Research into Antibacterial Activity of Novel Disinfectants Derived from Polyhexamethylene Guanidine Hydrochloride

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Abstract. It is common knowledge that microorganisms cause biological damage to structures and facilities within various buildings and constructions. One of the most effective ways to increase the biological resistance of construction and industrial materials is the introduction of biocides into their composition. This article presents the results of research on the inactivation of various types of microorganisms with new disinfectants of the Teflex group derived from polyhexamethylene guanidine hydrochloride. In the course of research, it was revealed that the specimens have biocidal (bactericidal), fungicidal and sporicidal activity when tested on bacterial suspensions and contaminated surfaces.

1. Introduction
The problem of increased durability of products and structures of buildings and constructions is currently paid considerable attention. It is known that more than 50% of the total volume of damage registered in the world is attributed to the activity of microorganisms. Almost all materials are subject to biological damage, including cement mortars and concretes, composite materials with binders, wood, etc. This is especially true when these materials are used in conditions conducive to the growth of microorganisms: in meat and dairy plants, in vegetable storehouses, livestock buildings, etc.[1, 2, 3, 4, 5]. Mold damage is also common on the interior walls of residential buildings, hospitals, religious buildings, architectural monuments and works of art.

Bacteria, filamentous fungi and actinomycetes constantly and everywhere inhabit the human environment, using organic and inorganic compounds as a nutrient substrate. In recent years, there has been an increase in the diversity and number of microorganisms that cause biological damage to materials and structures. The aggressiveness of known species has enhanced.
Bio-contamination of buildings and structures disturbs ecological situation. The combination of extreme environmental changes, manifested in the form of various processes of infection and biodegradation of building materials and structures, poses a serious threat to intrastate measures aimed at the safety of humans’ life and protection of their health. To expand the durability of building structures and improve the environmental situation, it is necessary to take measures that reduce or eliminate aggressive biological effects.

The following works deal with development of methods improving the bio resistance of building materials [6, 7, 8, 9]. The increase in biological resistance can be achieved through the use of special binders, biocidal additives, etc. [10, 11, 12, 13, 14].

The current sanitary and epidemiological situation in the modern world requires a constant search for new, more effective, scientifically proved methods of disinfection, as well as for the development of highly active, cost-effective environmentally friendly disinfectants with a wide range of antibacterial (antimicrobial) activity.

The relevance of this work is determined by the necessity to develop such biocidal agents which do not pollute the environment; are able to resist microorganisms of various systematic groups (bacteria, moldy fungi, etc.); have a long protective effect, are available and cheap.

Of particular interest in this regard are polymer derivatives that include guanidine, which is part of the amino acids (arginine and creatine) and vitamin B. The guanidine molecule contains three active nitrogen atoms, which allows you to introduce almost any substituents into it and get the positive charge necessary for biocidal activity. The presence of a double bond expands the spectrum of action of this group of preparations [15].

Among above disinfectants are «Teflex» group agents recently developed by CJSC «Soft Protector». These preparations are water-soluble polymer biocides, derivatives of polyhexamethylene guanidine hydrochloride, with transition metal salts and other functional additives introduced into their composition. The products are environmentally friendly disinfectants of the latest generation, safe for humans and animals (4 class of hazard) have low exposure operating concentrations of the active substance, are pH neutral, without color and smell, low toxic, corrosion-resistant and have high storage stability.

The objective of research: to assess the disinfection activity of the Teflex group preparations on the basis of select preparation - «MultiDez» Teflex.

2. Materials and methods
Disinfectant: «MultiDez» Teflex

Test objects: Candida albicans ATCC 10231; Aspergillus niger; ATCC 16404 ; Pseudomonas aeruginosa ATCC 15442; Staphylococcus aureus ATCC 6538; Escherichia coli ATCC 10536; Bacillus subtilis ATCC 6633 spor form; Bacillus cereus 619 spor form.

The «MultiDez» disinfection properties were tested on bacterial suspensions and assessed as follows.

Bacterial suspensions at a concentration of 5x10^8 CFU/ml were mixed in equal volumes with «MultiDez». In the control sample, sterile distilled water was added to the bacterial suspension instead of a disinfectant. Contact of microorganisms with the disinfectant was carried out under constant stirring in room conditions (temperature 20 ± 2°C, relative humidity 50-60 %). The contact time was 1 hour. After completion of incubation, the action of the disinfectant was stopped by a neutralizer solution (30 g/l of polysorbate 80 + 3 g/l of lecithine). The number of viable microorganisms in the suspension was determined by double dilution followed by inoculation on solid Hottinger’s nutrient agar. The disinfecting activity of the agent was evaluated in 24 hours by comparing the number of viable cells with the control sample.

The evaluation of sporocidal properties by radial diffusion technique was performed with respect to the lysed zone of microbial growth after applying drops of tested solutions of various concentrations to the B. Segei lawn. To obtain a lawn culture, B. cereus suspension at a concentration of 1x10^6 CFU/ml was applied to plates with Hottinger agar in the volume of 0.3 ml. The suspension was evenly
distributed with a spatula over the agar surface, the lawn was dried a little, then treatment disinfectant solutions with the following concentrations of the active substance were applied to the lawn surface by drip method - 2%; 1.5%; 1.3%; 1%; 0.8%; 0.5%; 0.4%; 0.2%; 0.1%; 0.05% in the amount of 20 µl. Sterile distilled water applied in the same volume to the lawn was used as a control sample. The degree of disinfecting activity of the tested solutions was evaluated against lysed zones in 24 hours.

Sporocidal properties were tested and assessed through treatment of contaminated coupons. Contamination of test surfaces (sterile glass coupons with an area of 2.5 cm²) was as follows: a spore suspension of B. segeus at a concentration of 5x10⁸ CFU/ml in a volume of 0.02 ml was applied to the coupon, evenly distributing the biological material over the entire area. The estimated contamination of coupons was ~1 x 10⁶ CFU/cm². The coupons were dried under room conditions (temperature 20 ± 20°C, relative humidity 50-60 %) until the full visual drying out of spore suspension. Then they were treated with solutions of disinfectants, applied to contaminated surfaces in the amount of 0.2 ml/coupon. The contact time of spores with disinfectants was 0.5 hours, 1 hour, 24 hours, 48 hours.

As a control sample, we used contaminated coupons treated in a similar way with sterile distilled water. After the completion of exposure, the number of viable spores was determined through washings from coupons using double dilution technique followed by inoculation on Hottinger’s solid nutrient agar. The efficiency of sporocidal exposure was evaluated in 24 hours by comparing the number of viable spores on control samples (which were not exposed to disinfectants) and experimental coupons.

Sporocidal properties were assessed with electronic microscopy the following way. 1 ml. of spore suspension 5x10⁸ spores/ml was mixed with 1 ml. of «MultiDez». In the control sample, 1 ml. of sterile distilled water was added to the spore suspension instead of a disinfectant. Contact of spores with disinfectants was carried out with constant stirring. The contact time was 0.5 hours, 1 hour, 24 hours, 48 hours. The disinfecting effect on Bacillus cereus spores after the contact time was suspended by washing in 10-time volume of sterile distilled water, followed by centrifugation. The resulting spore precipitate was used in preparing agents for electron microscopy.

Samples of B.cereus spores were placed for 10-12 hours in 2,5% glutaraldehyde solution in a phosphate buffer (0,15 M, pH – 7,2), washed in the same buffer, and then put in 1% of OsO4 solution in water. After 4 hours of exposure, the samples were washed with water, dehydrated in ethanol and propylene oxide, and enclosed in Araldide. Ultrathin films were obtained using a diamond knife on an LKB ultratome and then contrasted with uranyl acetate and lead citrate. The finished preparations were examined using a JEM-100 C electron microscope. All experiments were repeated thrice.

Research results:
Table 1 shows the results of disinfection treatment of bacterial suspensions of test cultures. The treatment time was 1 hour.

| Preparation | Test-object | Lg CFU/ml |
|-------------|-------------|-----------|
| Control samples | E.coli | 8,04±0,21 |
| | S. aureus | 8,17±0,12 |
| | Pseudomonas aeruginosa | 8,15±0,13 |
| | Bacillus subtilis | 8,05±0,06 |
| | Candida albicans | 7,71±0,10 |
| | Aspergillus niger | 7,75±0,21 |
| 0,4% of «MultiDez» solution | < 2,0 | < 2,0 |
| | < 2,0 | < 2,0 |
| 1% of «MultiDez» | < 2,0 | < 2,0 |
| | < 2,0 | < 2,0 |
The research results prove that the preparation had a wide spectrum of antimicrobial action. For vegetative forms, the number of viable microorganisms after 1 hour of treatment is reduced by more than 6 orders of magnitude. Sporocidal and fungicidal activity of the preparation is satisfactory, although somewhat lower – did not exceed 5 orders of magnitude. Maximum resistance was detected for the Aspergillus niger culture.

When treating the lawn of the B. cereus culture with «MultiDez», it was revealed that the preparation suppressed the growth of the test culture in a wide range of concentrations (see table.2, Fig.1.).

**Table 2.** The bactericidal activity of "MultiDez" in relation to B. cereus spores on Hottinger’s agar medium.

| Concentration of active substance in the preparation | Lysed zone, cm² |
|-----------------------------------------------------|-----------------|
| 2,00                                                | 4,78±0,46       |
| 1,50                                                | 4,22±0,46       |
| 1,30                                                | 4,16±0,36       |
| 1,00                                                | 3,74±0,26       |
| 0,80                                                | 3,19±0,09       |
| 0,50                                                | 3,08±0,21       |
| 0,40                                                | 2,74±0,09       |
| 0,20                                                | 2,36±0,21       |
| 0,10                                                | 1,97±0,14       |
| 0,05                                                | 1,69±0,08       |
| 0                                                   | 0               |

**Table 3.** Disinfection activity of «MultiDez» against B. cereus spores applied to glass, depending on the time of exposure.

| Preparation | Lg KOE/cm² | Lg CFU/ml |
|-------------|------------|-----------|
| Control     | 5,95±0,26  | 6,00±0,30 | 5,90±0,35 | 5,7±0,54 |
| 0,4% of «MultiDez» solution | 4,44±0,21 | 4,27±0,24 | 3,99±0,21 | - |
| 1% of «MultiDez» solution | 4,08±0,12 | 3,71±0,20 | 3,09±0,19 | Growth not detected |

It was found that the preparation manifests disinfection activity against B. cereus spores on contaminated glass coupons. Thus in 24 hours after exposure to a 1% solution of «MultiDez», the number of viable spores decreased by 3 orders of magnitude compared to the control group. Therefore, the level of contamination of coupons has reduced by 99.9%. After 48 hours of treatment with a 1% solution of «MultiDez», no viable spores were found on contaminated coupons.

The figure below displays changes in the morphological structures of B. cereus spores exposed to a 1% solution of «MultiDez».
Figure 1. Morphological changes in B. cereus spores after treatment with 1% solution of «Multidez».
a – control, b – time of exposure to the disinfectant – 2 hours; c - time of exposure to the disinfectant – 24 hours; d - time of exposure to the disinfectant – 48 hours

On ultrathin spore slide mounts, exposed to MultiDez, the capsule was gradually loosened, its subsequent thinning and delamination in the form of concentric formations, as well as partial damage to the membranes and exosporium took place. Upon long exposures (24 and 48 hours), these processes expanded and led to significant destructive changes in the cell walls and other membrane structures of the spore – fragmentation of membranes, destruction of the cortex and core of most of the spores, which eventually led to the final destruction of bacterial cells.

As of today, guanidine derivatives that combine good biocidal properties with relative low toxicity are one of the most prospective groups of disinfectants (GB 821113, 1959; SU 1184296, 1983, P. A. Gembitsky. Synthesis of metacid, Chem. Ind. 1984, No. 2, pp. 18-19; SU 1687261,1991). Their popularity largely rests on the fact that they are much more effective and safer than quaternary ammonium compounds, surfactants, phenol derivatives and chlorine containing disinfectants. Among the derivatives of guanidine, the most famous are polyhexamethylene guanidine (PGMG) salts (the most famous are chlorhexidine and polyhexamethylene biguanidine), in particular, hydrochloride (PGMG-CH), proposed for the control of bacterial contamination, as well as its derived compositions. However, existing technologies of PGMG-CH production lead to the formation of a rather toxic mixture due to the presence of a significant amount of impurities in its composition.

At the same time, the use of PGMG-CH derived compositions to combat the most resistant forms of microorganisms (mold fungi, spore forms, etc.) requires sufficiently high concentrations of active compound (up to 7% of its weight). The developed technology for obtaining PGMG-CH, which is the basis for the development of Teflex group preparations, has significantly increased the biocidal activity of disinfectants and decreased the environmental burden by reducing concentration of the active substance in the preparation. Improved disinfecting properties of Teflex preparations are achieved by creating a novel PGMG polymer composition and by introducing transition metal salts – nickel chlorite, manganese or iron sulfate, and others.

The above mentioned technological process ensures the creation of a new coordination compound of transition metals with polyhexamethylene guanidine chloride of a linear structure, able to interact with surface molecular complexes of microorganisms’ cells and form a polymer layer on their surface. In this case, some of the amino groups of PGMG-CH interact with the cell surface by forming hydrogen bonds or by electrostatic interaction, through which PGMG-CH is attached onto the surface. The other part of the amino groups is initially bound to metal ions.
Therefore, the cell surface and sorbed PGMG-CH are a supramolecular complex in terms of coordination chemistry. It should be noted that such supramolecular complexes differ in principle from chemically modified complexes obtained through forming covalent bonds. Functional groups of supramolecular surface ensembles are not rigidly fixed on the surface and their mobility remains, including the one after the mechanism of lateral diffusion. The intermediate layer between the cell surface and polymer molecules’ functional groups, through which the compound is fixed onto the cell surface, prevents its rigid interaction with the microorganism’s surface, which allows to retain the reagents’ coordinating ability and their bactericidal properties.

It is known that membranes are the target for polycationic polymers of the PGMG-CH type. As a result of membranes’ deformation caused by the excessive concentration of positive charges accumulated on the external surface of a microorganism (an elementary electrical breakdown or loss of elastic properties may occur), the internal content of the cells leaks out. Then metals come into action, attacking the cell along several biochemical routes simultaneously, including autocatalytic formation of reactive oxygen/nitrogen forms, oxidation of thiol-containing proteins, etc., disrupting the normal biological functions of cells and causing their death. Therefore, the components being the part of the developed preparations produce a synergistic effect, that is a greater effect than the sum of the consequences produced by each separately. This allows us to significantly enhance the bactericidal properties of the disinfectant, without increasing the concentration of active components of the preparation. Differences in the mechanisms of the biocidal action between metals and polycationic polymer ensure the absence of formation of resistant forms of microorganisms when using the above disinfectants.

3. Conclusion
The presented technology for obtaining PGMG-CH made it possible to create agents with a wide spectrum of antimicrobial action and decrease the environmental burden owing to a lower concentration of the active substance in the preparation. The research findings proved the biocidal, fungicidal and sporocidal activity of the «Teflex» group preparations. The "MultiDez" preparation showed it efficiency when tested on bacterial suspensions and in disinfection of contaminated surfaces. The obtained positive results allow us to consider the "Teflex" group preparations as one of the most viable preparations for disinfection measures.

4. References
[1] Erofeev V, Smirnov V, Myshkin A 2019 The study of species composition of the mycoflora, selected surface samples poliferation composites in humid maritime climate IOP Conference Series: Materials Science and Engineering 698(2) 022082 DOI: 10.1088/1757-899X/698/2/022082
[2] Dergunova A, Piskaykina A, Bogatov A, Salman Aa.D.S.D, Erofeev V 2019 The economic damage from biodeterioration in building sector IOP Conference Series: Materials Science and Engineering 698(7) 077020 DOI: 10.1088/1757-899X/698/7/077020
[3] Erofeev V, Smirnov V, Myshkin A 2019 The study of polyester-acrylate composite's stability in the humid maritime operating conditions Materials Today: Proceedings Vol 19 pp 2255-2257 DOI: 10.1016/j.matpr.2019.07.547
[4] Shafigullin L, Treschev A, Telichko V, Erofeev V 2017 Calculation of reinforced concrete shell of positive Gaussian curvature, given different resistantance of concrete and cracking Astra Salvensis pp 77-91
[5] Startsev O, Makhonkov A, Erofeev V, Gudojnikov S 2017 Impact of moisture content on dynamic mechanical properties and transition temperatures of wood Wood Material Science and Engineering 12(1) pp 55-62 doi: 10.1080/17480272.2015.1020566
[6] Erofeev V, Smirnov V, Dergunova A, Bogatov A, Letkina N 2020 Development and research of methods to improve the biosistability of building materials Materials Science Forum 974 MSF pp 305-311 DOI: 10.4028/www.scientific.net/MSF.974.305
[7] Erofeev V, Rodin A, Kravchuk A, Kaznacheev S, Zakharova E 2018 Biostable silicic rock-based glass ceramic foams *Magazine of Civil Engineering* **84**(8) pp 48-56 doi: 10.18720/MCE.84.5

[8] Erofeev V, Rodin A, Yakunin V, Bogatov A, Bochklin V, Chegodajkin A 2018 Alkali-activated slag binders from rock-wool production wastes *Magazine of Civil Engineering* **82**(6) pp 219-227. doi: 10.18720/MCE.82.20

[9] Travush V, Karpenko N, Erofeev V, Rodin A, Smirnov V, Rodina N. 2017 Development of Biocidal Cements for Buildings and Structures with Biologically Active Environments *Power Technology and Engineering* **51**(4) pp 377-384 doi: 10.1007/s10749-017-0842-8

[10] Erofeev V, Rodin A, Rodina N, Kalashnikov V, Erofeeva I 2016 Biocidal Binders for the Concretes of Unerground Constructions *Procedia Engineering* 165 pp 1448-1454 doi: 10.1016/j.proeng.2016.11.878

[11] Bazhenov Y, Erofeev V, Rimshin V, Markov S, Kurbatov V 2016 Changes in the topology of a concrete porous space in interactions with the external medium *Engineering Solid Mechanics* **4**(4) pp 219-225 doi: 10.5267/j.esm.2016.5.001

[12] Erofeev V, Bobryshev A, Lakhno A, Shafigullin L, Khalilov I, 2016 Sibgatullin K, Igtisamov R Theoretical evaluation of rheological state of sand cement composite systems with polyoxyethylene additive using topological dynamics concept *Solid State Phenomena* 871 pp 96-103 doi: 10.4028/www.scientific.net/MSF.871.96

[13] Erofeev V, Kalashnikov V, Emelyanov D, Balathanova E, Erofeeva I, Smirnova O, Tretiakov I, Matvievsksiy A 2016 Biological resistance of cement composites filled with limestone powders *Solid State Phenomena* 871 pp 22-27 doi: 10.4028/www.scientific.net/MSF.871.22

[14] Erofeev V, Kalashnikov V, Emelyanov D, Balathanova E, Erofeeva I, Smirnov V, Tretiakov I, Matvievsksiy A 2016 Biological resistance of cement composites filled with dolomite powders *SolidStatePhenomena* 871 pp 33-39 doi: 10.4028/www.scientific.net/MSF.871.33

[15] Erofeev V, Svetlov D, Smirnov V, Kraeva L, Lubchenkov M, Svetlov D 2020 Creation of «Teflex» biocidal preparations from the synthesis of a novel polymer to the product line Part 1 Development of technology for obtaining «Teflex» biocidal preparations *Academia Architecture and construction engineering* **2** pp 135-142