# Reply to "Letter to Editor regarding the article "Evaluation of the phytotoxicity of conventional and biodegradable plastic bags using seed germination tests" by Balestri et al. (2019) published on Ecological Indicators 102(2019):569–580"

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We thank Dr. Degli-Innocenti and colleagues for reading our article published on Ecological Indicators, whose correct title is “Phytotoxicity assessment of conventional and biodegradable plastic bags using seed germination test” (Balestri et al., 2019), and for taking the time to express concerns regarding the paper in a letter to the Editor (Degli-Innocenti et al., 2019). Most of these concerns could be derived from misunderstandings of the rationale of the study. We also thank the authors and the Editor for giving us the opportunity to elucidate some points that probably were not enough clear in the manuscript.

Firstly, we would like to clarify that the study was designed to evaluate the possible impact of both non-biodegradable (conventional) bags and compostable plastic bags incorrectly deposited on coastal environments through rainwater leaching i.e., migration of water-soluble organic compounds from bags into water, on early life plant stages. In species with non-dormant seeds, the uptake of available pore water by seed is rapid (hours) and activates the process of germination under favorable conditions (Oracz et al., 2012; Wolny et al., 2018). Since in beaches and sand dunes rainwater rapidly penetrates through sand due to high substrate porosity and can be readily imbibed by seeds (Maun, 2009), our study specifically referred to the possible effects of the water percolating from bags soon after precipitation on seed germination. Therefore, the kinetics of leaching, the biodegradation rate of leached substances into soils, and their potential effects on plants were outside the purpose of the study. Secondly, a “globally” established standard procedure to determine the potential toxicity of littered plastic items such as bags on the germination of seeds of species inhabiting coastal habitats is still not available. So, seeds of a terrestrial plant (*Lepidium sativum* L.) commonly used in phytotoxicity tests were tested, and some procedures prescribed in currently available standard ecotoxicological tests were adjusted to the study purpose.

Below there are our responses to the specific concerns expressed by Degli-Innocenti and colleagues in their letter to the Editor.

**(1) First reason of concern "communication of the results is sometimes inaccurate and misleading"**

*The abstract by mistake refers that "both conventional and biodegradable bags released into water intentionally added chemicals, such as the noxious bisphenol A, and other phytotoxic substances probably generated during bag manufacturing". This is not true for biodegradable bags, as then reported in Section 3 (Results and discussion), but the sentence is very misleading for those readers who only read abstracts.*

We partially agree with Degli-Innocenti and colleagues. Indeed, the study has shown that both non-biodegradable and compostable plastic bags have released into water intentionally added chemicals and other substances with phytotoxic activity. However, bisphenol A was released only from non-biodegradable bags, as reported in section 3. In the abstract, the results of the leaching experiments performed on the two bag types were combined in a sentence in order to not exceed the word limit for the abstract indicated by the Journal. However, we recognize that this could be misleading for those readers who only read the abstract, and hence we apologize for the inconvenience. We hope the Editor of the journal could help us correct this in our online article in Ecological Indicators.

**(2) Second reason of concern "the tested dose was arbitrary but it is described as if it were a realistic pollution degree"**

*The authors mention studies on quantification of litter deposition in the marine environment (Munari et al., 2015; Alshawafi et al., 2017; Pasternak et al., 2017; Schmuck et al., 2017). However, it is obvious that such deposits were formed in many years of accumulation and not in just one simultaneous discharge. Therefore, the concentration of soluble substances in the area surrounding the deposits cannot be simply deduced by the number of littered items found per $m^2$, because diffusion from litter occurred in different times in the past with different kinetics. Therefore, the number of plastic bags used in the extraction procedure does not mimic any realistic*

*pollution occurring in natural environments. Naming the extracts as "low, medium and high pollution degree" is misleading because there is no link with any specific real or potential pollution event.*

In fact, in our study the name attributed to the bag extracts (low, medium and high pollution) was clearly not referred to the concentrations of water-soluble substances released from plastic bags in natural environments, but rather to different degrees of "bag pollution" previously detected in nature, as explained in the section 2.1 of the paper. These bag pollution degrees were estimated from published data on the abundance of plastic items collected on beaches periodically subjected to clean-up operations (Munari et al., 2016; Alshawafi et al., 2017; Pasternak et al., 2017). This means that bag deposits on beaches were not formed in many years of accumulation, as stated by Degli-Innocenti and colleagues, but presumably only after few, single deposition events. Naming the extracts as "low, medium and high pollution degree" is not misleading since it is linked with realistic "bag" pollution events.

**(3) Third reason of concern "no information on rate and level of migration from bags to soil and on the subsequent fate in soil, but statements on the potential impact of bags because of leakage are present all over in the article."**

*The unsystematic nature of this pre-treatment makes it impossible to consider it as a simulation of the fate a bag can undergo in case of littering." …"In particular, the diffusion rate and the total amount of the water-soluble substances migrated from the bags into the soil, and the fate of the substances in soil after migration (including persistence) were not determined. The lack of all this highly relevant information means no conclusions on "soil pollution" can be drawn."*

Our statements on the potential effect of bags in the article were based on the results of leaching laboratory tests which demonstrated the capacity of bags to readily release water-soluble compounds into water, a phenomenon that can occur in nature during precipitations. For the reasons mentioned in the premise, assessing the diffusion rate, the amounts and the persistence of the

substances migrated from bags into soils was outside the scope of our study even if it is a further interesting issue. We also point out that the weathering bag treatment, i.e. the exposure of bags in sand dune, was not a "*simulation"*, but one of the possible fates that a bag could really undergo once entered natural habitats. Indeed, many plastic items entering natural coastal environments, including bags, can be transported by winds, or directly deposited on sand dunes where they are exposed to natural weathering (Poeta et al., 2014; Andrady, 2015; Šilc et al., 2018). Artificial weathering experiments are unlikely to reproduce the complexity of the interactions occurring among littered plastic items and natural abiotic/biotic factors in such habitats. In our study, the natural weathering treatment was performed to discriminate between the possible effects of bag content and those of natural abiotic/biotic factors on rainwater quality, as we claimed in the Material and methods section. This information is highly relevant from an ecological point of view. Indeed, observing a phytotoxic effect of the leachate from bags abandoned on dunes, one cannot establish if this effect is due to the release of substances used in bag manufacturing or the interplay of the bag and abiotic factors, for example the deposition of sea salts that can influence germination and seedling growth (Maun, 2009). In addition, the main aim of this treatment was not to determine the amount and fate of compounds released by bags into sand during dune exposure, rather to establish if weathered bags might still retain the capacity to affect rainwater quality, and thus seeds.

**(4) Fourth reason of concern "the germination test was carried out on filter paper and not in soil as prescribed by standard global procedures"**

*In subsection 2.2 the authors describe the Lepidium sativum germination test. L. sativum was used as indicator of bag leachate phytotoxicity, in agreement with specific standard ecotoxicity tests (UNICHIM, 2003; OECD, 2006; ISO, 2012a,b). However, the authors did apply only the UNICHIM procedure. Seeds were tested on filter paper soaked with the extracts rather than in soil, as prescribed by the globally established test methods. This is a relevant deviance from the International standards.*

As mentioned above in our premise, “globally” established standard methods designed to test the potential phytotoxicity of leachates from plastic items on the germination of seeds of coastal plant species have not been established, to our knowledge. Current international standard tests such as OECD (2006) and ISO (2012 a,b) prescribe the use of soils as plant growing substrates, but they are mainly designed to assess the toxic effects of soil-incorporated solid or liquid chemical substances on seedling emergence and growth. However, some countries have also established own procedures. We chose to use the Italian standard test, UNICHIM (2003), and to adjust it for the study purpose. This test can be applied not only to solid but also to liquid matrices such as rainwater and leachates, and it allows to assess their effects on earlier plant life history stages, i.e., seed germination and root elongation (UNICHIM, 2003; Baudo et al., 2013). The UNICHIM procedure involves the use of cellulose filter paper as germination medium, and this substrate has also been used in previous ecological studies and toxicological tests (Hoekstra et al., 2002; Pavel et al., 2013; Manãs and De las Heras, 2018; Bosker et al., 2019). We obviously agree with Degli-Innocenti and colleagues that seeds germinate in soil, and not on filter paper under natural conditions. However, the use of this inert germination substrate allows to readily observe the seed germination process and to overcome some problems related to the interpretation of the results of tests with natural soils, due to the variability of physical/chemical soil properties and microbial populations (OECD, 2006). *The potential effects of this deviance should have been investigated or at least discussed because under natural conditions seeds germinate in soil and not on filter paper. The exposure of biodegradable water-soluble substances to soil can lead to a fast biodegradation and thus to a substantial reduction of their permanence time in the environment”.*

We point out that the study was not a test on the biodegradation effect of bags in soils on seeds, rather it was aimed at assessing the potential effects of the water that might percolate from bags during rainfalls on seeds. As mentioned in our premise, this water is readily available in sandy soils and the uptake of soil pore-water by non-dormant seeds is rapid and activates the process of

157 germination in few hours (Oracz et al., 2012; Wolny et al., 2018). Organic compounds dissolved in
158 soil pore-water can be also easily absorbed by plant roots (Zhang et al., 2017). Therefore, if the
159 release of water-soluble biodegradable substances from bags into rainwater occurred in periods
160 favorable to seed germination (for example in spring and autumn), these substances would affect
161 seeds by contaminating rainwater (at least transitorily), much prior to be biodegraded in sandy soil.
162
163 **(5) Fifth reason of concern "the analysis of abnormalities is not a validated ecotoxicity test**
164 **and needs proper controls"**
165 *The problem is that the detection of abnormalities in the development of L. Sativum seedlings is not*
166 *an ecotoxicity test which has previously been systematically applied to chemicals. It is a new*
167 *approach. Therefore, the conclusions drawn by the authors are largely flawed because they not are*
168 *obtained through a validated test.*
169 Degli-Innocenti and colleagues focused their concerns on the use of developmental
170 abnormalities in *L. sativum* seedlings as endpoint in the phytotoxicity test, but they neglected other
171 responses to bag leachate observed in the study, such as hypocotyl and root growth inhibition,
172 which are endpoints widely applied in standard ecotoxicity tests (OECD, 2006; UNICHIM, 2003;
173 ISO 2012a,b). We also point out that the detection of seedling abnormalities in ecotoxicity tests is
174 not a new approach. Indeed, *L. sativum* is among those species reported in Annex 2 (List of Species
175 historically used in plant testing) of the OECD (2006), and the section of this document concerning
176 the visual assessment of phytotoxicity, reports "At the end of the test, measurement of percent
177 emergence and biomass of surviving plants should be recorded, as well as visible detrimental
178 effects on different parts of the plant. The latter include abnormalities in appearance of the emerged
179 seedlings, stunted growth, chlorosis, discoloration, mortality, and effects on plant development".
180 Although the detection of seedling abnormalities in *L. sativum* has not routinely been applied to
181 assess the ecotoxicity of chemicals, this semi-quantitative/qualitative endpoint provides important
182 additional information on sample's toxicity. An irregular germinated seed has not the ability to

develop in a normal plant even when growing in soil under favorable conditions (ISTA, 2003). In addition, a recent study, probably escaped the attention of Degli-Innocenti and colleagues, provided evidence of the suitability of abnormalities as an endpoint in phytotoxicity tests (Manãs and De las Heras, 2018).

*The test is affected by severe methodological flaws. No reference substances are applied. Preliminary studies should have been carried out testing substances already characterized for ecotoxicity/phytotoxicity to determine whether the development of abnormalities in L. sativum were consistent with the expected ecotoxicity and to determine the sensitivity and the reproducibility of the test method.*

However, the use of a positive reference substance may be required periodically in some procedures (OECD, 2006), and it serves to verify the sensitivity of the method (ISO 5667-16, 2017). Alternatively, historical growth measurement of controls could be used to evaluate the performance of the test system, and can serve as an intra-laboratory quality control measure (OECD, 2006). In our study, the sensitivity of the method was proven by the observed adverse effects of seeds to leachates obtained not only from compostable bags but also from conventional bags made of high-density polyethylene. The abnormalities observed in our study were consistent with those found in *L. sativum* seedlings following the exposure to a reference toxicant (zinc sulphate heptahydrate) reported in a previous study (Manãs and De las Heras, 2017). The validity and reproducibility of our test were ensured by high seed germination ( > 90%) obtained with distilled water as a negative reference substance (or control), a procedure adopted in previous ecological and ecotoxicological studies (Hoekstra et al., 2002; OECD, 2006; Josko et al., 2017; Manãs and De las Heras, 2018; Bosker et al., 2019).

*Finally it should have been clarified if natural GRAS substances could elicit the response if tested at very high doses."* Again, *"For example, the application of immature compost onto soil causes negative effects on seed germination, plant growth and development (Morel et al., 1985). The*

*possibility that biodegradable substances present in the extracts in high dose could elicit the response must be verified. Under these conditions, the results should be considered as preliminary.*

We agree with Degli-Innocenti and colleagues that high amounts of natural GRAS substances as well as immature compost could negatively affect seed germination and plant/root development. However, "*that biodegradable substances present in the extracts in high dose could elicit the response"* could be verified, but it is irrelevant from an ecological point of view. Indeed, coastal habitats are not cultivated fields nor composting environments, and hence no GRAS substances in high amounts should not enter these environments, especially in oligotrophic zones. Therefore, even if biodegradable substances were present in high dose in bag extracts and elicited the responses observed in *L. sativum* seedlings, this would not alleviate the impact that bags could cause on seeds when dispersed in natural environments. In addition, similar negative effects on seeds were found with leachates from conventional non-biodegradable bags.

Our study provided a first experimental evidence of the potential impact of the introduction of both compostable and conventional non-biodegradable bags in coastal habitats through leaching by rainwater. This information is a key requirement to assess the ecological risk derived from plastic items if dispersed in coastal environments, and the preliminary nature of the results of our study was explicitly claimed in the discussion section "*Clearly, further studies are needed to confirm our hypotheses about the effects of these compounds on higher plants*.", and again in the conclusion section "*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*". However, we take this opportunity to inform that the consistency of the effects of both compostable and non-biodegradable bags on coastal plants has also been proven by results of a recent field study (Menicagli et al., 2019) that demonstrated the relatively long-term persistence (more than 12

233 months) of small (10x10 cm) bag macro-fragments once buried on sand dunes, as well as their
234 adverse effects on seed emergence and growth.
235 We disagree with Degli-Innocenti and colleagues that our statement "*current standards on*
236 *compostability cannot exclude the occurrence of adverse environmental effects of bags when*
237 *abandoned on natural habitats" is pointless.* Obviously, current standards are not designed to
238 assess the behavior of littered bags in natural environments. But this issue was posed because the
239 meaning of the certification "compostability" (i.e. biodegradable in industrial composting facilities)
240 is not probably clear to all scientists (Harrison et al., 2018). In addition, there is confusion among
241 terms like bioplastics, bio-based and biodegradable and compostable plastics. The average
242 consumer and certain media often do not distinguish between the meaning of biodegradable and
243 compostable, so many people have assumed that biodegradable and compostable plastics can break
244 down completely in the environment without affecting organisms (Harrison et al., 2018). It is
245 sufficient to conduct a research on internet using the terms "plastic or compostable or
246 biodegradable" to ascertain it. On the other hand, in a Novamont press release (2 July 2019,
247 https://www.novamont.com/eng/leggi_press.php?id_press=53 ,
248 https://www.polimerica.it/articolo.asp?id=22100), Degli-Innocenti himself stated that "*All products*
249 *must be collected and recycled, including biodegradable products made of Mater-Bi, which must be*
250 *recovered in the form of compost together with kitchen waste. Nothing must be discarded*
251 *irresponsibly whether on the ground or in the sea, as this creates a potential ecological risk. The*
252 *intrinsic biodegradability of Mater-Bi products is a factor that can mitigate ecological risk. This,*
253 *however, must not become a commercial message but a further element to help assess the*
254 *environmental profile of biodegradable products*".
255 Finally, we would like to point out that our study was not a criticism to the environmental
256 benefits provided by biodegradable/compostable plastic items. We also appreciated the recent
257 efforts of Degli-Innocenti and colleagues to assess the biodegradability of Mater-Bi samples on
258 marine sediments and their impact on marine organisms

(https://www.novamont.com/eng/leggi_press.php?id_press=53) and we hope our studies could aid to develop more eco-sustainable items and new standard toxicity tests to further reduce "*the potential ecological risk*" of an introduction of bags on coastal environments. We can understand the controversial issues raised from the results of our research because of their potential commercial relevance. However, we are independent scientists without conflicts of interest, and we are not responsible for how the results of the study, which was published on an international peer-reviewed scientific Journal destined to a specialized audience (scientists, policy-makers, and resource managers investigating or applying ecological and environmental indicators), and of other our related published studies, could be misunderstood and/or manipulated by media to divulgate messages (i.e., either that compostable items minimize the problem of marine litter or that they are a false solution to the marine litter problem).

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