Short communication

Toxic effect of poly(hexamethylene guanidine) on diatom algae

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ABSTRACT. Poly(hexamethylene guanidine) has been used for several decades as a disinfectant for various surfaces and water, including drinking water. Its use has increased significantly due to the COVID-19 pandemic. The toxicity of poly(hexamethylene guanidine) has been studied on warm-blooded animals and fish, but there is little data on its action on planktonic organisms. For the first time the effect of poly(hexamethylene guanidine) (Anavidin preparation) on diatom algae which are one of the main producers of oxygen and photosynthesized organic substances was studied. The obtained data indicate complete suppression of the growth of the diatom *Ulnaria ferefusiformis* (formerly known as *Synedra acus*) at a concentration of 0.5 mg/L. Diatom growth is suppressed by more than 30% at 0.1 mg/L Anavidin, which corresponds to the MAC for water bodies for household and domestic use. Addition of polymeric acids, for example, poly(acrylic acid) partially neutralizes the toxic effect of poly(hexamethylene guanidine). Thus, due to the widespread use of poly(hexamethylene guanidine), it is necessary to thoroughly study its effect on various inhabitants of aquatic ecosystems.

Keywords: poly(hexamethylene guanidine), diatom algae, toxicity, poly(acrylic acid)

1. Introduction

Poly(hexamethylene guanidine) (PHMG) as hydrochloride or phosphate has been used for decades as a disinfectant for various surfaces and water, including drinking water. Its consumption has increased significantly due to the COVID-19 pandemic. The toxicity of PGMG has been studied on warm-blooded animals and fish (Kim et al., 2013; Kim and Ji, 2019; Song et al., 2020), but there are almost no data on its effect on planktonic organisms. PGMG is a polymeric cation with a positively charged nitrogen atom in each polymeric unit:
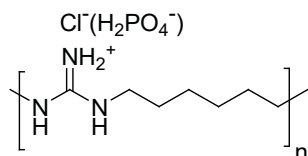
Due to its structure, PGMG is able to bind to cell membranes, disrupting their permeability. A number of studies indicate that the bactericidal activity of PGMG is associated with cell membrane damage (Zhou et al., 2010; Feng et al., 2011). PHMG not only affects membrane permeability but, more importantly, mechanically damages the cell membrane, causing leakage of cell contents into the external environment and subsequent cell death. The authors (Feng et al., 2011) also show that PGMG first damages the outer membrane (if the bacterium has one), and only at higher concentrations also damages the cell wall. The minimum bacteriostatic concentration of the polymer depends on its molecular weight, being, for the most toxic fractions, 2.4 (*Escherichia coli*, 21.8 kDa) and 7.0 (*Pseudomonas aeruginosa*, 15.8 kDa) mg/L.

The acute toxicity of PHMG hydrochloride is 600 mg/kg (rats, (Asiedu-Gyekye et al., 2014)), while liver and cardiovascular system lesions were observed at subchronic (90 days) doses of 0.006 mg/kg. In this regard, the safety of the 3 mg/L concentration recommended by manufacturers for disinfection of drinking water is questioned.

The action of PGMG on living cells depends significantly on the structure of the membranes of these cells. While practically harmless to human skin, it actively destroys lung alveoli when aerosol particles of its aqueous solutions are inhaled, which has led to a number of fatal cases when PGMG was used for water disinfection in household humidifiers (Jung et al., 2014; Kim et al., 2016; Zhua et al., 2020). PGMG entering natural water bodies with sewage and during disinfection of swimming pools can potentially pose a threat to hydrobionts, which makes it necessary to study its toxicity to various organisms. In this work, we first studied the effect of PGMG (Anavidin preparation) on diatom algae, which are one of the main producers of oxygen and photosynthesized organic substances.

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2. Materials and methods
2.1 Chemical reagents

Thiourea and KBrO$_3$ (Sigma Aldrich) were used without preliminary preparation. Hexane was purified by distillation. Dioxane was distilled under sodium. 2,2-Azobis(isobutyronitrile) (Sigma Aldrich) was crystallized from ethanol. Acrylic acid and acryloyl chloride (AC, Sigma Aldrich) were distilled in vacuum. Anavidin (poly(hexamethylene guanidine) phosphate 20%) was purchased from JSC "SPK IrIOCh" (Irkutsk, Russia), molecular weight was 6.1 kDa (viscosimerty according to (Bazaron and Stel'makh, 2008)) which corresponds to polymerization degree as 32. NaOH was preliminarily purified from carbonate impurities by filtering its 50% aqueous solutions. All solutions were prepared with deionized water.

2.2 Instrumentation

Deionized water was obtained with a Vodoley deionizer (JSC Khimelektronika, Moscow, Russia). pH meter/ionomer Multitest IPL-113 and conductometer Multitest KSL-101 (JSC Semico, Novosibirsk, Russia) were applied in physicochemical investigations. Fluorescence microscopy was carried out using a MOTIC AE-31T inverted microscope with an HBO 103 W/2 OSRAM mercury-vapour lamp.

2.3 Synthesis of poly(acrylic acid)
2.3.1 Polymerization of acrylic acid in dioxane

Poly(acryloyl chloride) was prepared via radical polymerization in dioxane (3 g of acryloyl chloride and 12 mL of dioxane) using AIBN as an initiator (2% of the monomer mass) at 60 °C in argon atmosphere in a hermetically sealed 50 mL vial during 72 h (Buruiana et al., 2007). The reaction mixture was dissolved in water, purified with dialysis against water and freeze-dried. The yield was 90%. Molecular weight of the obtained poly(acrylic acid) (PAA) was 20 kDa according to viscometry data (Newman et al., 1954).

2.2.2 Polymerization of acrylic acid in n-hexane

PAA was prepared via radical polymerization in n-hexane (5% w.t.) using AIBN as an initiator (2% of the monomer mass) at the boiling point of n-hexane in a round bottom flask with stirring during 3.5 h. The polymer was washed with n-hexane several times, purified with dialysis against water and freeze-dried. The yield was 86%. Molecular weight was 285 kDa.

2.2.3 Polymerization of acrylic acid in aqueous medium

Acrylic acid (5 g), 0.1 M thiourea (1 mL), 0.1M KBrO$_3$ (2.5 mL) and 0.1 M HCl (1.5 mL) were added to 50 mL of water. Polymerization was carried out for 10 days in a dark place at room temperature. The polymer was purified with dialysis against water and freeze-dried. The yield was 73%. Molecular weight was 2,000 kDa.

2.4. Cultivation of diatoms

A clonal culture of diatoms *U. ferefusiformis* was grown in DM medium (Thompson et al., 1988). It was cultured in 100-ml glass flasks in a New Brunswick G25 incubator (USA) at 12°C and light intensities of 13-16 μmol·m^{-2}·s^{-1} by day/night intervals of 16/8 h. Toxicity experiments were performed in plastic 96-well microculture plates, the wells had a flat bottom and their volume was 400 μL. Synchronous cultures of diatoms were diluted with DM medium to a final concentration of 1000-3000 cells/mL, and 90 μL of suspension was added to each well. Then 10 μL of the corresponding PGMG solution in DM medium was added to each well, and 10 μL of DM medium was used as a control. Experiments with each concentration of this reagent were repeated five to six times. Cultivation was performed in a microincubator (Safonova et al., 2007) at 18°C. The illumination was switched on and off at 12 h intervals, the light intensity at "daytime" was 16 μmol·m^{-2}·s^{-1}.

3. Results and discussion

The data obtained (Fig. 1) show the complete suppression of *U. ferefusiformis* growth at a concentration of 0.5 mg/L. Diatom growth was suppressed by more than 30% at 0.1 mg/L Anavidin, which corresponds to the maximum permissible concentration (MPC) for water bodies for household and cultural-domestic use. A study of diatom cells after exposure to a toxic concentration of PGMG (0.5 mg/L Anavidin) showed (Fig. 2) that chloroplasts retained their shape and fluorescence. It can be assumed that PGMG does not lead to the death of diatom cells, but inhibits their growth and ability to divide. As it is known, diatom cells, after the mitosis stage occurring inside the siliceous shell, must complete two new valves using silicic acid from the environment.
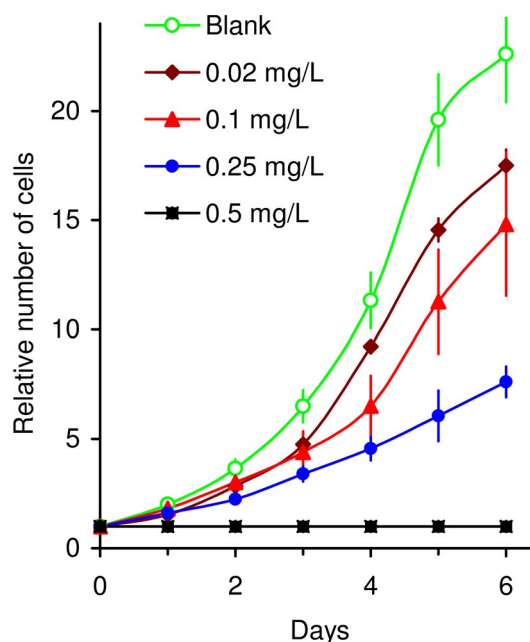

Fig.1. Results of cultivation of diatom *U. ferefusiformis* in the presence of different concentrations of Anavidin.
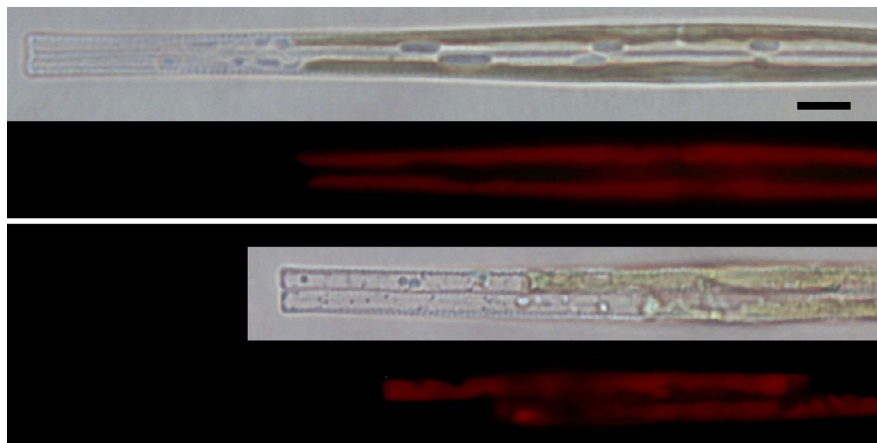
Fig.2. Light and fluorescence images of *U. ferefusiformis* cells before (top) and after cultivation in the presence of 0.5 mg/L Anavidin for 5 days (bottom). The red luminescence is chloroplasts. Scale bar represents 5 μm.

We have recently shown (Annenkov et al., 2020) that the assimilation of silicic acid by diatoms can proceed through its oligomerization stage followed by oligosilicate endocytosis. PGMG, like all polymeric bases, can bind to oligosilicates, hindering or blocking the supply of silicic acid into diatom cells. At the same time, complete cell death may not occur because its cell membrane is protected by a siliceous shell, which, in turn, is covered by polysaccharides.

Considering that polymeric amines form strong interpolymer complexes with polyacids, we suggested that the inhibitory effect of PGMG can be reduced by introducing poly(acrylic acid) (PAA). As shown by the electrochemical data (Fig. 3), PAA forms an interpolymer complex with Anavidin containing about a triple excess of PAA.

Culturing diatoms in the presence of 0.5 mg/L of Anavidin and PAA (Fig. 4, Fig. 5) showed that PAA partially prevented the inhibitory effect of Anavidin, with the low molecular weight PAA (20 kDa) being less active than the higher molecular weight samples (285 and 2000 kDa). It should be noted (Annenkov et al., 2020) that pure PAA suppresses diatom growth only at very high concentrations of tens of mg/L.

4. Conclusions

Thus, for the first time the effect of a widespread antiseptic poly(hexamethyleneguanidine) on the growth of diatoms was studied, the potential danger of disinfectants of this type for aquatic ecosystems was established and the possibility of neutralizing poly(hexamethyleneguanidine) in reaction with polymeric acid was demonstrated.
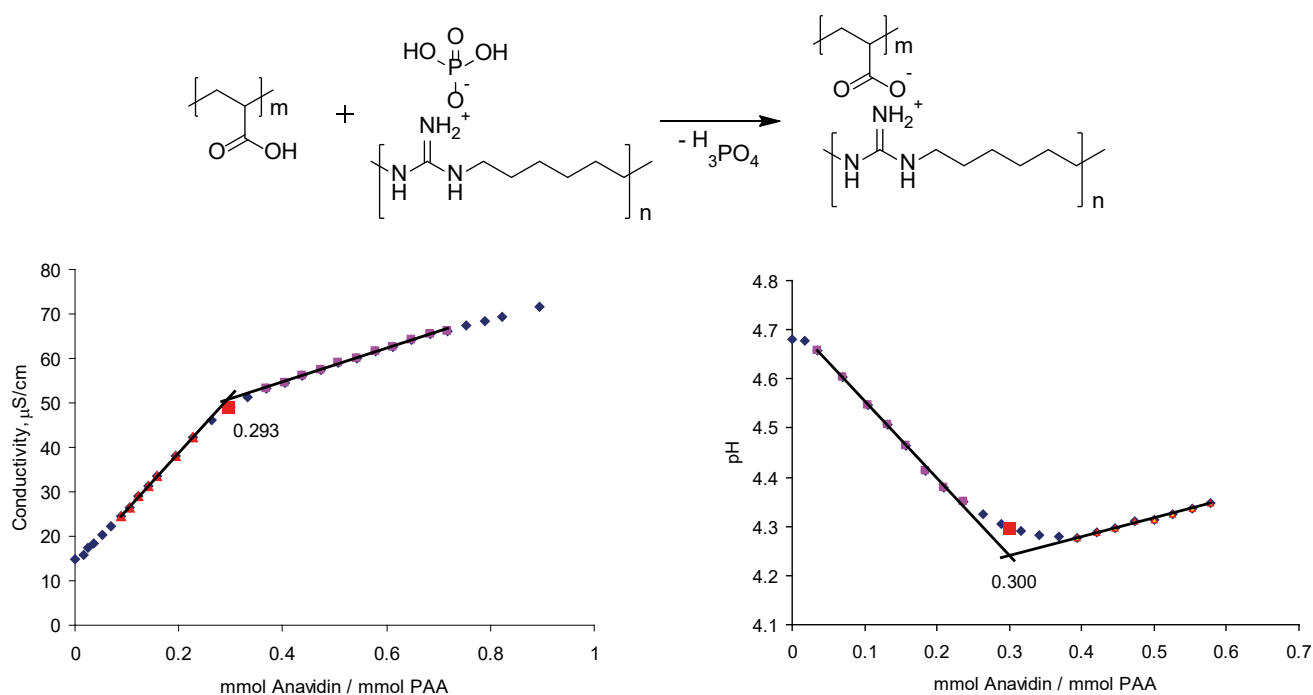

Fig.3. Conductometric (top) and potentiometric (bottom) titration curves of 0.5 mM PAA solution (285 kDa) with 0.1% Anavidin solution.

Acknowledgements

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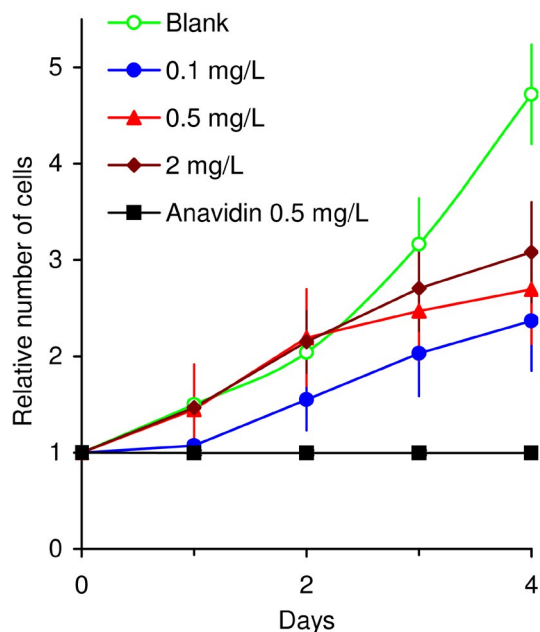
Fig.4. Results of cultivation of diatoms *U. ferefusiformis* in the presence of 0.5 mg/L of Anavidin and different concentrations of PAA (20 kDa).
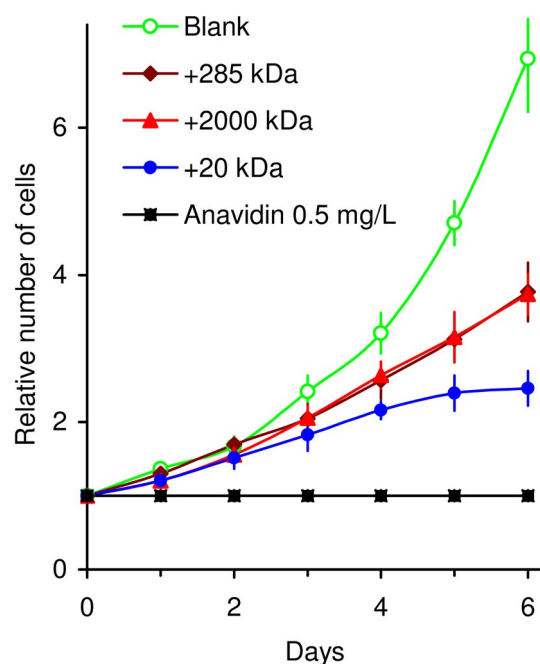

Fig.5. Effect of polyacrylic acid of different molecular weights at a concentration of 1 mg/L on the growth of diatoms in the presence of 0.5 mg/L Anavidin.

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