Land-Based Solutions for Plastics in the Sea

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D6.3 Ecotoxicological characterization for selected plastic samples

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2	UNIVERSIDADE DA CORUÑA	UDC	SPAIN	HES
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4	LABORATORIO IBERICO INTERNACIONAL DE NANOTECNOLOGIA	INL	PORTUGAL	RTO
5	KATHOLIEKE UNIVERSITEIT LEUVEN	KUL	BELGIUM	HES
6	HELMHOLTZ ZENTRUM FUR OZEANFORSCHUNG KIEL	GEOMAR	GERMANY	RTO
7	NATIONAL OCEANOGRAPHY CENTRE	NOC	UNITED KINGDOM	RTO
8	SORBONNE UNIVERSITE	SU	FRANCE	HES
9	OPEN UNIVERSITEIT NEDERLAND	OUNL	NETHERLANDS	HES
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11	ASSOCIACAO PARA O DESENVOLVIMENTO DO ATLANTIC INTERNATIONAL RESEARCH CENTRE	AC	PORTUGAL	RTO
13	BASF SE	BASF	GERMANY	LE
14	TG ENVIRONMENTAL RESEARCH	ER	UNITED KINGDOM	SME
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17	RADBOUD UNIVERSITEIT	RU	NETHERLANDS	HES
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Executive summary	This document corresponds to Deliverable 6.3. *Ecotoxicological characterization of 1st-year selected field plastic samples.* The toxicity of environmental plastics obtained from three different kinds of habitat (soil, riverbanks, coastline) assessed by testing serial dilutions of leachates using the freshwater and marine water test species described in LABPLAS Project Deliverables D6.1 and D6.2 ranged from very low to none, and some positive effects were even reported. Results do not support any relevant environmental risk of these materials at current loads of plastics found in the aquatic natural environment. In terrestrial tests, both particles and leachates did show relevant toxicity to earthworms, with the chronic reproduction test conducted with the plastic particles themselves being the most sensitive test of the battery. Future assessments should standardize leachate production at 1 g/L, and comparability of results could improve by standardizing serial dilutions at x2 geometric steps. Assessment is greatly limited by the availability of natural microplastics and/or obtaining SMNP from meso and macroplastics in the laboratory by micronization using mills. Therefore, throughput may improve by using microscale ecotoxicological methods.

Version	Date	Comments
1.0	27-Jul-22	First draft
2.0	25-Nov-22	Revised and corrected version
3.0	29-Nov-22	Final version

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ABBREVIATIONS AND ACRONYMS

Abbreviation / Acronym	Description
EC10	10% Effect Concentration
EC50	50% Effect Concentration
ELS	Early Life Stage
LABPLAS	LAnd Based solutions for PLAstics in the Sea Project
LOEC	Lowest Observed Effects Concentration
n.c.	not calculable
n.s.	not significant
NOEC	No Observable Effects Concentration
SET	Sea-urchin Embryo Test
SMNP	Small, micro- and nano-plastics
SOP	Standard Operating Procedure
STP	Sewage Treatment Plant
TU	Toxic Units
WP	Work Package

1 INTRODUCTION

Plastic is pouring from land into our oceans at a rate of nearly 10 million tonnes a year (Jambeck et al. 2015). Once in the sea, plastics fragment into particles moving with the currents and ocean gyres before washing up on the coastline or sinking on the seafloor (Barnes et al. 2009). The smaller the size the higher the risk posed by these particles to organisms and human health (Beiras and Schönemann 2020). Because small, micro- and nano-plastics (SMNP) cannot be removed from oceans, proactive action regarding research on plastic alternatives and strategies to prevent plastic from entering the environment should be taken promptly (Oberbeckmann and Labrenz 2020). The LABPLAS project is a 48-month project whose vision is to develop new techniques and models for the detection and quantification of SMNPs and to determine reliable methods for the assessment of the impact of plastics and associated chemicals in the environment. It will also develop practical computational tools that should facilitate the mapping of plastic-impacted hotspots and promote scientifically sound plastic governance.

During its first year, the LABPLAS Project conducted during months 7-12 field plastic sampling cruises in the Great North Sea (including the Thames and Elbe river basins), in the Baltic coast of Germany, and the Mero-Barcés river basin (NW Iberian Peninsula), including the Cecebre drinking water reservoir (Task 2.2 in WP2). Since the amount (mass) of SMNP present in these field samples was not sufficient to carry out the ecotoxicity tests originally planned in the project proposal, additional ad-hoc field sampling targeting large-micro, meso and macroplastics was conducted, and SMNP were obtained by grinding the collected plastic items down to SMNP size according to the SOP described in Deliverable 6.1 (see also Beiras et al. 2019). This process does not affect the chemical composition of the samples and mimics plastic fragmentation in the environment. Field sampling covered terrestrial, freshwater, and marine habitats. This report describes the ecotoxicological characterization of these samples using the aquatic and terrestrial ecotoxicological tests summarized in Fig. 1.

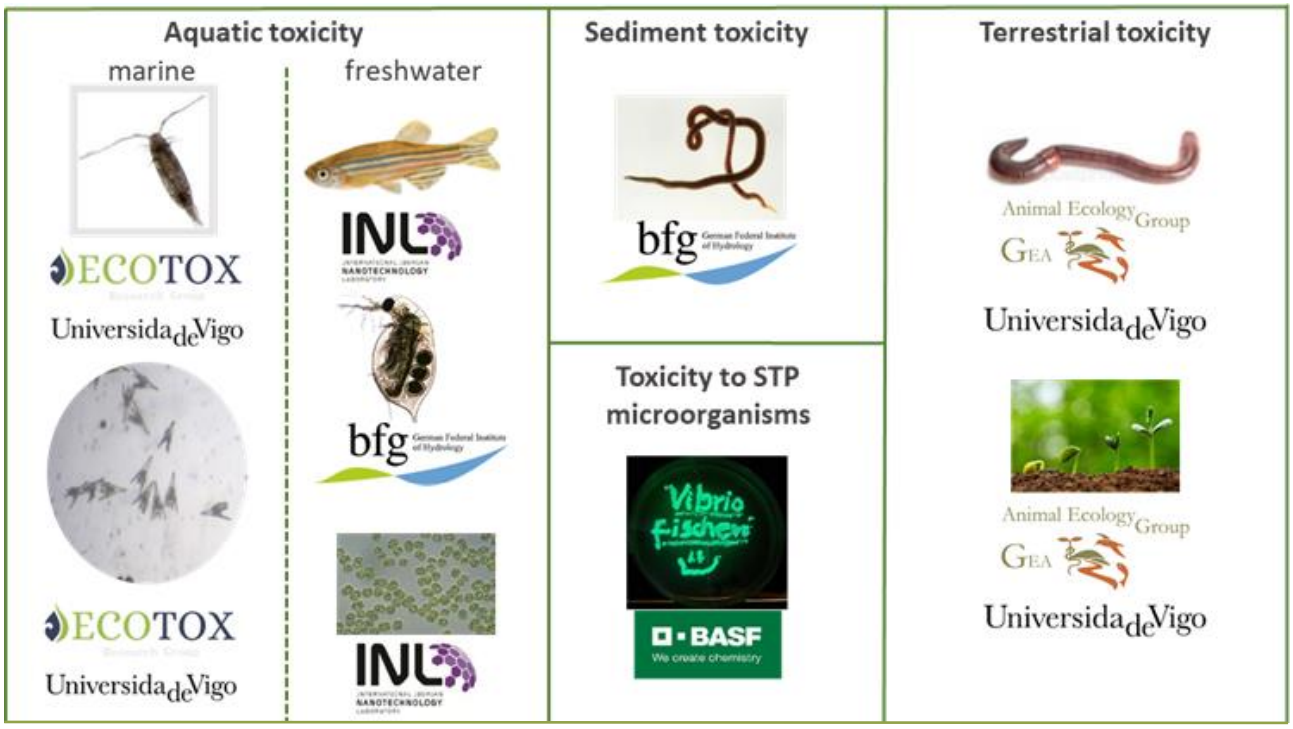


Figure 1. LABPLAS plastic toxicity test battery applied to test environmental field samples.

2 METHODOLOGY

Field plastic samples were obtained from three different environmental compartments: marine, freshwater, and terrestrial. All samples were sent to UVI, where they were cleaned with a brush under tap water, rinsed with abundant distilled water, dried at room temperature, and weighed. Photographs were taken (see below). Representative aliquots in mass of the different plastic typologies were manually cut to ca. 2 mm pieces with scissors or side-cutting pliers, mixed with dry ice (approximately 3 parts of CO_2: 1 part of plastic), and ground in an ultra-centrifugal mill (Retsch ZM 200) or a cryogenic ball mill (Retsch Cryomill) down to <250µm particles.

Micronized plastic stocks from each environmental compartment were then distributed to the different partners for compartment-related toxicity testing according to the test/species used as per Table 1: UVI-Ecotox and BASF for marine tests, INL and BfG for freshwater tests, and UVI-GEA for terrestrial tests. An additional subsample of the mixture obtained from each habitat was sent to UDC for chemical analyses within WP3.

A single pool of ground material representative of the plastic composition of each sampling site was tested with each bioassay, and bioassays were conducted with the species representative of each habitat, as per Table 1. As an advantage, this strategy warrants that the materials tested are relevant for the test species used, since samples were specifically collected from the habitats of those species. This pooled habitat-specific testing strategy also enhances the environmental relevance of the results and limits the number of tests that must be run, making feasible the use of a broad battery of species, which allows the potential detection of selective toxic effects. However, this strategy also has limitations such as reducing the potential to detect individual items particularly toxic and preventing associating potential toxicity with individual items. For this reason, in case overall toxicity is detected in one pool, additional testing with individual typologies from that pool suspected to be particularly toxic can be introduced in the future testing strategy for the second year's campaign. An attempt to anticipate this issue was already conducted in the case of the cigarette butts found in the marine samples since this typology was described to carry trace metals and other acutely toxic chemicals (Santos-Echeandía et al. 2021).

Habitat	Location	Test/sp	Partner
terrestrial	Abegondo (Mero-Barcés basin)	*Eisenia* survival rate	UVI-GEA
		Eisenia chronic reproduction	UVI-GEA
		Plant seedling emergence	UVI-GEA
aquatic – freshwater	Cecebre (Mero-Barcés basin)	Zebrafish ELS test	INL
		Daphnia chronic test	BfG
		Microcystis aeruginosa	INL
aquatic - marine	Kiel (German coast) and Ferrol (Iberian coast)	*Acartia*	UVI-Ecotox
		Paracentrotus lividus SET	UVI-Ecotox
		Vibrio fischeri	BASF

Table 1. Table of samples tested per test per partner

2.1 Terrestrial samples

On 19th July 2022, field plastics, as well as soil samples, were collected from agricultural fields in the Mero-Barcés rivers basin by UVI, including samples from fields with ecological and conventional agriculture techniques (Figure 2).

Figure 2. Field macro and meso plastics and soil samples collected in terrestrial habitats at the Mero-Barcés basin

Plastic items selected for terrestrial testing were those considered more representative of intentional and accidental input of plastics into the soil as a result of agriculture-related activities. With this aim, we followed the advice of Dr J. Castro Insua, a senior researcher from CIAM (Abegondo Agriculture Research Center, Xunta de Galicia). The selected items included mulch films from greenhouses and plastic used for hay and grass silage, as presented in Figure 3.

Figure 3. Macro plastics collected in terrestrial habitats from the Mero-Barcés basin and the CIAM greenhouses and silage facilities on 19th July 2022

A selection of the most representative plastic items from these samples (Figure 4.) was cleaned as described above and ground down to SMNP size according to the SOP described in Deliverable 6.1. This micronized plastic stock was then used for the terrestrial toxicity tests conducted by UVI-GEA as per Table 2.

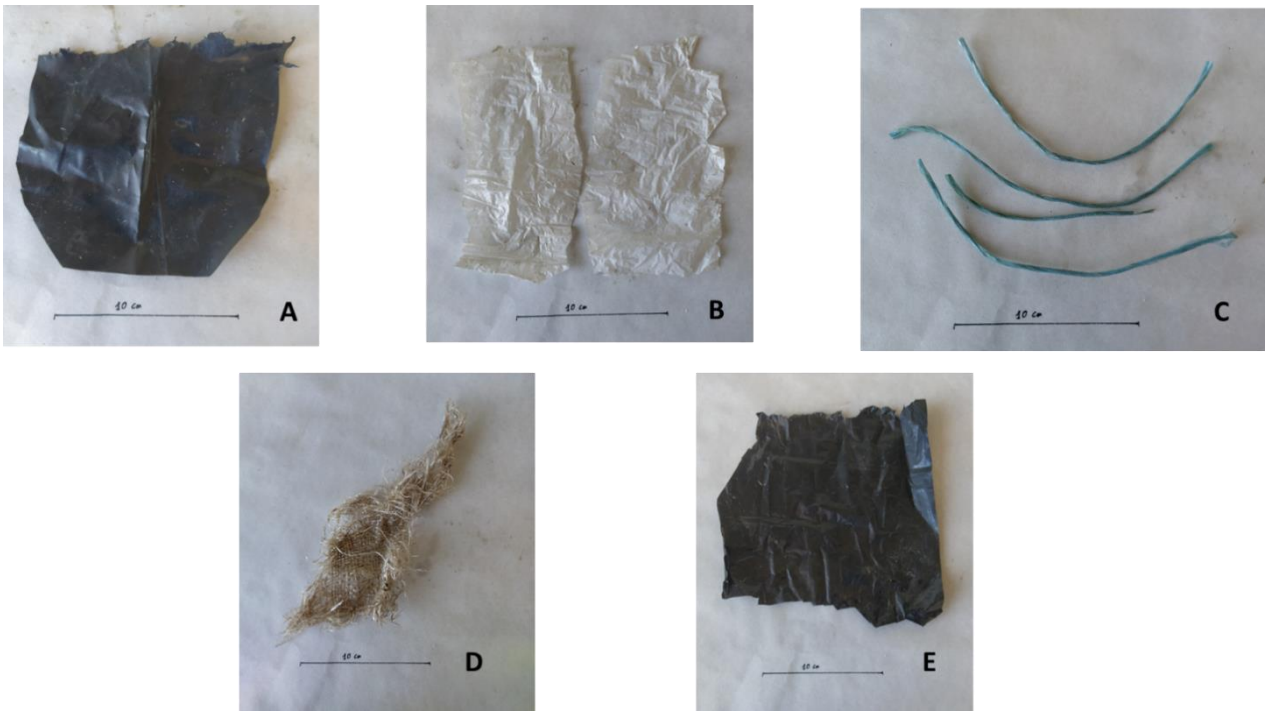


Figure 4. Selected plastic items collected from the Mero-Barcés basin and the CIAM greenhouses and silage facilities on 19th July 2022, were used to make up the terrestrial plastic sample (ID 110_LBP_Mix CIAM)

Item	Mass (g)	Per unit
A-Thick dark mulch	8.177	0.55
B-White mulch	4.544	0.31
C-Blue rope	0.897	0.06
D-White bag mesh	0.670	0.05
E-Thin dark mulch	0.497	0.03
TOTAL	14.875	1.00

Table 2. Composition of plastic stock used for terrestrial toxicity tests at UVI-GEA (ID 110_LBP_Mix CIAM)

2.2 Freshwater samples

Samples from freshwater habitats were collected on 9th June by UDC at the Spanish Mero-Barcés rivers basin (Figure 5, 43°16'38.1"N 8°17'44.5"W), pooled, and labelled as 095_LBP_Mix CEC (Figure 6).

Figure 5. Mero-Barcés rivers basin sampling points (marked as 1 and 2) on 9th June 2022

Figure 6. Large micro, meso and macro plastics collected from the Mero-Barcés river basins on 9th June 2022 (095_LBP_Mix CEC)

A selection of the most representative plastic items from these samples (Figure 7) was cleaned and ground down to SMNP size according to the SOP described in Deliverable 6.1. This micronized plastic stock was then distributed for freshwater toxicity tests to INL (i.e., zebrafish and *Microcystis*) and BfG (i.e., *Daphnia*) as per Table 3.

Figure 7. Selected items pooled to obtain sample 095_LBP_Mix CEC for freshwater toxicity tests

Item	Samples sent in June (to INL for zebrafish ELS test and BfG for Daphnia tests)		Samples sent in August (to INL for *Microcystis* tests)	
	Mass (g)	Per unit	Mass (g)	Per unit
A-Insulating tape	1.095	**0.09129**	0.282	**0.03802**
B-Plastic cup	1.198	**0.09966**	0.778	**0.10488**
C-Plastic bag	0.761	**0.06340**	0.583	**0.07859**
D-Candy wrapper	0.228	**0.01904**	0.300	**0.04044**
E-Fragments of flexible plastic	1.858	**0.15481**	1.576	**0.21246**
F-Bottle (veterinary use -mastitis)	6.287	**0.52395**	3.299	**0.44473**
G-Black plastic bag	0.572	**0.04768**	0.600	**0.08088**
TOTAL	**11.984**	**1.00000**	**7.418**	**1.00000**

Table 3. Composition of plastic stock used for freshwater toxicity tests

Additional samples of riverine origin were obtained from the Elbe river banks, downstream of the city of Hamburg (Figure 8). The largest (in mass) object collected was preliminarily tested by using the sea-urchin embryo test (SET) according to previously described methods. Results are shown in Section 3.3.1.

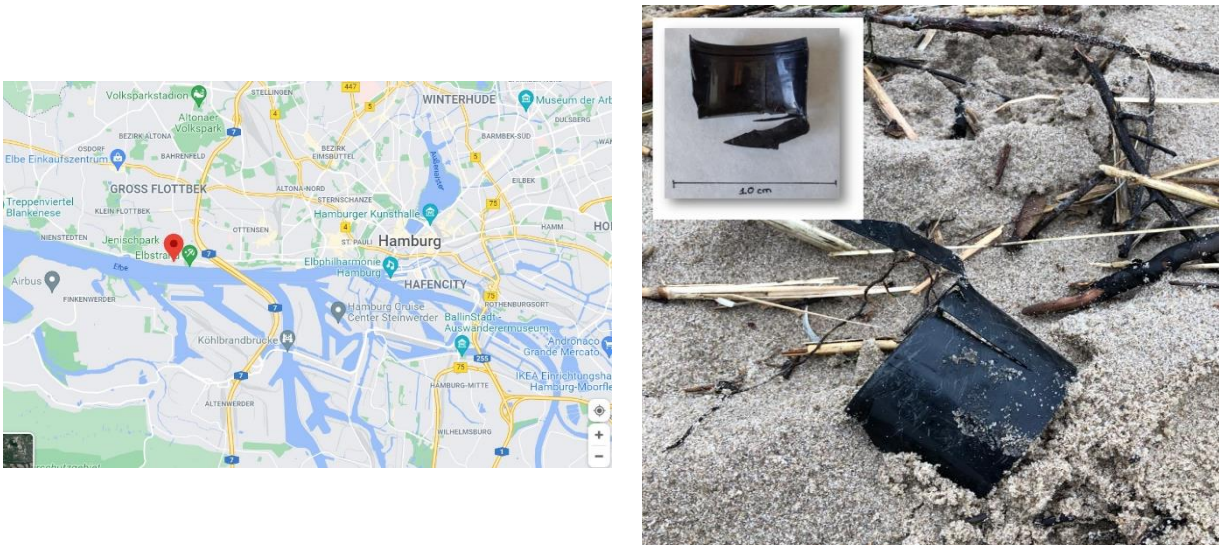


Figure 8. The location sampled on February 2022 (left) and item (ID084_LBP) tested for potential toxicity using SET.

2.3 Marine samples

Marine samples were taken by GEOMAR from field meso and macro plastics collected at Kalifornien Beach (Kiel, Germany). A selection of the most representative plastics from these samples (Figure 9, Table 3) was mixed and ground down to SMNP size according to the SOP described in Deliverable 6.1. This micronized plastic stock (093_LBP_Mix KIEL) was then distributed for marine toxicity tests to UVI – Ecotox and BASF. In the case of butts, only filters stripped from the paper cover were taken.

Figure 9. Selected items for marine toxicity tests collected on the coast of Kiel (Germany)

Item	Mass (g)	Per unit
A-Orange lid	26.67	0.652876
B-Coffee lid	2.09	0.051163
C-Polystyrene	6.76	0.165483
D-Black foam/rubber	4.73	0.115789
E-Butts filters	0.60	0.014688
TOTAL	**40.85**	**1.00000**

Table 4. Composition of plastic stock used for marine toxicity tests obtained from Kiel (Germany) (093_LBP_Mix KIEL)

Additional samples of marine origin were obtained from the Galician coast after the occurrence of stranded plastic pellets on the beaches of Ferrol (NW Iberian Peninsula) in January 2022 (Figure 10). Two main typologies could be identified: lenticular and spherical. The composition of each typology was identified at UVI by Fourier Transformed Infrared (FTIR) spectroscopy using a Nicolet 6700 ATR equipment and resulted to be polyethylene (lenticular, ID 080B_LBP_MixFerrol_L) and polypropylene (spherical, ID 080A_LBP_MixFerrol_P). The potential toxicity of each kind of pellet was assessed using the SET according to the standard methods previously described.

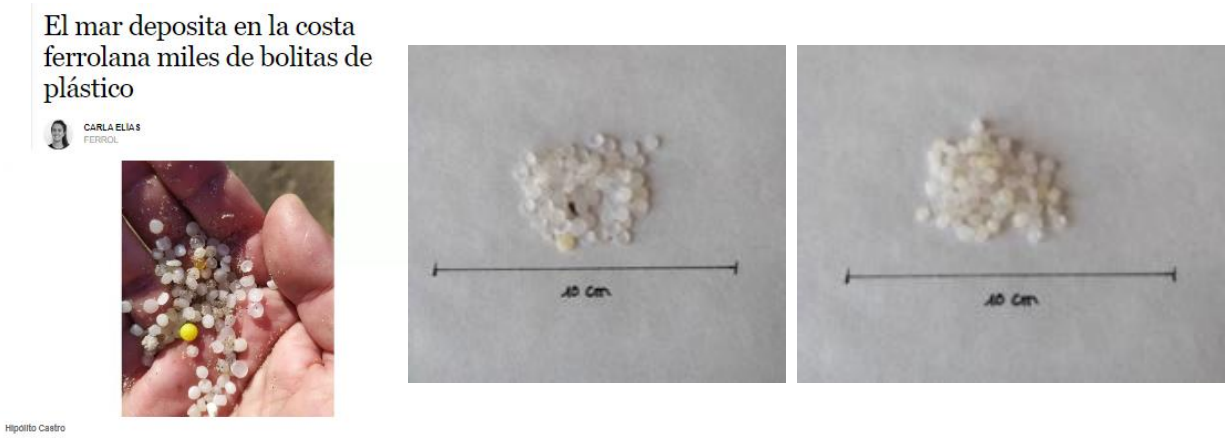


Figure 10. Plastic pellets found stranded in Ferrol beach: left PE (ID080B_LBP_MixFerrol_L), right PP (ID 080A_LBP_MixFerrol_P)

Samples from fine-grain marine sediment were obtained from a remote area with low anthropogenic pressure (Cabo Verde, Africa) and used as natural particle control. With this aim SOP described in Deliverable 6.1 were used. Briefly, sediments were sieved by 250 µm using metal sieves of certified mesh size, and leachates of 10 g/L were obtained.

3 TOXICITY TESTS RESULTS

3.1 Terrestrial toxicity tests results

3.1.1 *Eisenia andrei* survival and chronic reproduction test

The potential impact of plastic particles on soil fauna was assessed using survival and reproduction of *Eisenia andrei* as parameters, after 28 and 56 days of exposure in soil respectively (OECD guideline no. 222).

3.1.1.1 Exposure to plastic mix

The selected plastic items collected from the Mero-Barcés basin and CIAM facilities (ID 110_LBP_Mix CIAM), representative of terrestrial plastics of agriculture source (Fig. 4 and Table 2) were micronized down to <250 µm and used to obtain a 10 g/L leachate. A standard soil LUFA 2.2 was contaminated with both powder (P) and serial dilutions of the leachate.

Powder < 250µm	Solution diluted from a 10g/L leachate (dilution factor)
0	0
250	250 (x40)
750	750 (x13)
2250	2250 (x4.4)

Table 5. Range of concentrations of micronized plastic stock used in E. andrei survival and chronic reproduction tests (mg/Kg)

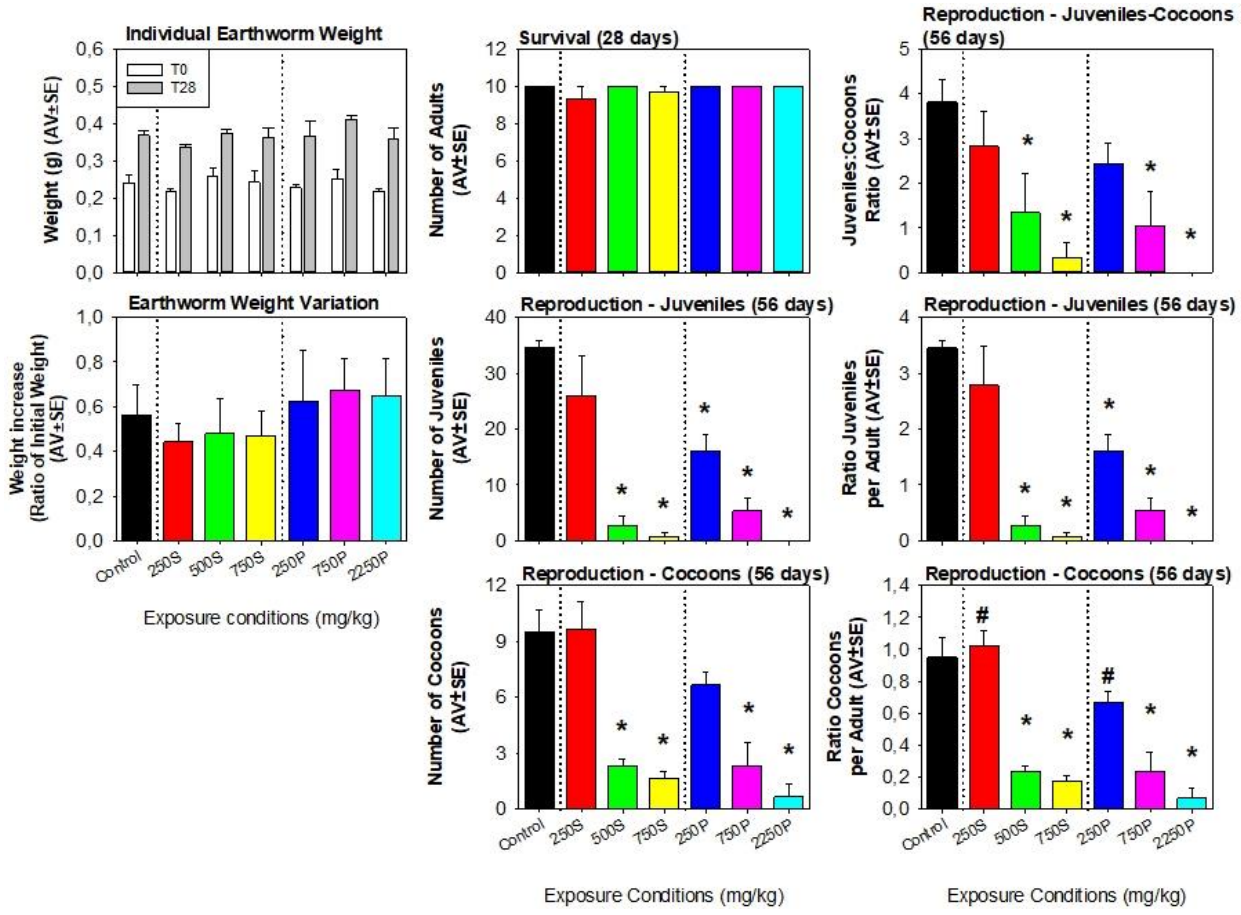


Figure 11. Eisenia andrei weight variation, survival after 28 days and reproduction after 56 days of exposure to plastic mix.
** - Significant difference to Control (non-contaminated LUFA 2.2 soil). # - Significant difference between solution and powder*

Exposure condition	Survival (Number of adults)		Reproduction (Number of juveniles per adult)		(Number of cocoons per adult)		(Number of juveniles per cocoon)	
	EC10	EC50	EC10	EC50	EC10	EC50	EC10	EC50
Powder	>2250	>2250	54 [19-159]	229 [164-321]	114 [38-338]	405 [257-637]	91 [18-451]	368 [200-677]
Solution	>750	>750	203 [146-281]	311 [247-390]	288 [143-581]	425 [323-559]	170 [62-466]	377 [247-576]

Table 6. Estimated 10% and 50% effect concentration (EC10 and EC50) (in mg/kg) for survival parameters after 28 days and reproduction parameters after 56 days

While no significant effect was observed in the *Eisenia andrei* survival parameter, considering the environmentally relevant concentrations used in the study. However, reproduction parameters, such as the number of juveniles per adult, number of cocoons per adult and number of juveniles per cocoon observed after 56 days of exposure, significantly decreased compared to the non-contaminated LUFA 2.2 soil. The lowest observed effect concentration (LOEC) for juveniles per adult was 500 mg/kg solution and 250 mg/kg powder. For both cocoons per adult and juveniles per cocoon significant differences were observed for 500 mg/kg solution and 750 mg/kg powder. For the EC50, the estimated values were similar for powder and solution for each parameter.

3.1.1.2 Exposure to soil with plastics

Soil samples from CIAM greenhouses that were in direct contact with plastics, as well as soil samples not in contact were retrieved from the field. As a reference, the standard LUFA 2.2 soil was used as a control.

No significant differences ($p<0.05$) in survival or reproduction as the number of juveniles were observed. Significant differences were observed for reproduction, as the number of cocoons per adult in contaminated soil increased compared to the control.

Soil ID	Soil Type
LUFA 2.2	LUFA 2.2 (control)
ID 111_LBP_soil CIAM clean	CIAM soil (non-contaminated soil)
ID 112_LBP_soil CIAM with MP	CIAM soil in direct contact with plastics

Table 7. Soil ID for Eisenia andrei exposure

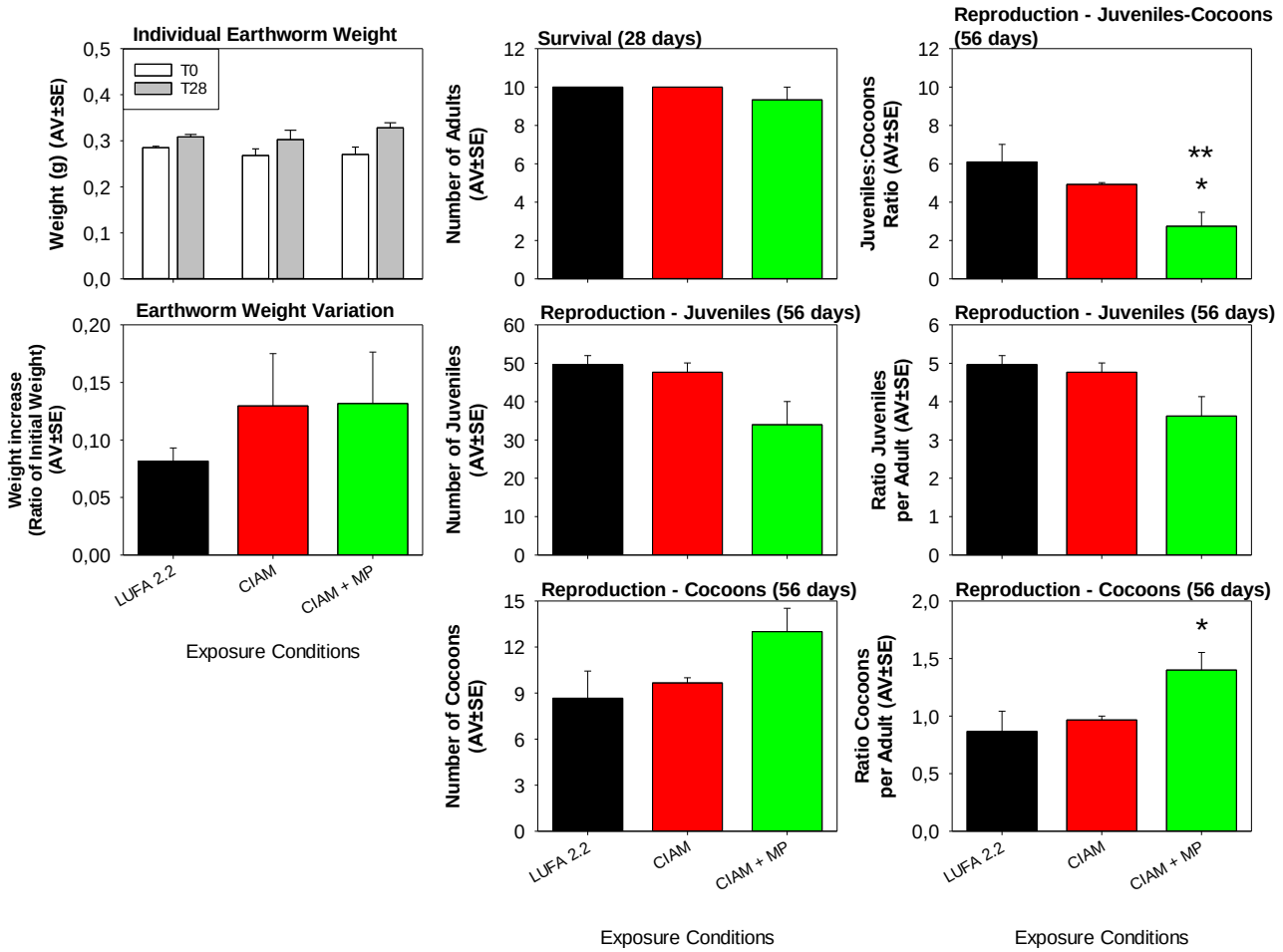


Figure 12. Eisenia andrei weight variation, survival after 28 days and reproduction after 56 days of exposure.
** - Significant difference to Control (LUFA 2.2), ** - Significant difference to respective non-contaminated soil*

3.1.2 *Lepidium sativum* germination test

3.1.2.1 Exposure to plastic mix

The selected plastic items collected from the Mero-Barcés basin and CIAM facilities (ID 110_LBP_Mix CIAM), representative of terrestrial plastics of agriculture source (Fig. 4 and Table 2) were micronized down to <250 µm and tested both directly as particles and as serial dilutions from a 10 g/L leachate, following the OECD no. 208 guideline.

Concentration used (mg/L)	
Powder < 250µm	**Solution diluted from a 10g/L leachate (dilution factor)**
0	0
250	250 (x40)
750	750 (x13)
2250	2250 (x4.4)

Table 8. Range of concentrations of micronized plastic stock used in L. sativum germination tests (mg/L)

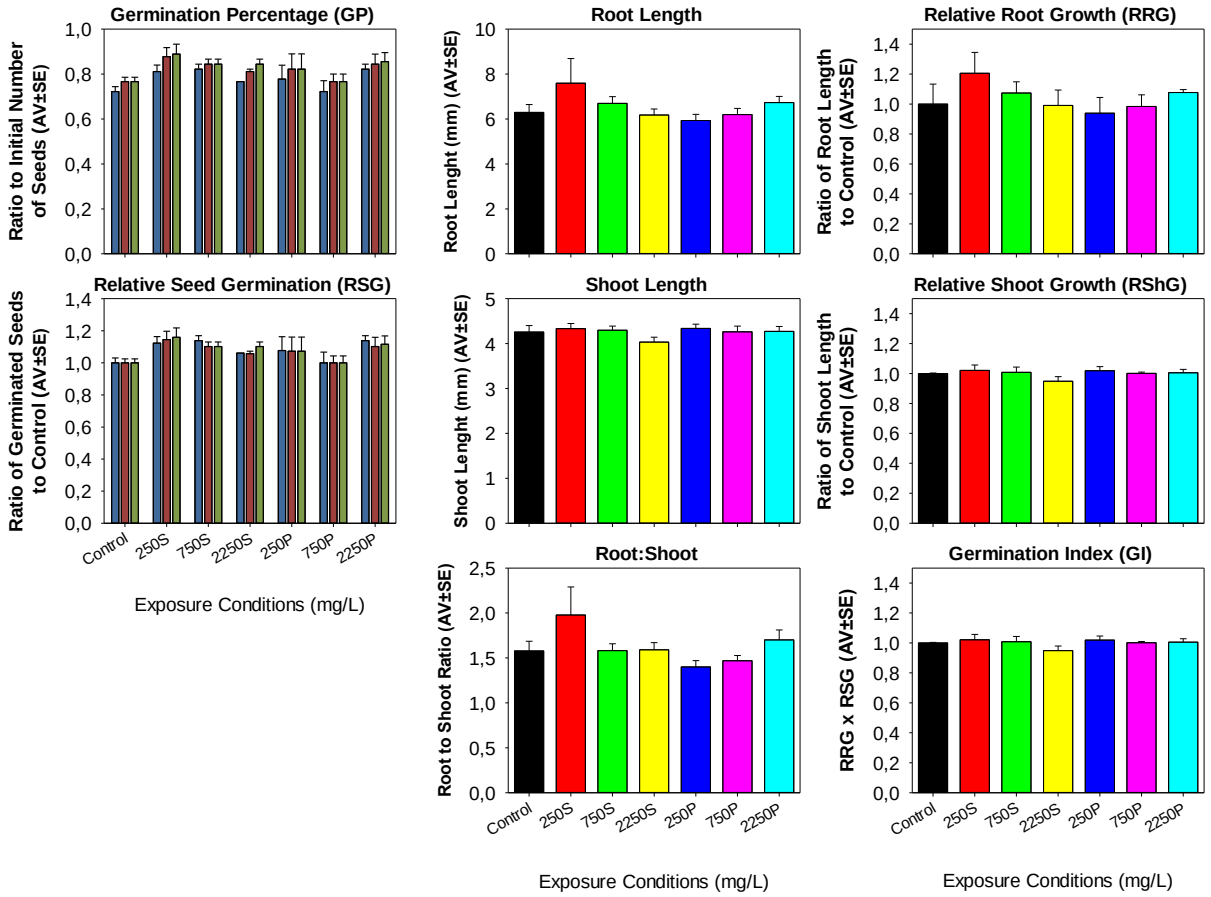


Figure 13. Lepidium sativum germination parameters after 7 days. S – Solution; P – Powder. No significant effects (p<0.05) were observed.

Exposure condition	Germination Percentage (GP)	Relative Seed Germination (RSG)	Relative Root Growth (RRG)	Germination Index (GI)
Powder	>2250	>2250	>2250	>2250
Solution	>2250	>2250	>2250	>2250

Table 9. NOEC estimation (in mg/L) for germination parameters after 7 days

No effects of any treatment on none of the endpoints studied were observed.

3.1.2.2 Exposure to soil extracts with plastics

From soil samples, both retrieved from the field (ID 111_LBP_soil CIAM and ID 112_LBP_soil + MP CIAM) and the control (LUFA 2.2), an extract was obtained, using a proportion of 1:10 (w/V) as a solution for the germination test. Apart from a significant increase in the shoot length after 7 days (p<0.05) in both field soil samples (contaminated and non-contaminated) compared to the control, no effects were observed.

Soil Type	Extract Proportion (soil : water)
LUFA 2.2 ID 111_LBP_soil CIAM clean ID 112_LBP_soil CIAM with MP	1:10

Table 10. Soil ID for extracts used for the L. sativum germination test

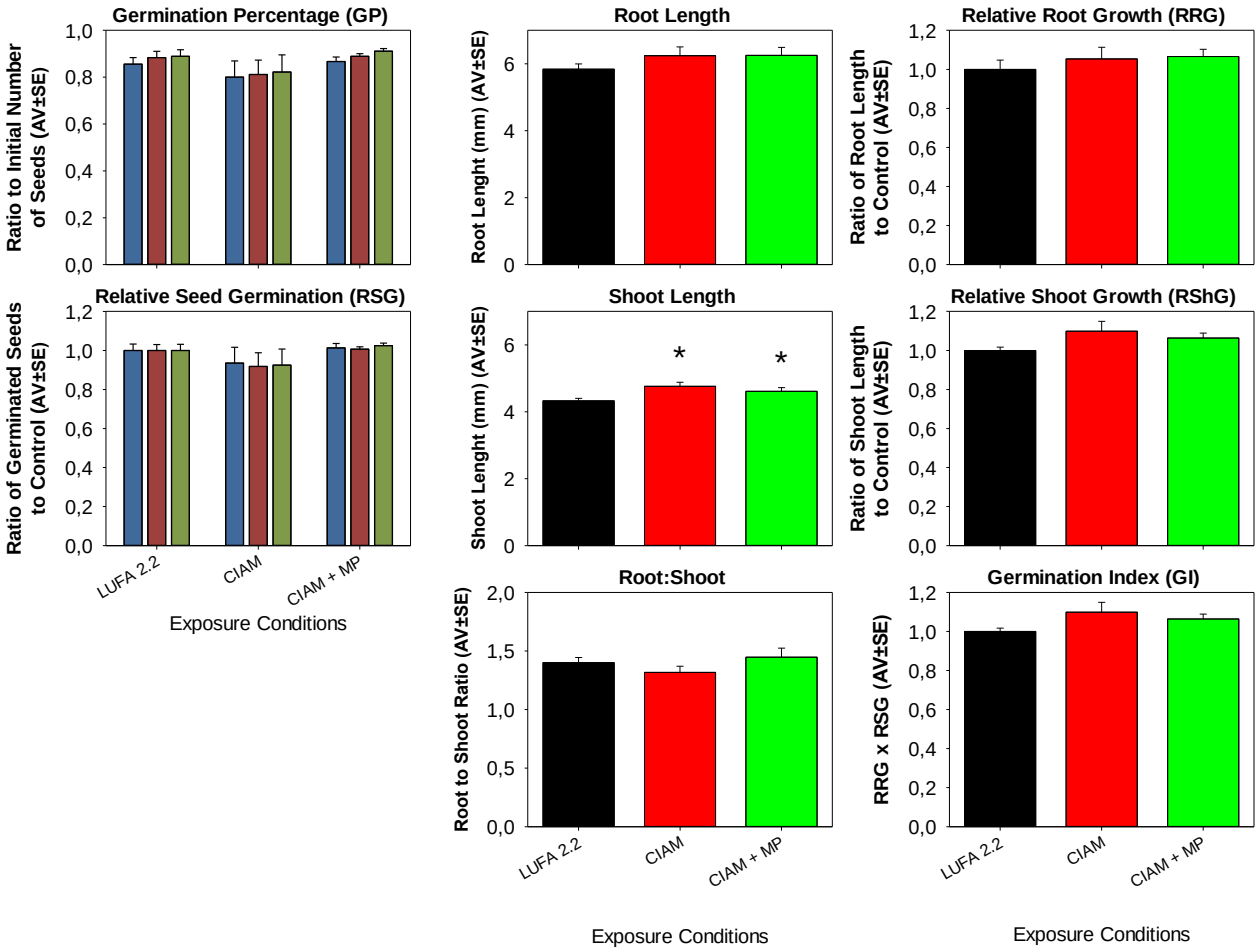


*Figure 14. Lepidium sativum germination parameters after 7 days. * - significant difference to control (p<0.05).*

3.2 Freshwater toxicity tests results

3.2.1 Aquatic freshwater toxicity tests results

3.2.1.1 *Daphnia*

The toxicity of the plastic leachates obtained at 1 g/L was investigated via a reproduction test with *Daphnia magna* (21 d, OECD 211). Therefore, juvenile daphnids (>24 h) were exposed to seven leachate concentrations at 20° C and a day/night cycle of 16:8 h in 75 ml glass beakers. We used seven replicates for each dilution (1:1, 1:2, 1:3, 1:4, 1:8, 1:16, 1:32) and 10 replicates for the control. According to the respective dilution series, the stock solution was placed in the beakers and filled up to a total volume of 65 ml with an M4 medium. The M4 medium was prepared once a week and stored at 20°C. The medium in the beakers was changed 2 times a week, and the pH value and conductivity were also checked. The feeding rations increased during the 21 days ranging from 0.13 to 0.23 mg/C/D of algae solution. Mortality and reproduction were checked daily for 21 days. Neonates were counted and removed from the beakers. At the start of the experiment 20 neonates of *Daphnia magna* were preserved in 70% ethanol as a size reference, after the end of the experiment the adult *Daphnia* were also placed in ethanol. The size was determined using a digital microscope.

The number of juveniles was analysed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, USA). All data were checked for normal distribution (Shapiro-Wilk test) and variance homogeneity (Bartlett's test). Normally distributed data were analysed with one-way ANOVA and Dunnett's post-hoc test. Significant

differences between the control and the treatments were indicated with stars (★ p < 0.05, ★★ p < 0.01, ★ ★ ★ p < 0.001).

All daphnids survived over 21 days. The reproductive output was slightly reduced in treatments containing leachates. Compared to the control (73.9 ± 6.17 (mean ± SD) neonates per female), we observed a significantly reduced reproduction in the dilutions 1/16 (63.7 ± 5.22, p < 0.01), 1/4 (62.7 ± 7.34, p < 0.01) and 1/3 (63.8 ± 2.79, p < 0.05). The growth of daphnids was not affected by leachates. Overall, the results do not indicate a dose-response relationship and may point to a minor effect level.

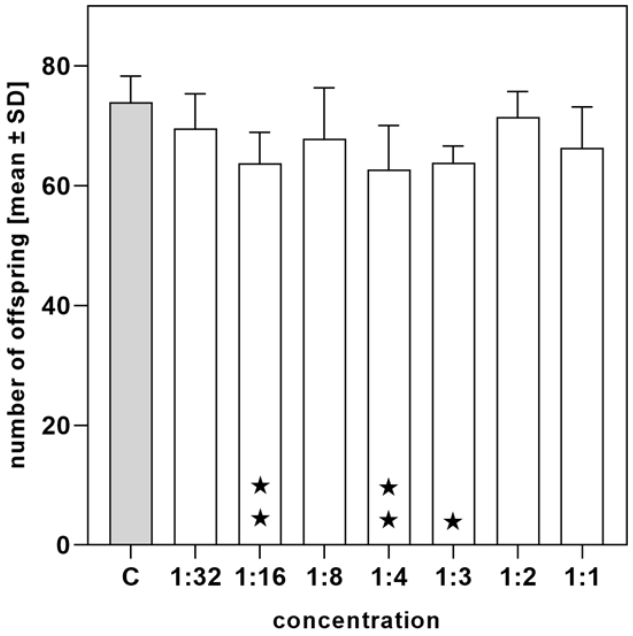


Figure 15. Effects of chronic exposure of Daphnia magna to microplastic-leachates obtained at 1 g/L. Asterisks indicate significant differences to control animals (★ p < 0.05, ★★ p < 0.01, ★★★ p < 0.001).

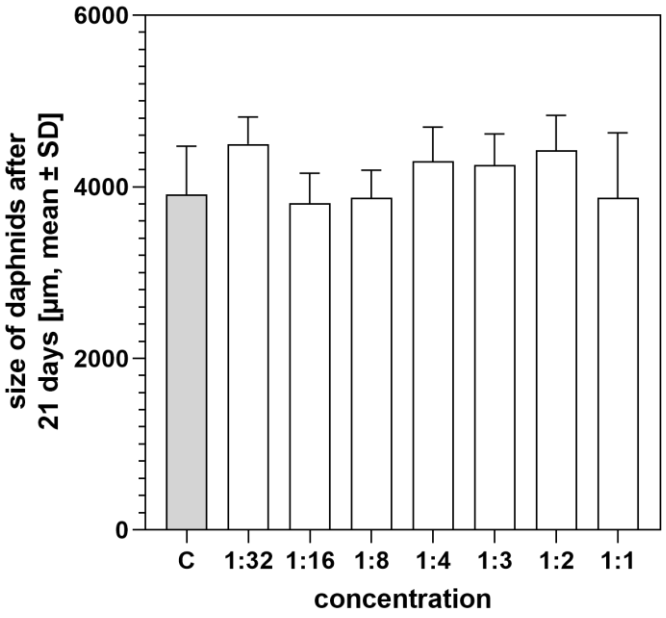


Figure 16. Effects of chronic exposure on the growth of Daphnia magna.

3.2.1.2 Zebrafish ELS

Leachates' preparations were carried out as established in D6.1 using a plastic load of 10 g/L. The tested dilutions were: 1 (or undiluted; represented as "UND" in the figures), 1/2, 1/5, 1/10 and 1/20. Two controls were always included: a negative control (eggs only exposed to their media, artificial freshwater (AFW); represented as "CTR" in the figures) and the true procedural control (eggs exposed to media that followed the same procedure as the leachates, but without plastics; represented as "F. CTR"). Two independent experiments were carried out following the guidelines established in D6.2 for semi-static zebrafish embryo toxicity. Given the fact that not many significant effects were observed, we showed the results of both independent experiments separately (N=1 and N=2).

No significant lethality was observed at any of the leachate's dilutions (figure 16), nor in any of the following sub-lethal toxicity endpoints; epiboly (figure 16), yolk volume (figure 17), eye surface (figure 18), head-trunk angle (figure 18), heart rate (figure 19), yolk extension (figure 19).

However, statistical differences were found between the control and the treatments for hatching, spontaneous movements and free-swimming. All of them are related to the development of neuro-muscular coordination, and all seem to be more advanced in the treatments than in the controls. No specific statistical significance was found with particular dilutions, so, no dose-response activity could be established.

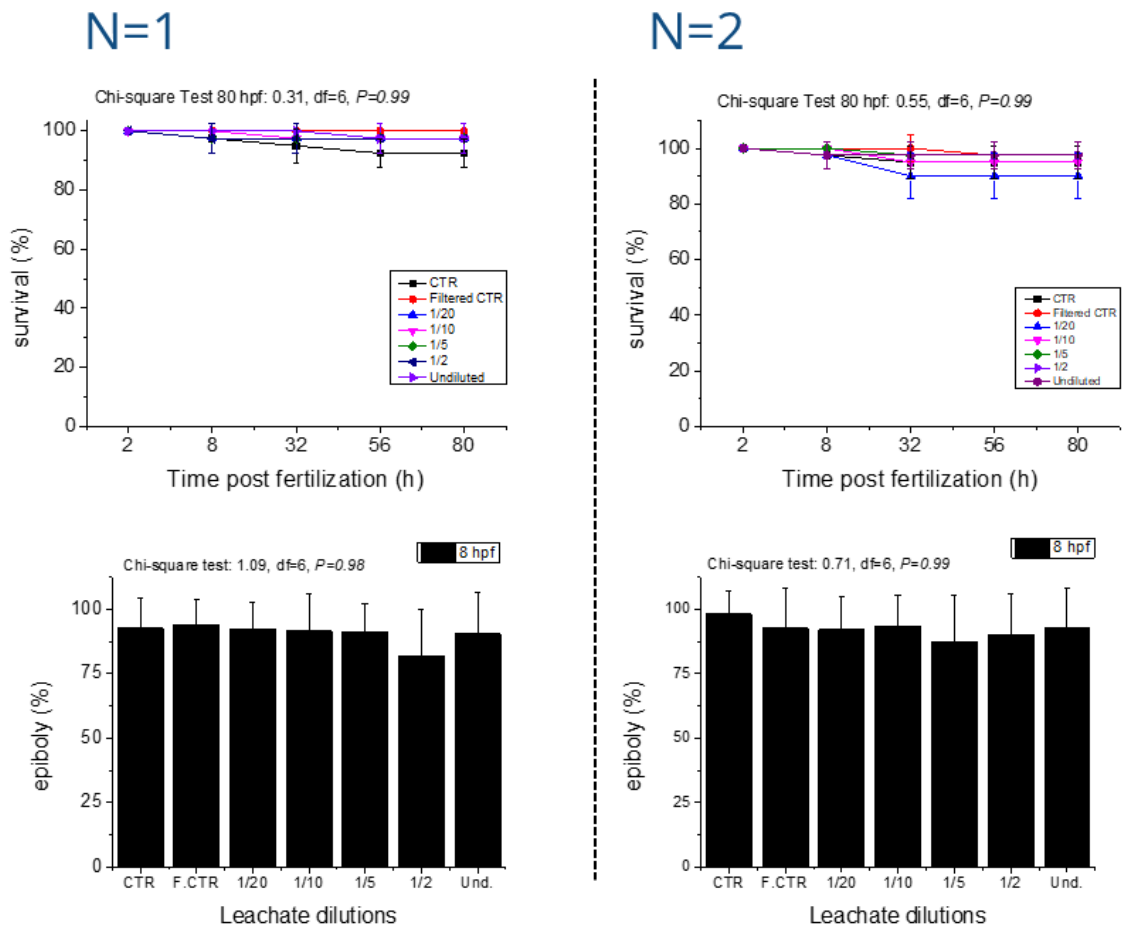


Figure 17. Results of zebrafish embryotoxicity tests for microplastic leachates, survival and epiboly. Results are expressed as mean ± standard deviation of 4 experimental replicates with 10 individuals each

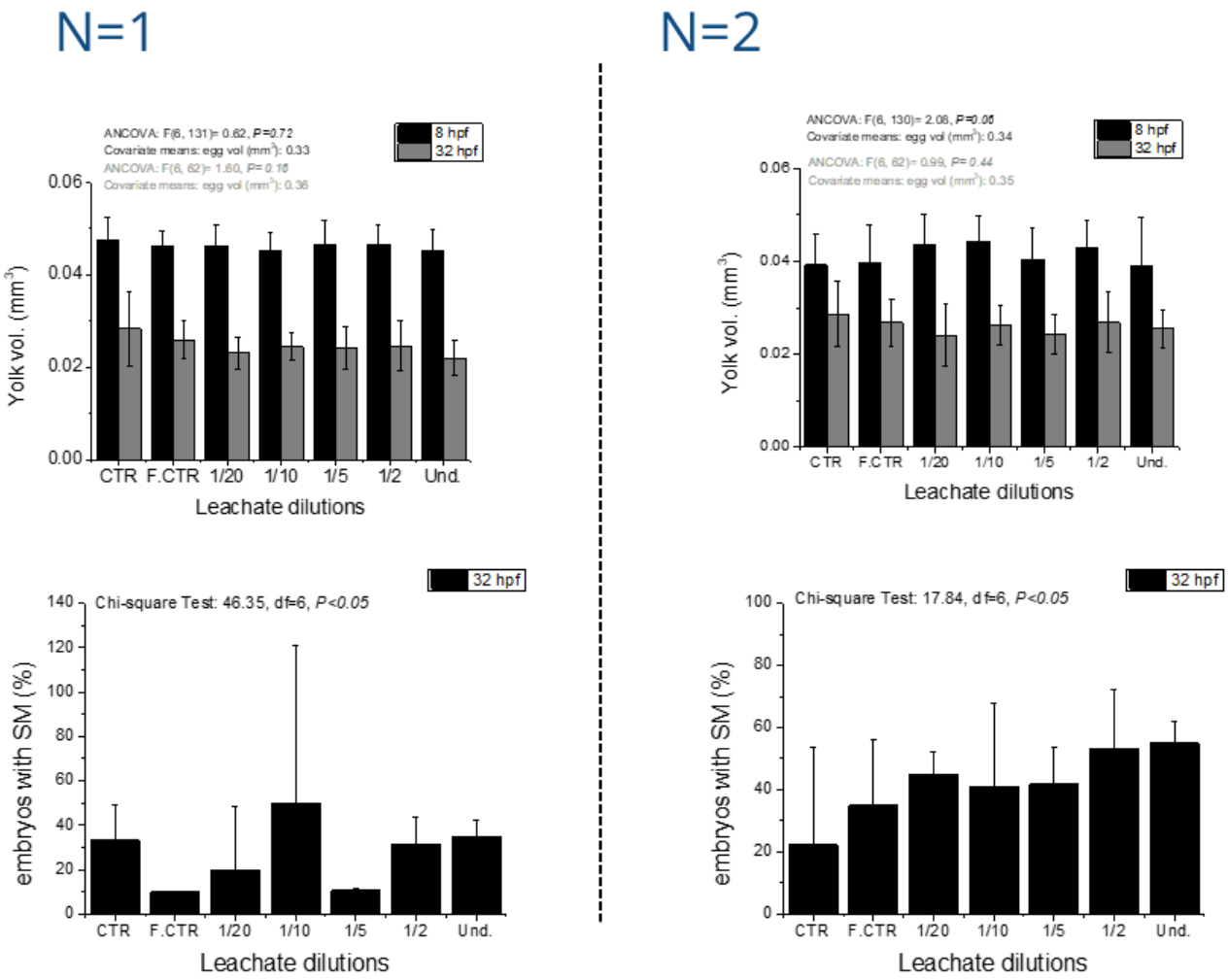


Figure 18. Results of zebrafish embryotoxicity tests for microplastic leachates, yolk volume and spontaneous movements. Results are expressed as mean ± standard deviation of 4 experimental replicates with 10 individuals each.

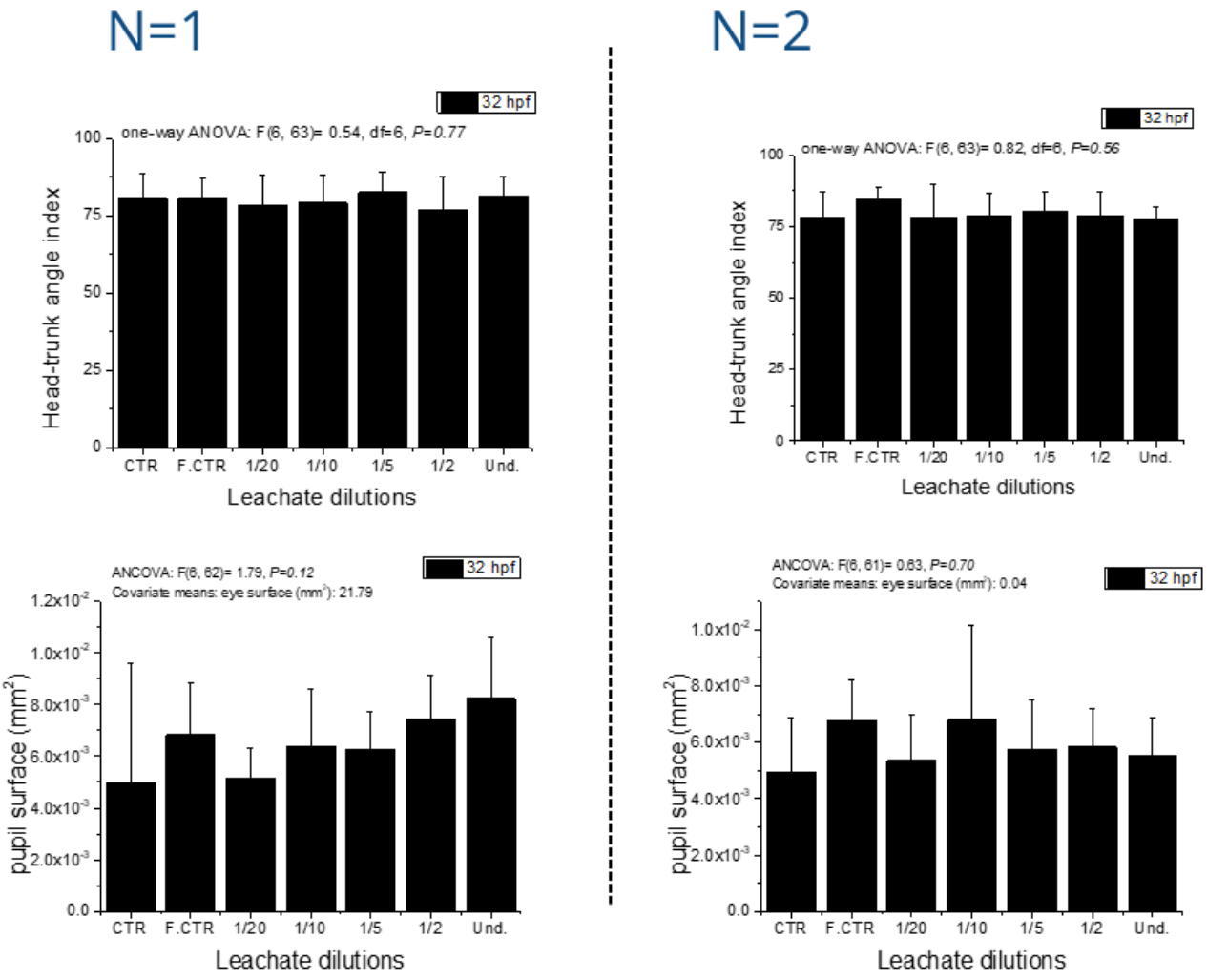


Figure 19. Results of zebrafish embryotoxicity tests for microplastics leachates; head-trunk angle and pupil surface. Results are expressed as mean ± standard deviation of 4 experimental replicates with 10 individuals each

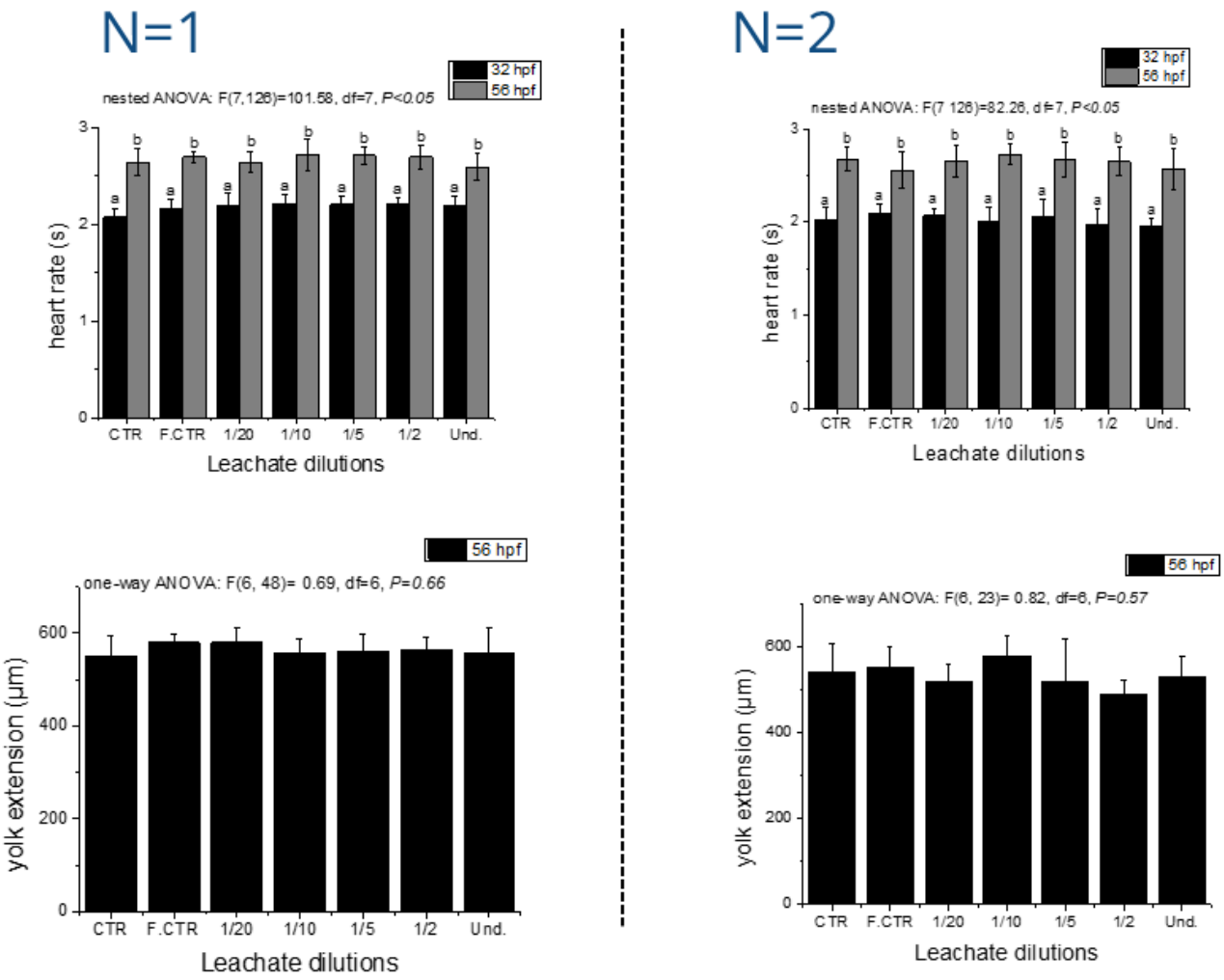


Figure 20. Results of zebrafish embryotoxicity tests for microplastics leachates; heart rate and yolk extension. Results are expressed as mean ± standard deviation of 4 experimental replicates with 10 individuals each.

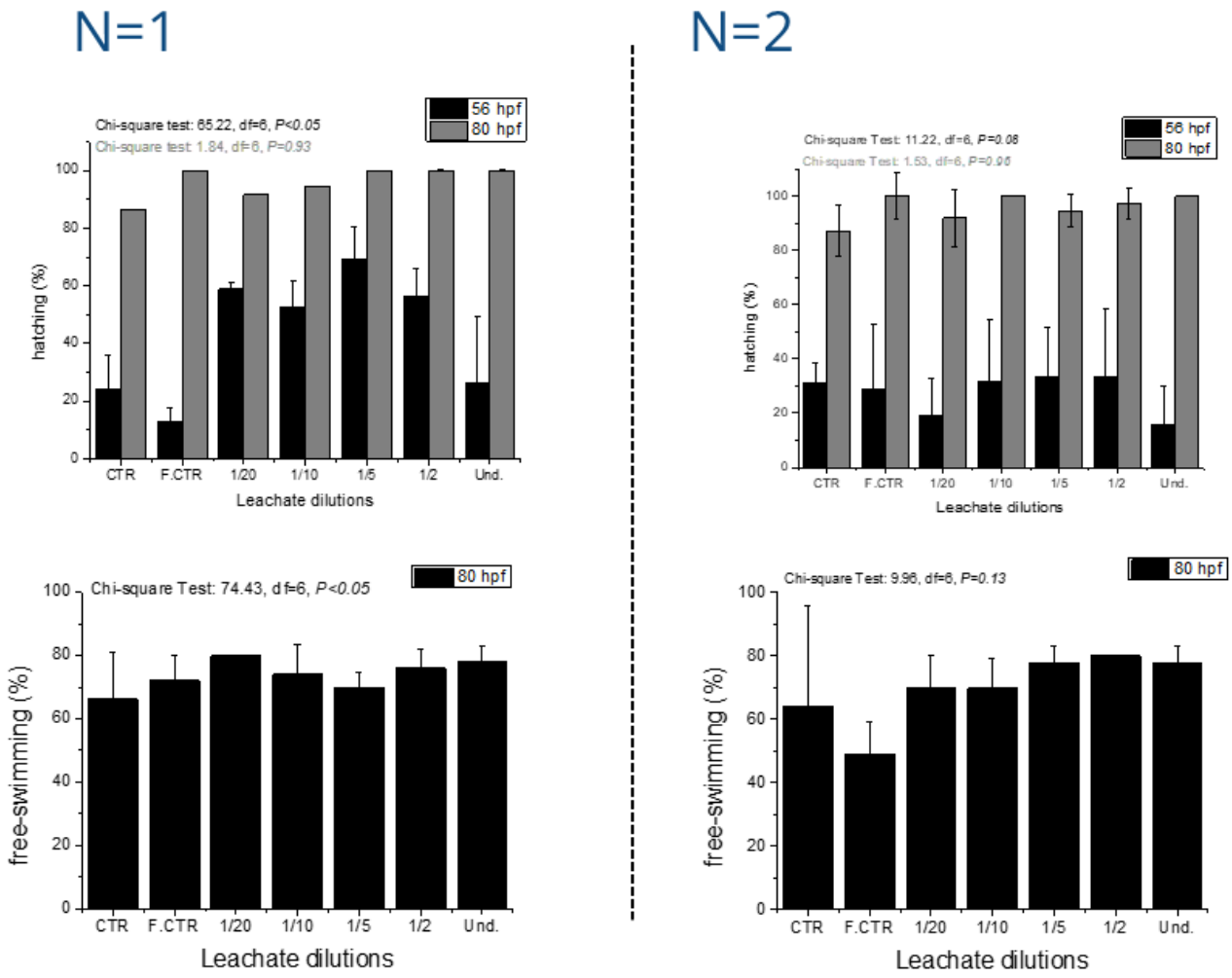


Figure 21. Results of zebrafish embryotoxicity tests for microplastics leachates; hatching and free-swimming. Results are expressed as mean ± standard deviation of 4 experimental replicates with 10 individuals each.

	hpf	Parameters	Leachate n=1	Leachate n=2
morphometric analysis	8	yolk volume	n.e.	n.e.
	32		n.e.	n.e.
	56	yolk extension	n.e.	n.e.
	8	epiboly	n.e.	n.e.
	32	head-trunk angle	n.e.	n.e.
	32	pupil surface	n.e.	n.e.
muscular coordination	32	spontaneous movements	Chi-square P<0.05	Chi-square P<0.05
	32	cardiac frequency	n.e.	n.e.
	56		n.e.	n.e.
	80	free-swimming	Chi-square P<0.05	Chi-square P<0.05
	56	hatching	Chi-square P<0.05	n.e
	80		n.e	n.e
	80	survival	n.e.	n.e.

n.e. - no effect observed compared with control

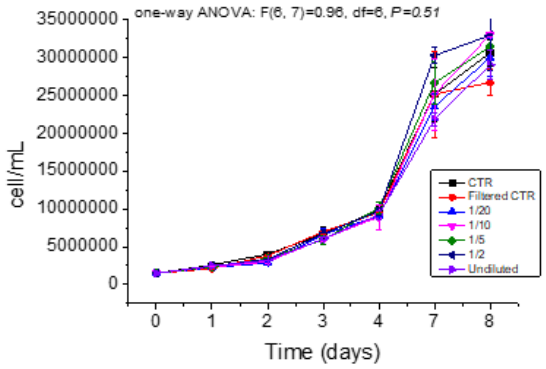
Table 11. Summary table of leachates sub-lethal toxicity against zebrafish embryos.

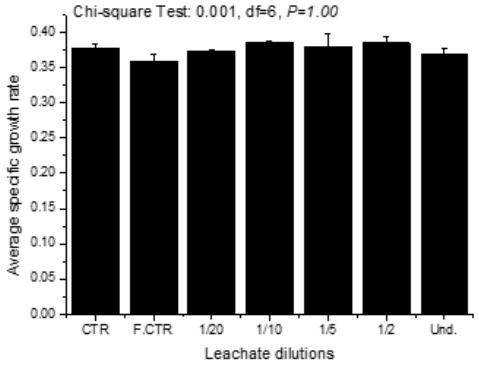
3.2.1.3 *Microcystis*

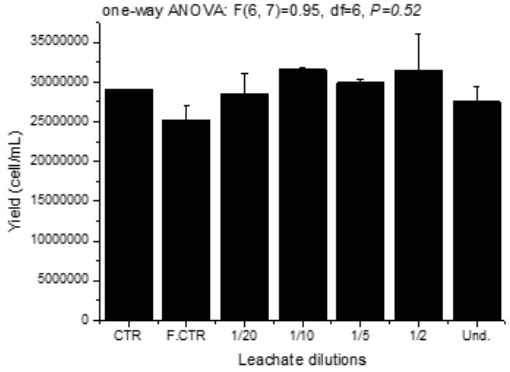
Leachates' preparations were carried out as established in D6.1. The tested dilutions were: 1 (or undiluted; represented as "UND" in the figures), 1/2, 1/5, 1/10 and 1/20. Two controls were always included: a negative control (*M. aeruginosa* only exposed to their media, AFW; represented as "CTR" in the figures) and the true procedural control (*M. aeruginosa* exposed to media that followed the same procedure as the leachates, but without plastics; represented as "F. CTR"). Two independent experiments were carried out following the guidelines established in D6.2 for the *Microcystis aeruginosa* growth inhibition test. Given the fact that not many significant effects were observed, we showed the results of both independent experiments separately (N=1 and N=2).

No significant inhibition of *M. aeruginosa* cell growth was observed at any of the leachate dilutions (Figure 21), in either of the evaluated toxicity endpoints; specific growth rate and yield.

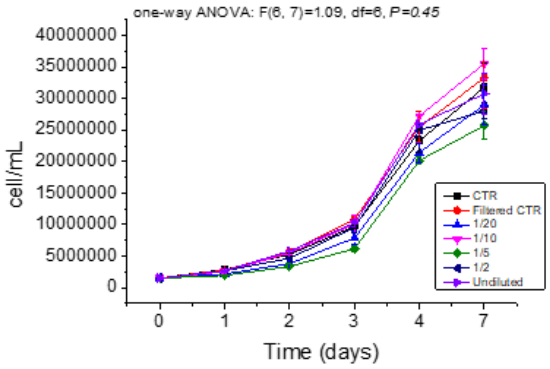
N=1

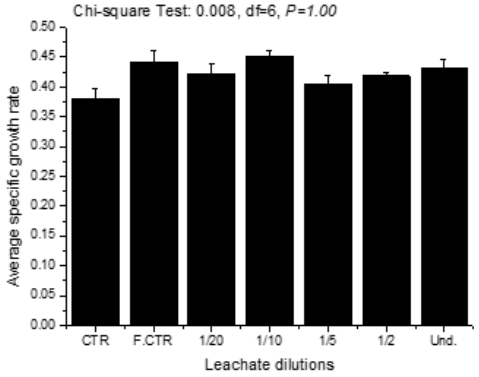






N=2





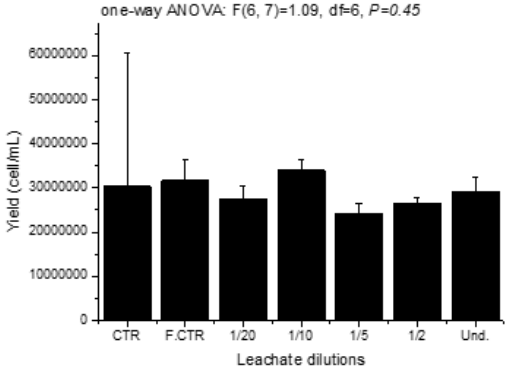


Figure 22. Results of M aeruginosa growth inhibition tests for microplastics' leachates; cell growth, specific growth rate and yield. Results are expressed as mean ± standard deviation of 2 replicates

Regarding microcystin production by the *M. aeruginosa* cells, no statistically significant differences were observed (Figure 22).

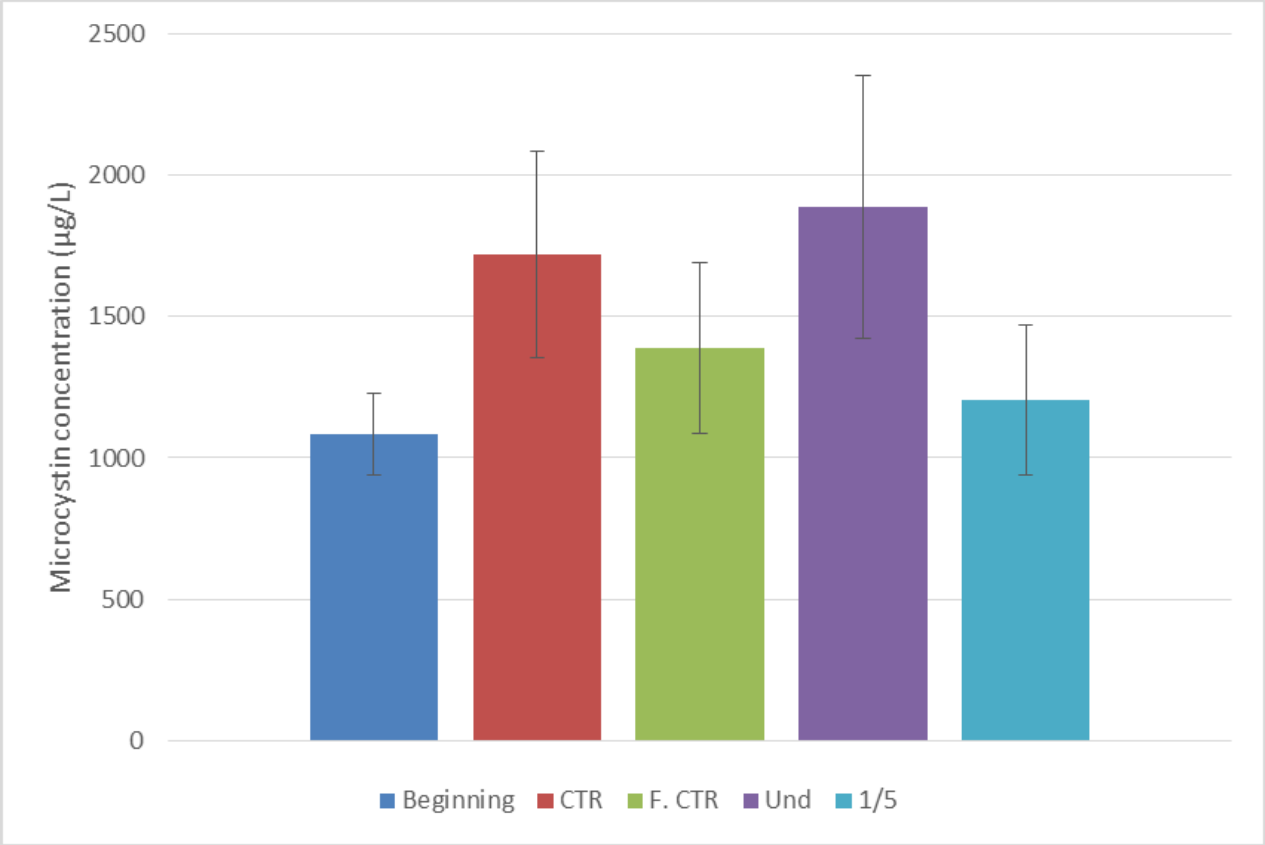


Figure 23. Results of M aeruginosa microcystin production upon exposure to microplastics' leachates; microcystin concentration was quantified using a commercial ELISA kit. Results are expressed as mean ± standard deviation of 3 replicates

3.3 Marine toxicity tests results

3.3.1 Paracentrotus lividus sea-urchin embryo test

Figure 23 shows the inhibition of larval growth caused by the leachate obtained from the Kiel plastic samples, and Table 10 shows the corresponding toxicity parameters obtained by fitting the data to a probit dose:response model. The material can be classified as slightly toxic according to the scale shown in Tab. 4 from D6.1. The Cecebre sample was also tested finding no toxicity at all.

The bioassay was conducted on 11/07/2022 according to internal SOP IT-ECTX-BE-0001 with a 10 g/L leachate of sample ID 093 corresponding to the Kiel plastic mix on 11/07/2022. The mean length of control larvae (377 µm) meets the acceptability criterion.

To explore the role of cigarette butts in the observed toxicity, an additional bioassay using 1 g/L leachate obtained from this material was conducted. For testing, the paper around the cigarette butt and any tobacco remaining was discarded, and only the filter was used. Results (Fig. 24 and Table 11) prove that cigarette butt filters are also classified as slightly toxic. However, their EC_{50} expressed in concentration units is more than 10 times lower, meaning that these materials are more than 10 times more toxic than the average plastic items found. This indicates a substantial contribution of cigarette butts to the overall toxicity of the Kiel plastics stock. However, since the contribution in mass of the butts to the total plastic collected is just 1.5%, we can also conclude that other plastic items may also be partly responsible for the slight toxicity recorded.

Item	Dilutions tested	NOEC	LOEC	EC_{10} (mg/L)	EC_{50} (mg/L)	TU
093_LBP_Mix Kiel	×1/30, ×1/10, ×1/3, ×1	×1/10	×1/3	2400 (2099, 2.685)	7107 (6562, 7752)	1,41
095_LBP_Mix Cecebre	×1/30, ×1/10, ×1/3, ×1	×1/3	×1	-	>10,000	<1

Table 12. SET results (NOEC, LOEC, EC10, EC50, TU) with 95% confidence intervals (in parentheses) for samples 093_LBP_Mix Kiel and 095_LBP_Mix Cecebre

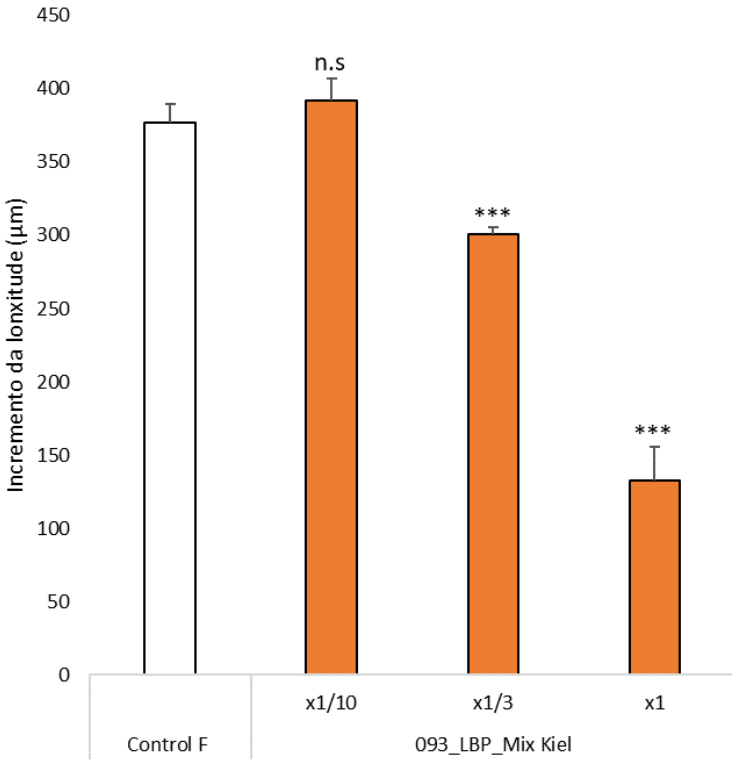


Figure 24. P. lividus larval length increase (µm) compared to filter control (Control F) for samples 093_LBP_Mix Kiel

The bioassay was conducted on 27/07/2022 according to internal SOP IT-ECTX-BE-0001 with a 1 g/L leachate of sample ID 093E corresponding to cigarette butt filters from Kiel plastics on 27/07/2022. The mean length of control larvae (317 µm) met the acceptability criterion.

Item	Dilucións	NOEC	LOEC	EC_{10} (mg/L)	EC_{50} (mg/L)	UT
093E_LBP_BUTTS	×1/30, ×1/10, ×1/3, ×1	×1/3	×1	426 (396 , 454)	606 (575 , 639)	1,65

Table 13. SET results (NOEC, LOEC, EC10, EC50, TU) with 95% confidence intervals (in parentheses) for samples 093E_LBP-cigarette butts (from Kiel)

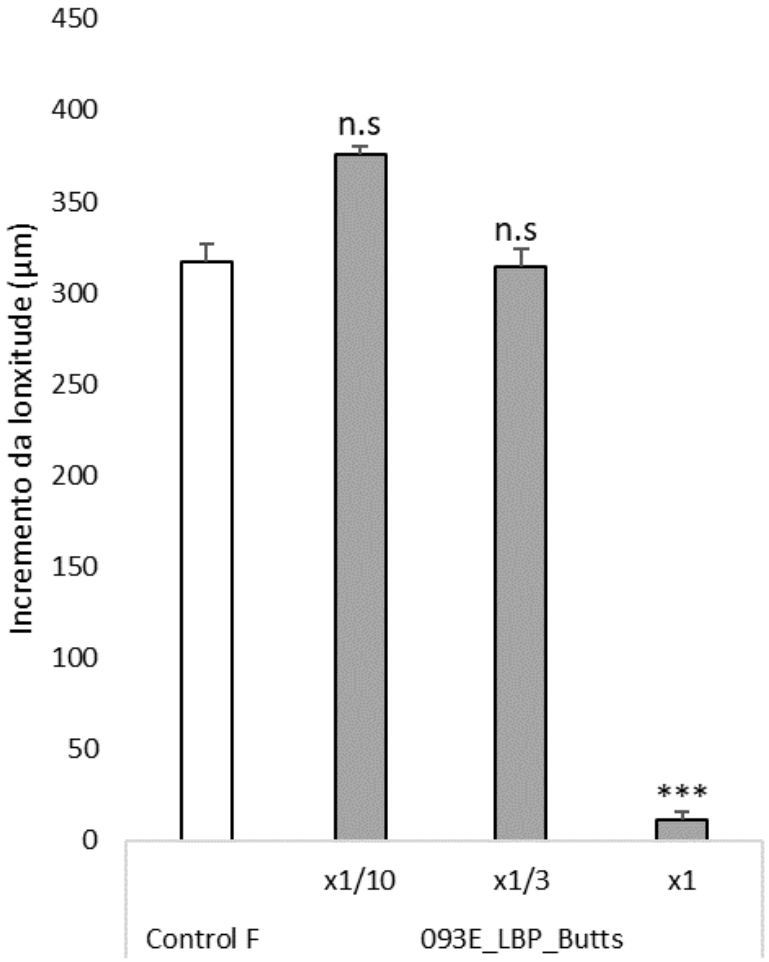


Figure 25. P. lividus larval length increase (µm) compared to filter control (Control F) for samples 093E_LBP_cigarette butts (from Kiel)

Figure 25 shows the inhibition of larval growth caused by the leachate obtained from the polypropylene (ID 080A) and polyethylene (ID 080B) plastic pellets sampled from a Ferrol (NW Iberian Peninsula) beach, and Table 12 shows the corresponding toxicity parameters obtained by fitting the data to a probit dose:response model. Both materials can be classified as non-toxic.

The bioassay was conducted on 08/04/2022 according to internal SOP IT-ECTX-BE-0001 with 1 g/L leachate of PP and PE pellets stranded on a beach in Ferrol (NW Iberian Peninsula) on 08/04/2022. The mean control length of larvae (403.9 µm) met the acceptability criterion.

Item	Dilutions tested	NOEC	LOEC	EC_{10} (mg/L)	EC_{50} (mg/L)	TU
80A_LBP_MixFerrol_P	×1/30, ×1/10, ×1/3, ×1	×1/3	×1	826,45 (689, 1020)	>1000	< 1
80B_LBP_MixFerrol_L	×1/30, ×1/10, ×1/3, ×1	×1	n.c	n.c	n.c	< 1

Table 14. SET results (NOEC, LOEC, EC10, EC50, TU) with 95 % confidence intervals (in parenthesis) for samples 80A and 80B

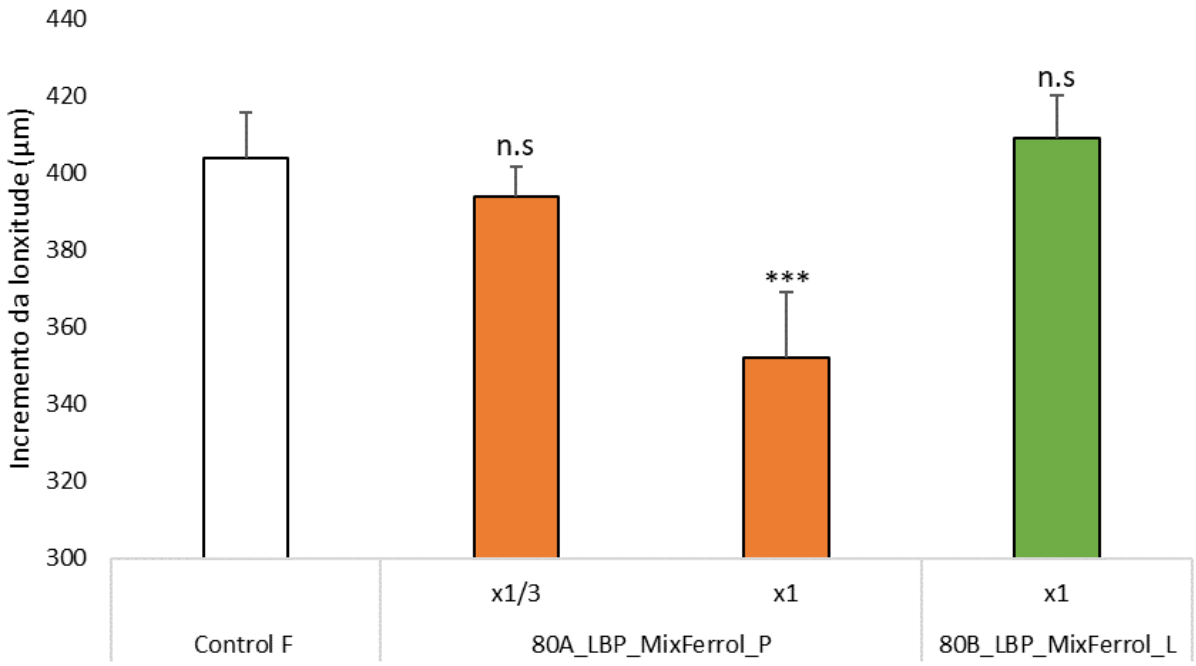


Figure 26. P. lividus larval length increase (µm) compared to filter control (Control F) for samples 80A and 80B

Sample ID084 from the Elbe River was tested on 28/04/2022 using 1 g/L leachate and no toxicity was found (NOEC = x1, TU<1).

3.3.2 *Acartia* nauplius test

Figure 26 shows the survival of copepod nauplius larvae incubated in leachates obtained from the Kiel plastic samples, and Table 13 shows the corresponding toxicity parameters.

The bioassay was conducted on 03/10/2022 according to internal SOP IT-ECTX-BE-0002 with a 10 g/L leachate. The mean larval survival in the control (100%) met the acceptability criterion.

Sample ID	Dilutions	NOEC	LOEC	EC_{10} (mg/L)	EC_{50} (mg/L)	TU
093_LBP_Mix Kiel	×1/30, ×1/10, ×1/3, ×1	×1	n.c.	n.c.	n.c.	**< 1**

Table 15. Results (NOEC, LOEC, EC10, EC50, TU) of the copepod larval survival test for sample 093

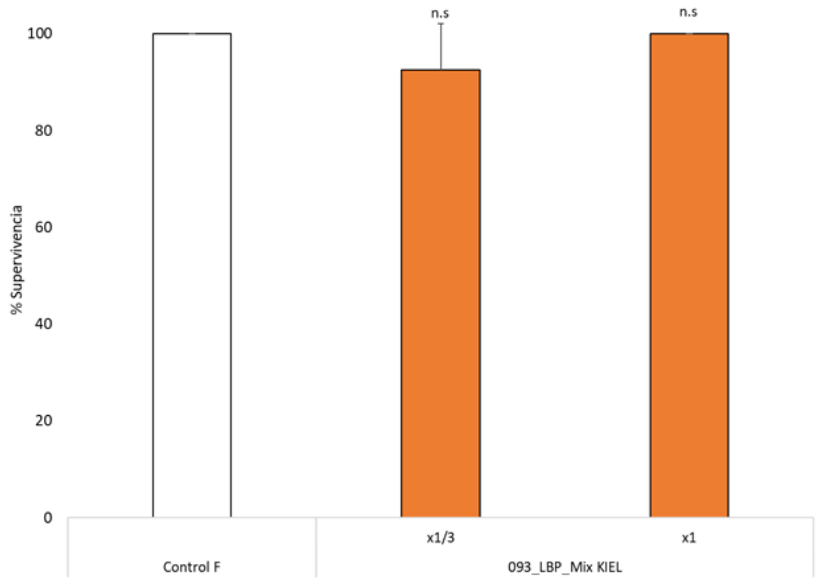


Figure 27. Copepod larval survival test for sample 093 (Kiel plastics)

Therefore, according to this bioassay, the sample can be classified as non-toxic.

3.3.3 *Vibrio fischeri* test

For testing the effect of the leachate of the 093_LBP_Mix Kiel environmental polymer sample on the bacterium *Vibrio fischeri* via inhibition of its autoluminescence, a dilution series of a lixiviate with a polymer loading of 10 g/L in the stock solution was measured. The test was performed according to DIN EN ISO 11348-2 (2009).

Coming from a stock lixiviate formulated with 10 g/L of the solid environmental polymer sample, the following dilutions were tested: 1:2, 1:3, 1:4, 1:6, 1:8, 1:12, 1:16 and 1:24. The pH ranged between 7.1 - 8.0 and the oxygen content was between 7.7-8.2 mg/L.

After 30 minutes of incubation at a constant temperature of 15°C, no significant inhibition was found. Hence, the EC50 could not be calculated. However, this results in an estimated EC50 > 1:2 dilution of the eluate from 1 g/L test substance in water. This corresponds to an EC50 > 0.5 g/L test substance nominally.

Dilution	Stock solution	Inhibition % (mean value of 2 replicates)
Reference substance (3,5-dichlorophenol)	--	34.45
1:2	Original sample undiluted	3.93
1:3	16.8 mL/25mL	1.16
1:4	12.5 mL/25mL	-2.09
1:6	8.33 mL/25mL	-7.49
1:8	6.25 mL/25mL	-4.55
1:12	4.17 mL/25mL	-8.74
1:16	3.13 mL/25mL	-7.08
1:24	2.08 mL/25mL	-2.83

Table 16. Dilutions for the DIN EN ISO 11348-2 (2009) Vibrio fischeri autoluminescence inhibition test

4 DISCUSSION

Environmental plastics showed slight to no toxicity in all the aquatic ecotoxicological bioassays conducted, including freshwater and marine species, and different trophic levels from bacteria to fish. Combining the moderate effects found with the high loads of plastic tested (1 to 10 g/L), in general orders of magnitude above environmental levels, these findings do not support a relevant impact of plastic leachates in the natural aquatic compartments.

In terrestrial tests, the earthworm chronic reproduction test conducted with the plastic particles themselves was the most sensitive test of the battery, and significant inhibition of reproduction endpoints was found at 500 mg/Kg for leachates, and 250 mg/Kg for particles. These findings deserve further research in future LABPLAS Project activities.

This report concerns exclusively the assessment of plastic particles below 250 µm obtained by fragmentation of macro and meso plastic. Future work should address the effects of smaller size fractions, and should include in the case of aquatic species not only leachates but also particles themselves.

This study relied on pools of environmental plastics directly sampled from the study areas to assess the overall effects of actual mixes of litter and to enhance the throughput and environmental relevance of the assessment. The current findings do not exclude the presence of particular plastic items with a higher impact than the average pool. This can be illustrated with the case of cigarette butts. None of the plastic mixes showed consistent indications of toxicity to any of the biological models for 1 g/L leachate. In contrast, leachates obtained from filters of cigarette butts made at 1 g/L did slightly but significantly reduced sea-urchin embryo development.

Future work may try to identify individual plastic materials particularly toxic to restrict their use, fight littering, or find environmentally safer alternatives. However, we must keep in mind that the present findings are a result of the current standard leaching methods used, and multiple variables (e.g. leaching period, temperature, particle size) are expected to affect leaching in natural environments.

As part of WP3 tasks, chemical analyses of the samples tested in this deliverable are ongoing. The results of those analyses in combination with the present report may contribute to establishing safe levels of plastics in the different environmental compartments.

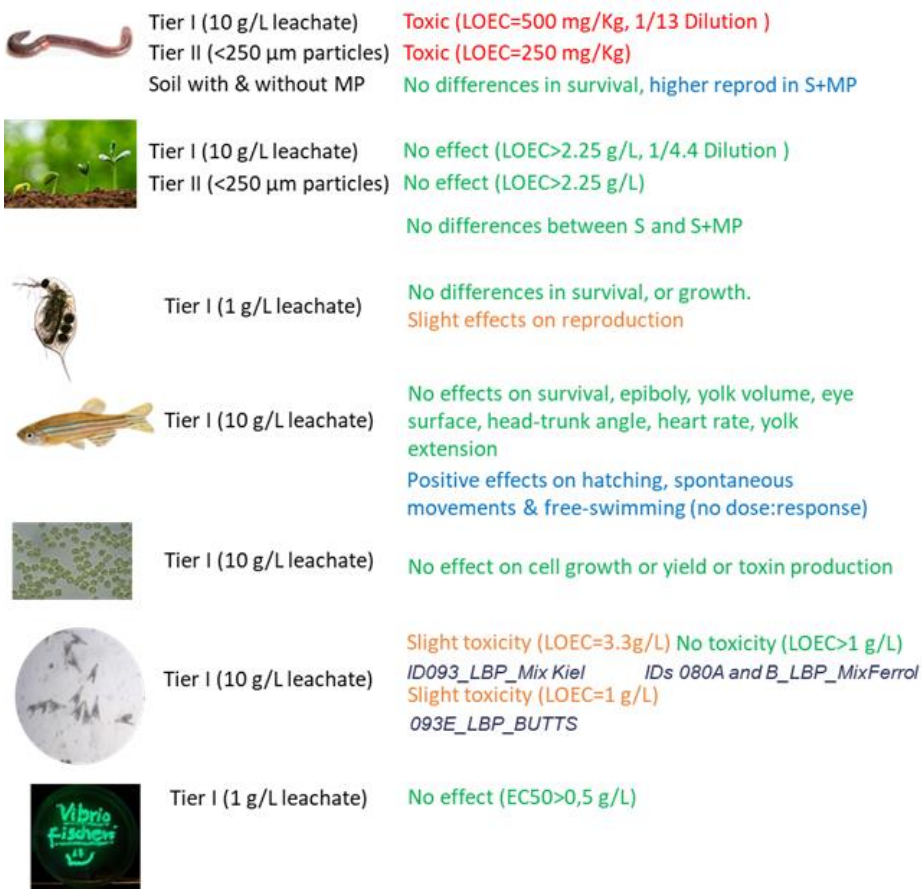


Figure 28. Summary of effects of ecotoxicological bioassays conducted on terrestrial, freshwater and marine species of different trophic levels. Green: no effects; orange: slight negative effects; red: toxicity; blue: positive effects.

Considering the logistical difficulties to sample amounts of environmental SMNP sufficient for toxicity testing – particularly for chronic tests- and even to produce those amounts in the laboratory by grinding meso and macroplastics, microscale methods are advised. In the case of aquatic species, Wells et al. (1998) provided a comprehensive review of microscale ecotoxicological tests useful for these purposes.

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