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# Assimilation of synthetic plastic nanoparticles by the oomycete *Pythium aquatile*

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**ABSTRACT.** The degradation of synthetic plastics in the environment proceeds through the stages of micro- and nanoparticles. Freshwater fungi and fungus-like organisms are key decomposers of organic material. The most fungi (including the fungus-like oomycetes) feed by osmotrophy, which involves the release of exoenzymes into the environment to break down complex biological polymers, followed by cellular uptake of simplified monomers. This article aims to cultivate oomycete *Pythium aquatile* Höhnk, 1953 in the presence of synthetic plastic nanoparticles as an only source of carbon compounds. The nanoparticles were bulk stained with water-insoluble fluorescent dye. We studied such widely used polymers as polystyrene (PS), poly(vinyl chloride), poly(methyl acrylate) (PMA) and poly(methyl methacrylate) (PMMA). This first experiment on exposure of plastic nanoparticles to oomycete hyphae showed the ability of these fungus-like organisms to capture these particles through a certain type of endocytosis. The fate of nanoplastics in the presence of *P. aquatile* depends on the chemical structure of the polymer. PMMA with a quaternary carbon atom is the most stable, PMA and PS containing the active α-CH atom are rapidly destroyed by oomycetes.

***Keywords***: oomycete, nanoplastics, polystyrene, poly(vinyl chloride), poly(methyl acrylate), poly(methyl methacrylate)

## 1. Introduction

Plastic waste has become a serious challenge for humanity in recent decades. The high stability of synthetic polymers in the environment was seen as the main problem. Years of research on building and household plastics show that these substances are not inert and can be involved in ecological chains (Amobonye et al., 2021). Microplastic particles (less than 5 mm and greater than 1 μm) can cause various damages to multicellular organisms, including fish, birds and mammals (Wang et al., 2021; Bhuyan, 2022; Zolotova et al., 2022). Nanoplastics, especially particles smaller than 200 nm, can potentially penetrate living cells through endocytosis (Liu et al., 2021). There are various organisms such as bacteria, fungi, algae, cyanobacteria and waxworms that can break down synthetic plastics (Nguyen et al., 2023). The study of biodestruction of plastic materials is a very topical task as a way to environmentally safe remediation of plastic waste.

Freshwater fungi and fungus-like organisms are key decomposers of organic material, playing an important role in nutrient cycling, bioremediation, and ecosystem functioning. Aquatic fungi can metabolize organic xenobiotics (Baker et al., 2019; Miglani et al., 2022), plastic polymers (Zeghal et al., 2021; Srikanth et al., 2022), heavy metals, dyes, industrial chemicals such as polychlorinated biphenyls (PCBs) and pharmaceuticals such as diclofenac (Assad et al., 2021). Most fungi (including the fungus-like oomycetes) feed by osmotrophy. This form of nutrition involves the release of exoenzymes into the environment to break down complex biological polymers, followed by cellular uptake of simplified monomers.

The study of the effects of plastic pollution, both macro- and microplastics, on various living organisms found several species capable to degrade the surface of plastics (Liu et al., 2022). Such fungi as *Zalerion maritimum* (Paço et al., 2017) and *Aspergillus flavus* (Zhang et al., 2020) can utilize polyethylene as a substrate and degrade it. Some fungal species are able to

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use plastic particles as a source of carbon for their growth (Sánchez, 2020). The degradation of synthetic plastics in the environment under the influence of mechanical, light and biological factors proceeds through the stages of micro- and nanoparticles. The question of interest is whether osmotrophs degrade polymers to low molecular weight compounds or can they assimilate nanoparticles as well? Feeding of osmotrophs with fluorescent stained plastic nanoparticles may answer this question.

Oomycetes are fungus-like heterotrophs, marine, freshwater, and terrestrial, capable of parasitizing a wide range of animals and plants, including algae (Beakes et al., 2012). Most oomycetes are saprotrophs and are responsible for the decomposition and recycling of organic materials in freshwater ecosystems. According to modern taxonomy, oomycetes belong to the Stramenopile group and, together with Rhizaria and Alveolates, form the SAR clade (Adl et al., 2012). Most oomycetes have the same modes of feeding and ecological roles as true fungi. For example, oomycetes grow by forming a network of filamentous hyphae that secrete digestive enzymes.

*Pythium* species are among the most common aquatic oomycetes. The genus currently includes 335 species and varieties (Index Fungorum, 2022), which generally maintain a parasitic (on plants or animals) or saprotrophic lifestyle. *Pythium* spp. are also known to be pathogens of algae (Lee et al., 2015; Herrero et al., 2020). More than 35 species of *Pythium* live in water, some species live in the soil (Nam and Choi, 2019). *Pythium* species capable of producing carbohydrate-active enzymes (CAZymes) are involved in the metabolism of glycoconjugates, oligosaccharides and polysaccharides and, in the case of plant pathogens, participate in the degradation of the host cell wall and storage compounds (Zerillo et al., 2013). Enzymes of this class are shown to play a key role in the biodegradation of plastic by fungi (Srikanth et al., 2022).

The first finding of *Pythium* in Lake Baikal was made by E.A. Kuznetsov in 1980 (Kuznetsov, 2003). Using classical morphological methods, he detected *Pythium debaryanum* in the Baikalsk Pulp and Paper Mill area. This species indicates a strong cellulose contamination. Later, *Pythium* was detected in Lake Baikal in the area of Listvennichny Bay and Bolshoi Ushkaniy Island (Bukin et al., 2022).

This article aims to cultivate *Pythium* in the presence of synthetic plastic nanoparticles as an only source of carbon compounds. The nanoparticles were bulk stained with water-insoluble fluorescent dye. In this work we studied such widely used polymers as polystyrene (PS), poly(vinyl chloride) (PVC), poly(methyl acrylate) (PMA) and poly(methyl methacrylate) (PMMA). *Pythium aquatile* Höhnk, 1953, isolated from the green alga *Draparnaldioides* spp. endemic to Baikal, was applied.

## 2. Materials and methods

### 2.1. Chemical reagents

Acetone, dichloromethane, chloroform, benzene, ethanol, tetrahydrofuran (THF) and sodium dodecyl sulfate (SDS) (reagent grade) were purchased from Alfa Aesar (Thermo Fisher Scientific Inc.). Acetone, chloroform, benzene, ethanol and dichloromethane were distilled before use. THF was refluxed with $LiAlH_4$ and distilled under argon. PS (MW 192 kDa), fluorescence dye dibenzylfluorescein (DBF), azobisisobutyronitrile (AIBN), methyl acrylate (MA), $K_3[Fe(CN)_6]$, methylene blue and sodium dodecyl sulfate (SDS) were purchased from Merck KGaA or Thermo Fisher Scientific Inc. Methyl acrylate (MA) was purified by distillation. Disposable spectrophotometric cuvettes (BRAND GMBH þ CO KG, Germany) were used as a source of PMMA. PVC (viscometry MW 1,600 kDa, measured in THF at 25°C (de Vries et al., 1971) was from OJSC Usoliekhimprom, Usolye-Sibirskoe, Russia. PMA was prepared by radical polymerization in boiling benzene (MA 24.8% w.t., AIBN 0.5% of the monomer weight) within 4 hours. The polymer was purified by multiple reprecipitation from dichloromethane into ethanol, yield was 85%. Molecular weight (285 kDa) was determined by viscometry in benzene at 30°C (Polymer Handbook, 1999).

### 2.2. Dispersions of nanoplastics

Dispersions of nanoplastics were prepared similarly (Annenkov et al., 2021). A solution of the polymer (30 mg) and dibenzylfluorescein (DBF, 0.25% of the polymer weight) in 10 ml THF (acetone for PMMA and PMA) was added by drops to an intensively stirred (2200 rpm) SDS solution (140 ml, 71.4 mg/L) during 20 min. Then stirring was continued for additional 10 min. The resulting turbid mixture was passed through a cotton filter to remove large pieces of polymer. The filtrate was treated with ultrasound for 10 min and centrifuged at 20,000 g for 30 min. The supernatant was carefully removed and the precipitate was resuspended in distilled water by ultrasonic treatment for 30 min followed by filtration through a 1.2 μm filter to obtain the final dispersion of nanoparticles.

The polymer concentration in the obtained dispersions was determined using UV spectroscopy for PS, IR spectroscopy for PVC and the gravimetric method for PMMA and PMA (Annenkov et al., 2021). PS dispersions were air dried and dissolved in methylene chloride to record UV spectra. The concentrations were found using the calibration curve. PVC concentrations were found by FTIR spectroscopy using calibration curves for signal intensity ratios of 1253 $cm^{-1}$ (PVC) and 2117 $cm^{-1}$ (CN group of $K_3[Fe(CN)_6]$). To prepare the calibration mixtures, PVC and $K_3[Fe(CN)_6]$ (internal standard) were mixed in different ratios, thoroughly ground with KBr, and pressed into pellets. The PVC dispersion was mixed with $K_3[Fe(CN)_6]$ solution, dried, mixed with KBr, thoroughly ground and pressed into pellets for the IR spectroscopy. SDS concentrations (Table) were determined according to the method given in (Hayashi, 1975; Palshin et al., 2020). The size of nanoplastics dispersions (Table) was measured by dynamic light scattering (DLS). All dispersions were diluted to 500 mg/L plastic and stored at 4°C.

**Table.** Characteristics of dispersions of nanoplastics with DBF dye.

| Samples | Polymer | SDS, mg/L | Particle radius, nm |
|---|---|---|---|
| PV22-M36 | PS | 6.1 | 89 |
| PV22-M37 | PVC | 30.1 | 39 |
| PV22-M38 | PMMA | 11.4 | 38 |
| PV22-M53 | PMA | 15.9 | 40 |

## 2.3. Oomycetes cultivation and molecular identification

Thallus pieces 5-10 mm in size of representatives of the Baikal endemic genus *Draparnaldioides* were placed in Petri dishes with potato-glucose agar and sprinkled with cefotaxime antibiotic to inhibit bacterial growth. The germinated mycelium was reseeded on fresh medium to obtain a pure culture.

Genomic DNA was extracted following a modified protocol of Doyle and Dickson (Doyle and Dickson, 1987). Species identification was carried out using morphological methods and the ITS1-4 barcode accepted for fungi (White et al., 1990) from the UNITE database (Nilsson et al., 2019).

For the experiment, the fungus was cultivated for 5 days in liquid Czapek-Dox medium (Dox, 1910) at 22°C with constant agitation. Then it was transferred to sugar-free Czapek-Dox medium and cultured under the same conditions for 10 days, then nanoparticles were added.

## 2.4. Instrumentation

The absorbance spectra were measured on an SM-2203 spectrofluorometer (CJSC Spectroscopy, Optics and Lasers – Modern Developments (SOLAR), Minsk, Republic of Belarus). IR spectra were recorded on an Infralum FT-801 instrument (SIMEX company, Novosibirsk) using KBr pellets. Fluorescence microscopy was carried out using a MOTIC AE-31T instrument with a HBO 103 W/2 OSRAM mercury lamp with blue filter (excitation 450 nm, emission from 520 nm). DLS experiments were carried out using a LAD-079 instrument designed at Kutateladze Institute of Thermophysics (Novosibirsk, Russia).

## 3. Results and discussion

Particles less than 200 nm in diameter prepared from industrial plastics (Fig. 1) were added to *P. aquatile* hyphae. The culture medium contained no carbon source other than plastic nanoparticles. The green fluorescence of the water-insoluble dye inside the nanoparticles was used to detect them in hyphae. Hyphae of *P. aquatile* show no fluorescence in the control experiment.

Fluorescent plastic nanoparticles become visible in the hyphae within the first hour of cultivation (Fig. 2). The green fluorescence in the hyphae persists for a week in the case of PVC and PMMA, but disappears in the experiment with PS during the first day (Fig. 2 – Fig. 5). We found no reliable presence of PMA

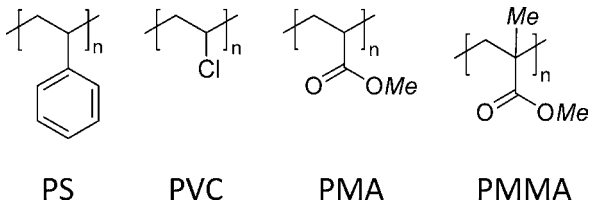


**Fig.1.** Structures of the studied plastics.

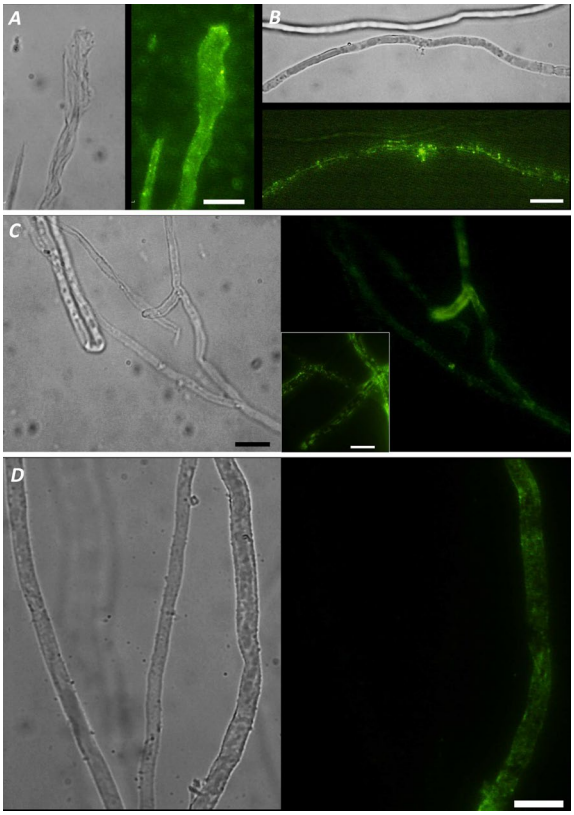


**Fig.2.** Light and fluorescence images of *P. aquatile* hyphae after cultivation in the presence of PVC nanoparticles. The concentration of plastic was 10 mg/L, cultivation time 15 min (A), 2 (B), 4 (C) and 24 h (D). Scalebar represents 10 μM.

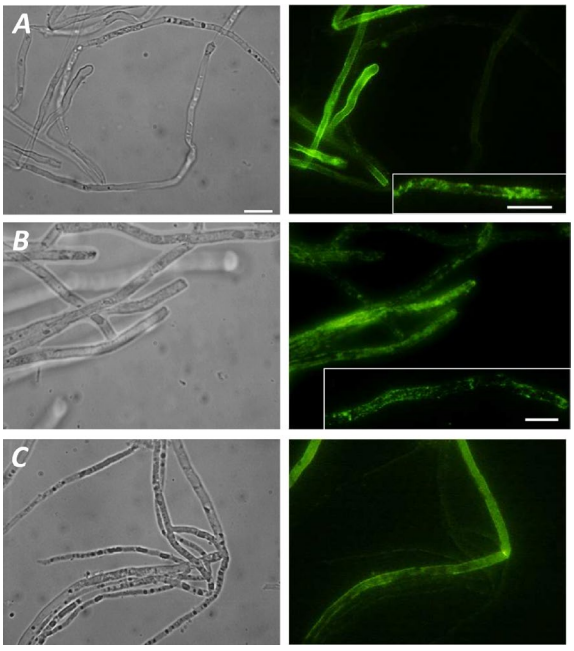


**Fig.3.** Light and fluorescence images of *P. aquatile* hyphae after cultivation in the presence of PMMA nanoparticles. The concentration of plastic was 10 mg/L, cultivation time 2 (A), 4 (B) and 24 h (C). Scalebar represents 10 μM.

nanoparticles in hyphae. The observed differences in the behavior of nanoplastics cannot be explained by the particle size: PMA particles have the same size as PVC and PMMA. All polymers are hydrophobic substances and the nanoparticles are stabilized by the same surfactant compound, so they are expected to be similar for the endocytosis system.

We hypothesize that the studied nanoparticles differ in their resistance to the destructive action of oomycete enzymes. Visible biodegradation of bulk plastic samples occurs within a few days (Kim et al., 2020; Zeghal et al., 2021; Zhang et al., 2022), while in the case of 100-200 nm particles, degradation can be expected within a few hours or sooner. The enzymatic degradation of vinyl-based polymers begins with the oxidation of the α-CH group and causes the macromolecule to break down into shorter chains (Gaytán et al., 2021 and references in this article). The first stage of this reaction proceeds through the formation of a radical $R\!\!>\!\!C\cdot$. The probability of the reaction increases with increasing radical stability. The phenyl and ether substituents stabilize the radical due to the conjugation effect, which is weaker in the case of chlorine. The α-oxidation pathway is not possible for PMMA due to the presence of a methyl group, and polymers of this type are more resistant to biodestruction (Sabatini et al., 2018). PMA and PS structures are the most sensitive to biodestruction, and the corresonding nanoparticles are rapidly degraded by oomycetes, possibly under the action of enzymes excreted into the cultural medium.

## 4. Conclusions

This first experiment on exposure of plastic nanoparticles to oomycete hyphae showed the ability of these fungus-like organisms to capture these particles through a certain type of endocytosis. The fate of nanoplastics in the presence of *P. aquatile* depends on the chemical structure of the polymer. PMMA with a quaternary carbon atom is the most stable, PMA and PS containing the active α-CH atom are rapidly destroyed by oomycetes. Our work raises some questions:

- What is the relationship between classical saprotrophic nutrition and intracellular assimilation in nanoplastics degradation by fungi and similar organisms?
- Can plastic nanoparticles be the only source of carbon for fungi? What types of plastics are toxic to fungi?
- Do fungi assimilate plastic nanoparticles by endocytosis in the presence of normal fungal food?

There are many other questions related to the biochemical aspects of plastic degradation by fungi, and plastic nanoparticles are a good tool for these studies. The large relative surface area of the nanoparticles accelerates any experiments compared to the use of bulk plastic samples. The plastic in the form of a stable dispersion contacts the surface of the fungus perfectly, which increases the reproducibility of the obtained data.

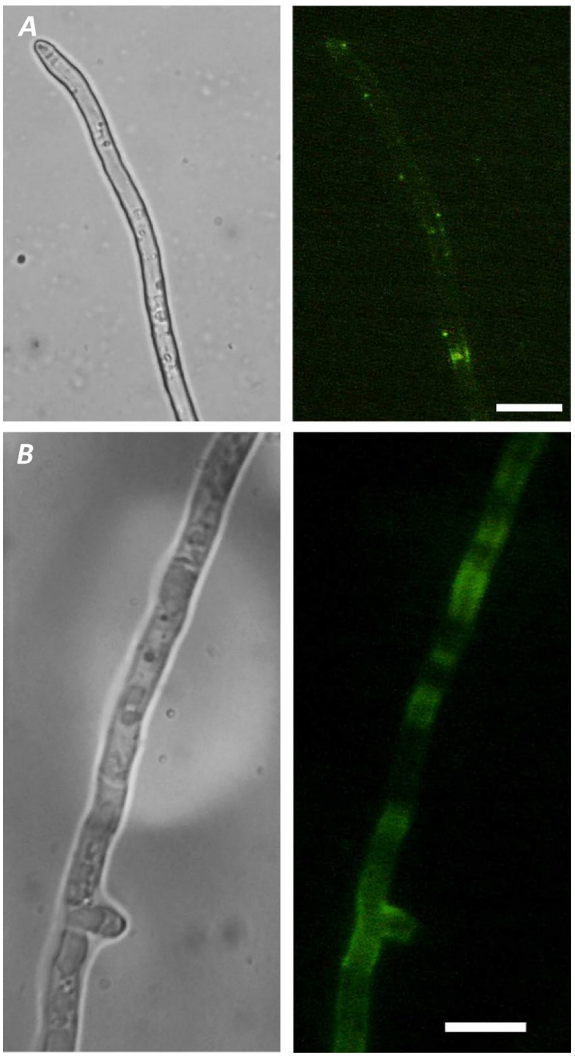


**Fig.4.** Light and fluorescence images of *P. aquatile* hyphae after cultivation in the presence of PS nanoparticles. The concentration of plastic was 10 mg/L, cultivation time 2 (A) and 4 h (B). Scalebar represents 10 μM.

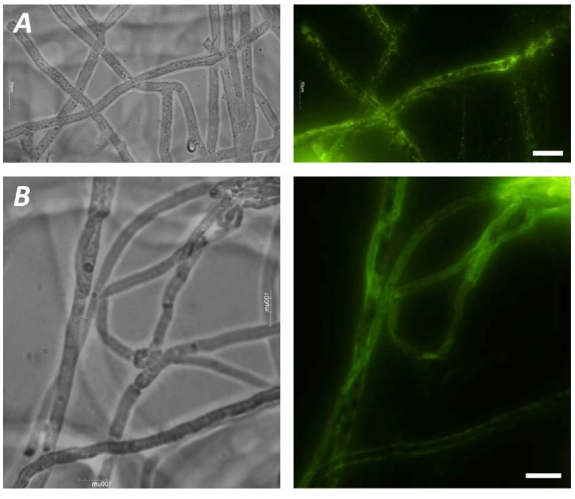


**Fig.5.** Light and fluorescence images of *P. aquatile* hyphae after cultivation in the presence of PVC (A) and PMMA (B) nanoparticles. The concentration of plastic was 10 mg/L, cultivation time 7 days with the addition of a new portion of plastic after 2 days of cultivation. Scalebar represents 10 μM.

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## Conflict of interest

The authors declare no conflict of interest.

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