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Soil monitoring of pentachlorophenol by bioavailability and ecotoxicity measurements

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Several approaches to monitor the bioavailability and ecotoxicity of pentachlorophenol (PCP) in sterile and non sterile soils as a function of aging are reported. Porapak resins and water were used to assess the bioaccessibility and the bioavailability of PCP in soil. Aging effects were observed mainly after 240 d of aging. Actual bioavailability, measured as PCP bioaccumulation in earthworms, decreased more markedly with time. The ecotoxicological biomarker neutral red retention time (NRRT) displayed a dose dependent effect but no aging effects after exposing the earthworms to polluted soils. Nevertheless, mortality of earthworms increased after 240 d at 150 mg kg^{-1} contamination. In contrast, the luminescent biosensor *Pseudomonas fluorescens* pUCD607 evidenced in non sterilized samples a slight reduction of ecotoxicity in time related to the degradation of the molecule. Once again, results highlight the necessity to study the fate of soil pollutants with different chemical and biological approaches. Different PCP degradation pathways and/or the different sensitivity of earthworms and bacteria could explain the different behaviours observed.

Introduction

PCP is an organochlorinated compound applied as biocide in agriculture and in the wood industry. PCP is a widespread environmental contaminant of soil, surface water and groundwater: it is known to bioaccumulate, to be very toxic and it is classified in the priority list of organic micropollutants because of its carcinogenity and toxicity.[1] PCP utilization as a pesticide is widely forbidden, but many countries still use it to prevent fungal attacks on wood.[2]

Soil extractions of PCP and other organic xenobiotics are usually carried out with exhaustive extraction methods such as Soxhlet, microwaves, sonication, supercritical CO_2.[3,4] These methods assess the total amount of the xenobiotic and usually overestimate the environmental risk because they do not take into account one of the major processes affecting the fate of xenobiotics in soils, *i.e.*, bioavailability.[5] With time, xenobiotic molecules migrate in soil micropores where they become unavailable for microbial degradation:[5] this time-dependent reduction of bioavailability is defined as aging.[5–7] The physico-chemical properties of both soil particles and the pollutant influence these processes.[8]

Different methods can be used to assess the bioavailability of PCP in soils. The fraction dissolved in the water phase at a given time can be considered as the bioavailable fraction for micro-organisms, especially because at neutral pH the weak acid PCP is mostly present as the more soluble phenolate ion,[9] while the bioavailability to earthworms can be assessed by employing earthworms that feed on and live in the soil and measuring the amount accumulated in their body tissue.[10] Solid phase resin extraction accelerates the desorption of the contaminant from the soil matrix by keeping the xenobiotic concentration in the aqueous phase almost equal to zero through the enhancement of the concentration gradient between the solid and the liquid phase. Some authors recently defined the fraction extracted by methods such as resins as the bioaccessible fraction.[11,12]

Ecotoxicity issues are becoming more and more prominent in the monitoring and risk assessment of contaminated soils and sediments. Coupling of chemical assessments of bioavailability and bioaccessibility with bioanalyses targeting the ecotoxicological effects of pollutants is a sound approach for an integrated monitoring of pollutants in soils.[13,14]

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Environmental impact

It is now widely accepted by the scientific community that the bioavailable rather than the total fraction of contaminants in soils is ecologically relevant. A scientifically sound monitoring and risk assessment of contaminated sites should thus be based on the coupling of bioavailable measurements with ecotoxicity measurements at different trophic levels. The paper reports a multi methodological approach to assessing the bioavailability and ecotoxicity of pentachlorophenol (PCP) in soil as a function of aging: this integrated monitoring approach is coherent with scientific evidences and can be adapted and used to monitor other contaminants too.

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Earthworms are widely employed not only for bioaccumulation assays[15,16] but also for the assessment of acute toxicity in international protocols.[17] Since they accumulate the pollutant in the tissues of the body they can be used as biomonitors. Their behaviour in soil polluted by chlorophenols has been extensively reported.[18,19] A most exact evaluation of the xenobiotic toxicity in the environment should be thus accomplished by joining chemical tests (bioaccumulation and potential bioavailability) to biological analyses by biomarkers.[20] The damage degree of earthworm immune cells (coelomocytes) is a biomarker of exposure and expresses the lysosomal membrane stability which have been evaluated by the neutral red retention time (NRRT) for TNT,[21] heavy metals,[22] fungicides,[23] PAH and organic phosphates,[24] and pentachlorophenol.[25]

Bacteria are extremely useful for environmental studies as biosensors or degraders because of their adaptation to different conditions, their capacity to degrade many organic chemicals and their ease of cultivation and genetic transformation. Microbial biosensors are based on bacteria genetically engineered with reporter genes (*e.g.*, luciferase, green fluorescent protein, galactosidase), whose promoters are activated by the presence of specific chemicals. Response levels (*e.g.*, amount of light or protein production) are a direct measure of the bioavailability and toxicity of pollutants.[26] *Pseudomonas fluorescens* 10586 pUCD607[27] is a luminescence-based biosensor constructed by genetic transformation involving the insertion of *lux* genes from the natural marine bacterium *Vibrio fischeri*. These genes are inserted upstream of a general metabolic promoter and thus reveal the organism's general metabolism by luminescence. *P. fluorescens* are ubiquitous bacteria of the soil environment: reduction of light due to the presence of contaminants is thus a measure of ecotoxicological effects in soil.[28]

An integrated approach in studying the bioavailability and ecotoxicity of PCP in soil as a function of compost amendment has been recently investigated.[29] It evidenced a significant influence of compost on the bioavailability and ecotoxicity of PCP in soils spiked with a high concentration (150 mg kg^{-1}) of the pollutant.

The aim of this study is to present an integrated monitoring of PCP bioavailability and ecotoxicity in soil by joint application of chemical and biological methods. Experiments were conducted in both sterile and non sterile microcosms, and samplings carried out at different times, up to 240 d, from spiking with two PCP pollution levels (15 and 150 mg kg^{-1}) to take into account possible aging effects.

Experimental

Experimental set-up

Experiments were carried out in microcosms spiked in the lab with 15 and 150 mg of PCP kg^{-1} of soil, sterilized by γ-irradiation and aged at room temperature in the dark for 20, 60, 120 and 240 d, in two replicates per treatment. Non sterilized microcosms were also studied.

Bioavailability of PCP was assessed by water batch extraction and earthworm body tissue accumulation, bioaccessibility by Porapak resin desorption, while the ecotoxicity was assessed by evaluation of the sub-cellular modifications of earthworm coelomycetes and through bioassays with the luminescent bacterial biosensor *P. fluorescens* pUCD607.

Test soil, artificial spiking

The soil used in this study was an agricultural soil with 2.4% of organic carbon sampled in Apulia (Italy) and sieved at 2 mm (Table 1). Soil samples were artificially spiked with PCP to final concentrations of 150 or 15 mg of PCP per kg of soil, using a dilution mixing method, with acetone as the carrier solvent.[30] A portion of each sample (10%) was spiked and mixed with PCP dissolved in acetone. The remaining soil was then added to the spiked portion and mixed. Acetone only was applied to the control. After removing the solvent by volatilization at room temperature, control and spiked soils (250 g) were placed in glass jars, and demineralized water was added to bring the moisture to 80% of field capacity. Soil samples were sterilized with 50 kGy of γ-irradiation from ^{60}Co source. Samples were then aged for 20, 60, 120, and 240 d at room temperature in darkness.

Each treatment was carried out in duplicate, and a total of 24 samples were setup, corresponding to a control, PCP treatment at 15 and 150 mg kg^{-1} and four aging periods (20, 60, 120, and 240 d).

Non sterilized soil samples contaminated with 150 mg kg^{-1} of PCP were also analyzed up to 120 d from contamination in order to assess the degradation and bioavailability of the contaminant under more realistic conditions.

PCP total extractable fraction

Three different methods were preliminarily compared for their ability to recover the total extractable fraction of PCP from soil samples: (i) shaking 2.5 g of soil and 10 mL of methanol for 14 h; (ii) shaking 2.5 g of soil and 10 mL of water–ethanol (1 : 1 v:v) solution for 14 h;[31] (iii) sonication of 5 g of soil with 50 mL of acetone–hexane (1 : 1 v:v) solution for 30 min at 220 W in a 28× UltraSONIK™ cleaner.

Methods were applied on three replicates per soil sample immediately after spiking with 15 and 150 mg kg^{-1} of PCP.

Assessment of the bioavailable and bioaccessible fractions

Bioavailability of PCP was assessed with water in batch extraction and with earthworm body tissue accumulation, while bioaccessibility was measured with Porapak P resin (desorption kinetics).

Table 1 Soil physico-chemical parameters and mineral composition

Physico-chemical parameters		Mineral Composition (% dw)	
pH (H_2O)	7.94	Illite and Smectite	19
pH (KCl)	6.88	Illite	53
Coarse sand (%)	2	Caolinite	21
Fine sand (%)	44	Calcite	2
Silt (%)	25	Quartz	3
Clay (%)	29	Plagioclasi	1
Organic Carbon (%)	2.36	Hematite	1
Total nitrogen (‰)	2.35		
Cation Exchange Capacity (meq/100 g)	21.1		
Electrical Conductivity (dS/m)	0.174		

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For water extraction, soil samples (1 g) were weighed in polycarbonate centrifuge tubes with 20 mL of bidistilled water at pH 7. The tubes were sealed, placed on an orbital shaker at 80 rpm for 12 h, and then centrifuged at 5000 *g* for 1 h. After centrifugation, supernatants were filtered and analyzed quantitatively for PCP using HPLC.

Adults of *Eisenia andrei* Bouché were obtained from Compagnoni farm (Lecco, Italy). They were acclimated in the laboratory at room temperature and reared on a standard diet, for at least 1 month before use in the experiments.

After each aging period, 12 mature earthworms were added to each glass container and exposed for 14 d at 24 ± 1 °C in the dark as recommended by the OECD protocol.[17] At the end of the exposure period, surviving worms were rinsed with tap water and left in a Petri dish for 24 h to reject the content of the gut. After the cell viability test and the lysosomal membrane stability evaluation, all earthworms were frozen at −80 °C and freeze dried. Pentachlorophenol was extracted in acetonitrile for 24 h and, after purification, the extracts were analyzed by HPLC.

The resin Porapak P was chosen to assess the PCP desorption kinetics from the soil matrix (bioaccessible fraction) among other resins for its good PCP affinity ($K_d = 1.47 \times 10^4$) and for its lower density than water, which makes it easily separable from the soil suspension.

At the end of each aging period (20, 60, 120, and 240 d), 0.5 g of contaminated soil was suspended in a solution of 8 mL of 0.005 M $CaCl_2$ and 1 mL of NaN_3 (18 mg mL^{-1}) in screw cap centrifuge tubes. Sodium azide was used as bacteria inhibitor. The Porapak resin (100 mg) was added to the soil suspension. Each tube was then incubated in an end-over-end agitator. The resin was separated from suspensions after 1, 3, 24, 72 and 168 h, (centrifugation at 720 *g* and recovering with a steel spatula) and changed with a fresh resin. The separated resin was then resuspended in 10 mL of acetonitrile and, after agitation for 24 h, the extract was analyzed by HPLC.

HPLC analyses

Samples were analyzed using an HPLC-UV Perkin-Elmer LC Mod. 410 high-performance liquid chromatograph and a diode-array detector (Perkin-Elmer, Series 200) set at 220 nm. Pentachlorophenol was eluted on a C-18 column (3.9 × 150 mm) at a flow rate of 1 mL min^{-1}. The mobile phase was a mixture of 0.05% phosphoric acid water solution (60%) and acetonitrile (40%). Concentrations of PCP were quantified from peak areas following linear interpolation of PCP standards injected at increasing concentrations between 0.5 and 5 mg L^{-1}.

Ecotoxicological assays—earthworms

After exposure to the contaminant for 14 d, half of the surviving earthworms were subjected to the ethanol extrusion of coelomocytes in phosphate buffered saline solution (PBS) (95%), ethanol (5%), GGE (10 mg mL^{-1}), EDTA (2.5 mg mL^{-1}). Samples were then centrifuged at 150 *g* for 10 min at 4 °C and coelomocytes were resuspended in LBSS Ca-free[32] two times. Cell viability was evaluated by Trypan blue dye exclusion (0.4%) under a light microscope (200× magnification) using a Neübauer slide.

The neutral red retention time (NRRT) assay indicates the lysosomal stability of coelomocytes. Each cell suspension was treated with the neutral red working solution.[23] Slides were examined under a light microscope (400× magnification) every 2 min. Cell counting ended when at least 50% of coelomocytes had fully stained cytosol. The duration of counting represents the NRRT of the lysosomes.[23]

Ecotoxicological assays—whole cell biosensors

Biosensor assays were carried out on water extracts from non sterilized soils at 20, 60 and 120 d of aging. Cultures of *Pseudomonas fluorescens* pUCD607 (*lux* CDABE from *Vibrio fischerii*, kan^r, amp^r[27]) were grown in 250 mL Erlenmeyer flasks containing 100 mL of LB broth at 25 °C, and rotated at 200 rpm. Kanamycin was added at 50 μg mL^{-1} to all cultures.

Before exposure to the samples, *Pseudomonas fluorescens* cells were harvested by centrifugation and resuspended in 5 mL of 0.1 M KCl. After 20 min of exposure, light output was measured using a SystemSure luminometer Model 18172 (Nova Biomedical Waltham MA, USA). Data were expressed in terms of % of luminescence of samples compared to that of the control (*i.e.*, water extracts from non contaminated soil).

Data handling and statistical analyses

The partitioning of PCP in different fractions was assessed with different methods according to the following definitions.

The bound fraction was defined as the difference between the total applied dose, set as 100, and the amount extracted by exhaustive method from the sterilized soils.

The bioavailable fraction was defined as the quantity extracted by water from sterilized soil.

The aged fraction was defined as the difference in the quantities extracted by exhaustive method and the bioavailable fraction from sterilized soil.

The bound, aged, and bioavailable fractions were assessed in samples from sterilized microcosms: the aim of this was to evaluate the physical partitioning of the molecule in these fractions without the influence of microbial activity. The degraded fraction was determined in the non sterilized microcosms and it was defined as the difference in the quantity extracted by exhaustive method from sterilized and non sterilized soils.

Data were expressed as the mean ± standard deviation. A parametric post-hoc Tukey test for multiple comparisons was used for the statistical analyses by Statistica software (StatSoft, Inc. Tulsa OK, USA).

Results and discussion

Assessment of chemical methods for recovery of the total fraction of PCP in soils

The water–ethanol batch extracted 86.4 ± 2.7% of the PCP applied dose, whereas the acetone–hexane sonication (65.5 ± 3.7%) and methanol batch extraction (64.5 ± 0.8%) appeared to be less efficient in PCP exhaustive extraction. Therefore, the water–ethanol batch extraction was adopted throughout the experiment, in accordance with Khodadoust *et al.*,[31] who

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showed also high recoveries of PCP with this method even after several months of aging.

Chemical assessments of PCP bioavailability

Water and Porapak extractions were tested for their recovery of PCP in sterilized soil at 20, 60, 120, and 240 d from contamination with 150 mg kg^{-1} of PCP. As a comparison, water–ethanol exhaustive batch extractions were also carried out (Fig. 1). 24 h cumulative desorption data with Porapak resin were considered an estimation of the bioaccessible fraction of PCP, in accordance with Harmesen[12] who indicates the first desorption phase as an estimation of the bioaccessible fraction. Results showed that the bioaccessible fraction of PCP was comparable to water–ethanol extractions and mixtures, and even higher up to 120 d of aging. However, after 240 d of aging the bioaccessible fraction reduced to 52.2 ± 3.3%. On the other hand, extraction with water at pH 7 was shown to be a mild non exhaustive extraction technique for PCP recovery. It also proved to be sensitive to aging processes: recovered amounts of PCP were respectively 41.6 ± 2.1%, 36.4 ± 3.2%, 30.8 ± 1.9%, and 27.2 ± 1.6% of the applied dose at 20, 60, 120 and 240 d, respectively. Therefore, the fraction extracted by water can be considered as the fraction of the xenobiotic immediately available and could represent an estimation of the PCP bioavailability for microorganisms.

Chemical data from sterilized microcosms were interpreted in terms of bound, aged and bioavailable PCP fractions, in accordance with the definitions previously discussed. Results are presented as bar graphs in Fig. 2, where the sum of the three fractions always equals to 100% of the applied dose. The bound fraction showed an increase from 20% at 20 d to 38% at 240 d, while the aged fraction was slightly reduced with time, passing from 38% to 34%. The reduction of the bioavailable fraction (from 41% to 27%) seems due to an increase of the bound fraction rather than of the aged fraction. It is possible to observe that the sum of the bioavailable and aged fraction is almost equal to the bioaccessible fraction measured by Porapak resin, except for the last aging time, when the measured bioaccessibility was lower. Therefore the bioaccessible fraction takes into account also the aged fraction that could become available again after a re-equilibrium of the system, for example after the removal of the bioavailable fraction.

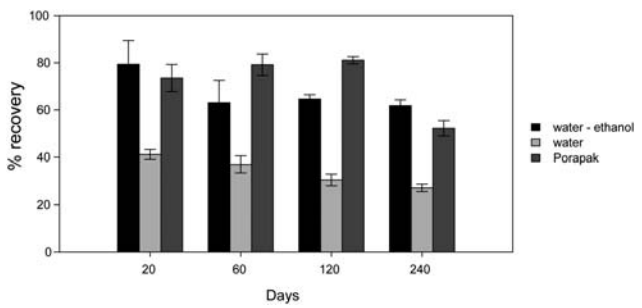

Fig. 1 Comparison of PCP extraction with water, Porapak, and water–ethanol from sterilized soils at 20, 60, 120, and 240 d from contamination with 150 mg PCP kg^{-1} soil.

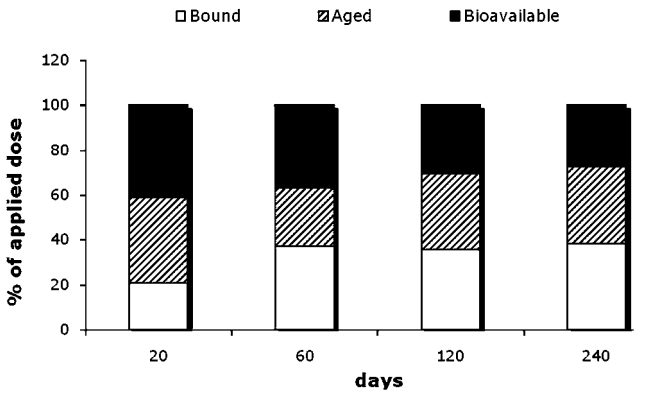

Fig. 2 Partitioning of PCP in bound, aged and immediately available fractions in sterilized microcosms at 20, 60, 120, and 240 d after contamination with 150 mg PCP kg^{-1} soil.

This concept is more evident if the desorption kinetics with Porapak are studied. The desorption kinetics are able to furnish more detailed information on the sequestration of the molecule in the soil matrix and give an idea on the "potential bioavailable fraction" of the molecule, that could become bioaccessible and/or bioavailable over long term periods of time. The total desorbed fraction (pooling the extraction up to 168 h) did not change up to 120 days of aging (about 90%) but reduced significantly to 77.3 ± 4.6% after 240 d (Fig. 3). Moreover, it is possible to observe a significantly slower release of the contaminant in the first 72 h of desorption with the Porapak resin from samples aged for 240 d (lower slope of the curve) that could be ascribed to the beginning of aging effects. So, as the incubation time is significantly increased, the aging effect is more valuable.

Desorption experiments conducted on soil spiked with 15 mg kg^{-1} of PCP with up to 240 d of aging showed that the percentage of PCP desorbed did not differ significantly within samples aged for 20, 60 and 120 d (about 80% after 168 h of desorption), while, it decreased significantly (56.6%) in samples aged for 240 d (Fig. 4). This difference, that could be ascribed to the aging effect, is larger in the first hours of desorption indicating

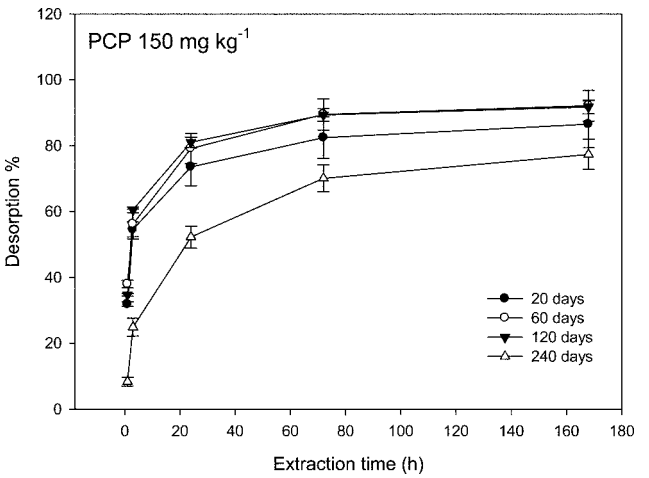

Fig. 3 Pentachlorophenol desorption from soil spiked with 150 mg PCP kg^{-1} soil and aged from 20 to 240 d by means of Porapak P resin.

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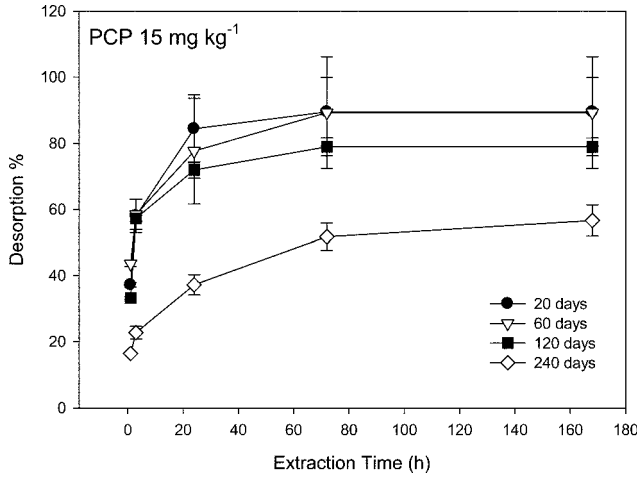

Fig. 4 Pentachlorophenol desorption from soil spiked with 15 mg PCP kg^{-1} soil and aged from 20 to 240 d by means of Porapak P resin.

a migration of the xenobiotic in soil regions more remote from the surface.

This contact time between the PCP and soil matrix probably allowed the beginning of aging processes, that is, the sequestration of pentachlorophenol in the nanopores of the soil matrix.

At 150 mg kg^{-1} of PCP loading, the reduction of the bioavailable fraction after 120 d of aging was less marked probably because it was hidden by the high contaminant concentration.

Actual bioavailability in earthworms

The measurement of the PCP bioaccumulation in earthworms has been carried out on samples aged up to 240 d with the two PCP concentration 15 and 150 mg kg^{-1}. These concentrations were chosen according to the LC_{50} of 87 mg kg^{-1} measured for *E. andrei* exposed to an artificial soil spiked with PCP.[33] Therefore, the lowest concentration was applied in order to observe sub-lethal effects of PCP over time, while the highest dose was chosen to estimate possible aging effects on acute toxicity. After

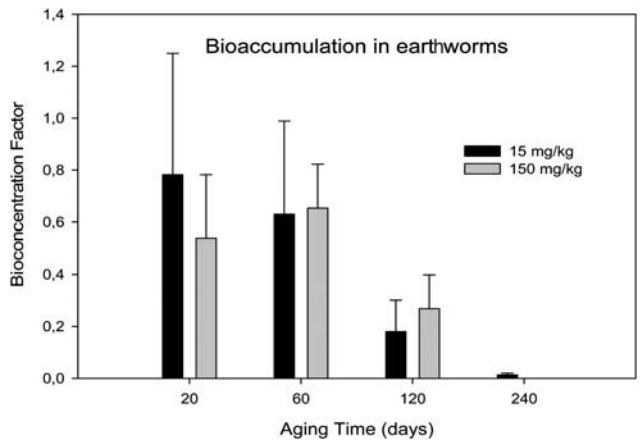

Fig. 5 Actual bioavailability of PCP in earthworms exposed for 14 d to differently aged and polluted soils. The bioconcentration factor is the rate between the PCP concentration in the worms and in soil, respectively.

240 d of aging, the survival rate of earthworms in soils spiked with 15 mg kg^{-1} of PCP was not significantly different to that observed after 20 d, while it decreased down to 0 at 150 mg kg^{-1} of PCP in 240 d.

The amount of PCP accumulated in the worm body was inversely related to the aging-time, as evidenced in Fig. 5, where the bioconcentration factor (BCF), expressed as ratio of the amount of PCP accumulated in worms and the PCP concentration in the soil, is reported in function of aging. After the exposure at 15 mg kg^{-1} of PCP, the BCF decreased from 0.78 ± 0.5 to $1.33 \times 10^{-2} \pm 0.6 \times 10^{-2}$ in samples aged at 20 and 240 d, respectively. Similarly, the BCF registered in the body of earthworms exposed to soils contaminated with 150 mg kg^{-1} of PCP decreased from 0.54 ± 0.2 to 0.27 ± 0.1 in samples aged at 20 and 120 d, respectively (Fig. 5). No earthworms survived after 240 d of aging at 150 mg kg^{-1} PCP.

The results herein obtained from chemical and bio-chemical assays stimulated an in depth study of the earthworm responses using specific biomarkers.

Ecotoxicological assays in earthworms

No significant differences were detected for the viability of coelomocytes collected by ethanol extrusion from earthworms exposed for 14 d to spiked soils (15 and 150 mg kg^{-1}) and the control. The aging of contaminated soils did not affect the cell viability as well (data not shown).

Therefore, in the presence of PCP, no relevant damage of the cell membrane were assessed by Trypan blue. In fact, PCP should modify the bulk lipid fluidity and decrease the phospholipidic phosphate levels, and no alterations in the membrane permeability have been reported.[1]

A significant toxic action on lysosomal membrane was pointed out in earthworms exposed for 14 d to soils spiked with 15 and 150 mg kg^{-1} of PCP and was reflected in a decrease of neutral red retention time.

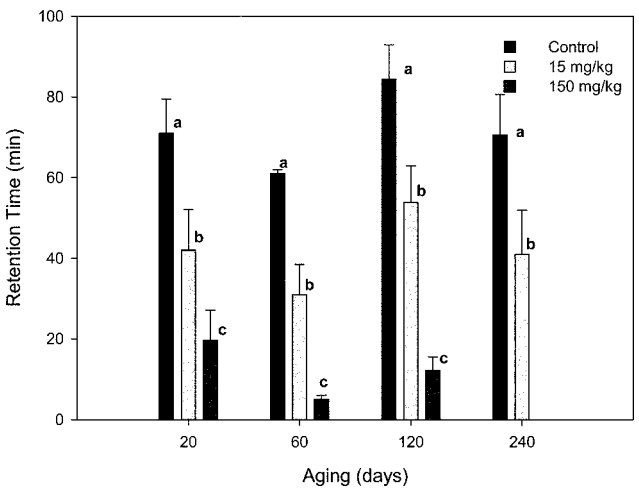

Fig. 6 Neutral red retention time (NRRT) assay on coelomocytes from *E. andrei* after 14 d of exposure in PCP aged soil. Different letters (a, b, c) indicate a significant difference among contamination levels and control within each aging period at the $p < 0.05$ level (parametric pos-hoc Tukey test).

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The NRRT index decreased proportionally to the PCP dosage and, in all aging periods, it was lower in soils spiked with 150 mg kg^{-1} of PCP than in those with 15 mg kg^{-1} and untreated, as reported before after an exposure for 7 d to the contaminated soils.[25] However, no significant aging effects were observed up to 240 d (Fig. 6).

In the present study, the NRRT assay seems to be an early, sensitive, and warning assay to detect toxicity also for PCP. In the cytoplasm of coelomocytes (pH close to 7) the PCP is partially dissociated towards the anion pentachlorophenolate, whereas it is moved towards the undissociated phenol (PCP) inside the lysosomes (acidic pH). The association of neutral and ionized weak acid molecules of PCP at the two sides of the lysosomal membrane could form heterodimers that may act as carriers of hydrogen ions across it. This activity should promote early membrane damage[34] which can be evidenced by the assay. This phenomenon could also explain the dose-dependent effect detected in all aging periods for the earthworms exposed to PCP in soils.

Even though no evidence of change in the ecotoxic response has been reported as a function of aging up to 240 d with the coelomocyte viability assay and NRRT assay, an increasing toxicity of the contaminated soil with aging has been observed, as evidenced by an increase of earthworm mortality at 150 mg kg^{-1} (data not shown). This was in contrast to the effects of other soil xenobiotics,[5,15,35] and with the observed decrease of the PCP bioavailability.

This fact might be related to the role played by the mineral and organic soil components in the abiotic transformation processes of organic pollutants by means of different interactions such as solubilization, adsorption and oxidation. The generation of radical intermediates by oxidation reaction of PCP with inorganic and organic soil components could in fact occur,[36] and the radical intermediates could be more toxic than PCP. Therefore even a very small amount of these intermediates could render the soil very toxic. For example, until now, evidence for variation in PCP toxicity was found in exposed mammalian cells, apparently influenced by the metabolite *p*-tetrachlorohydroquinone.[37,38]

Fate of PCP in non sterilized microcosms: degradation, bioavailability and ecotoxicity

Non sterilized soil microcosms spiked with 150 mg PCP kg^{-1} soil were studied up to 120 d from spiking in order to monitor the amount of the degraded (difference in the quantity extracted by an exhaustive method from sterilized and non sterilized soils), and bioavailable fraction of the contaminant. The ecotoxicity of water extracts was also assessed with the general metabolic reporter *P. fluorescens* pUCD607 (Fig. 7).

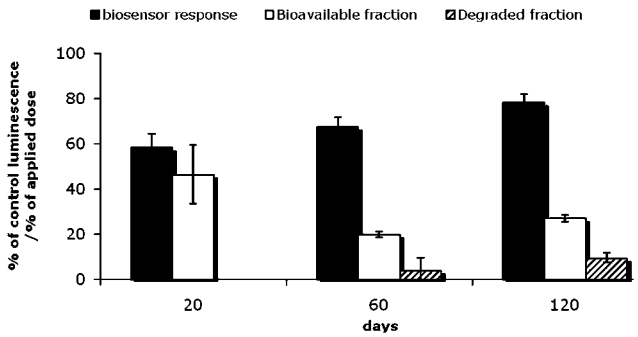

Fig. 7 Biosensor response, bioavailable fraction and degraded fraction from non sterilized microcosms at 20, 60 and 120 d after contamination with 150 mg PCP kg^{-1} soil.

Results showed a very poor degradation of PCP in 120 d, in accordance with literature evidence.[39] At 20 d, no PCP was degraded, and at 60 and 120 d the degraded amount was about 9% of the applied dose. On the contrary, the bioavailable fraction in non sterilized soils, as estimated by water batch extraction, was quite high: 46.5 ± 12% of the applied dose at 20 d, 19.7 ± 8% of the applied dose at 60 d, and 32.9 ± 11% of the applied dose at 120 d. Two important considerations arise from these results: in soils contaminated with PCP the main constraints for remediation are not related to a low availability of the molecule but to its recalcitrance to degradation, at least in soil not inoculated with degrading bacteria or amended with compost.[9] Secondarily, PCP desorption from the soil is not very difficult: after the 60 d sampling a first degradation and a reduction of the bioavailable fraction has been reported. However, at 120 d the bioavailable fraction increased again, probably because of the re-establishment of a new equilibrium distribution of the PCP in soil.

Ecotoxicity was assessed by means of the *lux*-marked whole cell biosensor *P. fluorescens* pUCD607. Results (Fig. 7) are expressed in terms of % of luminescence of water extracts from contaminated samples compared to the luminescence of water extract from a non contaminated sample, so higher values refer to lower toxicity. The highest toxicity was observed at 20 d (52.4% of control luminescence), which was then reduced at 60 d (72.7% of control luminescence) and at 120 d (74.4%). This could be due to a formation of low persistent and soluble metabolites of higher toxicity toward *P. fluorescens* just after spiking. These results are partly in contrast with the observed increase in mortality of earthworms after 240 d of aging. However it should be underlined that this assay is conducted on water extract and therefore could underestimate the presence of metabolites poorly soluble in water differently from the assay with earthworms conducted directly in soil.

Conclusions

In this report, several approaches were studied to assess the different fraction of PCP in soil as a function of aging coupled to ecotoxicological evaluation of the polluted soil based on earthworm immune system response and luminescent biosensors. The bioaccessibility of PCP in soil is very high at least in the first 120 d of aging. Starting from 240 d a noticeable aging effect was evident. On the contrary, the bioavailable fraction of PCP, measured by water extraction and with the PCP bioaccumulated in the worm body is low and decreased constantly with time.

However, the increase in earthworm mortality and obviously of toxicity observed at the highest PCP concentration with aging (after 240 d) was in contrast with the reduction of the potential bioavailability. Probably, this negative aging effect might be due to the partial conversion of PCP into more toxic compounds in soil or within the body of the earthworms.

With the aim to overcome the limits of chemical and/or biochemical analyses (chemical-mineral soil parameters and animal sensitivity towards the PCP) which do not supply

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a complete understanding of the process, and in order to carry out an integrated monitoring of chemical and ecotoxicological assessment of PCP in soil, biological tests to estimate the effective toxicity of this pollutant were also conducted.

The NRRT assay, which was carried out on the earthworm cells exposed to PCP in sterilized soils, displayed a dose dependent effect. On the contrary, this index was not able to assess any aging effects.

In order to evaluate the rate of PCP degradation and the toxicity towards *P. fluorescens* pUCD607 in an environment possibly more closed to the field, the biosensors were applied on non sterile soils. In this case, an aging effect was observed, with a decrease of the toxicity and an increase of the degraded fraction of PCP, after 60 and 120 d.

The biological results suggest that the different behaviour observed in sterile and non sterile soils could be due to different degradation pathways of PCP (abiotic degradation *versus* biodegradation) and to the different sensitivity of earthworms and bacteria to PCP and its metabolites. Therefore, once again this study highlights the necessity to carry out an integrated approach with different chemical, biochemical and biological assays for a correct risk assessment of a contaminated site.

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