littering. Many of the compostable shoppers currently marked in Europe are manufactured with environmental-friendly polymers such as starch-based polymers (Shah et al., 2008). These new bags have specifically been developed to be disposed at the end of their life in industrial or “home” composting systems under specific conditions of temperature, moisture, and pH (Song et al., 2009) to totally biodegrade and generate carbon- and nutrient-rich compost for agriculture applications. According to international standards, the final compost has to meet fundamental requirements including safety, and it is expected to have no impact or any ecotoxicity effects on the soil environment and plants (OECD, 2006; ISO, 2012a; ISO, 2012b). However, studies have shown that also biodegradable/compostable bags are entered into natural environments, and their possible impact on the receiving ecosystems is of increasing concern (Balestri et al., 2017; Sharma and Chatterjee, 2017; Harrison et al., 2018). In fact, once left in natural habitats these items can require over than six months to biodegrade (Accinelli et al., 2012; Muller et al., 2012; UNEP 2015; Balestri et al., 2017), causing alterations of chemical-physical characteristics of the substrate and water (for example pH, temperature and redox potential) that may be relevant to organisms (Carson et al., 2011; Green, et al., 2015), including plants (Balestri et al., 2017). Unfortunately, the exact chemical composition of biodegradable bags is often secret, but there is evidence that during degradation these bags can release some additives potentially toxic to organisms (Bejgarn et al., 2015). However, current biodegradability test methods do not take into account the potentially adverse ecological impacts of plastic bags, additives and intermediate polymer degradation products that can leach out of these items. Since the market of biodegradable bags is expected to greatly increase in the next decades (European Bioplastic, 2017), assessing the effects of new generation of bags not only on animals but also on plants should be extremely useful.

The aim of this study was to evaluate the impact of plastic bags via leaching on terrestrial higher plants through a rapid and cost-effective standard phytotoxicity test. We hypothesized that (i) when exposed to rainwater both types of bags would affect water quality by releasing processing compounds and that (ii) bag leachates would influence early life plant stages. To test these hypotheses, we examined the effects of bags on water chemical/physical properties that regulate the availability of nutrients to plants (Hinsinger, 2001; Shrivastava and Kumar, 2015), and we identified potentially phytotoxic compounds released by bags in leachates. Then, the phytotoxicity of bag leachates was assessed by using a laboratory seed germination and root elongation test. The application of standard phytotoxicity tests to plastic bag leachates could be critical for developing alternative more eco-sustainable materials.

2. Materials and methods

To assess the effects of different types of plastic bags, conventional and compostable bags, on water quality, leachates from bags were prepared in laboratory and chemical/physical analyses of the leachates were performed. Both virgin bags and bags previously exposed to natural environmental conditions were used to discriminate between the effects of plastic chemical content and bag interaction with abiotic/biotic factors. A qualitative screening of the leachates from virgin bags was also carried out by using gas chromatography-mass spectrometry to detect the processing compounds leached out from bags. Then, the phytotoxicity of the leachates was evaluated in a separate seed gemination and seedling growth experiment by using *Lepidium sativum* L. (garden cress, Brassicaceae) as test species.

2.1. Leachate preparation, chemical/physical analysis and qualitative additive screening

To prepare bag leachates for chemical/physical analyses and qualitative screening, two different types of commercially available shopper bags, a high-density polyethylene (HDPE) bag and a compostable and biodegradable bag purchased from local supermarkets in Italy, were used. This latter type of bag was made of Mater-bi®(MB), a biopolymer based on starch and vinyl-alcohol copolymers (Sforzini et al., 2016), and it is certified for industrial composting (EN13432) and "home" composting scheme (OK Compost Home).

A group of bags (hereafter referred to as virgin or not-exposed bags, NE) was left in laboratory at a temperature of 22 ± 1 °C. Another group of bags (hereafter referred to as exposed bags, E) was placed outdoor (average daily temperature of 13 ± 2 °C, total amount of precipitations of 57 mm and mean daylength of 10 hours) in a back-dune area at Rosignano Solvay (43°23'N 10°26' E, Italy) over a period of 10 days for natural weathering. The duration of the exposure period was similar to that of a previous study on the toxicity of bag leachates to a marine organism (Bejgarn et al., 2015). To avoid their dispersion, the bags were individually placed on the soil substrate and fixed with pebbles. At the end of exposition period, the bags were collected and transported to the laboratory.

All plastic bags were then cut into pieces of approximately 1 cm^2. Different amounts of pieces obtained from each type of bag were placed into glass flasks containing sterilized deionized water to obtain liquid (water)- to- solid (plastic) ratios of 100, 10 and 5, corresponding respectively to approximately 4.1×10^{-4}, 4.1×10^{-3} and 8.3×10^{-3} bag/mL, hereafter referred to as low (L), medium (M) and high (H) pollution degrees. These ratios were chosen to mimic various degrees of bag pollution, in terms of bag density, occurring in natural environments (up to 1 bag m^{-2}; Munari et al., 2016; Alshawafi et al., 2017; Pasternak et al., 2017; Schmuck et al., 2017). For each bag type, sterile deionized water with no bag material was used as control (no pollution). Each treatment was performed in triplicate. Flasks were wrapped in aluminum foil and placed in a culture chamber on a rotary shaker (95 rotations per minute) for 72 h (Bejgarn et al., 2015) at a temperature of 24 ± 1 °C. Thus, the experiment was a full factorial design consisting of three factors, plastic type (fixed, two levels: HDPE and MB), exposure (fixed, two levels: not-exposed or virgin bags and exposed) and pollution degree (fixed, four levels: control or no pollution, low, medium and high). After the incubation period, plastic pieces were separated from the liquid phase by filtration using nylon mesh filter (200 µm) and the filtered aqueous phase obtained from each treatment were used for water quality analysis, qualitative additive screening and seed germination test.

To examine the effect of bag leaching on water quality, a sample (30 mL) of the filtered aqueous solution obtained from each replicate of the MB and HDPE treatments was collected, and water chemical/physical indicators such as pH, salinity and total dissolved solids (TDS) were measured by a multiparameter meter (HI98194, Hanna Instruments). To detect the volatile organic compounds, present in leachates, samples of the filtered aqueous phases obtained from HDPE and MB virgin bags at the highest concentration were collected. About 30 mL of aqueous solutions were extracted for three times with 6 mL of an organic solvent in a separation funnel. Chloroform and n-hexane were employed for the extraction of solution deriving from HDPE bags, whilst methyl isobutylketone (MIBK) was used for the extraction of leachates from MB bag leachates. The aqueous and organic phases were separated, and the organic extracts were concentrated under reduced pressure and analyzed through gas chromatography-mass spectrometry (GC-MS, Agilent 7890B-5977A) equipped with HP-5MS capillary column (30 m×0.25 mm×0.25 µm) (5%-phenyl)-methylpolysiloxane. The carrier gas was helium with a flow of 1 mL/min. The injector and detector temperatures were maintained at 250°C and 280°C, respectively, and the following temperature program was adopted for the chromatographic run: 60 °C isothermal for 2 min; 10 °C/min up to 260 °C; 260 °C isothermal for 10 min. This non-polar column has an excellent inertness and low bleed characteristics and has been successfully adopted under similar chromatographic conditions for the analysis of different types of plasticizers and of a wide range of organic contaminants recovered by extraction from the aqueous phase (Hardesty et al., 2015). Organic compounds were identified on the basis of the NIST 2.0 Mass Spectral Database and by the comparison with literature data. Identification was restricted to the most abundant and representative organic constituents of the leachates.

2.2. *Lepidium sativum seed germination test*

Lepidium sativum was used as indicator of bag leachate phytotoxicity. This species is considered as a biological indicator for toxicity of wastes, soils and water contaminated with chemicals, including heavy metals, petrochemical compounds and polycyclic aromatic hydrocarbons (Gong et al., 1999). The fast growth and root sensitivity of the species to different compounds enable its use in standard ecotoxicity tests on environmental matrices (UNICHIM, 2003; OECD, 2006; ISO, 2012a; ISO, 2012b). In the presence of cytotoxic compounds, inhibition of the dividing processes of the root meristematic cells causes a reduction of roots length relative to control (Gong et al., 1999). Commercially available seeds of *L. sativum* (Italsementi s.n.c, Italy) were rinsed with sterile deionized water and placed in Petri dishes (12 seeds per dish) containing a layer of cellulose filter paper (Whatman N°1), previously moistened with 1.5 mL of one of the obtained filtered leachates, following the same design of the leaching experiment described above. Petri dishes were sealed with parafilm to prevent water evaporation and randomly placed in a culture chamber for 72 hours at 24 ± 1 °C in darkness, as recommended in standard phytotoxicity tests (UNICHIM, 2003). For each treatment there were five replicate dishes. At the end of the incubation period, the number of germinated seeds in each dish was counted and the percentage of germination was calculated. A seed was considered to have germinated when the length of the radicle had reached at least 2 mm (Luo et al., 2017). A visual evaluation of developmental abnormalities in seedlings (ISTA, 2003; Chandler, 2008) was also carried out, and the percentage of abnormal seedlings was calculated. Then, a sample of the remaining normal seedlings ($n = 5$) was collected at random from each Petri dish, and the seedlings carefully placed on squared paper and photographed for morphological measurements. The length of the radicle and the hypocotyl of each seedling was measured with an image analysis software (ImageJ 2, Rueden et al., 2017). The radicle to hypocotyl length ratio was also computed as it is considered an indicator of relative allocation of biomass to belowground and aboveground organs (Poorter et al., 2011). Before the germination test, seeds were examined under a stereomicroscope (Wild M3C, Leica) and pressure tested with forceps. Moldy or empty seeds, and those that did not resist the pressure were considered non-viable and thus discarded. A sample of seeds was tested using distilled water for rapid and homogeneous germination under the assay conditions. This test was considered valid as the end of the incubation period seed germination was higher than 90%.

2.3. Data analysis

Non-metric multidimensional scaling (MDS) based on the Euclidean distance was conducted on chemical-physical variables of leachates (pH, salinity and total dissolved solids) from each bag type separately (HDPE or MB) to visualize differences among samples. To reduce the leachate variables and determine the relationship among them, principal component analysis (PCA) was performed for leachates from each bag type. Three-way permutational multivariate analysis of variance (PERMANOVA) was also performed on chemical-physical data to examine the overall effect of treatments (plastic type, bag exposure and pollution). Then, univariate three-way PERMANOVAs were conducted on each individual chemical-physical variable.

For each bag type, separate MDS graphical representations based on the Euclidean distance were conducted on *L. sativum* data (percentage of germination, percentage of abnormal seedlings, hypocotyl and radicle length, and radicle to hypocotyl ratio) to visualize differences among samples. A three-way multivariate PERMANOVA was also conducted on all plant variables to compare the effects of the two different types of plastic bags (HDPE and MB), exposure and pollution degree on *L. sativum* performance, followed by separate univariate PERMANOVAs. Prior to the analyses, data were appropriately transformed and normalized when necessary. In PERMANOVAs, dissimilarities were calculated as Euclidean distances from 9999 permutations of the residuals under a reduced model, and when significant effects were detected posteriori pair-wise comparisons using 9999 random permutations were performed. Statistically significant terms were checked for differences in multivariate group dispersion through permutational analysis of multivariate dispersion (PERMDISP). All analyses were performed using PERMANOVA + add on to PRIMER 6 statistical software (Clarke and Warwick, 2006; Anderson et al., 2008).

3. Results and discussion

3.1. Leachate chemical/physical analysis and qualitative additive screening

MDSs performed on chemical-physical variables of water extracts (pH, salinity and TDS) obtained from each type of bag (MB or HDPE) showed an overlapping of samples belonging to controls (no pollution) and a clear segregation of these samples from those of the pollution treatments (Fig. 1). These findings suggest that the presence of the tested bags altered the quality of water. For MB bag leachates, a segregation between samples from virgin and exposed bags was also observed (Fig. 1), indicating that the examined characteristics were influenced by abiotic/biotic factors.

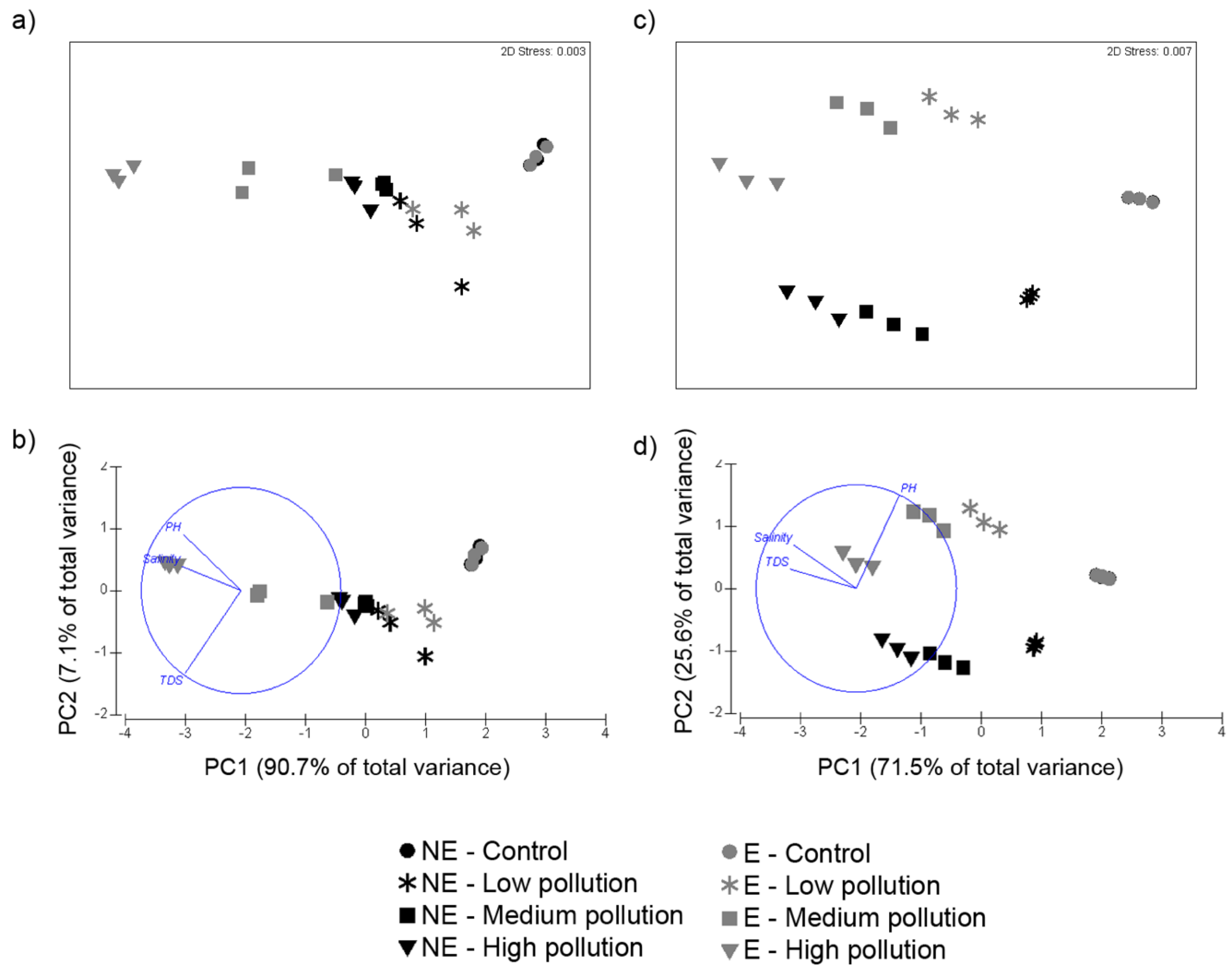


Fig. 1. Non-metric multidimensional scaling ordination (MDS) and principal component analysis biplots of chemical-physical variables of leachates from different concentrations of not-exposed (NE) and exposed (E) HDPE (a, b) and MB (c, d) plastic bags.

2-column fitting image, color image in online version and black-and-white image in printed version

Similar graphical representations were observed in principal component analyses (Fig. 1). The first (PC1) and the second (PC2) principal components accounted for 97.8% and 97.1% of the total variance for HDPE and MB leachates, respectively (Table 1). For both the materials, all the examined variables contributed to the construction of PC1 (Table 1), and thus they were consistently responsible for the observed differences in leachate quality among treatments.

Table 1

Results of principal component analysis performed on chemical/physical data of leachates from high-density polyethylene and Mater-bi® bags. Explained (a) and cumulative (b) variance, eigenvalues and eigenvectors of leachates variables are reported.

Principal components	1	2	3	1	2	3
	High-density polyethylene			Mater-bi®		
a) Variance explained						
Eigenvalues	2.720	0.213	0.065	2.140	0.768	0.087
% of variance	90.7	7.1	2.2	71.5	25.6	2.9
b) Cumulative variance %	90.7	97.8	100	71.5	97.1	100
Eigenvectors						
pH	-0.579	0.544	0.607	0.425	0.891	-0.158
Salinity	-0.591	0.233	-0.773	-0.622	0.414	0.664
TDS	-0.562	-0.806	0.187	-0.658	0.184	-0.730

Results of multivariate PERMANOVA analyses revealed a significant effect of all investigated factors (plastic type, exposure and pollution degree), as well as of their interaction, on the quality of leachate (Table 2). A consistent effect of the interaction among all factors was also detected by separate univariate PERMANOVAs for all examined variables (Table 2).

Table 2

Results of multivariate (a) and univariate (b) PERMANOVA analysis on pH, total dissolved solids (TDS) and salinity of leachates. Significant results are in bold, and pair-wise comparisons are reported. NE: not-exposed bag, E: exposed bag, H: high pollution, M: medium pollution, L: low pollution, C: control, no pollution, HDPE: high-density polyethylene bag, MB: Mater-bi$^{®}$ bag.

a) Multivariate PERMANOVA analysis

Source	df	Pseudo-F	P
Plastic (Pl)	1	223.11	**<0.001**
Exposure (E)	1	187.80	**<0.001**
Pollution (Po)	3	308.46	**<0.001**
Pl x E	1	45.57	**<0.001**
Pl x Po	3	45.27	**<0.001**
E x Po	3	34.38	**<0.001**
Pl x E x Po	3	26.02	**<0.001**
Residual	32		
Transformation		Log (x+1), normalized	

b) Univariate PERMANOVA analysis

		pH		Salinity		TDS	
Source	df	Pseudo-F	P	Pseudo-F	P	Pseudo-F	P
Plastic (Pl)	1	6215.3	**<0.001**	3.33	0.075	2.97	0.09
Exposure (E)	1	1606.1	**<0.001**	112.13	**<0.001**	248.9	**<0.001**
Pollution (Po)	3	556.4	**<0.001**	136.09	**<0.001**	1343.6	**<0.001**
Pl x E	1	997.85	**<0.001**	0.53	0.464	10.61	**<0.001**
Pl x Po	3	1218.2	**<0.001**	1.82	0.160	1.13	0.34
E x Po	3	214.42	**<0.001**	26.36	**<0.001**	36.91	**<0.001**
Pl x E x Po	3	205.24	**<0.001**	13.96	**<0.001**	55.48	**<0.001**
Residual	32						
Transformation		Normalized		Normalized		Log (x+1), normalized	
Pair-wise comparisons		MB ≠ C: NE, E HDPE ≠ C: E (H, M) MB ≠ HDPE: E (H, M), NE NE ≠ E: HDPE (H, M), MB MB (NE, E): H ≠ M ≠ L HDPE (E): H, M ≠ L		HDPE ≠ C: E MB ≠ C: NE, E (H, M) MB ≠ HDPE: NE, E (H) NE ≠ E: HDPE (H, M), MB HDPE (E): H ≠ M ≠ L		HDPE ≠ C: NE, E MB ≠ C: NE, E MB ≠ HDPE: NE (H), E NE ≠ E: HDPE (H, M); MB HDPE: H ≠ M ≠ L MB: H ≠ M ≠ L	

The pH of MB leachates was significantly lower that of control (pH 7.29 -7.31), regardless of exposure and pollution degree, while that of HDPE leachates was slightly higher than that of control but only with exposed bags and at medium and high pollution degree (Fig. 2). For MB leachates, the pH decreased with increasing pollution degree regardless of exposure, while that of HDPE leachates increased with increasing pollution but only with exposed bags (Fig. 2; Table 2). The pH of MB leachates was lower than that of HDPE irrespectively of pollution for virgin bags, and only at high and medium pollution degree for exposed bags. Leachates from virgin bags had significantly lower pH values than those from exposed bags, except that with HDPE bags at the low pollution degree (Fig. 2; Table 2). These findings are in accordance with results of a study by Bejgarn et al. (2015) on the leachate produced from plastic bags, showing an increase of pH in leachates from HDPE bags and a decrease of pH with compostable/biodegradable bags. However, Bejgarn et al. (2015) found a lower pH in the leachates obtained from compostable bags previously exposed to artificial weathering (exposure to UV radiation) than that measured in virgin bag leachates, and thus the opposite of that observed in the present study with MB bags. This discrepancy could be due to the different composition of the employed biodegradable bags. In addition, in our study exposed bags were subjected to real natural conditions for 10 days, and during this period abiotic factors (solar UV-radiation, rain and temperature) and microorganisms (Andrady, 2015) might have promoted plastic decomposition, causing the release of acid cations from plastics.

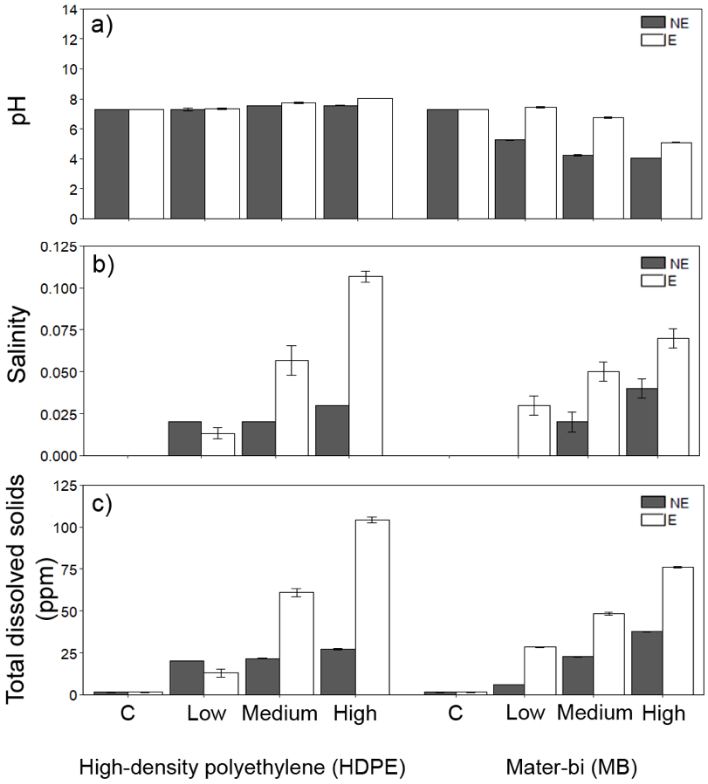


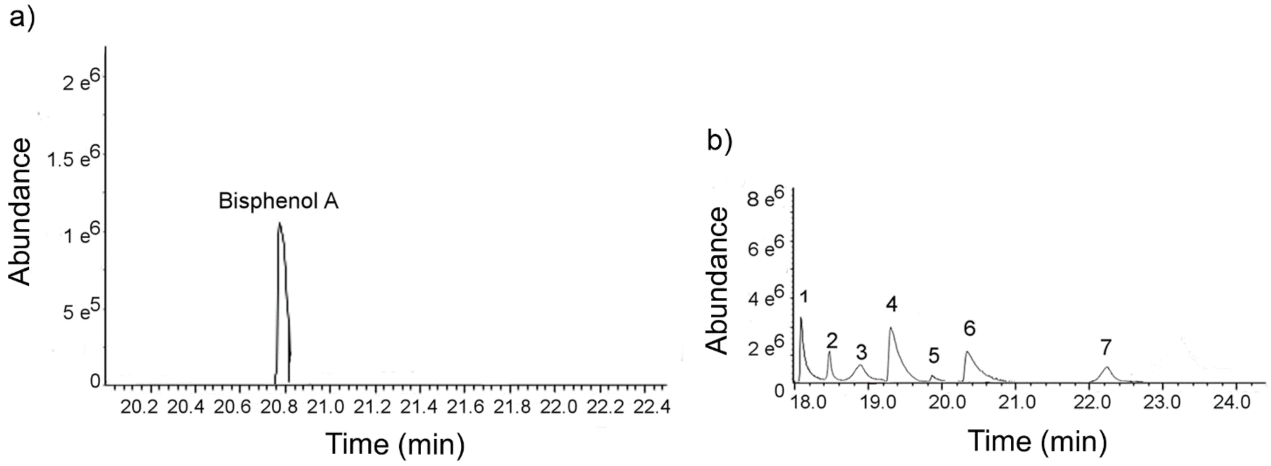
Fig. 2. Values of pH (a), salinity (b) and total dissolved solids (c) of leachates from different concentrations of not-exposed (NE) and exposed (E) HDPE and MB plastic bags. Data are mean ± SE, n = 3.

1.5- column fitting image

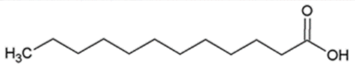
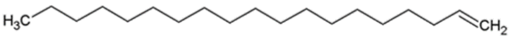
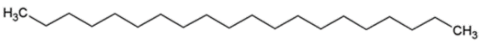
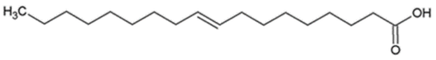
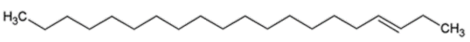
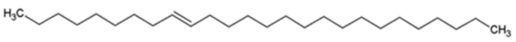
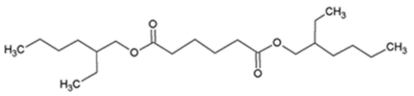
The salinity of HDPE leachates was significantly higher compared to that of control treatments but only with exposed bags, while that of MB leachates was higher than controls with virgin bags regardless of pollution degree, and only at high and medium pollution levels with exposed HDPE bags. For both types of bag, salinity increased with increasing pollution, and HDPE leachate salinity was higher than that of MB regardless of exposure at the high pollution degree. Leachates from exposed bags had significantly higher salinity than those from virgin ones irrespectively of pollution for MB bags, and at high and medium pollution degrees for HDPE bags (Fig. 2; Table 2). This finding suggests that MB bags contained or might have released more salts in water than HDPE ones, and that both bag types might have adsorbed salts deposited with salt spray from the adjacent coastal habitat during the exposure period.

The values of TDS in plastic leachates were significantly higher than controls and increased with increasing pollution degree irrespectively of exposure. The amount of TDS of MB leachates was significantly higher than that of HDPE ones at the highest pollution degree for virgin material, while was lower at the medium and high pollution degree for exposed materials (Fig. 2; Table 2). Significantly higher TDS amounts were found in leachates from exposed than virgin bags, except that for HDPE at low pollution level, indicating that weathering might have increased plastic embrittlement, and that MB bags were more prone to fragmentation than HDPE ones due to their greater susceptibility towards biodegradation.

Identifying potential toxic compounds in plastic bags is difficult, since their additive content can vary widely, even for the same type of item, depending on the manufacturer and the process of production (Groh et al., 2018; Hahladakis et al., 2018). In most previous studies, the chemical screening of plastic leachates has failed to identify chemical structures (Lithner et al., 2012; Bejgarn et al., 2015; Li et al., 2016). Here, in total twelve compounds have been identified in virgin plastic bag leachates. Mass spectra interpreted by NIST library were well matched and a qualitative analysis of the extracts has been performed. The largely prevailing compound in the chromatogram of HDPE chloroform extract (Fig. 3) was identified as BPA, an antioxidant, flame retardant commonly added to plastics. On the other hand, the extraction of HDPE leachate with n-hexane allowed to reveal the presence of seven different organic compounds with a long alkyl chain, soluble in this alkane (Fig. 3). Linear long-chain alkanes and alkenes resulted the prevailing components. These products can derive from the addition of a wax, by-product of polyethylene manufacture, which gives to the polymer more pronounced plasticization (AlMaadeed et al., 2015).



c)

Peak	Organic compound	
1	n-Dodecanoic acid	
2	1-Nonadecene	
3	Eicosane	
4	Oleic acid	
5	3-Eicosene	
6	9-Hexacosene	
7	di-(2-ethylhexyl)adipate	

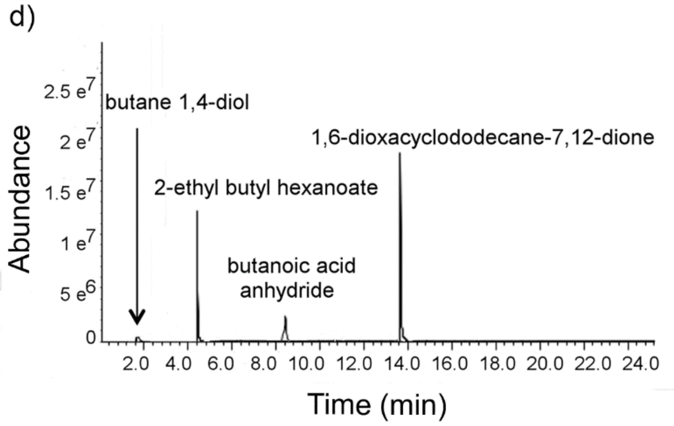


Fig. 3. Chromatograms of the chloroform extract (a) and of the hexane extract (b) from HDPE virgin bag leachate, and organic molecules present in the chromatogram of n-hexane extract (c). Chromatogram of the methyl isobutylketone extract (d) from the MB virgin bag leachate.

1.5- column fitting image

Dodecanoic and oleic acids have been also ascertained, probably deriving from ester plasticizers used as co-processing additives for the polyolefin (Mantese Sander et al., 2012). In fact, also di-(2-ethylhexyl)adipate, also known as DEHA, a largely used plasticizer, was identified. Instead, the four organic compounds extracted from MB bag leachate with MIBK were identified as traces of butane1,4-diol, 2-ethylbutyl hexanoate, 1,6-dioxacyclododecane-7,12-dione and traces of butanoic acid anhydride (Fig. 3). The first compound, in very low amount is the co-monomer of adipic acid in the polyester poly(butylene adipate), employed with corn starch for the preparation of Mater-bi®. The second compound is a hydrolytically stable ester used as volatile plasticizer or coalescing agent for coatings (Patent EP0026982A1, 1979). The third and largely prevailing compound could be a non-intentionally added substance or it could derive from decomposition of the initial components, or because of chemical interactions between them (Watanabe et al., 2007). It has been indeed found as new formed molecule from the reaction between butane 1,4-diol and adipic acid, the monomers of the polyester poly(butylene adipate) present in Mater-bi® (Canellas et al., 2015). Thus, the significant presence of 1,6-dioxacyclododecane-7,12-dione, as well of low amounts of free butane 1,4-diol, can suggest the depolymerization of the polyester with release of the free monomers, that could be probably responsible for the observed higher acidity and salinity of MB leachates (Rychter et al., 2010). Finally, traces of butanoic acid anhydride can be a residue of the starch esterification reaction. Butyrated corn starch is often present in Mater-bi® because this modified starch is able to increase the hydrophobicity and the flexibility of the biopolymer (Rahim et al., 2012).

3.2 Lepidium sativum seed germination test

After three days of incubation, total percent of germination of *L. sativum* seeds treated with plastic leachates was similar to that of the controls (Fig. 4). However, a significant number of germinated seeds treated with plastic leachates produced deformed seedlings (Fig. 5) and inhibitory growth effects were detected in many normal seedlings. MDS plots performed on all plant variables showed a segregation between samples belonging to plastic treatments and controls, more evident with MB bag samples than with HDPE ones (Fig. 6). Results of multivariate PERMANOVA revealed a significant effect of the interaction among plastic type, exposure and pollution, and this effect was effectively ascribable to the investigated factors and not to heterogeneity in multivariate dispersion (Table 3). Results of univariate PERMANOVA for all examined plant variables are reported in Table 4.

Table 3

Results of multivariate PERMANOVA analysis performed on *L. sativum* variables (percentage of germination, percentage of abnormal seedlings, hypocotyl length, radicle length and radicle to hypocotyl ratio). The transformation applied to data is also reported. Significant results are in bold.

Source	df	Pseudo-F	P
Plastic (Pl)	1	4.43	**0.003**
Exposure (E)	1	3.44	**0.015**
Pollution (Po)	3	22.33	**<0.001**
Pol x E	1	6.25	**<0.001**
Pl x Po	3	1.79	0.063
E x Po	3	1.94	**0.044**
Pl x E x Po	3	4.07	**<0.001**
Residual	64		
Transformation		Log (x+1), normalized	

Table 4

Results of univariate PERMANOVA analysis performed on *L. sativum* variables (percentage of germination, percentage of abnormal seedlings, radicle length, hypocotyl length and radicle to hypocotyl ratio). Significant results are in bold. Transformation applied to data and pair-wise comparisons are reported. NE: not-exposed bag, E: exposed bag, H: high pollution, M: medium pollution, L: low pollution, C: control or no pollution, HDPE: high-density polyethylene bag, MB: Mater-bi® bag.

		Germination (%)		Abnormalities (%)	
Source	d.f.	Pseudo-F	P	Pseudo-F	P
Plastic (Pl)	1	0.13	0.713	13.99	**<0.001**
Exposure (E)	1	0.54	0.461	8.44	**0.004**
Pollution (Po)	3	1.08	0.359	30.98	**<0.001**
Pl x E	1	0.14	0.724	0.53	0.465
Pl x Po	3	0.86	0.471	1.93	0.125
E x Po	3	1.99	0.122	1.14	0.342
Pl x E x Po	3	0.86	0.466	1.19	0.322
Residual	64				
Transformation		None		Fourth root	
Pair-wise comparisons				MB ≠ HDPE ≠ C ; NE ≠ E	

		Radicle length (cm)		Hypocotyl length (cm)		Radicle to hypocotyl ratio	
Source	d.f.	Pseudo-F	P	Pseudo-F	P	Pseudo-F	P
Plastic (Pl)	1	10.83	**0.001**	16.85	**<0.001**	4.37E-2	0.830
Exposure (E)	1	0.80	0.374	12.32	**<0.001**	4.83	**0.032**
Pollution (Po)	3	96.15	<**0.001**	138.4	**<0.001**	1.58	0.201
Pl x E	1	2.26	0.130	7.30	**0.009**	22.84	**<0.001**
Pl x Po	3	6.19	<**0.001**	8.43	**<0.001**	0.23	0.870
E x Po	3	3.99	**0.013**	5.82	**0.001**	0.92	0.444
Pl x E x Po	3	2.23	0.092	10.43	**<0.001**	9.85	<**0.001**
Residual	64						
Transformation		Log (x+1)		Square root		None	
Pair-wise comparisons		MB, HDPE ≠ C MB ≠ HDPE: H HDPE, MB: H ≠ M, L		HDPE ≠ C: NE, E (M, L) MB ≠ C MB ≠ HDPE: E (H) NE ≠ E: HDPE (H) MB (NE): M ≠ L		HDPE ≠ C: E (H) MB ≠ HDPE: NE, E (H) NE ≠ E: HDPE, MB (H)	

There was no consistent effect of plastic leachate on percent seed germination, probably because the germination stage is independent from substrate and the radicle does still not contribute to pollutant-uptake (Milberg and Lamont, 1997).

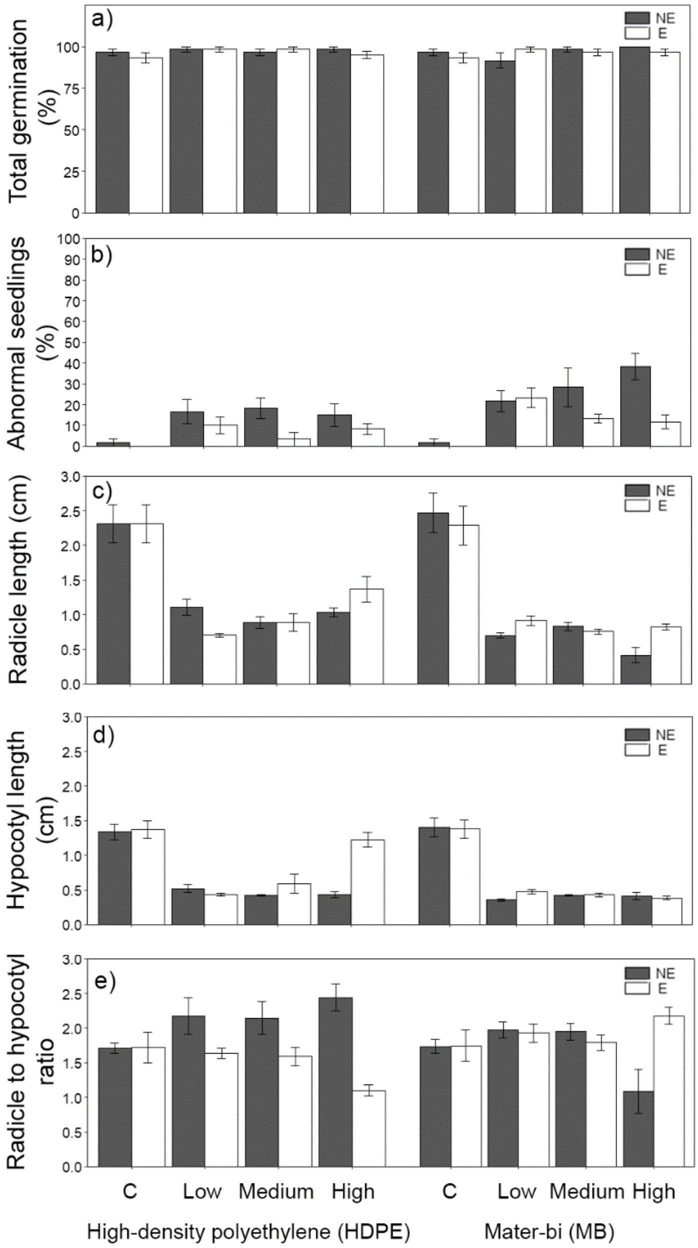


Fig. 4. Percentages of seed germination (a) and abnormal seedlings (b), radicle length (c), hypocotyl length (d), and radicle to hypocotyl ratio (e) of *L. sativum* seedlings treated with different concentrations of leachates from not-exposed (NE) and exposed (E) HDPE and MB plastic bags. Data are mean ± SE, n = 5.

1.5-column fitting image

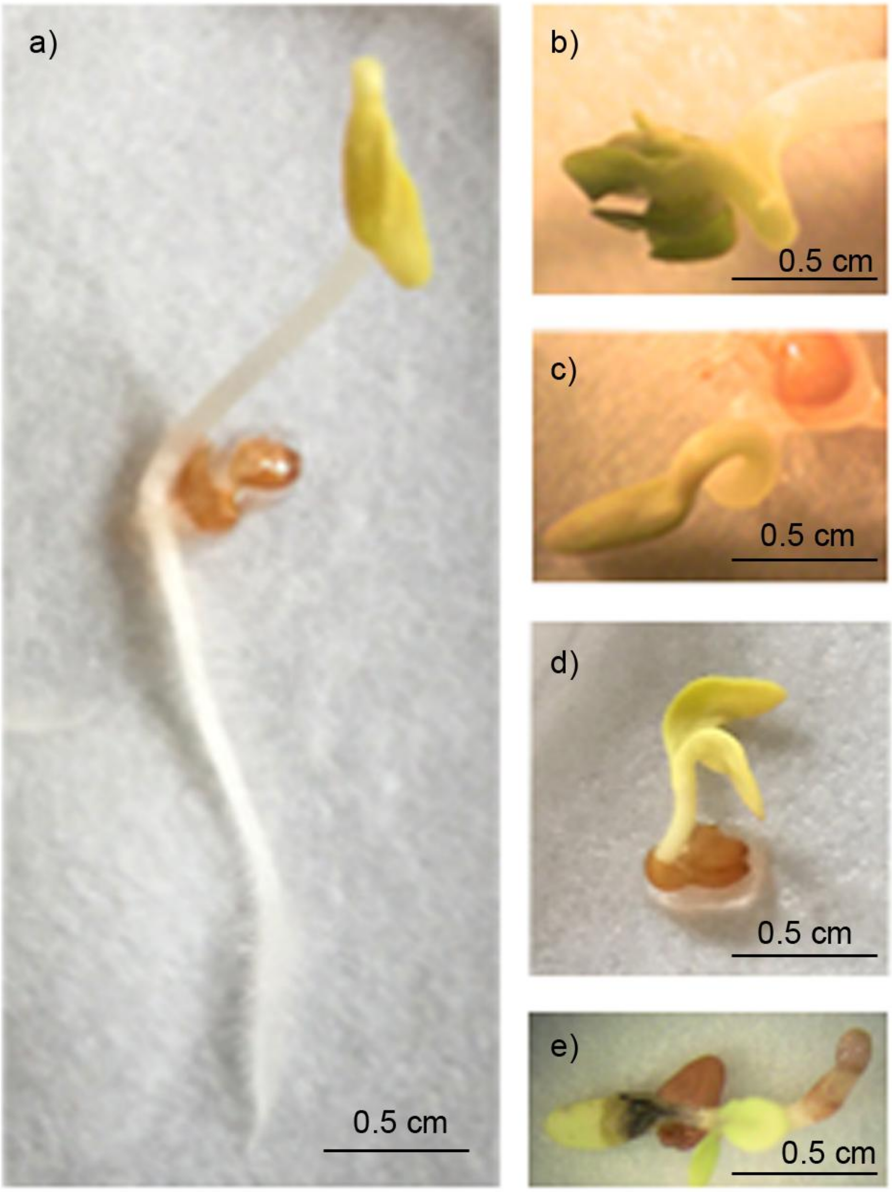


Fig. 5. *Lepidium sativum* seedlings. A normal seedling (a), and abnormal seedlings grown with bag leachates showing deformed cotyledons (b), a twisted hypocotyl (c), missing radicle (d) and deformity of the whole seedling (e).

1.5-column fitting image

Color image in online version and black-and-white image in printed version

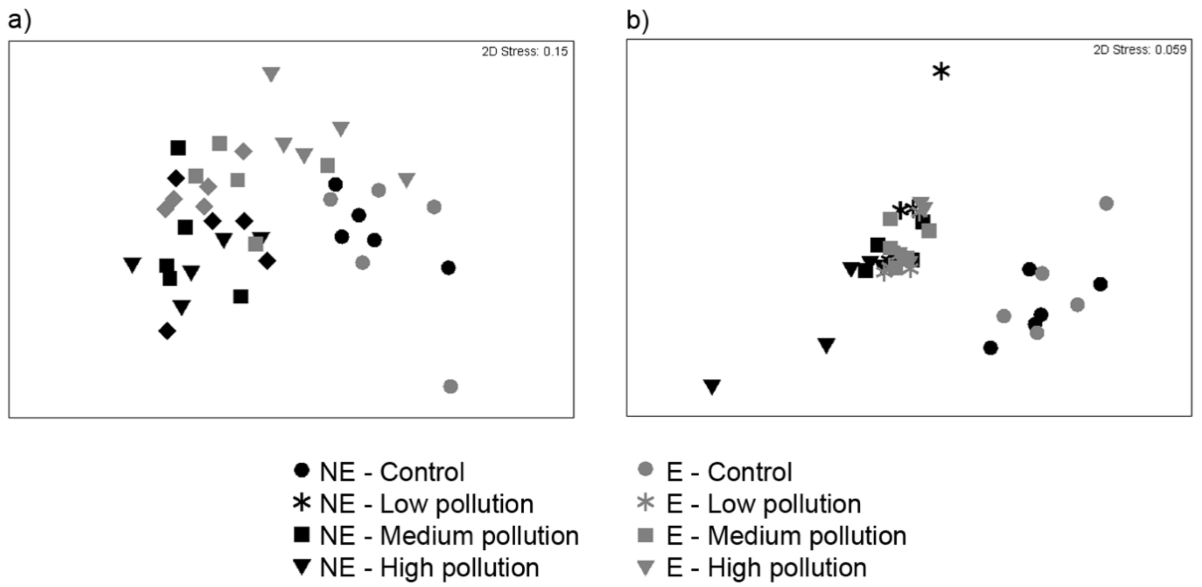


Fig. 6. Non-metric multidimensional scaling ordination of *L. sativum* seed germination and seedling growth data recorded with different pollution degrees of leachates from not-exposed (NE) and exposed (E) HDPE (a) and MB (b) bags.

2-column fitting image

However, up to 40% of the seeds germinated in the presence of plastic leachates showed developmental abnormalities, such as a short or stubby radicle, twisted hypocotyl and malformed and/or supernumerary cotyledons (Fig. 5; Table 5), indicating leachate phytotoxic effects (ISTA, 2003; Chandler, 2008; De Barro, 2008).

Table 5

Number of normal and abnormal *L. sativum* seedlings observed after incubation of seeds with leachates obtained from not-exposed and exposed high-density polyethylene bags (a) and Mater-bi® bags (b) at the different pollution degrees, low, medium and high.

		Total number of seeds	Total number of abnormal seedlings		
			Short/ stubby radicle	Twisted hypocotyl	Malformed supernumerary/ cotyledon
a) High-density polyethylene					
Not-exposed	High pollution	60	5	2	1
	Medium pollution	60	5	2	4
	Low pollution	60	3	2	3
Exposed	High pollution	60	3	1	0
	Medium pollution	60	0	1	0
	Low pollution	60	3	1	1
b) Mater-bi®					
Not-exposed	High pollution	60	18	3	2
	Medium pollution	60	9	4	4
	Low pollution	60	8	3	2
Exposed	High pollution	60	4	0	1
	Medium pollution	60	4	2	2
	Low pollution	60	5	3	5

Indeed, similar abnormalities have been observed in crops species and are considered as characteristics of seedlings exposed to toxic chemicals (Mitchell et al., 1988; De Barro, 2008). Overall, a higher number of abnormal seedlings, as well as of normal seedlings with reduced growth, was detected with leachates from not-exposed bags compared to exposed ones (Fig. 4; Table 4). These findings demonstrate that the chemicals responsible for such detrimental effects were present in virgin material, and were not absorbed by bags from the environment. They also indicate that a fraction of these chemicals was migrated into the environment during the exposure period. Available data on the effects of additives used in the manufacturing of traditional bags on plants indicate that BPA can have a clastogenic activity and induce morphological alterations of roots and shoots through the inhibition of both cell elongation and cell division, as well as alteration of gene expression and hormone function (Ferrara et al., 2006; Chandler, 2008; Weizbauer et al., 2011; Gupta et al., 2012). Instead, DEHA was ascertained as toxic at high concentration to some aquatic organisms (Lambert et al., 2010). Therefore, the developmental abnormalities observed in the present study in seedlings grown with HDPE bag leachates could be mainly related to the interference of BPA released from bags with plant hormonal metabolism and signaling. However, also the other identified compounds with phytotoxicity effects, such as for example oleic acid (Jyothi et al., 2014), might have contributed. Instead, the abnormalities detected in seedlings treated with MB leachates could mainly be attributed to the presence of 1,6-dioxacyclododecane-7,12-dione. Its presence, as well as that of traces of butane 1,4-diol, suggests a certain extent of depolymerization of the polyester poly(butylene adipate) with the release of butane 1,4-diol and adipic acid. This latter monomer is known to be toxic to aquatic organisms, including algae (Kennedy, 2002). Clearly, further studies are needed to confirm our hypotheses about the effects of these compounds on higher plants.

As concerning early seedling growth, the radicle of seedlings treated with leachates was significantly shorter than that of control groups, regardless of bag type, exposure and pollution degree (Fig. 4; Table 4). At the highest pollution level, the radicle of seedlings treated with MB leachates was reduced compared to that of seedlings grown with HDPE leachates, regardless of exposure condition (Fig. 4; Table 4). Also, the hypocotyl of seedlings treated with plastic leachates was shorter than that of controls, except that of those exposed to HDPE bags at the highest pollution degree. Leachates from exposed MB bags were more effective in reducing hypocotyl elongation than those from HDPE bags, even if only at the highest pollution degree (Fig. 4; Table 4). Here, seedlings treated with virgin MB bag leachates showed lower radicle to hypocotyl ratio than those grown with HDPE at the high pollution degree, while the reverse occurred in seedlings treated with leachates from exposed materials (Fig. 4; Table 4). This could be due to differential effects of the two type of bags on biomass allocation to belowground and aboveground organs (Poorter, 2011). With HDPE leachates, significantly lower radicle to hypocotyl ratios were detected with exposed materials compared to virgin ones, suggesting that the chemicals migrated from bags to the environment before the leaching experiment could have greater growth inhibitory effect on the hypocotyl than on the radicle (Fig. 4; Table 4). Instead, with MB leachates higher radicle to hypocotyl ratios were observed with exposed materials compared to virgin ones, thus the compounds released in the environment could have larger adverse effects on radicle growth (Fig. 4; Table 4).

Previous studies have shown that the growth of *L. sativum* seedlings is not influenced by variations of substrate pH between 4.4 and 8.8, while the presence of relatively high salt concentrations (about NaCl 50 mM) can inhibit the growth of the radicle and the plumule (El-Darier and Youssef, 2000; Bonanomi et al., 2006). In our study, the pH of leachates was lower than 4.4 only for MB virgin bags at the highest pollution level, and the highest values of salinity measured in leachates (about 2 mM) was lower than that reported to affect *L. sativum*. Therefore, the inhibitory effects on radicle growth observed with MB bag leachates could be explained by both water acidification and presence of released compounds. Instead, the suppression of hypocotyl growth observed with HDPE leachates could be mainly related to the presence of BPA. Overall, the results of this study show that leachates of traditional and compostable bags are toxic to the test species *L. sativum,* and provide new experimental evidence that leaching can occur in natural habitats causing contamination of soils and water available to plants.

4. Conclusions

The substitution of conventional plastic bags with biodegradable ones is a widely accepted strategy to reduce the environmental impact of plastic litter. Results of the present study demonstrate, however, that both types of bags can release processing compounds when incorrectly discarded in natural terrestrial environments due to precipitations, and hence can contaminate soils and waters. The *Lepidium sativum* seed germination test reveals that bag leachates can adversely affect seedling growth, and they could be thus potentially toxic to other higher plants. These findings are of particular ecological and managerial relevance. They indicate that international standards currently used to certify the compostability of bags, although more stringent than those developed for testing their biodegradability, cannot exclude the occurrence of adverse environmental effects of bags when abandoned on natural habitats. This is because in composting facilities phytotoxic water-soluble compounds, intentionally or non-intentionally added to plastics, can be gradually eliminated from litter as the degradation proceeds. Instead, in natural environments, these chemicals can quickly migrate out of plastic and be absorbed by roots affecting plant development. Thus, people and managers need to be adequately informed about the potential environmental impact of an incorrect disposal of bags.

The presence of additives used to manufacture plastic items, such as for example BPA, in natural environments is of great concern, due to their notorious adverse effects on human health and on marine and terrestrial organisms (Vandenberg et al., 2007; Talsness et al., 2009; Bejgarn et al., 2015). Many efforts have recently been made to develop eco-friendly, biodegradable substances (including bio-based compounds) as alternative to traditional additives. Our results suggest that in screening new plastic bag additives, special attention should be also paid in the future to their eventual transformation during the manufacturing process in products toxic to animals and plants. In this context, simple, rapid standard phytotoxicity tests performed on bag leachates, based on higher plants such as the *Lepidium sativum* seed germination and radicle elongation assay, could be useful tools.

Acknowledgements

We sincerely thank Flavia Vallerini for her support in the laboratory experiment. This work is part of the PhD research project of Virginia Menicagli funded by the University of Pisa (PRA and FA).

References

Accinelli, C., Saccà, M.L., Mencarelli, M., Vicari, A., 2012. Deterioration of bioplastic carrier bags in the environment and assessment of a new recycling alternative. Chemosphere. 89, 136-143. https://doi.org/10.1016/j.chemosphere.2012.05.028.

Alam, O., Billah, M., Yajie, D., 2018. Characteristics of plastic bags and their potential environmental hazards. Resour Conserv Recycl. 132, 121-129. https://doi.org/10.1016/j.resconrec.2018.01.037

AlMaadeed, M.A., Labidi, S., Krupa, I., Ouederni, M., 2015. Effect of waste wax and chain structure on the mechanical and physical properties of polyethylene. Arab J Chem. 8, 388-399. http://dx.doi.org/10.1016/j.arabjc.2014.01.006

Alshawafi, A., Analla, M., Alwashali, E., Aksissou, M., 2017. Assessment of marine debris on the coastal wetland of Martil in the North-East of Morocco. Mar Pollut Bull. 117, 302-310. https://doi.org/10.1016/j.marpolbul.2017.01.079

Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. PERMANOVA+ for PRIMER: Guide to software and statistical methods. PRIMER-E: Plymouth, UK.

Andrady A.L., 2015. Persistence of plastic litter in the oceans. In Bergmann M., Gutow L., Klages M. (Eds.), Marine Anthropogenic Litter, Berlin: Springer.

Avio, C.G., Gorbi, S., Regoli, F., 2017. Plastics and microplastics in the oceans: From emerging pollutants to emerged threat. Mar Environ Res. 128, 2-11. https://doi.org/10.1016/j.marenvres.2016.05.012

Balestri¸ E., Menicagli, V., Vallerini, F., Lardicci C., 2017. Biodegradable plastic bags on the seafloor: A future threat for seagrass meadows? Sci Total Environ. 605-606, 755-763. https://doi.org/10.1016/j.scitotenv.2017.06.249

Barnes, D.K.A, Galgani, F., Thompson, R.C, Barlaz, M., 2009. Accumulation and fragmentation of plastic debris in global environments. Philos Trans R Soc London B Biol Sci. 364, 1985-1998. https://doi.org/10.1098/rstb.2008.0205

Beare, M.H., Coleman, D.C., Crossley, D.A., Hendrix, P.F., Odum, E.P., 1995. A hierarchical approach to evaluating the significance of soil biodiversity to biogeochemical cycling. Plant Soil. 170, 5-22. https://doi.org/10.1007/BF02183051

Besseling, E., Wang, B., Lürling, M., Koelmans, A.A., 2014. Nanoplastic affects growth of *S. obliquus* and reproduction of *D. magna*. Environ Sci Technol. 48, 12336-12243. https://doi.org/10.1021/es503001d

Bejgarn, S., MacLeod, M., Bogdal, C., Breitholtz, M., 2015. Toxicity of leachate from weathering plastics: An exploratory screening study with *Nitocra spinipes*. Chemosphere. 132, 114-119. https://doi.org/10.1016/j.chemosphere.2015.03.010

Bonanomi, G., Sicurezza, M.G., Caporaso, S., Esposito, A., Mazzoleni, S., 2006. Phytotoxicity dynamics of decaying plant materials. New Phytol. 169, 571-578. https://doi.org/10.1111/j.1469-8137.2005.01611.x

Canellas, E., Vera, P., Nerin, C., 2015. UPLC-ESI-Q-TOF-MS(E) and GC-MS identification and quantification of non-intentionally added substances coming from biodegradable food packaging. Anal Bioanal Chem. 407, 6781-6790. https://doi.org/10.1007/s00216-015-8848-2

Carson, H.S, Colbert, S.L., Kaylor, M., McDermid, K.J., 2011. Small plastic debris changes water movement and heat transfer through beach sediments. Mar Pollut Bull. 62, 1708-1713. https://doi.org/10.1016/j.marpolbul.2011.05.032

Ceccarini, A., Corti, A., Erba, F., Modugno, F., La Nasa, J., Bianchi, S., Castelvetro, V, 2018. The hidden microplastics: new insights and figures from the thorough separation and characterization of microplastics and of their degradation by-products in coastal sediments. Environ Sci Technol. 52, 5634-5643. https://doi.org/10.1021/acs.est.8b01487

Chandler, J.W., 2008. Cotyledon organogenesis. J Exp Bot. 59, 2917-2931. https://doi.org/10.1093/jxb/ern167

Clarke, K.R., Gorley, R.N., 2006. *PRIMER* v6: User manual/tutorial. PRIMER-E: Plymouth.

De Barro, J., 2008. Understanding and managing the causes of abnormal seedlings in Lucerne. Rural Industries Research and Development Corporation.

Domene, X., Ramirez, W., Solà, L., Alcañiz, J.M., Andrés, P., 2009. Soil pollution by nonylphenol and nonylphenol ethoxylates and their effects to plants and invertebrates. J Soils Sediments. 9, 555-567. https://doi.org/10.1007/s11368-009-0117-6

El-Darier, S.M., Youssef, R.S., 2000. Effect of soil type, salinity, and allelochemicals on germination and seedling growth of a medicinal plant *Lepidium sativum* L.. Ann Appl Biol. 156, 273-279. https://doi.org/10.1111/j.1744-7348.2000.tb00035.x

European Bioplastic, 2017. Bioplastic market data 2017.

Ferrara, G., Loffredo, E., Senesi, N., 2006. Phytotoxic, clastogenic and bioaccumulation effects of the environmental endocrine disruptor bisphenol A in various crops grown hydroponically. Planta. 223, 910-916. https://doi.org/10.1007/s00425-005-0147-2

Galloway, T. S., Cole, M., Lewis, C., 2017. Interactions of microplastic debris throughout the marine ecosystem. Nat Ecol Evol. http://dx.doi.org/10.1038/s41559-017-0116

Gong, P., Wilke, B.-M., Fleischmann, S., 1999. Soil-based phytotoxicity of 2,4,6-trinitrotoluene (TNT) to terrestrial higher plants. Arch. Environ. Contam. Toxicol. 36, 152–157. https://doi.org/10.1007/s002449900455

Green, D.S., Boots, B., Blockley, D.J., Rocha, C., Thompson, R.C., 2015. Impacts of discarded plastic bags on marine assemblages and ecosystem functioning. Environ Sci Technol. 49, 5380-5389. https://doi.org/10.1021/acs.est.5b00277.

Groh, K.J., Backhaus, T., Carney-Almroth, B., Geueke, B., Inostroza, P.A., Lennquist, A., Leslie, H.A., Maffini, M., Slunge, D., Trasande, L., Warhust, A.M., Muncke, J., 2018. Overview of known plastic packaging-associated chemicals and their hazards. Sci Total Environ. 651, 3253-3268. https://doi.org/10.1016/j.scitotenv.2018.10.015

Gupta, A., Singh, M., Jones, A.M., Laxmi, A., 2012. Hypocotyl directional growth in Arabidopsis: A complex trait. Plant Physiol. 159, 1463-1476. https://doi.org/10.1104/pp.112.195776

Hahladakis, J.N., Velis, C.A., Weber, R., Iacovidou, E., Purnell, P., 2018. An overview of chemical additives present in plastics: Migration, release, fate and environmental impact during their use, disposal and recycling. J Hazard Mater. 334, 179-199. https://doi.org/10.1016/j.jhazmat.2017.10.014

Hardesty, B.D., Holdsworth, D., Revilland, A.T., Wilcox C., 2015. A biochemical approach for identifying plastics exposure in live wildlife. Methods Ecol Evol 6, 92–98. https://doi.org/10.1111/2041-210X.12277

Harrison, J.P., Boardman, C., O' Callaghan, K., Delort, A.-M., Song, J., 2017. Biodegradability standards for carrier bags and plastic films in aquatic environments: a critical review. R Soc open sci. 5, 171792. http://dx.doi.org/10.1098/rsos.171792

Hinsinger, P., 2001. Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. Plant Soil. 237, 173-195. https://doi.org/10.1023/A:1013351617532

ISO 11269-1: 2012a. Soil quality — Determination of the effects of pollutants on soil flora — Part 1: Method for the measurement of inhibition of root growth. International Organization for Standardization.

ISO 11269-2: 2012b. Soil quality — Determination of the effects of pollutants on soil flora — Part 2: Effects of contaminated soil on the emergence and early growth of higher plants. International Organization for Standardization.

ISTA 2003. International rules for seed testing. Edition 2003, International seed testing association, Zurich, Switzerland.

Jyothi, G., Reddy, K.R.K., Reddy, K.R.N., 2014. Oleic acid: a mycoherbicide from *Cochliobolus* lunatus for management of *Echinochloa crusgalli* in paddy. JEBAS. 2, 405-409.

Kennedy, G.L. Jr., 2002. Toxicity of adipic acid. Drug Chem Toxicol. 25, 191-202.

Kuzyakov, Y., Blagodatskaya, E., 2015. Microbial hotspots and hot moments in soil: Concept & review. Soil Biol Biochem. 83, 184-199. http://dx.doi.org/10.1016/j.soilbio.2015.01.025

Lambert, N., Rostock, C., Hetland, S., 2010. Environmental risks associated to plasticizers and sweeteners in the Nordic countries. Bergfald miljørådgivere, kongens gate 3 0153 Oslo.

Li, W., Khan, M.A., Yamaguchi, S., Kamiya, Y., 2005. Effects of heavy metals on seed germination and early seedling growth of *Arabidopsis thaliana.* Plant Growth Regul. 46, 46-50. https://doi.org/10.1007/s10725-005-6324-2

Li, H.X., Getzinger, G.J., Lee Ferguson, P., Orihuela, B., Zhu, M., Rittschof, D., 2016. Effects of toxic leachate from commercial plastics on larval survival and settlement of the barnacle *Amphibalanus amphitrite*. Environ Sci Technol. 50, 924-931. 10.1021/acs.est.5b02781.

Li, W.C., Tse, H.F., Fok, L., 2016. Plastic waste in the marine environment: A review of sources, occurrence and effects. Sci Total Environ. 566–567, 333-349. https://doi.org/10.1016/j.scitotenv.2016.05.084

Li, X., Wang, L., Wang, S., Yang, Q., Zhou, Q., Huang, X., 2018. A preliminary analysis of the effects of bisphenol A on the plant root growth via changes in endogenous plant hormones. Ecotoxicol Environ Saf. 150, 152-158. https://doi.org/10.1016/j.ecoenv.2017.12.031

Lithner, D., Nordensvan, I., Dave, G., 2012. Comparative acute toxicity of leachates from plastic products made of polypropylene, polyethylene, PVC, acrylonitrile–butadiene–styrene, and epoxy to *Daphnia magna.* Environ Sci Pollut Res. 19, 1763-1772. https://doi.org/10.1007/s11356-011-0663-5

Luo, Y., Liang, J., Zeng, G., Chen, M., Mo, D., Li, G., Zhang, D., 2018. Seed germination test for toxicity evaluation of compost: Its roles, problems and prospects. Waste Manag. 71, 109-114. https://doi.org/10.1016/j.wasman.2017.09.023

Ma, T., Christie, P., Teng, Y., Luo, Y., 2013. Rape (*Brassica chinensis* L.) seed germination, seedling growth, and physiology in soil polluted with di-*n*-butyl phthalate and bis(2-ethylhexyl) phthalate. Environ Sci Pollut Res. 20, 5289-5298. https://doi.org/10.1007/s11356-013-1520-5

Macoustra, G.K., King, C.K., Wasley, J., Robinson, S.A., Jolley, D.F., 2015. Impact of hydrocarbons from a diesel fuel on the germination and early growth of subantarctic plants. Environ Sci Process Impacts, 17, 1238-1248. http://dx.doi.org/10.1039/C4EM00680A

Mantese Sander, M., Nicolau, A., Guzatto, R., Samios, D., 2012. Plasticiser effect of oleic acid polyester on polyethylene and polypropylene. Polym Test. 31, 1077-1082. http://dx.doi.org/10.1016/j.polymertesting.2012.08.006

Martin-Closas, L., Botet, R., Pelacho, A.M., 2014. An in vitro crop plant ecotoxicity test for agricultural bioplastic constituents. Polym Degrad Stab. 108, 250-256. http://dx.doi.org/10.1016/j.polymdegradstab.2014.03.037

Milberg, P., Lamont, B.B., 1997. Seed/cotyledon size and nutrient content play a major role in early performance of species on nutrient- poor soils. New Phytol. 137, 665-672. https://doi.org/10.1046/j.1469-8137.1997.00870.x

Mitchell, R.L., Burchett, M.D., Pulkownik, A., McCluskey, L., 1988. Effects of environmentally hazardous chemicals on the emergence and early growth of selected Australian plants. Plant Soil. 112, 195-199. https://doi.org/10.1007/BF02139995

Moore, S.L., Gregorio, D., Carreon, M., Weisberg, S.B., Leecaster, M.K., 2001. Composition and distribution of beach debris in orange county, California. Mar Pollut Bull. 42, 241-245. https://doi.org/10.1016/S0025-326X(00)00148-X

Müller, C., Townsend, K., Matschullat, J., 2012. Experimental degradation of polymer shopping bags (standard and degradable plastic, and biodegradable) in the gastrointestinal fluids of sea turtles. Sci Total Environ. 416, 464–467. https://doi.org/10.1016/j.scitotenv.2011.10.069

Munari, C., Corbau, C., Simeoni, U., Mistri, M., 2015. Marine litter on Mediterranean shores: Analysis of composition, spatial distribution and sources in north-western Adriatic beaches. Waste Manag. 49, 483-490. https://doi.org/10.1016/j.wasman.2015.12.010

OECD 2006. *Test No. 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264070066-en

Oehlmann, J., Schulte-Oehlmann, U., Kloas, W., Jagnytsch, O., Lutz, I., Kusk, K.O., Wollenberger, L., Santos, E.M., Paull, G.C., van Look, K.J.W., Tyler, C.R., 2009. A critical analysis of the biological impacts of plasticizers on wildlife. Philos Trans R Soc Lond B Biol Sci. 364, 2047-2062. 10.1098/rstb.2008.0242

Pan, W.J., Xiong, C., Wu, Q.P., Liu, J.X., Liao, H.M., Chen, W., Liu, Y.S., Zheng, L., 2013. Effect of BPA on the germination, root development, seedling growth and leaf differentiation under different light conditions in *Arabidopsis thaliana*. Chemosphere. 93, 2585-2592. https://doi.org/10.1016/j.chemosphere.2013.09.081

Pasternak, G., Zviely, D., Ribic, C.A., Ariel, A., Spanier, E., 2017. Sources, composition and spatial distribution of marine debris along the Mediterranean coast of Israel. Mar Pollut Bull. 114, 1036-1045. https://doi.org/10.1016/j.marpolbul.2016.11.023

Poorter, H., Niklas, K.J., Reich, P.B., Oleksyn, J., Poot, P., Mommer, L., 2011. Biomass allocation to leaves, stema and roots: meta-analyses of interspecific variation and environmental control. New Phytol. 193, 30-50. https://doi.org/10.1111/j.1469-8137.2011.03952.x

Rahim, A., Haryadi, Cahyanto, M.N., Pranoto, Y., 2012. Characteristics of butyrylated arenga starch prepared at different reaction time and butyric anhydride concentration. Int Food Res J. 19, 1655-1660.

Rani, M., Shim, W.J., Han, G.M., Jang, M., Al-Odaini, N.A., Song, Y.K., Hong, S.H., 2015. Qualitative analysis of additives in plastic marine debris and its new products. Arc Environ Contam Toxicol. 69, 352-366. https://doi.org/10.1007/s00244-015-0224-x

Rychter, P., Kawalec, M., Sobota, M., Kurcok, P., Kowalczuk, M., 2010. Study of aliphatic-aromatic copolyester degradation in sandy soil and its ecotoxicological impact. Biomacromolecules. 11, 839-847. https://doi.org/10.1021/bm901331t

Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., Eliceiri, K.W., 2017. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics 18:529, https://doi.org/10.1186/s12859-017-1934-z

Schmuck, A.M., Lavers, J.L., Stuckenbrock, S., Sharp, P.B., Bond, A.L., 2017. Geophysical features influence the accumulation of beach debris on Caribbean islands. Mar Pollut Bull. 121, 45-51. https://doi.org/10.1016/j.marpolbul.2017.05.043

Sforzini, S., Oliveri, L., Chinaglia, S., Viarengo, A., 2016. Application of biotest for the determination of soil ecotoxicity after exposure to biodegradable plastics. Front Env Sci. 4: 68. https://doi.org/10.3389/fenvs.2016.00068

Shah, A.A., Hasan, F., Hameed, A., Ahmed, S., 2008. Biological degradation of plastics: A comprehensive review. Biotechnol Adv. 26, 246-265. https://doi.org/10.1016/j.biotechadv.2007.12.005

Sharma, S., Chatterjee, S., 2017. Microplastic pollution, a threat to marine ecosystem and human health: a short review. Environ Sci Pollut Res. 24, 21530-21547. https://doi.org/10.1007/s11356-017-9910-8

Shrivastava, P., Kumar, R., 2015. Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. Saudi J Biol Sci. 22, 123-131. http://dx.doi.org/10.1016/j.sjbs.2014.12.001

Song, J.H., Murphy, R.J., Narayan, R., Davies, G.B.H., 2009. Biodegradable and compostable alternatives to conventional plastics. Philos Trans R Soc Lond B Biol Sci. 364, 2127-2139. https://doi.org/10.1098/rstb.2008.0289

Staples, C., Friederich, U., Hall, T., Klečka, G., Mihaich, E., Ortego, L., Caspers, N., Hentges, S., 2010. Estimating potential risks to terrestrial invertebrates and plants exposed to bisphenol A in soil amended with activated sludge biosolids. Environ Toxicol Chem. 29, 467-475. https://doi.org/10.1002/etc.49

Talsness, C.E., Andrade, A.J.M., Kuriyama, S.N., Taylor, J.A., vom Saal, F. S., 2009. Components of plastic: experimental studies in animals and relevance for human health. Phylos Trans R Soc B. 364, 2079-2096. https://doi.org/10.1098/rstb.2008.0281

Vandenberg, L.N., Hauser, R., Marcus, M., Olea, N., Welshons, W.V., 2007. Human exposure to bisphenol A (BPA). Reproductive Toxicol. 24, 139-177. https://doi.org/10.1016/j.reprotox.2007.07.010

UNEP, 2015. Biodegradable plastics and marine litter: misconceptions, concerns and impacts on marine environments. United Nations Environment Programme, Nairobi, 2015.

UNEP, 2018. Single-use plastics: A Roadmap for Sustainability. United Nations Environment Programme, Nairobi, 2018.

UNICHIM 1651: 2003. Qualità dell’acqua - Determinazione dell’inibizione della germinazione e allungamento radicale in *Cucumis sativus* L. (Cetriolo), *Lepidium sativum* L. (Crescione), *Sorghum saccharatum* Moench (Sorgo) – (saggio di tossicità cronica breve). UNICHIM.

Watanabe, M., Nakata, C., Wu, W., Kawamoto, K., Noma, Y., 2007. Characterization of semi-volatile organic compounds emitted during heating of nitrogen-containing plastics at low temperature. Chemosphere. 68, 2063-2072. https://doi.org/10.1016/j.chemosphere.2007.02.022

Weizbauer, R., Peters, W.S., Schulz, B., 2011. Geometric constraints and the anatomical interpretation of twisted plant organ phenotypes. Front Plant Sci. 2, 62. https://doi.org/10.3389/fpls.2011.00062

Wu, Z., Zhang, X., Wu, X., Shen, G., Du, Q., Mo, C., 2013. Uptake of di(2-ethylhexyl) phthalate (DEHP) by plant *Benincasa hispida* and its use for lowering DEHP content of intercropped vegetables. J Agric Food Chem. 61, 5220-5225. https://dx.doi.org/10.1021/jf401378u

Zhang, C., Feng, Y., Liu, Y., Chang, H., Li, Z., Xue, J., 2017. Uptake and translocation of organic pollutants in plants: A review. J Integr Agric. 16, 1659-1668. https://doi.org/10.1016/S2095-3119(16)61590-3