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TEST REPORT

ECOTOXICOLOGICAL TESTS AGAINST TARGET ORGANISMS FOR UNAGED AND AGED PLASTIC FILM FRAGMENTS

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RADICAL PLASTICS INC.
100 Cummings Center, Suite 240D
Beverly, MA 01915

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OBJECT

Evaluation of ecotoxicological effects of oxidized plastic fragments eluates on different organisms from different trophical levels.

CLIENT

RADICAL PLASTICS INC.
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REFERENCE PERSON

Dr. Yelena Kann

METHOD AND INSTRUMENTS

PREPARATION OF THE SAMPLE

Samples were fragmented manually in small pieces (dimension less than 2 mm).

The eluates were prepared by mixing the samples in the test medium for 24 h at the maximum concentration equal to 16 g / l. A limit test was performed at this concentration.

This concentration is certainly greater than that which can be found in the real case of use. This concentration is selected to guarantee a high safety limit.

The sample was treated following the OECD document n. 23: 2019 "*GUIDANCE DOCUMENT ON AQUEOUS-PHASE AQUATIC TOXICITY TESTING OF DIFFICULT TEST CHEMICALS SERIES ON TESTING AND ASSESSMENT*" for the execution of the tests.

Samples were stored at 2-5 °C, the pH of eluate solutions were 7.20.

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ECO-TOXICOLOGICAL TEST

- ***VIBRIO FISCHERI* LIGHT EMISSION INHIBITION - TEST ON LUMINESCENT BACTERIA**

The inhibition of light emission by cultures of *Vibrio fischeri* is determined by means of a batch test. This is accomplished by combining specified volumes of the test sample or the diluted sample with the luminescent bacteria suspension in a test tube.

The test criterion is the luminescence, measured after a contact time of 30 min and, taking into account a correction factor (*fkt*), which is a measure of intensity changes of control samples during the exposure time. Test data and conditions were reported in Table 1.

Table 1. Test conditions and characteristics

STANDARD METHOD	UNI EN ISO 11348-3: 2019 Limit test at one concentration
AGE AND ORIGIN OF THE ORGANISMS	original lyophilized bacteria - Azur- batch number 19G4153A
STORAGE TEMPERATURE	- 20 ° C
RESULT OF TEST WITH REFERENCE TOXIC SUBSTANCE (3.5-DICHLOROPHENOL)	30 minEC_{50} = 3.6 mg / l
EXPERIMENTAL CONDITIONS	Number of replicates per concentration / control: 2 Concentrations / dilutions tested (dilution factor): 90% v / v Temperature (° C): 15 ° C

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	Test duration: 30 minutes Dilution / control water: diluent Azur batch number 19F4140
TEST LIMIT	not applicable
CALCULATION METHOD FOR DETERMINING THE EC_{50}	30 min EC50: not applicable
RESULTS VALIDITY CRITERIA	– The value of *kt* for 30 min of incubation is between 0.6 and 1.8; – Standard deviation from the mean of each individual control determination <= 3%; – reference substance (3,5-dichlorophenol) for the batch of bacteria delivered, causes from 20% to 80% of inhibition after 30 minutes of contact time (concentrations in the final test suspension 3.4 mg / l) – The test was conducted in compliance with the validity criteria provided

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- **ACUTE TOXICITY TEST WITH CRUSTACEANS – *DAPHNIA MAGNA* (24H / 48H)**

Young daphnids, aged less than 24 hours at the start of the test, are exposed to the test substance for a period of 48 hours. Immobilization is recorded at 24 hours and 48 hours and compared with control values. The animals should be less than 24 hours old at the start of the test, and, to reduce variability, it is strongly recommended they are not first brood progeny. They are derived from a healthy stock (i.e. showing no signs of stress such as high mortality, presence of males and ephippia, delay in the production of the first brood, discoloured animals, etc.). All organisms used for the test have originated from cultures established from the same stock of daphnids. The stock animals are maintained in culture conditions (light, temperature, medium) similar to those to be used in the test. Test data and conditions were reported in Table 2.

Table 2. Test conditions and characteristics

STANDARD METHOD	OECD Guideline n. 202: 2004 Limit test for one concentration
AGE AND ORIGIN OF THE ORGANISMS	Ephippia stabilized MicroBiotest inc. batch number DM181120
EXPERIMENTAL CONDITIONS	– Type and volume of containers: Containers with a volume of 20 ml, made of transparent plastic to avoid adsorption processes or the release of substances that can interfere with the test. – Volume of sample used: 12.5 ml

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	– Number of organisms and replicates by concentration / control: 20 daphnias divided into 4 replicates of 5; – Concentrations / dilutions tested (dilution factor): 8 g / l – Dilution / control water: medium Microbiotest inc. batch ISOD121020 – Temperature (° C): 20 ° C +/- 1 ° C – Lighting and photoperiod: test performed in the dark; – Nourishment: absent;
TEST LIMIT	24 and 48 hours
RESULT OF TEST WITH REFERENCE TOXIC SUBSTANCE ($K_2Cr_2O_7$) 24h EC_{50}	1.41 mg /
CALCULATION METHOD FOR DETERMINING THE EC_{50}	software used: "EPA Probit Analysis Program used for calculating LC / EC values" - version 1.5 48hEC_{50}: not applicable Dose-response curve: not applicable
RESULTS VALIDITY CRITERIA	a) dissolved oxygen concentration at the end of the test> = 3 mg / l; b) percentage immobilization of controls <= 10%; c) 24hEC_{50} of $K_2Cr_2O_7$ in the range 0.6 - 2.1

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	mg / l. The test was conducted in compliance with the validity criteria provided

- **FRASH WATER GREEN ALGAE GROWTH INHIBITION TEST (72H)**

The purpose of this test is to determine the effects of a substance on the growth of freshwater microalgae and/or cyanobacteria. Exponentially growing test organisms are exposed to the test substance in batch cultures over a period of normally 72 hours. In spite of the relatively brief test duration, effects over several generations can be assessed.

The system response is the reduction of growth in a series of algal cultures (test units) exposed to test substance. The response is evaluated as a function of the exposure concentration in comparison with the average growth of replicate, unexposed control cultures. The cultures are allowed unrestricted exponential growth under nutrient sufficient conditions for full expression of the system response to toxic effects (optimal sensitivity), and continuous light for a sufficient period of time to measure reduction of the specific growth rate.

Growth and growth inhibition are quantified from measurements of the algal biomass as a function of time. Algal biomass is defined as the dry weight per volume, e.g. mg algae/litre test solution. However, dry weight is difficult to measure and therefore surrogate parameters are used. Of these surrogates, cell counts are most often used. Other surrogate parameters include cell volume, fluorescence, optical density, etc. A conversion factor between the measured surrogate parameter and biomass should be known.

The test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure period. Test data and conditions were reported in Table 3.

Table 3. Test conditions and characteristics

STANDARD METHOD	OECD Guideline n. 201: 2011 Limit test for one concentration
AGE AND ORIGIN OF THE ORGANISMS	stabilized spores MicroBiotest inc. batch number SC290920
EXPERIMENTAL CONDITIONS	– type and volume of containers: transparent plastic containers to avoid adsorption or release processes of substances that can interfere with the test. – Inoculated cells / ml: 10000 cells / ml – Number of replicates per concentration / control: 6/6 – Concentrations analyzed: 8 g / l – Temperature (° C): 23 ° C +/- 2 ° C – Illumination: 6000 - 10000 lux – Dilution / control water: reconstituted water MicroBiotest inc. batch number SC180920
TEST LIMIT	72 h
RESULT OF TEST WITH REFERENCE TOXIC SUBSTANCE ($K_2Cr_2O_7$) 72h EC_{50}	0.59 mg/l

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CALCULATION METHOD FOR DETERMINING THE EC_{50}	Not applicable
RESULTS VALIDITY CRITERIA	a) increase in growth of the control population by a factor> = 16 in 72 h b) variation in the coefficient of variation of the growth rate between replicates of the control for each day of incubation <= 35% c) variation in the coefficient of variation of the growth rate between replicates of the control over the entire period <= 7% d) increase of the pH value of the control during the test <= 1.5 The test was conducted in compliance with the validity criteria provided

- **ACUTE TOXICITY TEST WITH PLANTS – *GERMINATION***
  INTERNAL METHOD

4 types of commercial were chosen for this test seeds based on their characteristics:

- Monocotyledon: Tall fescue *(Festuca Arundinacea*)
- Dicotyledons: San marzano Tomato (*Solanum lycopersicum 'San Marzano'*), Rio Grande tomato (*Lycopersicum esculentum*) and Cress (*Lepidium sativum*)

The test was carried out on aged and unaged plastic film and in presence and absence of mineral sent by committee.

The seeds were left overnight in 10 ml of distilled water for revitalize dry seeds.

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The eluates were prepared by stirring 10 g / L of samples for 48 hours at 250 rpm and filtered through a syringe filter (PVDF - 0.22 µm). Thereafter 0.625 ml of elutes were diluted in 50 ml of bi- distilled water.

The tests in presence of mineral were carried out by dispersing the mineral in water in a ratio of 1:3 mineral/samples .

10 seeds were placed on filter paper in 2 Petri-dishes.

The filter paper was moistened with 1 ml of diluted solution and then Petri-dishes were transferred to a dark growth room at 18 ±2 °C for 14 days.

Seed germination was observed daily. Each seed was removed from the Petri-dish as soon as the sprout is visible (approximately 3 mm long) and placed in an ethanol solution to stop the growth processes.

The maximum duration of a test was 14 days and counting continued until no germination occurred for 5 successive days.[1]

Each germination toxicity test must include the negative control as one of the experimental treatments.

The negative control is essentially double distilled water without of contaminants that adversely affect seed performance during testing. The use of the negative control provides a measure of the acceptability of the test and the health and performance of the seeds used.

However, the range of application of cress seed is proved to be limited, and there has been a few works to determine whether there exists a species of plant seed that is more sensitive than cress seed to the toxicity of compost.

[1] J. P. Grime, G. Mason, A. V. Curtis, J. Rodman, S. R. Band A comparative study of germination characteristics in a local flora - Journal of Ecology, 69(3), 1981, pp. 1017–1059.

Measure and calculate the indicators related to the test results including:[2]

→ Seed germination (SG)

$$SG\ \% = \frac{Number\ o\ germinated\ seeds}{Number\ of\ total\ seeds} \times 100$$

→ Relative seed germination (RSG)

$$RSG\ \% = \frac{Number\ of\ germinated\ seeds\ (Sample)}{Number\ of\ germinated\ seeds\ (Control)} \times 100$$

- **BIOLOGICAL ASSAY WITH *EISENIA FETIDA* - TEST OF ACUTE TOXICITY - DETERMINATION OF EFFECTS OF MORTALITY AT 7-14 DAYS OF EXPOSITION**

STANDARD METHOD : ISO 11268 - 1: 2012.

This part of ISO 11268 specifies one of the methods for evaluating the habitat function of soils and determining the acute toxicity of soil contaminants and chemicals to *Eisenia fetida* by dermal and alimentary uptake. It is applicable to soils and soil materials of unknown quality, e.g. from contaminated sites, amended soils, soils after remediation, agricultural or other sites concerned, and waste materials.

Effects of substances are assessed using a standard soil, preferably a defined artificial soil substrate. For contaminated soils, the effects on survival are determined in the test soil and in a control soil.

[2] Y. Luo, J. Liang, G. Zeng, M. Chen, D. Mo, G. Li, D. Zhang Seed germination test for toxicity evaluation of compost: Its roles, problems and prospects - Waste Management, 71, 2018, pp. 109-114.

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– *EXPERIMENTAL DESIGN*

The sample is represented by oxidized LPDE reduced to fragments and stored in the dark at + 4 ° C until the tests were set up.

The execution of the experimental protocol involved mixing the sample with an artificial substrate; a specific quantity of organisms, adult earthworms of the *Eisenia fetida*, was placed in each replica of the sample and control to evaluate, 7 and 14 days the effects induced by the pollutants derived or content in samples themselves.

The edaphic fauna used in the monitoring of pollutants includes Nematodes, Enchitreidae, Oligochetes, Gastropods, Collemboli, Isopods, Arachnids[3] . The observed endpoints may vary according to the species used, but effects on survival, reproduction, body growth, bioaccumulation of toxic substances are generally evaluated.

Among all these organisms, earthworms (about 90 species recognized in Italy) interact positively in soils with plants, they are useful and often fundamental organisms in the recycling of organic materials such as dead roots and litter and in their transformation into humic and nutrient compounds easily assimilable by plants.

The organism used in this study is *Eisenia fetida* worm. This worm represents specie that is widespread in their specific environment, it is relatively easy to breed and it is able to give as differentiated answers to the various environmental problems. In fact, earthworms lend themselves well to highlighting the conditions of a soil both through survival or mortality, and through parameters that denounce sub-lethal toxicity, such as burial time and reproductive capacity.

[3] Cortet, J., Gomot de Vauflery, A., Poinsot-Balaguer, N., Gomot, L., Texier, C., Cluzeau, D. The use of invertebrate soil fauna in monitoring pollutant effects. Eur. J. Soil Biol. 35 (3), 2000, 115- 134.

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Earthworms (**Figure 1**) are insufficient hermaphroditic species: they must therefore fertilize each other, discharging the sperms into the partner's seminal receptacles, remaining united by the clitellum, a thickened cylindrical formation that appears in adults located between 32 and 37 metamers. The fertilization of the eggs takes place in the cocoon. The individual released from the cocoon is similar to the adult, but thin and very small in size. Each adult sexually developed individual produces 6 cocoons per week, for a total of 19 young worms on average.

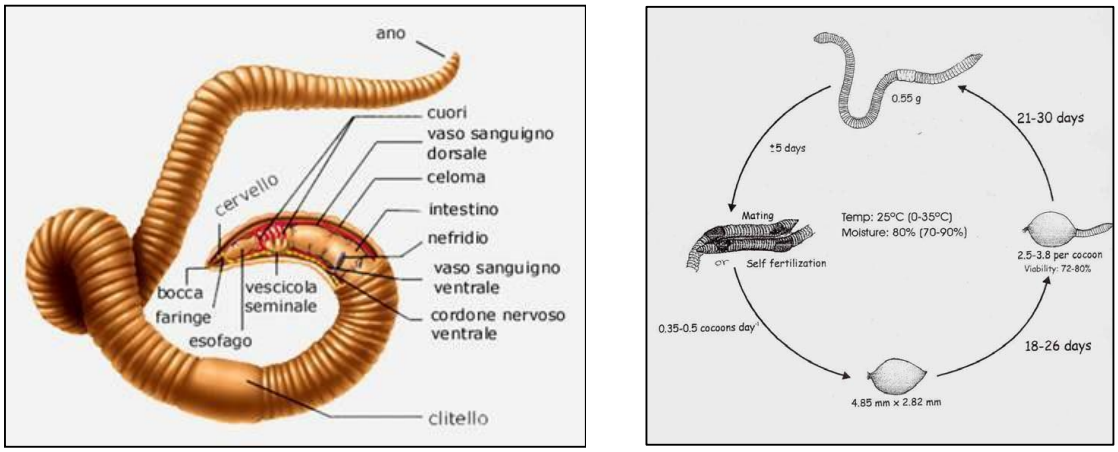

Figure 1 (Left) adult earthworm - outline of morphology and anatomy. (Right) *Eisenia fetida* Sav, biological cycle[4]

The suitability of earthworms to play the role of bioindicators of soil excellence has led to the development of official OECD and ISO standards, to monitor, through standardized acute and chronic toxicity tests, the state of soils and the effects of substances poured into it.

[4] Edwards, C. A., Arancon Q. N., Sherman R., 2011. Vermiculture technology. Earthworms, organic wastes and Environmental Management. Taylor and Francis Group, LLC. Pp 601 Fondazione Lombardia per l'Ambiente – Bioindicatori Ambientali - 1998

Some of them provide for the testing, with more repetitions, of soils using groups of 10 earthworms each belonging to the species *Eisenia fetida* or *Eisenia andrei*, and of controls represented by standardized artificial soils.

For the sample and control, 4 replicates with 10 organisms per replica were prepared. In the case of the acute toxicity test, after 2 weeks of exposure the mortality percentage of exposed individuals to the samples is recorded and compared with individuals unexposed to contaminated soil ("Blanc").

– *DOSAGES APPLIED*

0.5 g of oxidized LDPE / kg of substrate was set up in 4 replicates

– *ARTIFICIAL SOIL COMPOSITION*

i. Sphagnum peat (dried), finely ground and without visible plant residues : **10 %**
ii. Kaolinitic clay (dried), containing not less than 30% of kaolinite: **20 %**
iii. Industrial quartz sand (dried) with a predominance of fine sand with more than 50% of the mass consisting of particles with a size between 0.00 and 0.2 mm : **69 %**
iv. Humidity : **40 – 60 %** of artificial soil water holding capacity.

– *EARTWORMS SELECTION AND PREPARATION*

The species used in the test are adult earthworms of the species *Eisenia fetida*. In particular, adult earthworms aged at least 3 months, with clitella and with a mass between 300 mg and 600 mg were used.

The earthworms come from a synchronized farm providing individuals with relatively homogeneous ages since, as required by the standard, the individuals used in the assay must not differ in age from each other for more than 4 weeks.

In a first phase of the experimental protocol, earthworms were selected starting from synchronized breeding and then acclimatized for 1 day in the artificial soil (Figure 2).

Figure 2. Acclimatization of selected earthworms.

At the beginning of the test earthworms were weighed individually (Figure 3) and divided into categories based on weight: the wet mass of the individual earthworms must be between 300 and 600 mg (Figure 4).

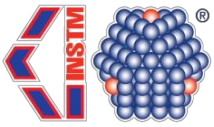

Figure 3. Earthworms washed and dried before weighing

Figure 4. Breakdown of earthworms into weight categories

This allows the preparation of the groups of 10 organisms that must be placed in the containers for the start of the assay to be carried out in a more correct and homogeneous way. To avoid systematic

errors in the distribution of earthworms in the containers, the homogeneity of the population to be tested is precisely determined by single weighing of the earthworms, sampled using a random method from the selected population and acclimatized in the artificial soil.

The test was carried out at 20 ± 2 ° C.

The containers were not ventilated during the execution of the tests. Once a week the containers were opened to moisten the soil and this is considered sufficient to allow gas exchanges.

The food was provided at the beginning of the test. About 5 g of dry food (had flour) was placed on the soil surface of each container and moistened with drinking water (about 5 ml per container).

– *MEASUREMENTS*

After 7 days of exposure the living organisms were counted and the dead ones removed.

After 14 days of exposure, the total number of live organisms and their mass were determined for each container.

– *VALIDITY OF THE ASSAYS AND EXPRESSION OF THE RESULTS*

For acute assays to be considered valid, the following criteria must be met in controls:

The observed mortality rate is ≤ 10%.

The average mass loss of earthworms must not exceed 20%

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SAMPLES DESCRIPTION

Sample identification	**Unaged CN22**	**AG235 Aged outdoors MA**
Material	LDPE unaged	LDPE aged
Sample name	Unaged	Aged
Thickness	0.023 mm ± 0.004	0.019 mm -± 0.002
Appearance	Transparent film	Transparent film

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RESULTS

ACUTE TOXICITY TEST WITH BACTERIA, ALGAE AND CRUSTACEOUS

Analysis	Test method	Result		Unit
		Unaged	*Aged*	
Vibrio fischeri light emission inhibition - test on luminescent bacteria	UNI EN ISO 11348-3: 2019	18	35	% I 30'
Green algae growth inhibition test by (72h)	OECD Guideline 201:2006	14	15	% - 72h
Daphnia magna mobility inhibition (24h / 48h)	OECD Guideline 202:2004	0	0	% - 48h

ACUTE TOXICITY TEST WITH PLANTS – *GERMINATION*

- Cress (Lepidium sativum)

Sample	Seed germination (SG) (%)	Relative Seed germination (RSG) (%)
Control	80	100
Unaged	40	50
Aged	70	88
Unaged min	70	88
Aged min	30	38

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- Tall fescue *(Festuca Arundinacea)*

Sample	Seed germination (SG) (%)	Relative Seed germination (RSG) (%)
Control	90	100
Unaged	100	111
Aged	100	111
Unaged min	100	111
Aged min	100	111

- San Marzano Tomato (*Lycopersicum esculentum*)

Sample	Seed germination (SG) (%)	Relative Seed germination (RSG) (%)
Control	80	100
Unaged	80	100
Aged	100	125
Unaged min	90	113
Aged min	100	125

- Rio Grande tomato (*Lycopersicum esculentum*)

Sample	Seed germination (SG) (%)	Relative Seed germination (RSG) (%)
Control	90	100
Unaged	100	111
Aged	100	111
Unaged min	90	100
Aged min	100	111

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BIOLOGICAL ASSAY WITH *EISENIA FETIDA*

The following table shows, in the first place, the data obtained for the acute "blank" to demonstrate the validity of the performed test, as required by the standard ISO 11268-1.

	Day 0		Day 14		Results	
	N° organisms	Mass (g)	N° organisms	Mass (g)	Mortality (%)	Body mass growth (%)
Blank 1	10	3.956	10	4.525	0	14
Blank 2	10	3.800	10	4.186	0	10
Blank 3	10	3.687	8	3.870	20	5
Blank 4	10	3.884	10	4.258	0	10
Average	-	3.832	-	4.210	5	10

	Day 0		Day 14		Results	
	N° organisms	Mass (g)	N° organisms	Mass (g)	Mortality (%)	Body mass growth (%)
Sample 1	10	3.67	10	4.333	0	18
Sample 2	10	3.71	10	4.802	0	30
Sample 3	10	3.26	10	4.004	0	23
Sample 4	10	3.58	10	4.442	0	24
Average	-	3.56	-	4.3953	0	24

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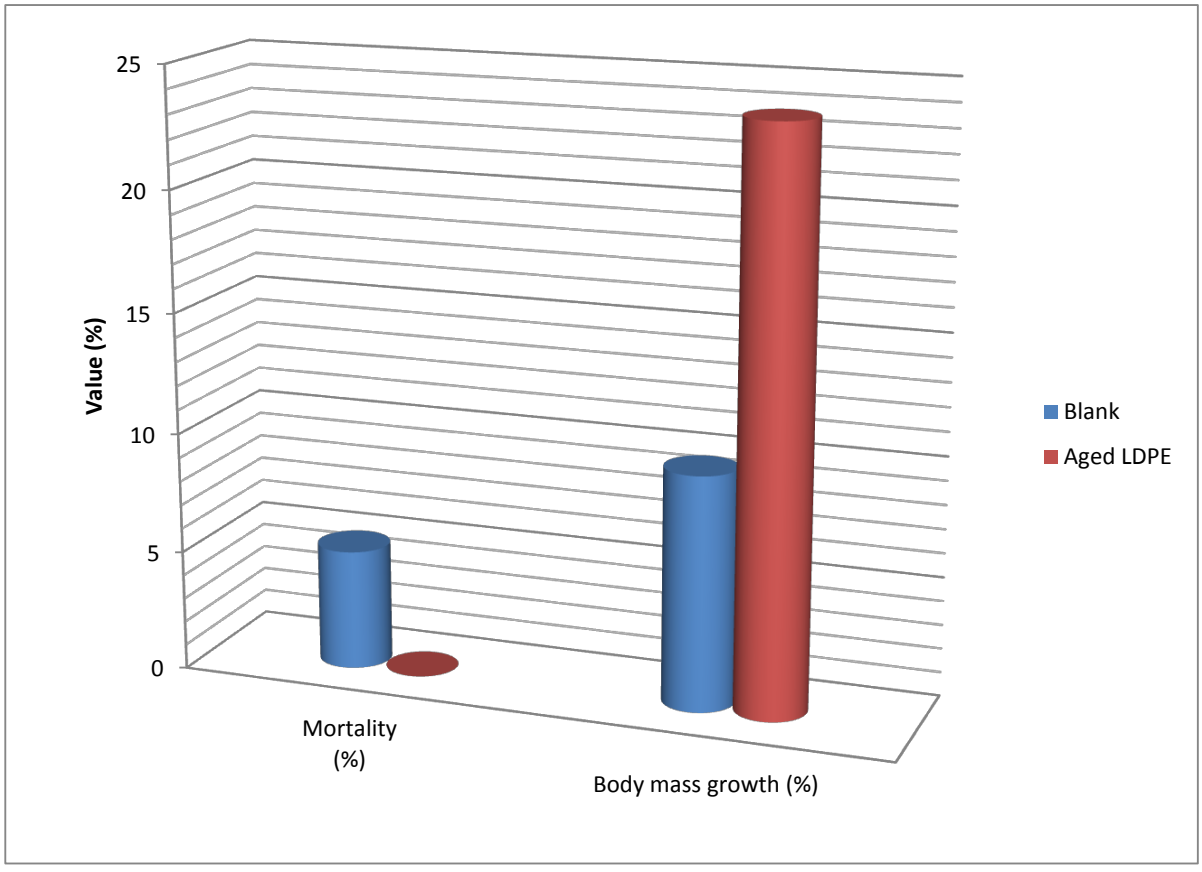
The conditions of validity of the test were met since:

- The average mortality rate of the control is less than 10%
- There was no loss of organisms mass, on the contrary there was an increase of 10%

Comparison mortality and body mass growth between blank and sample (figure reported below) illustrate that the sample, at the concentrations analyzed during the test, did not show significant effects on the survival of the earthworm and an increase in body mass of 24% occurred.



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CONCLUSION

No significant ecotoxic effects for both samples (unaged and aged plastic film) were highlighted.
The eluates obtained from the aged plastic film showed a slight increase in the effects towards the luminescent bacteria compared to the non-aged ones. However, these effects are below the alarm threshold.

No negative effect was registered in germination test (excluding cress).
No negative effect was also observed in case of presence of mineral in the dispersion. On the contrary, a slight increase in germination was found in the presence of the eluates, especially if oxidized.

No negative effect was detected against crustaceous and worms, but indeed for the latter organisms a positive effect was detected in terms of body mass increase.

Review
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Approved
CEO
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