Physiological responses to nanoCuO in fungi from non-polluted and metal-polluted streams

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HIGHLIGHTS
• NanoCuO inhibited fungal biomass production in a dose- and time-dependent manner.
• NanoCuO was more toxic to fungi from clean than from metal-polluted streams.
• Mycelial alterations and nanoCuO adsorption were higher in fungi from clean streams.
• Laccase activity induced by nanoCuO was correlated with adsorbed Cu and/or Cu2+.
• Laccase activity depended on the presence of gene fragments in Cu-binding regions.

ABSTRACT
Nanocopper oxide (nanoCuO) is among the most widely used metal oxide nanoparticles which increases their chance of being released into freshwaters. Fungi are the major microbial decomposers of plant litter in streams. Fungal laccases are multicopper oxidase enzymes that are involved in the degradation of lignin and various xenobiotic compounds. We investigated the effects of nanoCuO (5 levels, ≤200 mg L−1) on four fungal isolates collected from metal-polluted and non-polluted streams by analyzing biomass production, changes in mycelial morphology, laccase activity, and quantifying copper adsorbed to mycelia, and ionic and nanoparticulate copper in the growth media. The exposure to nanoCuO decreased the biomass produced by all fungi in a concentration- and time-dependent manner. Inhibition of biomass production was stronger in fungi from non-polluted (EC50 (10 days) ≤31 mg L−1) than from metal-polluted streams (EC50 (10 days) ≥65.2 mg L−1). NanoCuO exposure led to cell shrinkage and mycelial degeneration, particularly in fungi collected from non-polluted streams. Adsorption of nanoCuO to fungal mycelia increased with the concentration of nanoCuO in the medium and was higher in fungi from non-polluted streams. Extracellular laccase activity was induced by nanoCuO in two fungal isolates in a concentration-dependent manner, and was highly correlated with adsorbed Cu and/or ionic Cu released by dissolution from nanoCuO. Putative laccase gene fragments were also detected in these fungi. Lack of substantial laccase activity in the other fungal isolates was corroborated by the absence of laccase-like gene fragments.

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1. Introduction
Nanocopper oxide (nanoCuO) is among the commercially used metal oxide nanoparticles having a broad range of applications in electronics, medical and pharmaceutical fields and daily-life products (Carnes and Klabunde, 2003; Dutta et al., 2003; Ren et al., 2009; Zhang et al., 2008). Metal oxide nanoparticles have special intrinsic properties, compared to their bulk forms, and have attracted attention in ecotoxicology (Navarro et al., 2008; Rousk et al., 2012) as their increased commercialization enhances the chance of these nanoparticles to reach the environment. Natural surface waters are likely to serve as the ultimate sink of nanomaterials, and there is some evidence on the occurrence of metal or metal oxide nanoparticles in running waters (e.g. ZnO, FeO and MnO, Wigginton et al., 2007; TiO2, Kaegi et al., 2008). Ions of many metals are known to be toxic to aquatic biota and the processes they drive (Beltman et al., 1999; Niyogi et al., 2002; Duarte et al., 2009; Batista et al., 2012). Recent studies have pointed to the potential ecotoxicity of nanometal oxides to aquatic organisms (Blaise...
et al., 2008; Lee et al., 2009; Miller et al., 2010; Pradhan et al., 2012) and nanoCuO are likely to be more toxic than their bulk particles (Heinlaan et al., 2008; Aruoja et al., 2009; Mortimer et al., 2010).

In streams, a polyphyletic group of fungi primarily with Ascomycetous affinities (Bärlocher, 2010) play an important role in organic matter turnover and energy transfer to higher trophic levels (Pascoal and Cássio, 2008). In an earlier study, Pradhan et al. (2011) showed that nanoCuO and Cu$^{2+}$ strongly affected the activity and diversity of aquatic fungal communities (Pradhan et al., 2011). However, there is little information on how fungal populations with different backgrounds respond to nanoCuO. Adaptive mechanisms underlying the tolerance/resistance against several metal ions, including Cu$^{2+}$, were shown in aquatic fungi helping to explain their survival in metal-polluted environments (Jaeckel et al., 2005; Azevedo et al., 2007; Guimarães-Soares et al., 2007; Krauss et al., 2011).

Laccases are extracellular multicopper-containing oxidoreductases, which catalyze one-electron oxidation of aromatic amines, phenolic and nonphenolic compounds with concomitant reduction of oxygen to water through its copper reduction center (Junghanns et al., 2005; Castilho et al., 2009). Due to their high redox potential ($\approx +800$ mV), fungal laccases have a wide range of applications in lignin degradation, a natural organic matter in soils or surface waters (Steinberg et al., 2006). Moreover, Cu$^{2+}$ stimulates the laccase activity in fungi, including those involved in plant litter decomposition in streams (Junghanns et al., 2005, 2008). Conversely, laccase activity of the terrestrial wood decomposing fungus Trametes versicolor decreased by short-term exposure to Cu nanoparticles, while no effects were observed in the presence of Cu$^{2+}$ (Shah et al., 2010). This suggests that nanoCu can have a different mode of action than its ionic form, making it important to better understand the effects of nanometal oxides on laccase activity.

We investigated the effects of nanoCuO on four fungal isolates belonging to three species: two isolates were collected from non-polluted streams and the other two isolates were collected from metal-polluted streams. We hypothesized that i) nanoCuO would induce toxicity to fungi by inhibiting biomass production and changing mycelial morphology, ii) fungal populations from non-polluted streams would be more affected by nanoCuO than those from metal-polluted streams, and iii) copper ions dissolved from nanoCuO would modulate laccase activity in fungi. To that end, we examined the morphology of fungal mycelia, biomass production and extracellular laccase activity after exposure to increasing nanoCuO concentrations for two time periods. In addition, we quantified total copper adsorbed to fungal mycelia, and dissolved ionic and nanoparticulate copper in the growth medium to better understand the effects of nanoCuO on fungi. Finally, because laccase activity in fungi is highly dependent on growth conditions, the presence of laccase-like multicopper oxidase genes was checked under non-exposure conditions.

2. Materials and methods

2.1. Fungal cultures and exposure conditions

Four fungal isolates were used for the experiment, namely Articulospora tetracladia UMB-072.01 (Ar72) and Phoma sp. UHH 5-1-03 (P5), collected from non-polluted streams, and A. tetracladia UMB-061.01 (A61) and Clavariopsis aquatic aquatica WD(A)-00-1 (Ca1), collected from polluted streams. The isolate Ar72 was collected in foam from the Mcllvaine buffer (pH 4.0) at 420 nm ($\epsilon_{240} = 36$ nmol$^{-1}$ cm$^{-1}$) in 96-well flat-bottom microtiter plates (VWR, Darmstadt, Germany), using a microplate reader (SLT Spectra, Tecan, Crailsheim, Germany). Each well contained 160 μL of buffer, 20 μL of ABTS (20 μM) and 20 μL of mycelium-free medium. The measured values were corrected with a blank containing 20 μL of buffer instead of mycelium-free medium. Enzyme activity was expressed as units (U), where 1 U equals 1 μmol product formed per minute.

2.2. Preparation and characterization of nanoCuO suspensions

A stock suspension was prepared by suspending nanoCuO powder ($<$50 nm, 99.5%, Sigma-Aldrich, St. Louis, MO, USA) in sterilized (121 °C, 20 min) Milli Q water, and the suspension was sonicated in a water bath (42 kHz, 100 W; Branson 2510, Danbury, CT, USA) for 30 min in the dark before use (Pradhan et al., 2012). NanoCuO in the stock suspension and growth medium was examined by scanning electron microscopy (SEM; Leica Cambridge S 360, Cambridge, UK) coupled to an energy dispersive X-ray (EDX) microanalysis setup (15 keV), as described in Pradhan et al. (2012). Briefly, 20 μL of nanoCuO suspension was loaded on a clean grease-free slide in the dark, air-dried and coated with gold in vacuum. Coated slides were scanned by SEM–EDX to confirm the presence of CuO nanoparticles. Size distribution of nanoCuO in stock suspension and growth medium was monitored by dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS, Malvern Instruments Limited, UK) to check nanoparticle agglomeration.

2.3. Visualization of mycelial morphology

Fungal mycelia were harvested by filtration (5 μm pore size; Millipore, Billerica, MA, USA), washed with Milli Q water and re-suspended in 2 mL phosphate-buffered saline (1 × PBS, GIBCO, pH 7.4). Mycelia were fixed in 2.5% (v/v) glutaraldehyde for 24 h, and dehydrated in ethanol (v/v) as follows: 20%, 8 h; 40%; 6 h; 60%; 4 h; 80%; 2 h; and 100%, 1 h. Mycelial suspensions (20 μL) were loaded on slides, coated with gold in vacuum, and scanned by SEM–EDX as above.

2.4. Activity of extracellular laccase

The activity of laccase (EC 1.10.3.2) was quantified by using the methodology described in Junghanns et al. (2008). Briefly, the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was followed in Mcllvaine buffer (pH 4.0) at 420 nm ($\epsilon_{420} = 36$ nmol$^{-1}$ cm$^{-1}$) in 96-well flat-bottom microtiter plates (VWR, Darmstadt, Germany), using a microplate reader (SLT Spectra, Tecan, Crailsheim, Germany). Each well contained 160 μL of buffer, 20 μL of ABTS (20 μM) and 20 μL of mycelium-free medium. The measured values were corrected with a blank containing 20 μL of buffer instead of mycelium-free medium. Enzyme activity was expressed as units (U), where 1 U equals 1 μmol product formed per minute.

2.5. Fungal biomass quantification

Fungal mycelia were harvested and washed, as above, dried at 80 °C to constant mass (± 8 h), and weighed to the nearest 0.001 g.
2.6. Biosorption and metal analysis

To quantify the biosorption of nanoCuO to fungal cell-walls, mycelia were harvested and washed as above, and soaked for 12 h at 60 °C in a mixture containing 4% HCl and 1% formic acid to dissolve nanoCuO to ionic Cu, and 1 mM EDTA as chelating agent. The solution was filtered through a polycarbonate membrane (0.2 μm pore size; Millipore, Billerica, MA, USA) before copper quantification.

To quantify ionic Cu dissolved from nanoCuO and the nano form of copper in the culture medium, the mycelium-free medium was centrifuged at 75,600 × g for 90 min (Beckman Avanti J-25i, USA). The supernatant containing the ionic Cu was filtered through a polycarbonate membrane (0.2 μm pore size). The filtered supernatant, the residue from filtration, and the pellet from centrifugation of each sample were treated, individually, with 5% HCl and 10% HNO3 at 60 °C for 8 h, before copper quantification by inductively coupled plasma mass spectrometry (ICP-MS, X Series 2, Thermo Scientific).

2.7. Screening of laccase-like multicopper oxidase genes

The genomic DNA was extracted from the four fungi grown in the absence of nanoCuO using the UltraClean Soil DNA kit (MoBio Laboratories, Solana Beach, CA, USA). Putative laccase gene fragments flanked by conserved sequences of laccase genes near the two pairs of histidines in two out of the four laccase copper binding regions of asco- and basidiomycetes (domains II and III; Lyons et al., 2003) were amplified with the degenerate primer pair Lac2for, 5′ GGI ACI WII TG0 TAY CAY WSI CA 3′ and Lac3rev, 5′ CCR TGI WKR TGI AWI GGR TGI GG 3′ (Lyons et al., 2003; Castilho et al., 2009). Ambiguous bases were defined as follows: R = A/G, W = A/T, Y = C/T, S = C/G, K = T/G and I = inosine. For polymerase chain reaction (PCR), 1 × Go Taq Green Master Mix (Promega Corporation, Madison, WI, USA), 60 μM of each primer and 2 μL DNA (5 ng/μL) were mixed gently with nuclease-free water in a final volume of 25 μL. DNA amplification program started with a denaturation for 3 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 95 °C, primer annealing for 30 s at 45 °C and elongation for 2 min at 70 °C, followed by a final elongation for 5 min at 70 °C. A PCR reaction without DNA template served as negative control. DNA amplification was performed in a Doppio thermal cycler (VWR International, Leuven, Belgium). Five microliters of each amplification product was loaded on 1.3% agarose gel (BioRad, Danbury, CT, USA) and electrophoresis was carried out for 45 min at 90 V in 1× Tris-acetate-EDTA (TAE) buffer. The GeneRuler™ 50 bp DNA ladder (Thermo Scientific, Wilmington, DE, USA) was used as a marker. GelStar (Lonza Rockland, Inc., USA) was used for detecting the bands on the gel. The gel images were captured under UV light in a transiluminator Eagle eye II (Stratagene, La Jolla, CA, USA).

2.8. Data analyses

Two-way ANOVAs (Zar, 2009) were used to assess how fungal endpoints (fungal biomass production and laccase activity) varied with the fungal isolate and nanoCuO concentration. Data were analyzed separately for both time periods. Bonferroni post-tests (Zar, 2009) were used to check which treatments differed significantly from the respective control. Percentage data were arcsine square root transformed to achieve normal distribution and homoscedasticity (Zar, 2009). The effective nanoCuO concentration inhibiting 50% of fungal biomass production (EC50) after 3 and 10 days of exposure was calculated using Probit 1.63 (Sakuma, 1998). Correlations were used to examine the relationships between fungal biomass or laccase activity and adsorbed copper to fungal mycelia or dissolved ionic copper or nanoparticulate copper in the growth medium. Analyses were done with Statistica 6.0 (Statsoft, Inc., Tulsa, OK, USA).

3. Results

3.1. Characterization of nanoCuO by SEM and DLS

SEM analysis of nanoCuO in the aqueous stock suspension showed that the size of CuO nanoparticles ranged from 50 to 50 nm (not shown). However, DLS showed a single peak ranging between 100 and 340 nm with a z-average of 216 nm (Fig. 1) and a polydispersity index (Pdi) of 0.196 in the stock suspension. In the growth medium (1% ME), the Pdi increased to 0.387 and an additional peak between 75 and 165 nm (z-average of 114.4 nm) with 7.2% of area intensity was observed (Fig. 1). Also, in the growth medium, the major peak shifted to 220–550 nm (z-average of 379.6 nm) corresponding to 92.8% of area intensity. This suggests that nanoparticle agglomeration increased in the growth medium compared to aqueous stock suspension probably due to interactions between components of the medium and nanoparticles and/or self-agglomeration. However, the presence of an additional smaller peak in the growth medium indicated a decrease in self-agglomeration of a small fraction of nanoparticles, probably due to the affinity of O groups from nanoCuO towards H+ under lower pH conditions (growth medium pH ≤ 5.5 and aqueous stock pH = 6.0).

3.2. Mycelial morphology and nanoCuO adsorption

SEM analysis of fungal mycelia revealed that the exposure to nanoCuO promoted alterations in mycelial morphology, namely shrinkage and degeneration of cell-walls in all fungal isolates (Fig. 2). The morphological changes in fungal mycelia increased with exposure time (from 3 to 10 days) and with increasing concentrations of nanoCuO. Adsorption of nanoCuO to fungal mycelia was detected (pointed arrows; Fig. 1). Size distribution of nanoCuO by dynamic light scattering in aqueous stock suspension and in 1% malt extract (ME) medium.
Fig. 2) and the presence of Cu was confirmed by EDX (Fig. S1). A clear difference in mycelial morphological alterations and nanoCuO adsorption was observed between fungi from non-polluted streams (At72 and P5) and metal-polluted streams (At61 and Ca1): the exposure to 200 mg L\(^{-1}\) of nanoCuO led to more severe effects on mycelia of At72 and P5 and to more nanoCuO adsorbed to mycelia (Figs. 2 and S1).

### 3.3. Copper in the growth medium and adsorbed to fungal mycelia

In the absence of nanoCuO, no copper was detected on the mycelial surface of any fungal isolate (Fig. 3A). Under nanoCuO exposure, copper adsorbed to mycelia increased with nanoCuO concentration, and the highest copper adsorption was found after exposure to 200 mg L\(^{-1}\) of nanoCuO.
Large amounts of Cu\(^{2+}\) released from nanoCuO were found in the growth medium of all fungal isolates, and the amount increased with the increase in concentration of nanoCuO in mycelia of fungal isolates from non-polluted streams (At72 and P5, 3418.9 and 1087.7 mg L\(^{-1}\)) than for fungi from metal-polluted streams (At61, 80.5 mg L\(^{-1}\) and Ca1, 108.7 mg L\(^{-1}\)) (Table 1). Although a similar pattern was observed at the longer exposure time (10 days), EC\(_{50}\) values decreased for all fungal isolates (Table 1).

After 3 days of exposure to nanoCuO, biomass of all fungal isolates was negatively correlated with copper adsorbed to mycelia (P < 0.05, Table 2). Fungal biomass was also negatively correlated with Cu\(^{2+}\) dissolved from nanoCuO in the growth medium, except in the case of P5 (Table 2). Apart from Ca1, biomass of the other fungi was not significantly correlated with nanoparticulate copper in the medium.

### 3.5. Activity of extracellular laccase

In the absence of nanoCuO, the activity of extracellular laccase at day 3 was only detected in P5 (3.2 U L\(^{-1}\); Fig. 5A). At day 10, laccase activity increased mainly in P5 (11.6 U L\(^{-1}\); Fig. 5B). The exposure to nanoCuO led to an increase in fungal laccase activity in a concentration-dependent manner (two-way ANOVAs, P < 0.05; Fig. 5). The highest laccase activity was observed in P5 (15 and 546.2 U L\(^{-1}\) after 3 and 10 days, respectively), followed by Ca1 (3 and 69.3 U L\(^{-1}\) after 3 and 10 days, respectively). Only minor laccase activity was detected in the other fungal strains (Fig. 5A and B).

After 3 days of exposure to nanoCuO, a significant correlation was found between laccase activity and adsorbed copper to P5 and Ca1 mycelia (P < 0.05, Table 2). Additionally, extracellular laccase activity of Ca1 was also correlated with both forms (ionic and nano) of copper in the growth medium (Table 2).

### 3.6. Laccase-like multicopper oxidase genes

Gel electrophoresis revealed the presence of laccase-like multicopper oxidase gene fragments in the laccase copper binding regions II and III in the PCR amplified products of the fungal isolates Ca1 and P5 when grown in the absence of nanoCuO (Fig. 6). We observed four less intense bands of about 900 bp, 750 bp, 350 bp and <200 bp and a highest nanoCuO concentration, the amount of dissolved Cu\(^{2+}\) in the medium was highest in P5 cultures (7838.6 μg microcosm\(^{-1}\)) and lowest in At72 cultures (3528.8 μg microcosm\(^{-1}\)) (Fig. 3C).

### Table 1

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Fungal biomass* (g dry mass L(^{-1}))</th>
<th>LOEC (mg L(^{-1}))</th>
<th>EC(_{50}) (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 days</td>
<td>10 days</td>
<td>3 days</td>
</tr>
<tr>
<td>At72</td>
<td>0.96 ± 0.05</td>
<td>2.48 ± 0.1</td>
<td>5</td>
</tr>
<tr>
<td>At61</td>
<td>1.11 ± 0.08</td>
<td>2.72 ± 0.17</td>
<td>25</td>
</tr>
<tr>
<td>Ca1</td>
<td>0.67 ± 0.07</td>
<td>2.1 ± 0.21</td>
<td>25</td>
</tr>
<tr>
<td>P5</td>
<td>0.86 ± 0.05</td>
<td>2.29 ± 0.12</td>
<td>5</td>
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* Mean ± SD, n = 3.
prominent band of about 600 bp in Ca1, while only a strong single band of about 500 bp was observed in P5 (Fig. 6). No DNA bands were found for At72 and At61 (Fig. 6).

4. Discussion

In this study, the exposure to nanoCuO led to a decrease in the biomass produced by all fungi in a concentration- and time-dependent manner. These results agree with a previous report in which nanoCuO inhibited biomass production by stream-dwelling fungal communities on decomposing plant litter (Pradhan et al., 2011). However, compared to fungal communities, fungal isolates in malt extract medium seemed to be more sensitive to nanoCuO. Indeed, the inhibition of biomass production by the four fungal isolates after 10 days exposure to 200 mg L\(^{-1}\) nanoCuO varied between 64.9 and 93.9%, whereas fungal biomass at the community level was only lowered by 16.1 and 19.3% after 7 and 14 days of exposure to a similar nanoCuO concentration. Two main reasons might account for these differences: i) the high number of fungal species or strains in stream-dwelling communities increases the chance of encountering more tolerant/resistant species and/or ii) fungal mycelia growing inside plant litter are somehow protected against direct contact with nanoCuO.

Our results clearly showed that two fungal isolates collected from non-polluted streams were more sensitive to nanoCuO than two fungal isolates from metal-polluted streams, as shown by the lower LOEC and EC50 values for biomass production. Although there is lack of information on the tolerance/resistance of fungi against nanoCuO, biomass production by fungi collected from metal-polluted streams was less affected by ionic metals than that of fungi from non-polluted streams (Jaekel et al., 2005; Miersch et al., 2005). The exposure to Cu\(^{2+}\) led to a lower inhibition of biomass production by Heliscus submersus isolated from a metal-polluted stream compared to Varicosporium elodeae from a non-polluted stream (Azevedo et al., 2007). Also, the growth of strains of A. tetracladia and Tetracladium marchalianum collected from copper-polluted streams was much less affected by Cu\(^{2+}\) than strains of the same species isolated from non-polluted streams (Miersch et al., 1997). Similarly, we found intraspecific differences in the biomass production under nanoCuO stress within isolates of A. tetracladia collected

Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fungi</th>
<th>Copper (μg microcosm (^{-1}))</th>
<th>Adsorbed Cu to fungi</th>
<th>Nanoparticulate Cu in the medium</th>
<th>Dissolved Cu(^{2+}) in the medium</th>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Fungal biomass (g L(^{-1}))</td>
<td>At72</td>
<td>–0.93</td>
<td>0.021</td>
<td>–0.87</td>
<td>0.053</td>
</tr>
<tr>
<td></td>
<td>At61</td>
<td>–0.99</td>
<td>0.002</td>
<td>–0.82</td>
<td>0.091</td>
</tr>
<tr>
<td></td>
<td>Ca1</td>
<td>–0.95</td>
<td>0.014</td>
<td>–0.94</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>–0.90</td>
<td>0.040</td>
<td>–0.86</td>
<td>0.060</td>
</tr>
<tr>
<td>Laccase activity (U L(^{-1}))</td>
<td>Ca1</td>
<td>0.99</td>
<td>–0.001</td>
<td>0.91</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>0.89</td>
<td>0.043</td>
<td>0.69</td>
<td>0.202</td>
</tr>
</tbody>
</table>

\(r\), coefficient of correlation.
from metal-polluted (At61) and non-polluted (At72) streams. Adaptive mechanisms of exposure to high levels of metal ions have been shown in aquatic fungi and include changes in the activity of antioxidant enzymes (Azevedo et al., 2007) and in the levels of glutathione (GSH) or other thiol-containing compounds (Guimarães-Soares et al., 2006, 2007; Braha et al., 2007). This may help to explain the ability of aquatic fungi isolated from metal-polluted streams to decompose plant litter under metal stress (Fernandes et al., 2011).

The differential inhibition pattern of biomass production after nanoCuO exposure in fungal isolates with different background was consistent with the change in mycelial morphology, with evidence of more severe cell-wall shrinkage and mycelial degeneration in fungi from non-polluted streams. A similar alteration in cell-wall morphology was previously shown in aquatic fungi after exposure to Cu²⁺ (Azevedo et al., 2007). SEM-EDX analyses showed higher biosorption of nanoparticulate copper to mycelia of fungi from non-polluted streams (P5 and At72) than from metal-polluted streams (Ca1 and At61). Moreover, our results agree with those found by others showing that Cu²⁺ biosorption in aquatic fungi increases in a dose-dependent manner (Braha et al., 2007).

In our study, the mean size of most nanoCuO in the growth medium was greater (379.6 nm with Pdl of 0.387) than that measured in the stream water (202 nm; Pdl. 0.186; Pradhan et al., 2012), and nanoCuO toxicity to fungal populations in the growth medium (this study) was higher than to fungal communities in the stream water (Pradhan et al., 2011). The presence of smaller nanoCuO (114.4 nm) in the growth medium found in our study might also have contributed to the increased toxicity because smaller nanoparticle species are generally more toxic to living organisms (Van Hooeck et al., 2009). Also, the lower pH (≤ 5.5) of the growth medium compared to the stream water (pH 5.8; Pradhan et al., 2012) might have played a role in nanoCuO toxicity by promoting the release of Cu ions from nanoparticles. In our study, negative correlations between fungal biomass (except for P5) and the amount of Cu²⁺ in the medium were found. However, some studies reported that dissolved Cu²⁺ alone could not fully explain nanoparticle toxicity (Griffit et al., 2008; Buffet et al., 2011). Negative correlations between adsorbed copper and fungal biomass were also found in our study in all fungi. The toxicity of nanoCuO may occur directly by adsorption of nanoparticles to cells or indirectly by the entry of nanoCuO followed by its degradation in the lysosomes leading to an intracellular accumulation of Cu²⁺ (Petersen and Nelson, 2010).

Laccases, as multicopper oxidoreductase enzymes, are modulated by copper availability in the medium (Junghanns et al., 2005; Castilho et al., 2009). Also, growth conditions such as nutrient availability and pH are recognized to affect the activity of these enzymes in aquatic fungi (Abdel-Raheem, 1997). In our study, Phoma sp. (P5) was the only fungus showing laccase activity (11.6 U L⁻¹) in 1% malt extract without nanoCuO, and laccase activity in this fungus did not exceed 20.9 U L⁻¹ in 2% malt extract (Junghanns et al., 2005). Earlier studies showed that Cu²⁺ stimulates laccase activity in P5 and C. aquatica (Ca1) (Junghanns et al., 2005, 2008), and depletions of these ions can inactivate the enzyme (Keum and Li, 2004). However, a reduction of laccase activity in the white-rot fungus T. versicolor was found after short-term exposure to highly aggregated nanoCu (Shah et al., 2010).

In our study, the exposure to nanoCuO stimulated laccase activity in P5 and Ca1, and laccase activity in these fungi was correlated with adsorbed nanoCuO to fungal mycelia. In addition, the highest Cu²⁺ amount was measured in the growth medium of P5 followed by Ca1, suggesting that dissolved Cu²⁺ might have contributed to the stimulated laccase activity. The clearly measurable extracellular laccase activities in P5 and Ca1 were well corroborated by the detection of one and five laccase-like gene fragments in these fungi, respectively. Moreover, our results regarding these putative laccase gene fragments are in agreement with the presence of one single and five putative laccase genes detected upon targeting laccase copper binding regions I and III in P5 and Ca1, respectively (Junghanns et al., 2009; Solé et al., 2012). Such differences in the laccase gene inventory might have contributed to the observed differences in laccase activities between P5 and Ca1. By contrast, even under nanoCuO exposure, only minor laccase activities (perhaps representing unspecific ABTS oxidation activities attributable to other factors than laccase) could be detected in the isolates of A. tenuifolia (At72 and At61). These findings agree with the absence of laccase-like gene fragments in the A. tenuifolia strains.

5. Conclusions

Overall results suggested that nanoCuO expresses its toxicity to fungi by inhibiting fungal biomass production and altering the mycelial morphology in a dose- and time-dependent manner. Laccase activity varied greatly among fungi and appeared to be related to the presence of laccase-like genes with a copper oxidase domain. Laccase activity and fungal biomass production were related to the amounts of nanoCuO adsorbed to mycelium and dissolved Cu²⁺ released by dissolution from nanoCuO to the growth medium. Different physiological responses to nanoCuO exposure were found among fungi as shown by i) the stronger inhibition in biomass production, ii) more pronounced alterations of mycelial morphology, and iii) higher nanoparticle biosorption in fungi from non-polluted streams than from metal-polluted streams. These differences were also observed at the intraspecific level (At61 and At72), further supporting higher tolerance/resistance to nanoCuO-induced stress in fungi from metal-polluted streams. Nevertheless, more studies using other fungal strains from different sites would be helpful to better support and generalize our conclusions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2013.07.073.

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