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Abbreviation list:
NP, nonylphenol, mixture of isomers; 4NP, 4-*n*-nonylphenol; SA, sludge from Ambares; SMF, spore multiplication factor; SP, sludge from Plaisir.

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Abstract

The impact of nonylphenol on fungi following the application of contaminated sewage sludge on agricultural soil was studied in laboratory experiments. Nonylphenol bioavailability and adsorption were determined in the soil alone and soil/sludge mixtures. Mixing the soil with sludge made it possible to measure the nonylphenol concentration in the soil solution comprised between 6.6 10^{-6} M and 3.8 10^{-7} M according to the sludge. We then examined the dose-response relationship between nonylphenol concentration in the culture medium and both biomass production and germination rate of the spores from several strains of filamentous fungi. When applied in this range of concentration, nonylphenol was without noticeable short-term impact on these endpoints. Long-term exposure of fungi to nonylphenol was also assessed. The most intensive effect was a strong stimulation of spore production and germination in *Fusarium oxysporum*. Biomass production by the *Fusarium* strains also increased. Finally, nonylphenol was shown to induce laccase production in *Trametes versicolor*. We conclude that the potential of nonylphenol to adversely impact soil fungi remains low.

Introduction

In many countries, application on cultivated land of sewage sludge produced from wastewater treatment plants is common practice. New risks are emerging today for both the environment and human health. Indeed, there may be adverse effects for micro-organisms and higher plants, as well as for consumers (animals or humans) because of soil, water, feed and food contamination (Bokern et al., 1998) by heavy metals, organic compounds and biological agents.

Among organic chemicals, wastewater entering treatment plants contain nonylphenols (NP, a mixture of branched isomers), an important compound in the production of many commercial and industrial chemicals. NP is used to produce nonylphenol polyethoxylates, which are nonionic surfactants widely used in domestic, agricultural and industrial applications.

NP is subsequently discharged into surface waters through the microbial biodegradation of these polyethoxylates in sewage treatments (Ahel *et al*., 1994a), and potential aquatic risks have been extensively studied (for reviews see Sumpter, 1998; Servos, 1999; Nilsson, 2000; Vos *et al*., 2000). In this context, it is important to make a distinction between 4-*n*-NP (a main compound present in the mixture of isomers) and NP (the mixture of isomers) because they

have some distinct physico-chemical properties. However, studies concerning the fate and toxic effects of nonylphenol compounds refer in most cases to 4-*n*-NP alone. Both *in vitro* and *in vivo* studies reported that this isomer is a potent endocrine disrupter, modulating steroidogenesis and activity of hormone-metabolizing enzymes, and inducing feminization. It binds onto cellular estrogenic receptors, thereby regulating the expression of estrogen-responsive genes (Machala and Vondracek, 1998), and can cause proliferation of breast cancer cells in women (Soto *et al.,* 1991).

However, most NP formed during wastewater treatment is associated with sludge, amounting to 1 g or more *per* kg of dry sludge (Ahel and Giger, 1985; Ahel *et al.,* 1994a and b; APE Research Council, 2002). NP is released onto terrestrial environments through sludge application on agricultural land. Nevertheless, only little work has been done on the fate of NP in soils (Topp and Starratt, 2000; Hesselsoe *et al.,* 2001, Dubroca *et al.,* 2003), and an assessment of the potential risk has rarely been conducted (APE Research Council, 2002). Yet, some studies revealed that NP can modify the structure of microbial communities of lake sediments (Jontofsohn *et al*., 2002). In the laboratory, production of carbon dioxide from soil is inhibited by concentrations of NP higher then 100 mg/kg (APE Research Council, 2002), which proved to be toxic to filamentous fungi and yeasts as the result of uncoupled respiration (Karley *et al*., 1997). Finally, it has been established that endocrine disrupters can interfere with flavonoid signalling during plant-bacterial symbiosis (Fox *et al*., 2001). To our knowledge, experiments have seldom been performed taking into account NP already present in the sludge, then the amendment of soil with the contaminated sludge. Using this experimental approach, Gejlsbjerg et al. (2001) were unable to demonstrate a negative impact of NP on bacterial denitrification, nitrification or aerobic respiration in soils.

The objective of the present study was to assess the impact of NP on soil fungal populations as a result of contaminated sludge application. Adverse effects on the soil ecosystem depend on the bioavailability, degradation and toxicity of the contaminant. The fate of NP in soil has been previously published (Dubroca et al., 2003). We examine here 1) the potential concentration of NP to which soil organisms may be exposed, 2) the relevant toxicity by studying endpoints related to fungal growth (biomass production) and reproduction (sporulation and germination of spores).

Materials and Methods

Chemicals

4-*n*-Nonylphenol (4-*n*-NP) was obtained from Lancaster, whereas technical NP (NP) was obtained from Fluka. Other chemicals were available from Sigma. Labelled 4-*n*-NP (2 GBq $mmol^{-1}$, radiochemical purity > 99%) was a generous gift of Dr J.-P. Cravedi (INRA, Toulouse).

Soil and sludge characteristics

The soil used in this study was a silt loam, comprising 25.5% sand, 55.0% silt and 19.5% clay. Its organic matter content was 1.65%. The soil pH was 8.1 and the cationic exchange capacity was 10.2 meq 100 g^{-1}. The soil, collected in the 10-20 cm layer in a field, was sieved (2-mm) and used immediately.

Two types of sludge exhibiting different characteristics were applied on soils (Table 1). The sludge from Ambares (SA) was formed following the treatment of both urban (90,000 equivalent inhabitants) and industrial wastewaters, whereas that from Plaisir (SP) collected mostly urban wastewater (42,000 equivalent inhabitants).

Bioavailability of NPs

Amounts of NP bioavailable for soil fungi were determined using the protocol described by Gaillardon (1995). Soil and soil/sludge samples (10 g equivalent dry matter) were placed in 5-cm diameter Petri dishes to give a 3-4 mm thick layer. Aqueous solutions of unlabelled NP and labelled (4.0 kBq) 4-*n*-NP were applied to the surface of the soil alone by pipette to ensure 80 % of the moisture holding capacity of the soil, and a final concentration of 40 mg kg^{-1}. Sludge samples were spiked with the mixture to allow the same final NP concentration in the soil. After adding the chemical, the solvent (acetone) was left to evaporate for 30 min. The chemicals were sorbed to the sludge for 24 hours at 4°C under nitrogen atmosphere before the sludge was mixed with the soil. Soil/sludge ratio was 95/5 on a dry weight basis. All the dishes were placed in the dark at 4°C to avoid biotransformation.

Concentrations of NP in the soil solution were determined 4 hours after treatment (soil alone) or mixing (with sludge). Two superposed 42.5 mm diameter glass micro fibre filters GF/A (Whatman) were laid on the soil surface and a slight pressure was applied for 10 s to favour wetting of the filters. The upper filter was then recovered. The volume of soil solution and the

dissolved radioactivity retained in the filter were determined by weighing and liquid scintillation counting.

Adsorption isotherms

Adsorption isotherms were obtained using the batch equilibrium method. Aliquots of 25 mL of 0.05, 0.10, 0.15, 0.20, 0.30 and 0.40 mg L^{-1} water solutions of NP (unlabelled NP and labelled 4NP) supplemented with 10^{-2} M $CaCl_2$ were added to 5 g of soil or soil/sludge mixture, in centrifuge glass tubes. Equilibration was achieved by stirring for 24 hours at 20°C. 5-ML aliquots of the supernatants were removed by centrifugation at 4220 g for 30 min and immediately analyzed. Concentrations of free NP in the supernatant (the equilibrium concentrations, Ce) were determined by liquid scintillation counting. Control blanks were run in parallel to measure tube-wall adsorption of NP. Adsorption data were fitted with the Freundlich equation, $x/m = KCe^{1/n}$, where x/m is the amount of NP adsorbed in mg kg^{-1}, and Ce is the equilibrium solution concentration of NP in mg L^{-1}. The constant K is a measure of the magnitude of adsorption, or adsorption capacity of the sorbent.

Fungi

The fungal strains *Fusarium oxysporum, Fusarium solani* and *Mucor racemosus* used in this study were all isolated from soil samples. The white-rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor* were obtained from the ATCC (referenced as 24725 and 32745, respectively). They were maintained at 4°C on agar plates.

Fungal liquid cultures and preparation of suspensions of spores

Fungal strains used to produce suspensions of spores were grown on a culture medium already published, containing glycerol (*P. chrysosporium*, Mougin *et al*., 1994) or maltose (all other strains, Lesage-Meesen *et a*l., 1996) and ammonium tartrate as carbon and nitrogen sources. A mycelial mat on agar plugs (10 mm diam) was inoculated into 10 ml of the culture medium in a 150-mL Erlenmeyer flask. Cultivation was carried out statically in the dark at 25°C. After 8-12 days of growth, the spores were harvested by shaking the cultures with glass beads, counted and conditioned as liquid suspensions containing $2.5 \pm 0.5\ 10^6$ spores mL^{-1} for inoculation.

Short-term toxicity assessment

Culture media were supplemented with NP ranging from 10^{-3} to 10^{-7} M immediately after inoculation (acetonic solutions, 50 µL per Erlenmeyer). Effect of NP on spore germination was determined after a 24-hour exposure by calculating the germinating rate. Fungal biomass was separated from the medium after 2, 4 and 8 days of culture and dried overnight at 105°C for dry weight determination. Effect of NP on sporulation was estimated by harvesting the spores formed from the cultures and inoculating them in liquid media not supplemented with NP. The germinating rate was also calculated after 24 hours.

Long-term toxicity assessment

Suspensions of spores were used to inoculate Erlenmeyers containing 10-mL liquid media supplemented with 5.0 10^{-6} M NP, or solutions of the chemical diluted by 2 at each inoculation to reach 3.1 10^{-7}M at the end of the experiment. The cultures were then allowed to grow and sporulate in the dark at 25°C for 10 days. Spores were harvested as described above and the experiments were still repeated 4-times. Fungal biomass was measured on a dry weight basis. Germination and sporulation were expressed as a spore multiplication factor (SMF), which reflects the ratio between the amounts of spores produced by a culture versus the amounts of spores used for inoculation.

Laccase induction

Cultivation was carried out as described above for 4 days. After this time, ethanolic solutions of NP or 4NP were added to the cultures to give 0.5 10^{-3} M (100 µl solution per Erlenmeyer flask). Aliquots were assayed for laccase activity after a further 3-day incubation.

Laccase activity measurements

Laccase production was assessed by measuring enzymatic oxidation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS) at 420 nm ($\varepsilon = 3.6 \times 10^4$ cm^{-1} M^{-1}) according to Wolfenden & Wilson (1982). The reaction mixture contained 20 µl extracellular fluid and 980 µl 1 mM ABTS in 0.1 M KH_2PO_4/citric acid buffer (pH 3.0) at 30°C. The buffer solution was saturated with air by bubbling prior to the experiment. One unit of enzyme activity is defined as the amount of enzyme that oxidises one µmol ABTS in one min.

Experimental design

Each experiment was carried out in triplicate. Results are expressed as means ± SD. IC_{50} were calculated by non linear regression analysis. In several experiments, a Student's *t*-test was performed to determine significant differences.

Results

Exposure assessment of soil fungi to NPs

The two sludges contained high levels of NP (Table 1). SA was spiked with additional NP to ensure a final concentration of 40 mg kg^{-1} chemical after sludge application. That experimental design allowed us to calculate the half-lives of NPs in soil, before and after sludge application (Dubroca et al., 2003). The value was 4 days in the non-amended soil, and it increased to 16 and more than 16 days after application of SA and SP, respectively. In the latter case, a lag phase of 8 days in NPs biotransformation suggested the complete inactivation of the endogenous microflora by the sludge.

These results incited us to measure NPs concentration in soil solutions for non-amended and amended soils. Values obtained were 1.93 ± 0.23 mg L^{-1} for the soil alone, 1.46 ± 0.73 mg L^{-1} for the soil/SA mixture, 0.083 ± 0.004 mg L^{-1} for the soil/SP mixture. Mean values corresponded to concentrations of 8.8 10^{-6} M NP for the non-amended-soil solution, 6.6 10^{-6} M (after SA application: A-limit) and 3.8 10^{-7} M (after SP application: P-limit) NPs in soil solutions (Table 2).

Experimental data for adsorption of NP to the soil and the soil/sludge mixtures resulted in a good fit according to the Freundlich equation (correlation coefficient significant at the 0.05 level). Adsorption data for the non-amended soil fitted with a non linear isotherm (L-type, $1/n < 1$), while data for the two amended soils were better described by a linear isotherm (C-type, $1/n \approx 1$)(Table 2). K values for the soil alone and the soil amended with SA were similar, suggesting no great change of adsorption of NP due to sludge A application. By contrast, SP application led to an increased adsorption of NP, with a 20-fold enhanced K constant.

Short-term toxicity of NP on fungi

We examined the dose-response relationship between NP concentration (ranging from 10^{-3} to 10^{-7} M) and the development of fungi in liquid cultures. Only 4 fungal strains, namely *P. chrysosporium*, *F. oxysporum, F. solani* and *M. racemosus* produced spores in our culture

conditions, whereas *T. versicolor* was unable to sporulate. This strain was inoculated as mycelial mats grown on agar plugs.

We first calculated the concentrations decreasing the germination of spores by 50% after 24 hours (IC_{50}) compared to untreated controls (Table 3). Our results showed that the "mother" spores obtained from *P. chrysosporium* were very sensitive to NP, with a IC_{50} of 7.5 10^{-6} M on germination rate. This value is similar to A-limit. The "mother" spores obtained from the 3 other strains were affected to a lesser extent.

We then studied the impact of NP on fungal growth by measuring biomass dry weight after 2, 4 and 8 days of culture in the presence of NP. Fungal sensitivities to NP varied according to the chemical concentration and the duration of the growing period (Table 3). The white-rot fungi *T. versicolor* and *P. chrysoporium* grew in all treatment conditions and were the least sensitive to NP, with concentrations causing 50% inhibition (IC_{50}) globally amounting to 10^{-4} and 5.0 10^{-4} M after 2 and 4 days for the second strain. Only a slight inhibiting effect of NP was observed concerning the growth of *T. versicolor* at 10^{-3} M, the higher concentration tested. The growth of *F. solan*i was also less sensitive to NP than that of *M. racemosus* and *F. oxysporum*. For these last two strains, CI_{50} were globally comprised between 5.0 10^{-6} and 5.0 10^{-5} M, respectively. In all cases, the adverse effect of NP on fungal growth observed on day 2, was decreased at day 4, and totally disappeared after 8 days of incubation. We were unable to observe a lethal effect (LC_{100}) of NP on *T. versicolor* (fungal growth from agar plugs), *P. chrysosporium* and *F. solani* (germination of "mother" spores) in our experimental conditions, whereas the growth of *M. racemosum* and *P. oxysporum* was totally inhibited with 10^{-4} M NP. Only IC_{50} calculated for *M. racemosum* and *P. oxysporum* were comprised between the A- and P-values measured for NP concentration in the soil solution.

In addition, high doses of NP (above 10^{-4} M) induced morphological defects in *F. solani* hyphae. In summary, NP treatment resulted in a swelling of hyphae, associated with the loss of hyphal apical dominance, and increased branching (data not shown).

Finally, we studied the ability of "daughter" spores produced by fungi grown in the presence of NP to germinate in media free of the chemical. Their sensitivity appeared somewhat increased regarding those of "mother" spores, as calculated IC_{50} values were 2- to 80-fold lower (Table 3).

In addition, the suspensions of "daughter" spores obtained from the two strains of *Fusarium* grown in the presence of high concentrations of NP included numerous small spores (microspores) which were not able to germinate within the 24-hour period taken into account (data not shown).

IC_{50} values influencing fungal growth and reproduction are in most cases higher than A-limit, the highest value measured for the amount of NP bioavailable in soil solutions.

Long-term toxicity of NP on fungi

We examined the effect of NP on fungi following 5 cycles of biological events that could affect the size of fungal populations, namely spore germination, biomass formation and spore production. Three culture conditions were used: controls without NP, assays under constant NP concentration of $5.0\ 10^{-6}$ M (below most of the IC_{50} values calculated above), and assays under decreasing concentrations of NP, thus reaching $3.1\ 10^{-7}$ M at the end of the experiment. These decreasing values mimic the natural disappearance of the chemical in the soil due to its transformation. These concentrations are consistent with P- and A-limits.

The calculated SMF, integrating both the production and germination of spores, varied according to the strains. As a general case, the SMF naturally decreased in control cultures after successive exposure of spores to NP, with a maximal effect between the two first generations (Figure 1). NP also affected diversely this endpoint. A slight negative impact of NP on *P. chrysosporium* SMF was significant only at the beginning of the experiments, whilst a stronger effect was noticed throughout the experiment regarding *M. racemosus* and *F. solani*. For these three strains, the effect did not seem related to NP level in the medium. The most intensive impact of NP on fungal SMF was observed on *F. oxysporum* cultures. The SMF corresponding to this strain dramatically decreased in the absence of the chemical. By contrast, it increased up to 100 times by NP, with an effect increasing with the number of biological cycles. In that case too, the pollutant level did not modulate the intensity of the effect.

Biomass production in the control cultures tended to decrease as the number of biological cycles increased (Figure 2). The presence of NP had a diverse effect on this decrease depending on the fungal strains considered. No significant impact (at the 0.05 level) could be observed when *P. chrysosporium* and *M. racemosus* were exposed to constant or decreasing levels of NP. By contrast, a significant effect of NP was observed for *F. solani* and *F. oxysporum*, whose growth seemed to be stimulated in the presence of NP at least at the end of the experiment, whatever the conditions of treatment.

Induction of laccases by NP and 4NP

Laccases are exocellular multicopper oxidases produced by numerous white-rot fungi. They can be induced by xenobiotics (Mougin et al., 2002). The dose-response relationship between

NP concentration and laccase induction was investigated in liquid cultures of *T. versicolor* by measuring ABTS oxidation. Laccase activity in the control amounted to 0.16 ± 0.02 U mL^{-1} after 3 days of treatment (Figure 3). It was enhanced by 3 to 4 for NP concentrations ranging from 10^{-7} to 10^{-5} M in the medium. These values were consistent with the A- and P-limits. At higher concentrations, laccase activity increased with NP doses. Among the isomers, 4NP was shown to be a potent inducer thus increasing laccase activity by 14 after 3 days at 5 10^{-4} M. Between 10^{-6} and 10^{-4} M, NP was also shown to increase lignin peroxidase production 3 fold in *P. chrysosporium* cultures (data not shown).

Discussion

Application of wastewater sludge on agricultural soils raises questions about the bioavailability of organic pollutants for living organisms and their possible negative impact on the exposed organisms.

The amounts of chemicals bioavailable for soil micro-organisms are rarely determined. Our results concerning the amount of NP measured in the soil solution under several adsorbents (soil or soil/sludge mixtures) depend on the physico-chemical properties of the sludges. They demonstrate the decrease in bioavailability of NP following the application of the sludge from Plaisir onto the soil. Nevertheless, these amounts remain easily measurable, and our experiments provide minimum and maximum values for the concentrations of NP that can be used in fungal liquid cultures to assess the impact of the chemical in realistic environmental situations.

Experimental data for adsorption of NP to the soil and the soil/sludge mixtures resulted in a good fit to the Freundlich equation. Nonlinear L-shaped isotherms indicated that our non-amended soil has a moderate affinity for NP in the initial stages of adsorption (Giles et al., 1960, 1974). Linear C-type isotherms obtained in the presence of the two sludges suggested a constant partition of NP between the solution and the adsorbent. The calculated adsorption coefficient (K) for NP onto the soil/SP mixture was 20-fold higher than the value obtained with the other adsorbents, evidencing a higher extent of adsorption of NP in SP. These results agree with the lower amount of NP (two orders of magnitude) measured in the soil solution using static measurement. In soils, it has been reported that phenolic compounds of moderate hydrophobicity adsorb onto organic matter. Application of the sludges with similar amounts of organic matter resulted in distinct effects on adsorption, thus suggesting the involvement of other parameters, such as their chemical composition. Taken together, our data provide

information for the first time on exposure of soil micro-organisms to NP. They show that the bioavailability of NP can be significant when applied to soil following incorporation into sludge. In the absence of measured data, calculated predicted environmental concentrations (PEC) amounted to 2-4 mg kg^{-1} (APE Research Council, 2002). The soil contamination used in our study was 10-fold higher, in order to take into account the worst case hypothesis, such as massive soil amendment with heavily contaminated sludge.

Impact of NP on fungi was then assessed using 3 strains isolated from soils (namely *F. solani*, ascomycete; *M. racemosus*, zygomycete, *F. oxysporum*, deuteromycete) and two white-rot basidiomycetes from our collection (*P. chrysoporium* and *T. versicolor*) that could also be found in soils. Endpoints taken into account concern reproduction (production and germination of spores) and growth (biomass production), both governing the size of the fungal populations. It is difficult to assess an impact on fungal populations directly grown in soils. For this reason, some experiments were performed using fungal liquid cultures.

Short-term (acute) toxicity of NP was assessed first. Generally, the germination rate of fungal spores seemed moderately sensitive to exposure to NP in environmentally-sound amounts. Nevertheless, even in the presence of higher amounts of NP, inducing a strong inhibition of germination, the consequences on the size of the resulting populations were slight. In fact, an adverse effect of NP on fungal growth was only detected in young fungal cultures, in the 2 days following the treatment with the chemical. This kind of lag phase in growth has already been reported concerning *Neurospora* cultures exposed to NP with similar IC_{50} values (Karley et al., 1997). This effect was reduced after 4 days of culture, and totally suppressed after 8 days. These results suggest that nutrient availability remains the main factor governing fungal growth, as soon as the spores germinate. In our experiments, most of the calculated IC_{50} for fungal growth (after 2 days of exposure) and spore germination were in the 1-10 10^{-6} M range. They compare well with the acute concentrations causing 50% lethality or inhibition in fish, amphibian, invertebrate, mollusc or algae species (Weinburger et al., 1987; Servos, 1999). They were below the values reported to inhibit bacterial nitrification (Gejlsbjerg et al., 2001).

However, exposure to NP at doses higher than these resulting from soil amendment under good agricultural practice is not without other effects on fungi. An uncoupling effect of NP on respiration has already been described in liquid cultures supplemented with high concentrations of the chemical (Karley *et al*., 1997). These authors also reported morphological defects in hyphae in *Neurospora crassa*, consisting in a swelling of hyphae

associated with the loss of hyphal apical dominance and increased branching. These abnormalities could be due to disruption of the hyphal free cytosolic Ca^{2+} gradient, the H^+ gradient, and the actin cytoskeleton of the apical cells (Jackson and Heath, 1993; Karley et al., 1997). We observed these abnormalities in *Fusarium solani* cultures (another ascomycete), as well as the formation of immature microspores. These results must be considered from an ecological point of view, because ascomycetes represent the main populations of soil fungi. In contrast, our results show that the white-rot basidiomycetes studied appeared less sensitive to NP than the other strains.

Possible long-term (chronic effects) were also investigated. We decided to integrate both the production and germination of the spores in a global index named the spore multiplication factor (SMF). The decrease often observed in the non treated cultures reflects the loss of performance of the fungal strains due to natural nutrients possibly missing in our synthetic culture media. Nevertheless, both an increase of the SMF or its decrease suggest a chemical stress caused by NP. In chronic toxicity tests, no observable effect concentration (NOEC) has been determined as low as 10^{-5} M in fish and 10^{-8} M in invertebrates (Servos, 1999). Threshold for chronic toxicity on organisms living in sediments was 10^{-5} M (APE Research Council, 2002). Our data showed that a constant exposure of some fungal strains to 5.0 10^{-6} M NP modified their sporulation.

How NP can modify the reproduction of fungi remains to be elucidated. It has long been established that inhibition of the germination rate is mainly due to an effect on respiration, a typical mode of action of fungicide compounds (Leroux, 1996). However, little is known on the endogenous factors affecting sporulation, such as sexual hormone signalling. On one hand, several fungi are able to produce or metabolize several steroid hormones, thus affecting fungal growth and development (Brasch, 1997; Rizner et al., 1999; Rizner and ZakeljMavric, 2000). On the other hand, trisporic acid is a sexual hormone of zygomycetes, which triggers the first steps of zygophore formation (Czempinski et al., 1996). It is likely that NP, a well-known endocrine disrupter, may interfere with these sexual hormone pathways. The growth of *P. chrysosporium* and *M. racemosus* was not affected by NP in our experimental conditions maintained during 5 biological cycles.

NP has also been shown to induce laccase production. 4NP is a more potent inducer than the complete mixture. This effect on extracellular oxidases may be considered as a positive effect, because it leads to a decreased bioavailability of the chemical and to its increased detoxication. This reaction may also lead to the stabilization of the chemical in the soil, thus

preventing groundwater contamination. As demonstrated earlier (Mougin et al., 2002), our present results confirm that xenobiotics can be potent inducers of extracellular enzymes of the ligninolytic pathway on fungi, even in low concentrations.

The relative tolerance to NP exhibited by several fungal strains could be related to their high efficiency in transforming NP. For example, white-rot basidiomycetes (*P. chrysosporium* and *T. versicolor*) are known to secrete extracellular oxidases (peroxidases and/or laccases) that are able to catalyze the rapid polymerization of NP through oxidative coupling (Tsutsumi et al., 2001; Dubroca et al., 2003). The reaction located in the culture medium efficiently reduced the bioavailability and toxic impact of the chemical. In addition, zygomycetes including *Cunninghamella* and *Mucor* sp. are known to possess intracellular P450 systems able to efficiently transform pollutants (Mougin, 2002).

Conclusions

These preliminary experiments show that the potential of NP from sewage sludge applications to have an adverse impact on soil fungi is rather low, because of reduced exposure to the chemical. Nevertheless, it remains necessary to develop extensive chemical monitoring and toxicity studies in order to evaluate much more accurately the possible impact of organic compounds present in sludge used as soil amendment. These points should be completed by physiological and biochemical studies to better identify the modes of action of NP on fungi.

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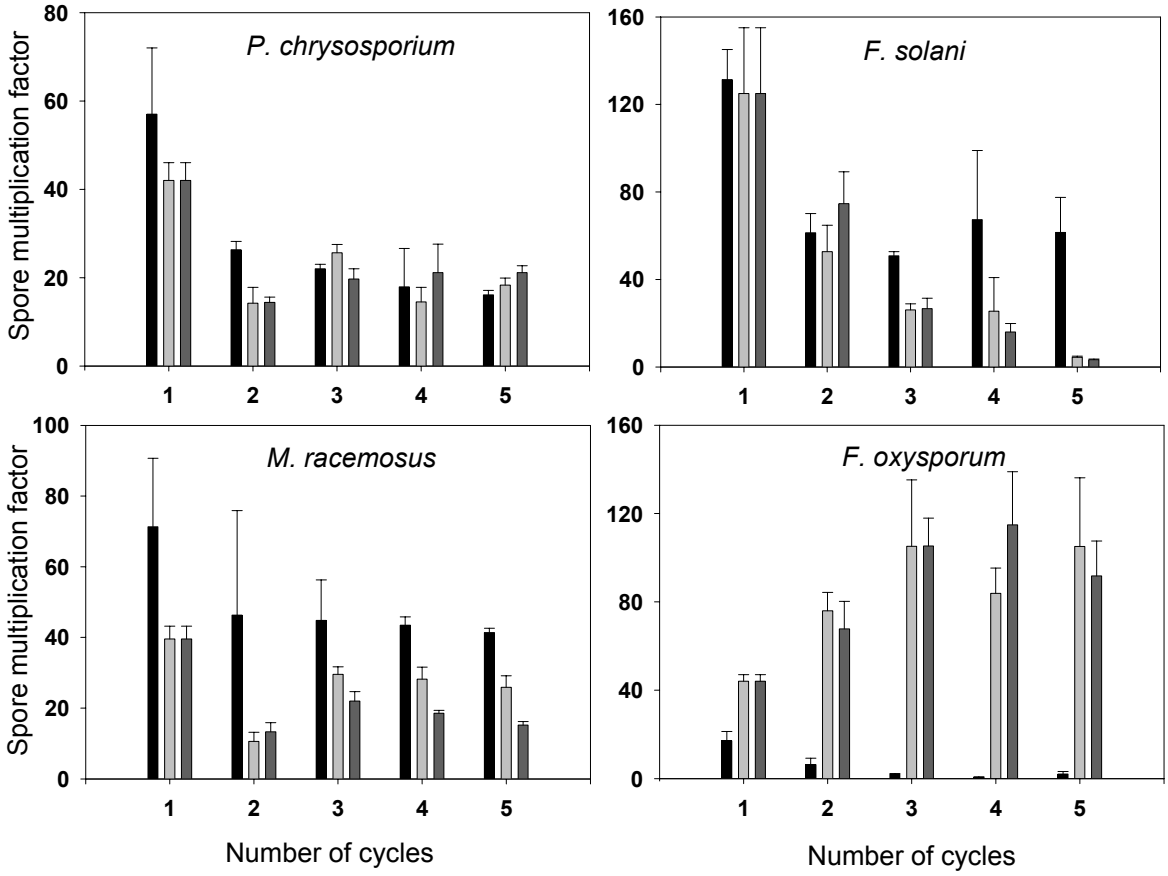
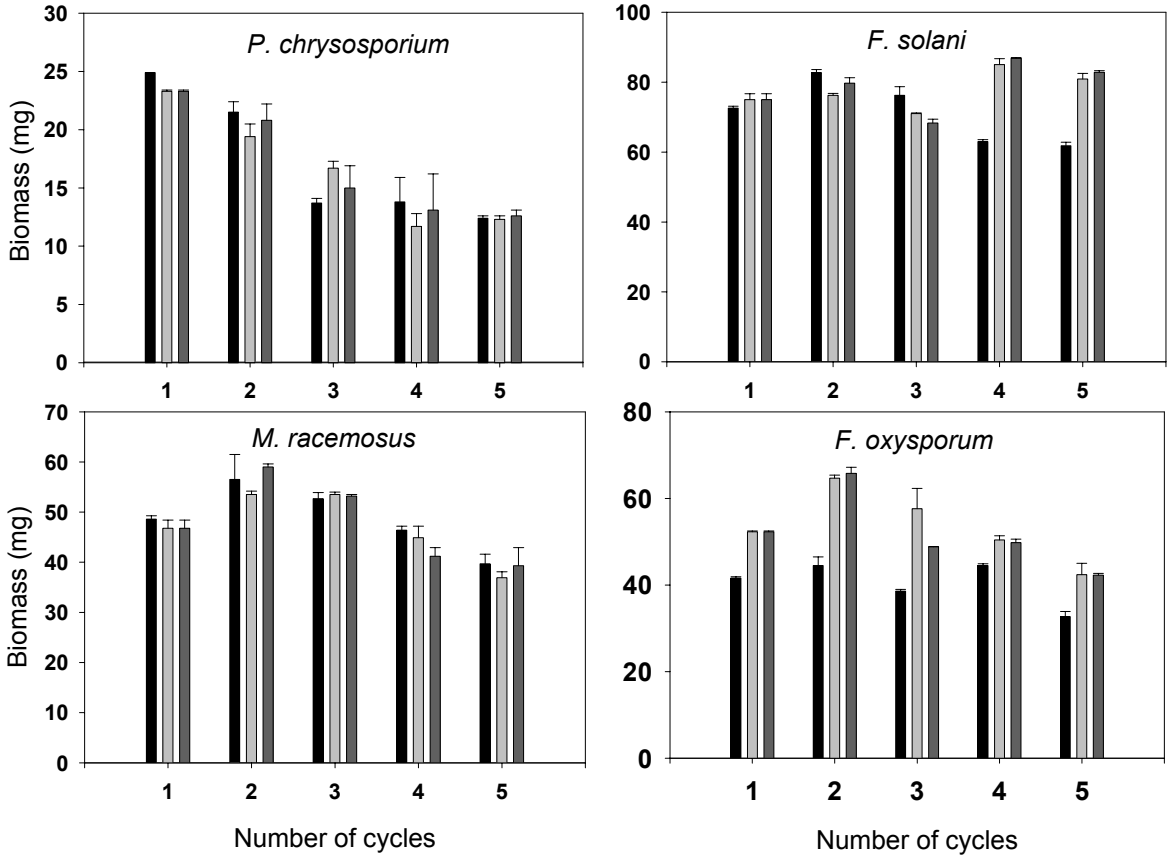
Legends to figures

Figure 1. Impact of nonylphenol on the spore multiplication factor during long-term exposure of fungal liquid cultures. Black bars refer to untreated controls, white bars to cultures treated with constant concentration of the chemical, grey bars to cultures treated with decreasing concentrations of the chemical.

Figure 2. Impact of nonylphenol on biomass production during long-term exposure of fungal liquid cultures. Black bars refer to untreated controls, white bars to cultures treated with constant concentration of the chemical, grey bars to cultures treated with decreasing concentrations of the chemical.

Figure 3. Impact of nonylphenol on laccase production in fungal liquid cultures of *Trametes versicolor* after 3 days of exposure to the chemical.


P. chrysosporium
F. solani
M. racemosus
F. oxysporum
Spore multiplication factor
Spore multiplication factor
Number of cycles
Number of cycles
0
20
40
60
80
0
40
80
120
160
0
20
40
60
80
100
0
40
80
120
160
1
2
3
4
5


P. chrysosporium
F. solani
M. racemosus
F. oxysporum
Biomass (mg)
Biomass (mg)
Number of cycles
Number of cycles

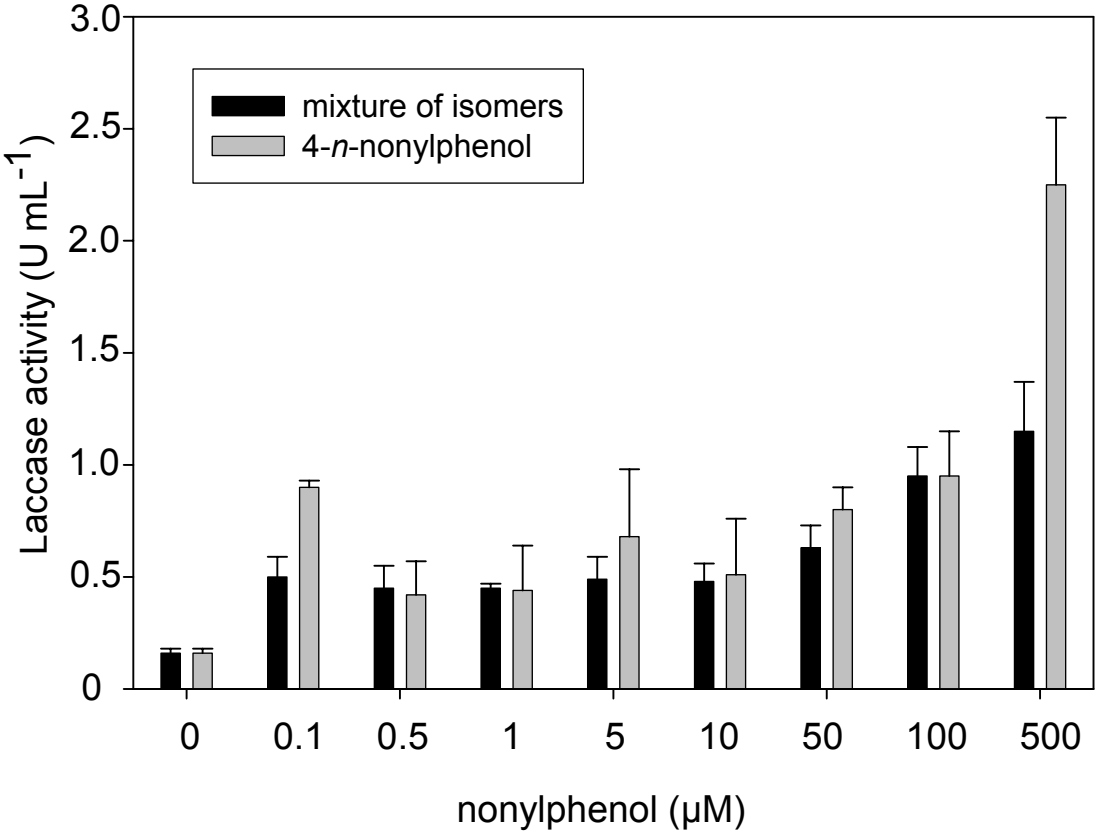

3.0
2.5
2.0
1.5
1.0
0.5
0
Laccase activity (U mL-1)
mixture of isomers
4-n-nonylphenol
0
0.1
0.5
1
5
10
50
100
500
nonylphenol (µM)

Table 1. Characteristics of the two types of sludge used in this study.

Parameter	Sludge from Ambares (SA)	Sludge from Plaisir (SP)
Origin	Ambares	Plaisir
Treatment of wastewater	aerobic digestion + anaerobic stabilization	aerobic digestion + anaerobic stabilization + adding of lime
Dry matter (%)	75.71	33.12
Organic matter of dry matter (%)	51.41	45.20
Total nitrogen of dry matter (%)	3.97	2.66
pH water	7.8	12.5
NPs content (mg kg^{-1} dry matter)	200.00	506.00
PAHs[†] content (mg kg^{-1} dry matter)	0.51	0.43
PCBs[††] content (mg kg^{-1} dry matter)	0.089	0.071
Metal ions[§] (mg kg^{-1} dry matter)	8880.71	113.86

[†]PAHs: fluoranthene, benzo[b]fluoranthene, benzo[a]pyrene.
[††]PCBs: 28,52, 101, 118, 138, 153, 180.
[§]Metal ions: Fe, Mn.

Table 2. Exposure of soil fungi to NP in soils and soil/sludge mixtures.

Adsorbent	NP concentration in the soil solution (M)	Nonlinear correlation coefficient (r^2)	K ($L\ kg^{-1}$)	1/n
Nonamended soil	$8.8\ 10^{-6}$	0.995	45.3	0.79
Soil + sludge A	$6.6\ 10^{-6}$	0.994	58.6	1.04
Soil + sludge P	$3.8\ 10^{-7}$	0.998	1083.6	1.03

Table 3. Acute toxicity of NP on fungal strains in liquid cultures

	IC_{50} (M)				LC_{100} (M)
Fungal strains	Germination of mother spores	Fungal growth after 2 days	Fungal growth after 4 days	Germination of daughter spores	Fungal development
T. versicolor	/	$> 10^{-3}$	$> 10^{-3}$	/	$> 10^{-3}$
P. chrysosporium	$7.5\ 10^{-6}$	10^{-4}	$5.0\ 10^{-4}$	$2.5\ 10^{-6}$	$> 10^{-3}$
F. solani	$4.1\ 10^{-4}$	$4.8\ 10^{-5}$	$> 10^{-4}$	$5.1\ 10^{-6}$	$> 10^{-3}$
M. racemosus	10^{-4}	$5.1\ 10^{-6}$	$5.3\ 10^{-5}$	$5.9\ 10^{-5}$	10^{-4}
F. oxysporum	$2.9\ 10^{-5}$	$5.0\ 10^{-6}$	$5.1\ 10^{-5}$	$5.1\ 10^{-6}$	10^{-4}

Table 4. Impact of NP on fungal reproduction in liquid cultures

Fungal strains	CI_{50} F1 (M)	CI_{50} F2 (M)	microspores (M)
P. chrysosporium	$7.5\ 10^{-6}$	$2.5\ 10^{-6}$	n.o.
F. solani	$4.1\ 10^{-4}$	$5.1\ 10^{-6}$	10^{-4}
M. racemosus	10^{-4}	$5.9\ 10^{-5}$	n.o.
F. oxysporum	$2.9\ 10^{-5}$	$5.1\ 10^{-6}$	$6.9\ 10^{-5}$

n.o.: not observed