Original Article

Fluorescent nanoplastic particles: synthesis and influence on diatoms

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ABSTRACT. A method for obtaining fluorescent labeled polyvinyl chloride nanoparticles of 60-110 nm by deposition of plastic and dye solution into water has been developed. These nanoparticles are suitable for testing effect of nanoplastic on living organisms. We have cultivated diatom algae *Ulnaria ferefusiformis* in the presence of polyvinyl chloride nanoparticles and revealed that the suppression of the vital activity in diatoms is not due to the penetration of nanoparticles into the cell but arises from their association with the outer surface of the cells near the labiate process (rimoportula).

Keywords: Nanoplastics, diatom, fluorescence, pollution

1. Introduction

Plastic waste is a global problem for ecosystems. The properties of plastic (low density and high strength) provide the increasing use of plastic products in everyday life, thereby leading to an increase in plastic production (Plastic Europe, 2019). However, these benefits make plastic materials hazardous to the environment (Ryan, 2015). Plastic can persist in nature for many years and spread easily to areas remote from the pollution source (Paul-Pont et al., 2018).

In the environment, plastic products are destructed under the influence of many factors, which leads to the formation of smaller plastic particles: macroplastics (> 25 mm), mesoplastics (5-25 mm) and microplastics (< 5 mm) (Young et Elliott, 2016; European Commission: group of chief scientific advisors, 2019). Microplastics also include particles that cannot be seen with the naked eye (< 50 μm) as well as nanosized particles (< 100 nm) (European Commission: group of chief scientific advisors, 2019).

An analysis of the studies over the past five years can indicate that practically microplastics have been found in every place on the planet (Paul-Pont et al., 2018; Peng et al., 2019). If plastic particles larger than tens and hundreds of microns can be easily found, this is not the case for nanoplastics. Particles smaller than 1000 nm are actively involved in Brownian motion that keeps them dispersed in the water column and facilitates interaction with microorganisms. The potentially high number of nanoplastic particles in the environment, the lack of their monitoring and technical difficulties in their detection together with small size that allows them to penetrate living organisms make nanoplastics one of the most pressing problems of our age in the field of environmental pollution and the vital activity of living organisms.

The effect of micro- and nanoplastics on living organisms is studied on various biological species, from unicellular organisms to mammals (Chang et al., 2019). Despite this, little attention has been paid to primary producers that are potentially more exposed to the threats from the smallest plastic waste. Such organisms include diatoms that produce more than 20% of oxygen and primary organic matter originating from all photosynthetic organisms (Tréguer et al., 1995) as well as influence the global carbon cycle (Field et al., 1998). There are only a few studies of the effect of micro- and nanoplastics on diatoms. The impact of amino-modified micro- and nanoparticles of polystyrene (PS) on the *Chaetoceros neogracile* diatoms resulted in a change in physiological parameters (Gonzalez-Fernandez et al., 2019; Seoane et al., 2019). The presence of PS microparticles reduced the activity of cellular esterase, whereas PS nanoparticles also influenced such parameters as chlorophyll content, esterase activity, cell growth, and photosynthetic efficiency. The impact of micro- and nanoparticles of polyethylene (PE) on the *Thalassiosira weissiflogii* diatoms during 10 hours already led to a significant decrease in the concentrations of diatoms (Baudrimont et al., 2020). PS nanoparticles of 50 and 100 nm in size at a concentration of 5 mg/L caused some toxic effects in the *Phaeodactylum tricornutum* marine diatoms, such as an increase in the oxidative stress, damage to the photosynthetic apparatus and DNA, as well as depolarization of mitochondria and cell membrane (Sendra et al., 2019). Despite the use of fluorescently labelled nanoparticles, the authors could not determine the ability of nanoparticles to pen-

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etrate diatom cells. The obtained data indicate only a certain association of plastic particles with cells. The small size of pores (areolas) in their silica shell comparable with the sizes of nanoparticles can hamper the penetration of plastic nanoparticles into diatoms. We have revealed (Annenkov et al., 2020) that diatoms can capture a water-soluble polymer with a coil diameter of several tens of nm from the environment through endocytosis. In that study, we used a flexible chain polymer, poly(acrylic acid), which coils can deform during the penetration into narrow pores. Therefore, it remains unclear whether plastic nanoparticles that have a toxic effect on diatoms can penetrate cells.

Most modern studies related to the effect of micro- and nanoplastics on living organisms use commercial synthetic plastic particles, both unmodified and with amino and carboxyl groups on the surface. As a rule, the modification degree and the exact structure of modifying groups are unknown, which hampers the use of such samples to simulate the impact of plastic particles on living organisms.

In this study, we developed the method for the synthesis of fluorescently labelled polyvinyl chloride (PVC) nanoparticles with a diameter of 65-110 nm, which were stabilized by the addition of sodium dodecyl sulfate (SDS). The *Ulnaria ferefusiformis* Kulikovskiy & Lange-Bertalot diatom was cultivated in the presence of the obtained nanoparticles monitored with fluorescence microscopy. The *U. ferefusiformis*, a freshwater diatom has been previously known as *Synedra acus* (Kulikovskiy et al., 2016) and is an appropriate model organism in genomic, cytological and biochemical experiments (Ravin et al., 2010; Kharitonenko et al., 2015; Annenkov et al., 2019).

2. Materials and methods
2.1 Chemical reagents

PVC (Russia, Usolie-Sibirskoye) had a molecular weight of 1600 kDa (measured viscometrically in THF at 25 ˚C (de Vries et al., 1971)). Benzyl chloride, sodium dodecyl sulfate (SDS), fluorescein, and K_2CO_3 (Aldrich) were used without preliminary preparation. Cyclohexane was refluxed and distilled over sodium. Tetrahydrofuran (THF) was refluxed with sodium under argon, filtered and distilled from $LiAlH_4$ under argon. Toluene, chloroform, ethyl acetate, methylene chloride, and methanol were purified by distillation. Dimethylformamide (DMF) was shaken for 30 minutes with anhydrous $CuSO_4$, filtered through a Büchner funnel and distilled in vacuum. NaOH was purified from carbonate impurities by filtration of its 50% aqueous solution.

2.2 Synthesis of Dibenzylfluorescein fluorescent probe (O-Benzylfluorescein benzyl ester)

Three grams (9.03 mmol) of fluorescein in the form of acid, 3.74 g (27.1 mmol) of K_2CO_3 and 3.43 g (27.1 mmol) of benzyl chloride were added to 20 ml of DMF at room temperature. The mixture was heated in a glycerol bath (60°C) with magnetic stirring for 3 hours 15 minutes (reaction scheme at Fig. 1). After cooling to room temperature, the reaction vessel was left at 10°C in a refrigerator for 24 hours. The resulting orange precipitate was filtered off on a glass filter, washed repeatedly with cyclohexane, distilled water, 0.1 M aqueous NaOH solution, and several more times with distilled water. The wet product was dried in a vacuum desiccator over anhydrous calcium chloride, and then under vacuum of an oil pump for three hours. Thin-layer chromatography (TLC) (silica gel, ethyl acetate : methylene chloride : methanol = 1:1:0.2) showed single yellow spot with R_f = 0.68. The yield was 2.73 g (59% of theoretical).

2.3 Preparation of PVC fluorescent nanoparticles

A solution of polymer (30 mg) and dibenzylfluorescein (0.25% of the polymer weight) in 10 ml of THF was added dropwise within 20 minutes to an intensively stirred (2200 rpm) solution of SDS (71.4 mg/L) in 140 g of distilled water. Then stirring was continued for additional 10 minutes. The resulting dispersion was filtered through a cotton filter to remove large polymer aggregates. The filtrate was processed with ultrasound for 10 minutes. Then, it was centrifuged at 15000 and 20000 g for 30 minutes. The supernatant was removed using a pipette; the precipitate was resuspended in distilled water under ultrasound for 30 minutes and filtered through a 1.2 µm filter to obtain final dispersion of nanoparticles.
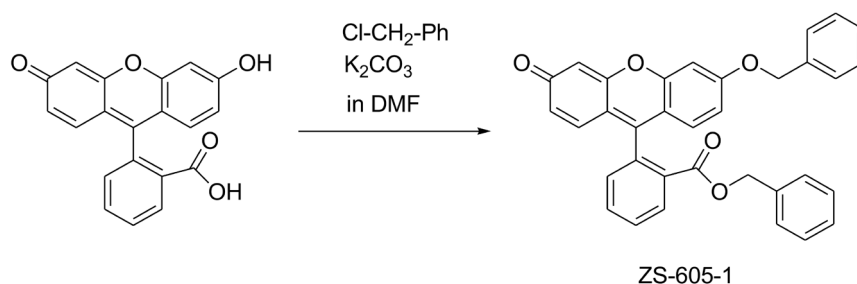
2.4 Determination of SDS

Fig.1. Synthesis of the fluorescent dye

SDS concentrations were determined according to the method given in (Hayashi, 1975). Calibration aqueous solutions of SDS (0.1-10 µg/ml) were first prepared. One ml of every solution was mixed with 0.5 ml of a methylene blue solution (156.3 µM in 0.7 mM phosphate buffer, pH 7.2) and 3 ml of chloroform in a glass vial (Diaem LLC, Moscow, 4ML-13-V1002DU, 15x45 mm, 4 ml) followed by 5 min intense shaking. The mixture was centrifuged to separate the layers for 20 minutes. Then the vial was placed in a spectrophotometer as a sample cuvette to measure the absorbance of the $CHCl_3$ layer at 655 nm using an analogues vial with chloroform as a reference one. The found linear dependence between the absorbance and concentration (Suppl. 2) was used as a calibration curve. Samples of the dispersions were centrifuged at 14000 g for one hour to precipitate interfering nanoparticles. The precipitation was considered completed if the dynamic light scattering (DLS) gave no autocorrelation function. Then 0.2 ml of the upper supernatant layer was made up to 1 ml by deionized water and SDS concentration was determined as described above.

2.5 Determination of PVC

Concentrations of PVC in dispersions were quantified with IR spectroscopy using $K_3Fe(CN)_6$ as an internal standard. Calibration mixtures with different ratio of salt and PVC were ground; the spectra were measured in KBr pellets (Suppl. 3). The absorption band of the CN group at 2117.7 cm^{-1} served as a signal of the salt; three signals at 1428, 1252 and 614.5 cm^{-1} were used for PVC.

The calibration mixtures gave linear dependences for molar ratio of PVC to $K_3Fe(CN)_6$ vs. absorbance ratio of PVC to $K_3Fe(CN)_6$ (Suppl. 4). To determine the concentration of PVC in working dispersions 1 ml of dispersion was mixed with 0.3 ml of a $K_3Fe(CN)_6$ aqueous solution of a known concentration in an agate mortar. The mortar was placed in a vacuum desiccator with anhydrous $CaCl_2$ for a night. The dried mixture was combined with KBr, ground and pressed into a pellet to the measure its IR spectrum. The calibration curves were used to calculate PVC concentrations in suspensions, and the obtained values were averaged.

2.6 Determination of nanoparticle sizes by dynamic light scattering (DLS)

The DLS method was used to determine the particle size of plastics. A PVC dispersion sample was 20-fold diluted with water purified by deionization and filtering through a 0.45 µm cellulose acetate membrane. Measurements were carried out with a 540-nm solid state laser at a 90° scattering angle and temperature of $20 \pm 0.02°C$. The sizes of particles are given in Table 1.

2.7 Spectral characteristics

The absorbance spectrum of the dye was measured for its toluene solution in a 1 cm cuvette using toluene as a reference. The absorbance, excitation and luminescence spectra of the PVC dispersions (100 mg/L) were measured in 1 cm cuvettes using deionized water as a reference (Suppl. 5). The spectral split width for excitation spectra was 10 nm (excitation) and 5 nm (emission), whereas for the emission spectra it was 5 nm (excitation) and 10 nm (emission).

2.8 Diatom cultivation

A culture of the *U. ferefusiformis* diatom was isolated from phytoplankton of Listvennichny Bay, Lake Baikal, Russia. The diatoms were cultivated in DM medium (Thompson et al., 1988) with a silicon content of 0.11 mM (in the form of sodium silicate). The cultivation details are described in (Annenkov et al., 2010). To assess the effect of PVC particles on diatoms, a PVC suspension was added to 5 ml of the culture. The mixture was cultivated for two days and was examined using light and fluorescence microscopy.

2.9 Instrumentation

Deionized water was obtained with a Vodoley deionizer (Khimelektronika company). During the preparation of PVC nanoparticles, solutions were stirred using a MIULAB overhead stirrer (50-2200 rpm).

To measure the absorbance of solutions for determining the SDS concentrations, a UNICO 2100 UV spectrophotometer was used. The absorbance, excitation and emission spectra were measured on an SM-2203 spectrofluorimeter (CJSC Spectroscopy, Optics and Lasers – Modern Developments (SOLAR), Minsk, Republic of Belarus). DLS experiments were carried out using a LAD-079 instrument designed at Kutateladze Institute of Thermophysics (Novosibirsk, Russia). IR spectra were recorded on an Infralum FT-801 instrument (Simeks company, Novosibirsk) for KBr pellets.

An FEI Quanta 200 scanning electron microscope (SEM) was used to study the nanoparticles. The samples were diluted with water, placed on aluminium sample holders, allowed to air dry and sputter coated with gold using SDC 004 (BALZERS) coater. Fluorescence microscopy was carried out using a MOTIC AE-31T inverted microscope with an HBO 103 W/2 OSRAM mercury-vapour lamp.

3. Results and discussion

When a solution of PVC and a hydrophobic dye in an organic solvent is added to an intensively agitated aqueous solution of SDS, it leads to the formation of a dispersion of coloured particles. During precipitation, the precipitated polymer captures the dye into particles. Slow addition of polymer solution and intensive stirring facilitate the formation of small particles, but there are also some visible particles and flakes of the polymer in the system. To remove large particles, we passed the suspension through the cotton filter, after which the filtrate was placed in an ultrasound bath to destroy aggregates of micro- and nanoparticles that could form during filtration. Subsequent centrifugation was carried out to concentrate particles and reduce the SDS concentration in the finished product. Solutions

Table 1. Characteristics of PVC nanoparticles

Sample	Centrifuge, g	Yield, %	Concentration, mg/L		R_h, nm
			PVC	SDS	
PV20-07M	20000	25.6	1017 ± 47	23.6	47
PV20-M31	20000	17.1	518 ± 39	23.3	55
PV20-21M3	15000	16.9	462 ± 41	23.6	43
PV20-22M4	15000	28.4	674 ± 40	24.0	44
PV20-23M*	20000	19.3	851 ± 63	27.1	32

* - obtained by repeated centrifugation of the supernatant mixture from PV20-21M3 and PV20-22M4

were centrifuged in portions in the same test tube. At the same time, the first portions of particles underwent a longer centrifugation than the last ones. Despite this, such a method for concentrating particles did not lead to their irreversible aggregation, and the subsequent ultrasound processing in distilled water and filtration through a 1.2 μm filter resulted in a stable suspension.

To obtain a fraction of smaller PVC particles, we centrifuged (20000g) supernatant remaining after the centrifugation at 15000 g (Table 1, the PV20-23M sample).

Determination of surfactant concentration in the final suspensions is based on the formation of a water-insoluble salt between SDS and methylene blue, which is extracted with chloroform from the aqueous phase. Extraction and measurement of absorbance were carried out in the same glass vial. This allowed us to avoid a possible measurement error caused by a change in the SDS concentration due to the evaporation of a volatile solvent when the solution was transferred to the cuvette. Table 1 shows the results of determining the SDS concentrations. It is noteworthy that dispersion require preliminary centrifugation to get rid of PVC particles interfering the measurement of the SDS concentration.

To determine the PVC concentrations in suspensions, we developed a method based on the IR spectroscopy using potassium hexacyanoferrate (III) as an internal standard. $K_3[Fe(CN)_6]$ has a strong signal at 2117 cm^{-1}, which is not overlapped with the PVC absorption bands at 1428, 1252 and 614.5 cm^{-1} (Fig. 2). The above results (Table 1) indicate that it is possible to prepare PVC dispersions with concentrations of 500-1000 mg/L; the yield was 17-28% of the initial PVC.

The excitation spectrum of nanoparticles in water (Fig. 3) exhibits a rather wide excitation band with several maxima in the range from 350 to 520 nm. In this region, excitation wavelength does not affect the position of the luminescence spectra; the highest luminescence intensity is observed at $\lambda_{ex} = 461$ and 492 nm. Such a wide excitation region enables us to apply optical microscopy with a fluorescence source using various optical filters to detect nanoparticles in the study of their effect on living organisms. There is a split signal in the emission spectra within the wavelength interval from 520 to 570 nm.

Based on DLS, particles precipitated by single centrifugation at 15000 g have a diameter of approximately 100 nm (Fig. 4). Re-centrifugation of the supernatant at higher g values yields smaller particles (Fig.
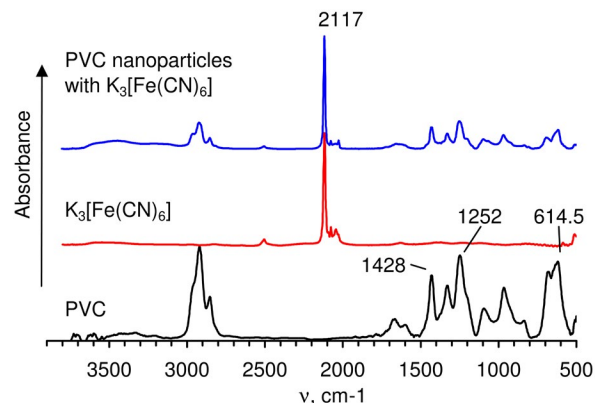

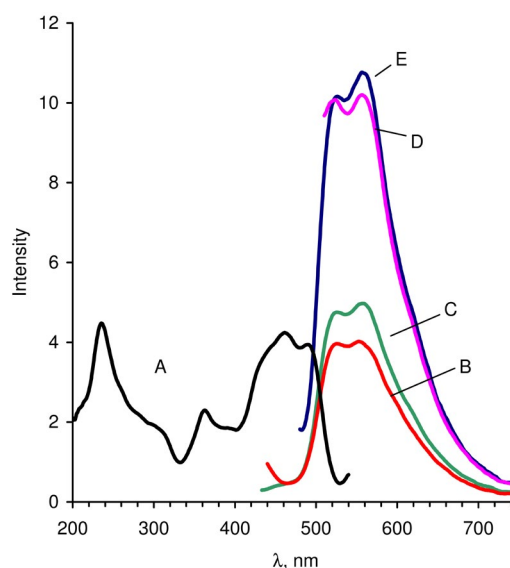**Fig.2.** IR spectra of PVC nanoparticles, $K_3[Fe(CN)_6]$ and their mixtures

Fig.3. Excitation (A) and luminescence (B – E) spectra of PVC nanoparticles in water. PV20-M31, C 100 mg/L. A – excitation at emission wavelength $\lambda_{em} = 555$ nm; luminescence during excitation: B – $\lambda_{ex} = 400$ nm, C – $\lambda_{ex} = 365$ nm, D – $\lambda_{ex} = 461$ nm, and E – $\lambda_{ex} = 492$ nm

4, PV20-23M). SEM Microphotographs confirm the DLS data, which show the particle sizes less than 100 nm (Fig. 5).

U. ferefusiformis were cultivated in the presence of 1 mg/L of PVC particles, the PV20-07M sample. Dilution of the concentrated dispersion to this concentration corresponds to the SDS concentration in the final medium of 0.023 mg/L, which is much lower than the maximum permissible concentration for anionic surfactants in fishery water bodies (0.1 mg/L, Order of the Ministry of Agriculture of the Russian Federation dated

13 December 2016 No. 552) and comparable with the concentration permissible for waters discharged into Lake Baikal (0.01 mg/L, Order of the Ministry of Natural Resources and the Environment of the Russian Federation dated 21 February 2020 No. 83). After cultivation for two days, a significant number of dead cells with degraded chloroplasts accumulated in the culture. We did not observe the penetration of PVC nanoparticles into diatom cells, but there were associations of plastic nanoparticles at the ends of diatom cells (Fig. 6). Labiate process (rimoportula, (Kaluzhnaya and Likhoshway, 2007)) is located there, one of the functions of which is to release polysaccharides and other organic substances that ensure the attachment of diatoms to plants and other surfaces (Sato et al., 2008). Rimoportula is also expected to have other functions, including endocytosis. We can assume that PVC nanoparticles, without penetrating diatom cells, interact with their secreted sticky substance and block the functioning of the cell near rimoportula, which ultimately leads to its death.

4. Conclusions

We have developed the method to prepare 60-110 nm PVC nanoparticles suitable for testing their effect on living organisms. It has been revealed that the suppression of the diatom vital activity is not due to the penetration of nanoparticles into the cell but arises from their association with the outer surface of the cells near the labiate process (rimoportula).

Acknowledgements

References

Annenkov V.V., Danilovtseva E.N., Zelinskiy S.N. et al. 2010. Novel fluorescent dyes based on oligopropylamines for the in vivo staining of eukaryotic unicellular algae. Analytical Biochemistry 407: 44-51. DOI: 10.1016/j.ab.2010.07.032

Annenkov V.V., Zelinskiy S.N., Pal'shin V.A. et al. 2019. Coumarin based fluorescent dye for monitoring of siliceous structures in living organisms. Dyes and Pigments 160: 36-43. DOI: 10.1016/j.dyepig.2018.08.020

Annenkov V.V., Gordon R., Zelinskiy S.N. et al. 2020. The probable mechanism for silicon capture by diatom algae: assimilation of
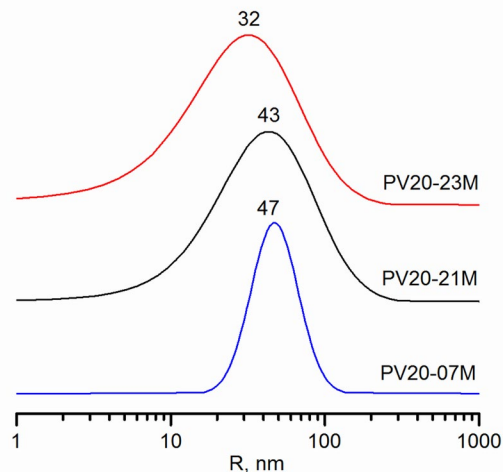

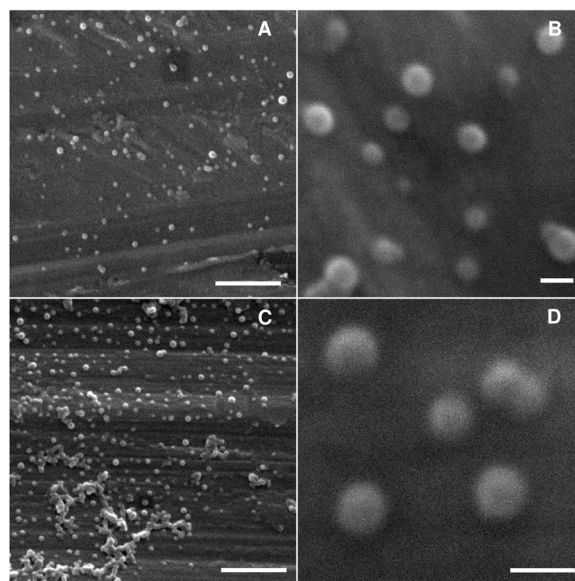**Fig.4.** Size distribution of PVC nanoparticles based on DLS

Fig.5. SEM microphotographs of PVC nanoparticles. A and B - PV20-21, C and D - PV20-23M. Scale: A and C - 1 μm, B and D - 100 nm
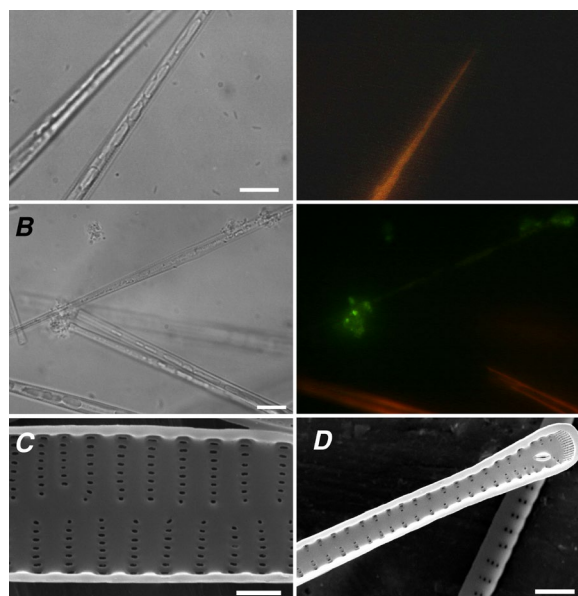

Fig.6. Microphotographs of the *U. ferefusiformis* cells grown in the presence of 1 mg/L of PVC nanoparticles, PV20-07M sample (A and B, left – optical images, right – fluorescent images), and of its silica valves (C and D, SEM). Red fluorescence corresponds to chloroplasts; green – PVC nanoparticles. The arrow (D) indicates labiate process (rimoportula). Scale: 10 (A and B), 1 (C) and 2 (D) μm

polycarbonic acids with diatoms, is endocytosis a key stage in building of siliceous frustules? Journal of Phycology. DOI: 10.1111/jpy.13062

Baudrimont M., Arini A., Guégan C. et al. 2020. Ecotoxicity of polyethylene nanoplastics from the North Atlantic oceanic gyre on freshwater and marine organisms (microalgae and filter-feeding bivalves). Environmental Science and Pollution Research 27: 3746-3755. DOI: 10.1007/s11356-019-04668-3

European Commission: group of chief scientific advisors. 2019. Environmental and health risks of microplastic pollution. SAM Scientific Opinions 6: 1-64. DOI: 10.2777/65378

Chang X., Xue Y., Li J. et al. 2019. Potential health impact of environmental micro- and nanoplastics pollution. Journal of Applied Toxicology 40: 4-15. DOI: 10.1002/jat.3915

Field C.B., Behrenfeld M.J., Randerson J.T. et al. 1998. Primary production of the biosphere: integrating terrestrial and oceanic components. Science 281(5374): 237-240. DOI: 10.1126/science.281.5374.237

Gonzalez-Fernandez C., Toullec J., Lambert C. et al. 2019. Do transparent exopolymeric particles (TEP) affect the toxicity of nanoplastics on *Chaetoceros neogracile*? Environmental Pollution 250: 873-882. DOI: 10.1016/j.envpol.2019.04.093

Hayashi K. A rapid determination of sodium dodecyl sulfate with methylene blue. 1975. Analytical Biochemistry 67: 503-506. DOI: 10.1016/0003-2697(75)90324-3

Kaluzhnaya O.V., Likhoshway Y.V. 2007. Valve morphogenesis in an araphid diatom *Synedra acus* subsp *radians*. Diatom Research 22: 81-87. DOI: 10.1080/0269249X.2007.9705696

Kharitonenko K.V., Bedoshvili Ye.D., Likhoshway Ye.V. 2015. Changes in the micro– and nanostructure of siliceous valves in the diatom Synedra acus under the effect of colchicine treatment at different stages of the cell cycle. Journal of Structural Biology 190: 73-80. DOI: 10.1016/j.jsb.2014.12.004

Kulikovskiy M., Lange–Bertalot H., Annenkova N. et al. 2016. Morphological and molecular evidence support description of two new diatom species from the genus *Ulnaria* in Lake Baikal. Fottea 16: 34-42. DOI: 10.5507/fot.2015.011

Paul-Pont I., Tallec K., Gonzalez-Fernandez C. et al. 2018. Constraints and priorities for conducting experimental exposures of marine organisms to microplastics. Frontiers in Marine Science 5: 1-22. DOI: 10.3389/fmars.2018.00252

Peng L., Fu D., Qi H. et al. 2019. Micro- and nano-plastics in marine environment: Source, distribution and threats — a review. Science of the Total Environment 698 (134254): 1-12. DOI: 10.1016/j.scitotenv.2019.134254

Plastic Europe. Plastics – the facts 2019: an analysis of European plastics production, demand and waste data. Technical Report. 2019. https://www.plasticseurope.org/download_file/force/3183/181

Ravin N.V., Galachyants Y.P., Mardanov A.V. et al. 2010. Complete sequence of the mitochondrial genome of a diatom alga *Synedra acus* and comparative analysis of diatom mitochondrial genomes. Current Genetics 56: 215-223. DOI: 10.1007/s00294-010-0293-3

Ryan P.G. 2015. A brief history of marine litter research. In: Bergmann M., Gutow L., Klages M. (Eds.), Marine anthropogenic litter. Cham: Springer, pp. 1-25. DOI: 10.1007/978-3-319-16510-3_1

Young A.M., Elliott J.A. 2016. Characterization of microplastic and mesoplastic debris in sediments from Kamilo Beach and Kahuku Beach, Hawai'I. Marine Pollution Bulletin 113(1–2): 477-482. DOI: 10.1016/j.marpolbul.2016.11.009

Sato S., Mann D.G., Matsumoto S. et al. 2008. *Pseudostriatella* (Bacillariophyta): a description of a new araphid diatom genus based on observations of frustule and auxospore structure and 18S rDNA phylogeny. Phycologia 47: 371-391. DOI: 10.2216/08-02.1

Sendra M., Staffieri E., Yeste M.P. et al. 2019. Are the primary characteristics of polystyrene nanoplastics responsible for toxicity and ad/absorption in the marine diatom *Phaeodactylum tricornutum*? Environmental Pollution 249: 610-619. DOI: 10.1016/j.envpol.2019.03.047

Seoane M., Gonzalez-Fernandez C., Soudant P. et al. 2019. Polystyrene microbeads modulate the energy metabolism of the marine diatom *Chaetoceros neogracile*. Environmental Pollution 251: 363-371. DOI: 10.1016/j.envpol.2019.04.142

Thompson A.S., Rhodes J.C., Pettman I. 1988. Culture collections of algae and Protozoa: catalogue of strains. Kendal: Titus Wilson & Son Ltd.

Tréguer P., Nelson D.M., Van Bennekom A.J. et al. 1995. The silica balance in the world ocean: a reestimate. Science 268(5209): 375-379. DOI: 10.1126/science.268.5209.375

de Vries A.J., Bonnebat C., Carrega M. 1971. Dilute solution properties and molecular characterization of polyvinyl chloride. Pure and Applied Chemistry 26: 209-240. DOI: 10.1351/pac197126020209