Nonylphenol (NP) is the most abundant environmental estrogen listed as one of the priority hazardous substances in the Water Framework Directive (EC 2000) and the priority pollutant of Baltic Sea (HELCOM 2010). The present study aims to compare the effects of technical nonylphenol (tNP) on the cellulase, amylase and protease activity of the terrestrial fungal strains played a significant role in aquatic ecosystems due to their high adaptive capacity and a large range of functional activity. The study also attempts to understand the mechanisms behind the varying sensitivity of the terrestrial fungi to tNP. The fungal strains were isolated from the bottom sediments of the coastal area of the eastern part of the Gulf of Finland. The terrestrial fungi were identified based on their morphological characteristics and nucleotide sequence analysis of internal transcribed space region. One reason for significant differences in sensitivity to the toxicant studied among the fungi is the change in the fungal cell permeability, in particular in cell membrane permeability, induced by NP. Environmentally relevant concentrations of tNP cause significant changes in activity of hydrolytic enzymes in the terrestrial fungi Aspergillus tubingensis, Penicillium expansum, Penicillium glabrum, and Cadophora fastigiata involved in organic matter degradation in bottom sediments. There can be increasing or decreasing trend, depending on both the type of enzyme and the tNP concentration. The revealed changes may disrupt the destructive processes in bottom sediments, as well as succession and stability of microbial communities functioning in the aquatic environment. It was found that tNP contributes to the activation of proteolytic enzymes, considered as potential fungal virulence factors. This may lead to emergence fungal strains with enhanced virulence in aquatic microbiocenoses. The investigations of the physiological responses of terrestrial fungi under nonylphenol will be important for biochemical processes dynamics and their environmental consequences evaluation.

Key words: coastal area, nonylphenol, fungi, bottom sediments, hydrolytic enzymes activity

I. INTRODUCTION

During many years the Baltic Sea is under an increasing anthropogenic pressure. The main problems of the Baltic Sea pollution, particularly in the eastern part of the Gulf of Finland, include the release of contaminants from wastewater discharges, development of navigation and
construction of oil terminals on the shores and raised beaches of the Gulf. The Gulf of Finland is affected by organic and metal pollution [1-3].

In recent decades, there has been increasing concern about environmental pollution with endocrine-disrupting chemicals (EDCs). Due to their widespread presence in the environment and toxic activity, EDCs have received increased attention in water quality management and health care. Among EDCs, nonylphenol holds a prominent place.

Nonylphenol is the most abundant environmental estrogen listed as one of the priority hazardous substances in the Water Framework Directive (EC 2000) and the priority pollutant of Baltic Sea (HELCOM 2010). It is used for the production of nonylphenol polyethoxylates (NPEOs) which have been widely used as surfactants in industrial processes and households [4].

Nonylphenol enters the environment primarily through wastewater pathways. On entering aqueous environment, NP with its high hydrophobicity (log $K_{ow}$ 4.8–5.3) and low water solubility (5.43 mg/L at 20°C) is transferred to the near-bottom layers and is accumulated in sediments and aquatic organisms [5]. As consequence, the transfer and accumulation of NP with increasing trophic level leads to serious ecotoxicological risk [6]. Organisms that are preferred in toxicity assessments of NP include algae, fish, and invertebrates. Among them, algae have the largest capacity for NP bioaccumulation, with NP concentrations ranging from 1.5 to 38 mg/kg and bioconcentration factors, from 200 to 10,000. In fish, the concentrations vary from 0.03 to 1.59 mg/kg, with bioconcentration factors ranging from 13 to 408 [7, 8]. The NP levels in bottom sediments vary from 0.01 to 1240 mg/kg sediments, reaching 3500 mg/kg in some cases [7].

Bottom sediments are sites of intense biogeochemical cycling regulated by microorganisms including terrestrial fungi. Fungi play a significant role in aquatic ecosystems due to their high adaptive capacity and a large range of functional activity. They show high efficiency in transforming organic substrates in aquatic ecosystems. Compared to other organisms, fungi are considered to be fairly resistant to toxicants. This is the reason why terrestrial fungi are often one of the dominant species in sediments contaminated with toxic chemicals [9].

At present, only a few studies have been conducted on NP’s toxicity to fungi. Nonylphenol exerts toxic effects on the growth of filamentous fungi *Neurospora crassa* [10], *Fusarium oxysporum* and *Fusarium solani* [11], and *Metarhizium robertsii* [12]. Under growth suppression conditions, inhibition of fungal respiration and changes in fungal morphology were observed. In *Neurospora crassa*, *Fusarium solani* and *Metarhizium robertsii* under NP treatment cell shapes were abnormal and hyphal apical dominance was lost. These abnormalities were presumably due to disruption of the hyphal free cytosolic Ca$^{2+}$ gradient, the H$^+$ gradient, and the actin cytoskeleton of the apical cells [10]. In NP-treated *Metarhizium robertsii* samples, fungal hyphae exhibited ultrastructural changes at the cytoplasmic level, with major differences detected in vacuoles, mitochondria and cell walls [12].

Long-term exposure to low NP concentrations of 0.004 to 0.06 mg/L caused increased biomass production in the fungi *Fusarium oxysporum* and *Fusarium solani*. Moreover, a strong stimulation of spore production and germination was observed for *Fusarium oxysporum* [11].

However, until now, there have only been a few reports on the effects of NP on the physiological activity of filamentous fungi. Our previous studies have noted the influence of technical nonylphenol on cellulase and amylase activity of some terrestrial fungal strains of genera *Aspergillus*, *Cladosporium*, *Exophiala*, and *Penicillium* [13].
The present study aims to compare the effects of NP on the cellulase, amylase and protease activity of the terrestrial fungal strains isolated from the bottom sediments of the coastal area of the eastern part of the Gulf of Finland, which have different sensitivities to NP. The study also attempts to understand the mechanisms behind the varying sensitivity of the terrestrial fungi to NP.

II. MATERIALS AND METHODS

Chemicals

Technical nonylphenol (CAS: 84852-15-3) was purchased from Sigma-Aldrich, USA. The other chemicals were obtained from Cryochrom, Russia.

Fungal strains and identification

The fungal strains *Aspergillus tubingensis* F11, *Cadophora fastigiata* F 17, *Penicillium expansum* F 44, and *Penicillium glabrum* F 41 used in this work were isolated from the bottom sediments of the coastal area of the eastern Gulf of Finland.

The fungal isolates were identified based on their morphological characteristics [14, 15] and a nucleotide sequence analysis of the internal transcribed space (ITS) region.

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’ [16]. 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 sec; 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 sec. Electrophoresis was carried out with 1% agarose gel (Invitrogen, USA) in TAE. A 100-bp GeneRuler™ and Lambda DNA/HindIII 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).

Experimental set-up

The fungal cultures were grown in liquid media at 25°C on a rotary shaker Certomat BS-1 (230 rpm). A spore suspension of the fungal cells with the titer of 1-2·10⁶ CFU/mL was used for inoculation of the culture media. The fungal biomass was determined by measuring its dry cell weight.

tNP dissolved in ethanol (125 mg/mL stock solution) was aseptically added to the culture media to reach the required concentration. The ethanol content, 0.04% v/v, was constant in all the variants. The control culture media were supplemented with the same amount of ethanol. The effect of ethanol (applied to dissolve tNP) on the growth of the fungi was found to be negligible (data not shown).
**Permeability assays**

For the analysis of the cell permeability, we used cultures of the fungi grown to stationary phase in a liquid Czapek medium with 2% glucose.

The changes of the cell permeability of the terrestrial fungi exposed to tNP were monitored from the "loss" by the cells of metabolites exhibiting an absorption band in the ultraviolet (220-350 nm) [17]. A 200-mg weighed portion of mycelium was resuspended in 20 mL of distilled water and incubated for 1 h on a rotary shaker (230 rpm) at 30°C. The supernatant was analyzed with a Genesys 10 UV scanning spectrophotometer (Thermo Spectronic, USA). The permeability was expressed as arbitrary units per gram of dry weight biomass (d.w.b.).

**Hydrolitic enzymes assays**

For the analysis of cellulytic enzyme activity we used 7-day cultures of the fungi grown in a liquid Hutchinson medium with 1% sodium carboxymethylcellulose (Na-CMC). The enzymatic activity of cellulase was determined with the use of Na-CMC according to the procedures described by Li et al. [18]. The results were expressed in micrograms of glucose per microgram of protein.

The biomass production in these experiments was estimated from the resultant protein content determined by the method of Lowry et al. [19].

For the assay of proteolytic enzyme activity, the strains were grown in a liquid medium containing (in g/L) MgSO₄ – 0.52; KCl – 0.52; KH₂PO₄ – 1.52; FeSO₄·7H₂O – 0.01; ZnSO₄·7H₂O – 0.01; glucose – 20.0; and albumin – 10.0 for 5 days. The extracellular proteolytic activity was determined according to the procedures described by Liu et al. [20]. The results were expressed as units per gram of dry weight biomass.

For the analysis of the amylase activity, we used 5-day cultures of the fungi grown in a liquid Czapek medium with 2% soluble starch at 28°C. The amylolytic activity was determined using the colorimetric procedure based on starch hydrolysis by amylolytic complex enzymes to dextrins of varying molecular weight according to Sandhu et al. [21]. The results were expressed in grams of hydrolyzed starch per gram of dry weight biomass.

**Statistical analyses**

All statistical analyses were performed with Statistica software (version 6; Statsoft). All of the data are presented as the mean ± SD of triplicates (n = 3). The data were tested with standard variance ANOVA, followed by Student’s t-test to determine significant differences. The differences were considered significant at P≤0.05

**III. RESULTS AND DISCUSSION**

In this study we used the terrestrial fungal strains isolated from the bottom sediments of the coastal area of the eastern part of the Gulf of Finland.

The fungi were identified based both on the morphological characteristics according to the most common criteria [14, 15] and on analysis of the sequences of the ITS region of DNA.

For the investigation we selected terrestrial fungal strains that exhibited different sensitivities to NP (Table 1).
The strains investigated can be arranged in increasing order of their sensitivity to NP in the following sequence: *Penicillium expansum* F 44 < *Penicillium glabrum* F 41 < *Aspergillus tubingensis* F11 < *Cadophora fastigiata* F 17. The EC$_{50}$ value for *Cadophora fastigiata* F 17 strain, which is the most sensitive to tNP is 10-20 times lower than that for the strains from the genera *Aspergillus* and *Penicillium*.

Table 1. Toxicity parameters of tNP for terrestrial fungi

<table>
<thead>
<tr>
<th>Fungal culture</th>
<th>*EC$_{50}$, mg/L</th>
<th>*EC$_{90}$, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus tubingensis</em> F11</td>
<td>10.0</td>
<td>&gt;100.0</td>
</tr>
<tr>
<td><em>Cadophora fastigiata</em> F 17</td>
<td>1.0</td>
<td>7.0</td>
</tr>
<tr>
<td><em>Penicillium glabrum</em> F 41</td>
<td>15.0</td>
<td>&gt;100.0</td>
</tr>
<tr>
<td><em>Penicillium expansum</em> F 44</td>
<td>20.0</td>
<td>&gt;100.0</td>
</tr>
</tbody>
</table>

*EC$_{50}$ and EC$_{90}$ are the effective concentrations of 50 and 90% toxicant inhibition of fungal growth, respectively.

The values of the toxicity parameters were calculated per 48 hours.

One reason for such significant differences in sensitivity to the toxicant studied among the fungi may be the change in the fungal cell permeability, in particular in cell membrane permeability, induced by NP.

The cytoplasmatic membrane is the primary target of negative impact of many chemical substances [22]. For all living cells, the ion transport through the cell membrane is essential for maintaining the ionic and osmotic homeostasis of the cell, as well as for information transfer, energy supply for cellular metabolism, substrate accumulation, and degradation products removal [23].

Permeability describes the ease with which ions can pass through a cell membrane to move substances into and out of the cell. Various toxicants have been reported to cause changes in permeability of fungal cell membranes [24, 25] in consequence of adaptation to the toxicant action. One factor that may be responsible for changes in cell membrane permeability is oxidation of membrane lipids [26]. Enhancement of membrane lipid peroxidation under NP-induced oxidative stress was observed in microalgae [27, 28].

Under tNP exposure, *Cadophora fastigiata* F 17 strain, the most sensitive to tNP, exhibited a 1.6-fold increase in the cellular permeability relative to the control (without tNP) (Table 2).

Table 2. Effect of nonylphenol on the cell permeability of the terrestrial fungi

<table>
<thead>
<tr>
<th>Fungal culture</th>
<th>tNP content, mg/L</th>
<th>Permeability, % to control</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus tubingensis</em> F11</td>
<td>50.0</td>
<td>45±10</td>
</tr>
<tr>
<td><em>Cadophora fastigiata</em> F 17</td>
<td>1.0</td>
<td>162±17</td>
</tr>
<tr>
<td><em>Penicillium expansum</em> F 44</td>
<td>50.0</td>
<td>85±18</td>
</tr>
<tr>
<td><em>Penicillium glabrum</em> F 41</td>
<td>50.0</td>
<td>91±14</td>
</tr>
</tbody>
</table>

Increased cellular permeability may facilitate the entry of toxicant into the cell, as well as the loss of vital metabolites. In tNP-resistant strains of the filamentous fungi the permeability of cell membranes either remained practically unchanged (*Penicillium expansum* F 44 and *P. glabrum* F 41) or significantly, by up to 55%, decreased (*Aspergillus tubingensis* F11) relative to
the control variants (without tNP). These findings suggested that one possible mechanism behind high resistance of terrestrial fungi of genera *Penicillium* and *Aspergillus* is a decrease in the cell membrane permeability, which complicates the toxicant penetration into the cell.

Terrestrial fungi, which are an important component of aquatic ecosystems, including bottom sediments, possess a wide range of extracellular hydrolytic enzymes which enable them to actively degrade organic matter in the aquatic environment [9]. Therefore, the impact of tNP on the hydrolytic enzymes involved in fungal degradation of organic matter in water and bottom sediments is an issue that deserves special attention.

The cellulolytic enzymes performing biodegradation of cellulose, the most abundant biopolymer on Earth, occupy the central position in the organic carbon cycle. Among the terrestrial fungi isolated, *Aspergillus tubingensis* F11, *Penicillium expansum* F 44, and *Penicillium glabrum* F 41 strains exhibited cellulase activities. As shown by our previous study [29], the trend in the cellulase activity in the *Penicillium expansum* F 44 strain under the tNP influence depends on the tNP concentration. At low tNP concentrations (up to 1.0 mg/L) that do not significantly affect the *Penicillium expansum* F44 strain growth, the enzyme activity increased by 125% relative to the control (without tNP). An increase in tNP concentration in the medium to >1.0 mg/L resulted in both the culture growth inhibition and reduction in the cellulase activity, to 71% of the control value at tNP concentration of 10.0 mg/L. The cellulase activity of the *Aspergillus tubingensis* F11 strain was affected by tNP in a similar way (Fig. 1). By contrast to *Penicillium expansum* F 44 and *Aspergillus tubingensis* F11 strains, *Penicillium glabrum* F 41 exhibited a significant reduction in the cellulase activity both at low tNP concentrations that left the fungal growth practically unaffected (up to 5 mg/L) and at the growth inhibiting tNP concentrations (10.0 mg/L) (Fig. 1).

![Fig.1. Effect of tNP on the cellulolytic enzyme activity of the terrestrial fungi. The samples were taken in three independent trials.](image-url)
Starch-hydrolyzing amylolytic enzymes were detected in all the terrestrial fungi investigated in this study. The tNP effect on the amylase activity of the terrestrial fungi was found to be species-nonspecific. Under tNP exposure, a decrease in amylase activity by 35 to 60%, depending on the species to which the strain belongs, was observed for all the strains investigated (Fig. 2). It should be noted that the inhibitory effect of tNP on the amylase activity of the filamentous fungi was observed both at tNP concentrations that have no effect on the micromycete growth and at the growth inhibiting tNP concentrations.

![Fig. 2. Effect of tNP on the amylase activity of the terrestrial fungi. The samples were taken in three independent trials.](image)

Our previous study [13] has revealed similar effects from tNP treatments on the cellulase and amylase activities of other fungal strains of the genera *Aspergillus, Penicillium, Cladosporium* and *Exophiala*.

Along with cellulase and amylase activity, the activity of proteolytic enzymes as influenced by tNP exposure of the terrestrial fungi seemed to be an important research subject. This is due to the fact that not only these enzymes are known for their participation in protein breakdown in bottom sediments but also the secreted proteases have been intensively investigated as potential virulence factors of fungi [30].

Using the *Aspergillus tubingensis* F11 and *Penicillium expansum* F44 strains as an example, we demonstrated that, under tNP exposure, the protease activity of the strains isolated increased 1.4-1.5 times (Fig. 3).
In our previous studies [13, 29] we have shown that tNP can also increase the synthesis of other pathogenicity factors of fungi, pigments and polysaccharides.

Our data suggest that tNP has a potential to enhance fungal pathogenicity, which may lead to adverse environmental impacts, namely, to emergence of strains with enhanced virulence.

IV. CONCLUSIONS

Thus, terrestrial fungal species having different resistances to tNP were isolated from the bottom sediments of the coastal area of the eastern part of the Gulf of Finland. One reason for the differences in sensitivity to tNP among the fungi is presumably the disturbance of the cellular permeability. Environmentally relevant concentrations of tNP cause significant changes in activity of hydrolytic enzymes (cellulases, proteases and amylases) in the terrestrial fungi *Aspergillus tubingensis*, *Penicillium expansum*, *Penicillium glabrum*, and *Cadophora fastigiata* involved in organic matter degradation in bottom sediments. There can be increasing or decreasing trend, depending on both the type of enzyme and the tNP concentration. The revealed changes may disrupt the destructive processes in bottom sediments, as well as succession and stability of microbial communities functioning in the aquatic environment. It was also found that, along with enhancement of the synthesis of such fungal pathogenicity factors as pigments and polysaccharides, developed in fungi as adaptive mechanisms, tNP contributes to the activation of proteolytic enzymes, also considered as potential virulence factors. This may lead to emergence fungal strains with enhanced virulence in aquatic microbiocenoses. The investigations of the physiological responses of terrestrial fungi under nonylphenol will be important for biochemical processes dynamics and their environmental consequences evaluation.

Fig. 3. Effect of tNP (20 mg/L) on the protease activity of the terrestrial fungi. The samples were taken in three independent trials.
V. REFERENCES


