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Fate and effects of nonylphenol in the filamentous fungus *Penicillium expansum* isolated from the bottom sediments of the Gulf of Finland

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ABSTRACT

Nonylphenol (NP) is the most abundant environmental pollutant that is classified as an endocrine disruptor, and it originates from the degradation of nonylphenol ethoxylates, which are widely used as industrial surfactants. It has been referred to in a list of substances of particular risk to the Baltic Sea, in a list of priority hazardous substances in the Water Frame Directive, and in the 3rd draft Working Document on Sludge, developed by the EU. In this study, the fate and effects of NP in the filamentous fungus *Penicillium expansum* isolated from the bottom sediments of the coastal zone of the eastern Gulf of Finland were investigated in laboratory experiments. This strain was more tolerant to technical nonylphenol (tNP) compared to other types of aquatic organisms, such as fish, protozoa, and algae. The toxicity concentration values of tNP in *Penicillium expansum* were EC_{50} 20 mg L^{-1} and EC_{90} > 100 mg L^{-1}. The activity level of hydrolytic enzymes, cellulases and amylases decreased significantly in the tNP treatments. Given the significant role played by terrestrial fungi in the transformation of organic substrate into bottom sediment, such an effect from tNP on fungi could disturb the regulatory mechanisms and balance between the biosynthesis and biodegradation of organic matter in aquatic ecosystems as well as the formation of cenotic relations in aquatic biocenoses. Oxidative stress induced by tNP has been found to increase the synthesis of enzymatic protection factors, such as superoxide dismutase, catalase and nonenzymatic factors (melanin-like pigments and extracellular polysaccharides). This research indicated that the malondialdehyde concentration (the biochemical marker of lipid peroxidation) in the cells of the fungus decreased with increasing antioxidation factors. *Penicillium expansum* was able to decrease the tNP concentration in the culture medium. The removal of tNP was mainly caused by fungal degradation rather than by simple sorption and accumulation in the cells. Because terrestrial fungi play a significant role in the function of the heterotrophic block of bottom sediments and because tNP has become an increasingly persistent toxic organic and endocrine disruptor, these results may be ecologically relevant for aquatic systems. The results from this study demonstrate a potential application of this fungal species for the removal of tNP from the environment.

1. Introduction

Nonylphenol (NP) is a xenobiotic compound that is widely used for the production of nonylphenol ethoxylate surfactants (NPEOs), which are commonly used as detergents, emulsifiers, wetting and dispersing agents, antistatic agents, demulsifiers and solubilizers (Varquez-Duhalt et al., 2006; Soares et al., 2008; De Weert et al., 2011).

Nonylphenol is persistent in the environment, highly toxic to various organisms and known to be an endocrine disruptor (Servos, 1999; Preuss et al., 2006; Gao and Tam, 2011; Puy-Azurmendi et al., 2014).

The use of nonylphenol in the European Union has been limited since 2005. Nonylphenol has been referred to in a list of substances of particular risk to the Baltic Sea, in a list of priority hazardous substances in the Water Frame Directive, and in the 3rd draft Working Document on Sludge, developed by the EU.

However, despite the restrictions imposed in many countries, NP is found in all ecosystems – in surface waters, sediments, groundwater, soil and air – and is mainly released into the environment from industrial wastewater. Because NP exhibits low solubility in water and high hydrophobicity, it has a great tendency to bioaccumulate and partition to organic-rich sediments.

The portion of NP in the environment has been determined to be >60% in sediments, >10% in soil and approximately 25% in water (Düring et al., 2002). The concentration of nonylphenol in sediment varies from 0.01 to 1240 mg kg^{-1} of sediment and, in some cases, can reach 3500 mg kg^{-1} (Soares et al., 2008). Nonylphenol exhibits high persistence in sediment. The degradation half-life of nonylphenol is estimated to be >60 years once it enters sediment (Shang et al., 1999).

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Considering the negative effects of NP on aquatic organisms, it is necessary to understand its environmental fate in aquatic ecosystems. The latter has been widely studied, and the organisms that are preferred in toxicity assessments of NP include algae, invertebrates and fish (Soares et al., 2008).

Presently, only limited studies have focused on the interactions between aquatic microorganisms and NP. It has been shown that NP may affect cyanobacterial growth. In the cyanobacteria strains of *Microcystis aeruginosa*, *Aphanizomenon flosaquae*, *Anabaena variabilis* (*Trichormus variabilis*), *Nodularia spumigena*, and *Oscillatoria agardhii* (*Planktothrix aghardii*) under stress induced by NP, changes in the formation of photosynthetic pigments have been observed. Furthermore, in the strains of *Microcystis aeruginosa* and *Oscillatoria aghardii* (*Planktothrix aghardii*), stimulation of synthesis of algotoxins and odorants (geosmin and 2-methylisoborneol) is possible (Wang and Xie, 2007; Zaytseva et al., 2015).

High concentrations of NP (>1–2 mg L^{-1}) have been found to cause increases in antioxidant enzymatic activity of superoxide dismutase, peroxidase, catalase, and glutathione S-transferase as well as the glutathione levels in microalgae, suggesting that the cells respond to stress caused by high NP levels (Wang and Xie, 2007; Liu et al., 2010; Gao and Tam, 2011).

The mitosporic fungal strain UHH 1-6-18-4 isolated from nonylphenol-contaminated river water and hyphomycete *Clavariopsis aquatic* are more resistant to nonylphenol. In both fungi, the addition of 55 mg L^{-1} NP had no effect on fungal growth (Junghanns et al., 2005).

The responses of terrestrial fungi isolated from the aquatic environment to NP remain unclear.

Aquatic microbial communities, including terrestrial fungi, are considered to be important ecological components of aquatic environments due to their performance in biogeochemical processes (Paerl et al., 2003). In bottom sediments, the biomass of mycelia may reach up to 88% of the total biomass of terrestrial fungi, which may indicate the activity of terrestrial fungi in aquatic ecosystems (Terekhova, 2007). The ability of terrestrial fungi to withstand or mitigate anthropogenic stresses is becoming increasingly important.

They can interact with a variety of toxic aquatic contaminants and metabolize them (Marco-Urrea et al., 2015). The fungus *Aspergillus sclerotium*, isolated from marine ecosystems, is able to degrade the very toxic, to aquatic organisms, organic pollutants pyrene and benzo[*a*]pyrene (Passarini et al., 2011). The ability to degrade polycyclic aromatic hydrocarbons (PAHs) was also observed by Ravelet (Ravelet et al., 2000) in relation to many different fungi that belong to the genera *Gliocladium*, *Penicillium*, *Scopulariopsis*, and *Mucor* that have been isolated from PAH_S-contaminated sediment.

Some species of terrestrial fungi, including *Aspergillus*, *Mucor*, *Fusarium*, and *Penicillium*, play an important role in the biodegradation process of petroleum hydrocarbons in marine environments (Xue et al., 2015).

The majority of the available data on the microbial degradation of NP refer to processes of biotransformation by bacteria, microbial consortia or ligninolytic fungi (Gabriel et al., 2008; Cajthaml et al., 2009; Lu and Gan, 2014). Only a few studies have reported non-ligninolytic fungi capable of degrading tNP (Rozalska et al., 2014; Janicki et al., 2016). Furthermore the ability of terrestrial fungi isolated from an aquatic environment to remove and degrade tNP has never been reported.

The present study has the following aims: (i) to investigate the toxic effect of tNP on the terrestrial fungus *Penicillium expansum*, including the alteration of the growth rate, cellular contents and antioxidants enzyme activities; (ii) to assess the ability of *Penicillium expansum* to degrade tNP.

Table 1
Growth parameters of *Penicillium expansum* F44 under tNP (100 mg L^{-1}).

Growth parameters	Control	tNP
Lag phase, h	10 ± 2	20 ± 3
Specific growth rate μ, h^{-1}	0.33 ± 0.04	0.14 ± 0.02
Fungal biomass yield, g L^{-1}	7.5 ± 0.5	6.0 ± 0.5

2. Materials and methods

2.1. Isolation and identification of the fungal strain

The fungal strain *Penicillium expansum* was isolated from the bottom sediments of the coastal zone of the eastern Gulf of Finland. Isolation of the fungus was performed on a standard agar nutrient media (Czapek's Agar with 2% glucose and Sabouraud Dextrose Agar) containing 100 U mL^{-1} benzylpenicillin.

The fungal isolate was identified based on its morphological characteristics (Domsch et al., 1980; Samson and Van Reenen-Hoekstra, 1988) and a nucleotide sequence analysis of the internal transcribed space (ITS) region.

The morphology of the fungal strain was examined under a fluorescence microscope MIKMED-6 LUM-LED (LOMO, Russia) with a 100× objective lens.

Genomic DNA was isolated using a reagent kit, an AxyPrep Multisource Genomic DNA Miniprep Kit (Corning, USA), in accordance with the manufacturer's instructions. The following PCR primers were used for sequencing the ITS1-5.8S-ITS2 region: ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ (White et al., 1990). PCR was performed in 25-μL reaction mixtures containing 200 μM dNTPs (Helicon, Russia), 5 pmol of each primer (Eurogen, Russia), 1 U of *Taq* polymerase (Helicon, Russia) and 20–50 ng of purified template DNA. For amplification, a C1000™ Thermal Cycler was used (BioRad, USA). The PCR conditions were as follows: initial denaturation at 95 °C for 3 min and 30 s; 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 2 min; and a final extension at 72 °C for 6 min and 10 s. Electrophoresis was carried out with 1% agarose gel (Invitrogen, USA) in TAE. A 100-bp GeneRuler™ and Lambda DNA/*Hin*dIII markers (Fermentas, USA) were used for the sizing and approximate quantification of the DNA fragments. Purification of the PCR products was usually performed using a PureLink™ Quick kit (Invitrogen, USA) according to the manufacturer's instructions. Direct sequencing of the PCR products was carried out using an ABI PRISM 3500xl genetic analyzer (Applied Biosystems, USA). The sequences were compared with related sequences available in the GenBank databases using BLAST analysis (http://www.ncbi.nlm.nih.gov).

2.2. Chemicals

tNP (technical nonylphenol) CAS: 84852-15-3 was obtained from Sigma-Aldrich, USA. All of the chromatographic solvents were purchased from Sigma-Aldrich, Germany. The other chemicals (pure or high purity) were purchased from Cryochrom, Russia.

2.3. Media and growth conditions

Spores from 14 days of fungal growth on Czapek's Agar with 2% glucose were used to inoculate 50 mL of a liquid Czapek medium with 2% glucose and a pH of 6.8 in 250-mL Erlenmayer flasks. After 48 h of incubation, the precultures were transferred to fresh medium in a ratio of 1:9. tNP dissolved in ethanol (stock solution of 250 mg mL^{-1}) was aseptically added to the cultures to reach the required concentration. Solvent control flasks, each containing 50 mL of liquid Czapek medium with ethanol at a final concentration of 0.04% (v/v), and a fungal culture without tNP were also made to ensure that the ethanol used to dissolve the tNP had no significant effects on the isolated strain. Furthermore, abiotic (uninoculated) controls were prepared to monitor the abiotic losses of tNP. Each treatment, as well as the control, was performed in triplicate.

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