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Ecofriendly calcium phosphate and calcium bicarbonate biogrouts

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1 5,603 words, 31,354 characters

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3 **Ecofriendly calcium phosphate and calcium bicarbonate biogrouts**

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14

15 **Abstract**

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17 An important direction of construction biotechnology is production and application of the
18 biogrouts to decrease the hydraulic conductivity of soil or fractured rocks. The most popular
19 biogrout is a mixture of calcium chloride, urea, and either ureolytic bacteria or enzyme urease. A
20 disadvantage of this biogrout is the release of a large quantity of toxic ammonia to air, as well as
21 harmful ammonium and hydroxide ions to water. The aim of this study was evaluation of two
22 new biogrouts that release less of harmful by-products to environment. First biogrout was a
23 mixture of acidified hydroxyapatite solution from the bone powder with an addition of two times

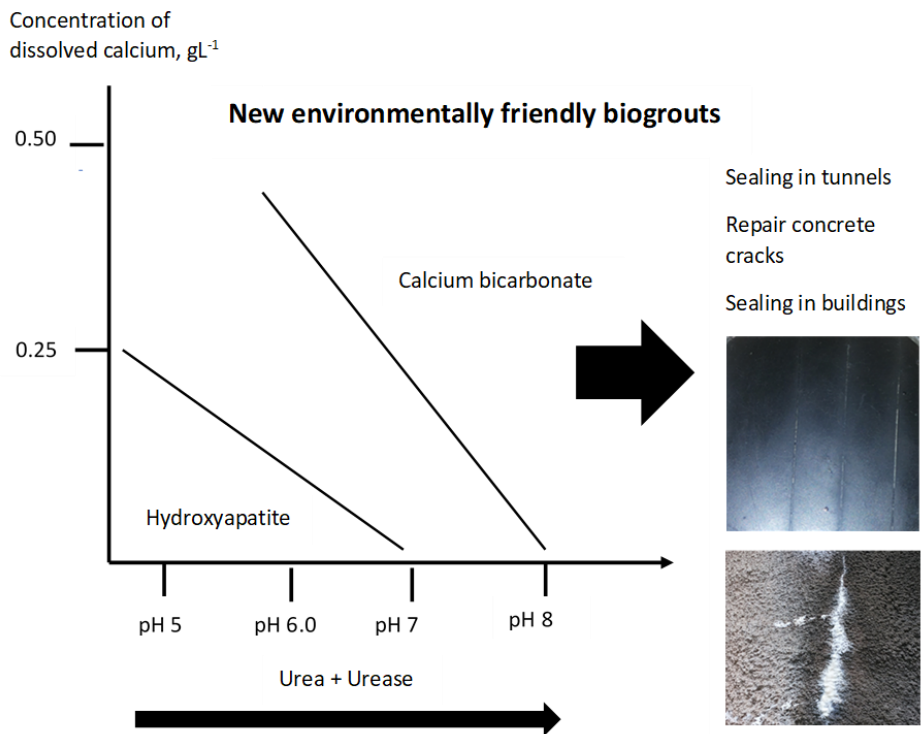
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3 24 less urea that is used in conventional biogrout. Final pH was 7. Second biogrout was a solution
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6 25 of calcium bicarbonate produced from chalk powder and carbon dioxide with an addition of urea
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8 26 and acidotolerant ureolytic bacteria. This biogrout released to environment four times less
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10 27 ammonium and ammonia than conventional biogrout. Final pH was 8. Precipitation of about 2 g
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12 28 of calcium carbonate per kg of the cracked stone diminished the water seepage through the
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14 29 specimen by three orders of magnitude. Geotechnical or environmental engineering applications
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16 30 of these environmentally friendly biogrouts are limited by the consumption of a large volume of
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18 31 the grouting solution. It is due to low concentration of dissolved calcium, about 200 - 400 g m⁻³,
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20 32 in these liquid biogrouts. So, these biogrouts could be most effective for the sealing and
21
22 33 strengthening of the materials with low porosity such as the fractured rocks or cracked concrete.
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31 **Key words:** biogrout; soil stabilization; fractured rocks sealing; calcium bicarbonate; calcium
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33 36 phosphate; hydroxyapatite.
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38 **Highlights**

- 39 - environmentally friendly biogrout can be produced from hydroxyapatite of the bones;
 - 40 - environmentally friendly biogrout can be produced from limestone and carbon dioxide;
 - 41 - new biogrouts can be used for the sealing of the fractured rocks and cracked concrete.
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7 **Graphical abstract**
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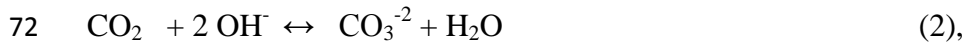
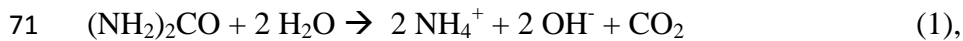


40 **1. Introduction**
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45 52 Construction Biotechnology, i.e. applications of biotechnology to civil and environmental
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47 53 engineering, includes production of the biogrouts (Stabnikov et al., 2015; Ivanov and Stabnikov,
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49 54 2017). The microbial grout has low viscosity and deeper penetration of the grouting solution in
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51 55 soil or fractured rocks than any conventional chemical grout. Its application produces *in situ*
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53 56 insoluble crystals that bind or clog the porous material. The biogrout can be used in geotechnical
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55 57 and environmental engineering for the ground improvement, soil stabilization, soil desaturation
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57 58 and mitigation of soil liquefaction after earthquake, stabilization of the slopes and dams, control
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4 59 of the wind and water erosion of soil, to diminish erosion of a shore, to diminish seepage in the
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6 60 channel, pond, landfill, reservoir, tunnel, and dam, to immobilize chemical, radioactive, and
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9 61 biological pollutants of soil (DeJong et al., 2013; Ivanov and Stabnikov, 2017; Haouzi and
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11 62 Courcelles, 2018). There are known at present several hundred experimental papers on these
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14 63 topics as shown in the reviews (DeJong et al., 2013; Stabnikov et al., 2015) and in a monography
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16 64 (Ivanov and Stabnikov, 2017). However, a few practical scale applications of biogrouts are
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18
19 65 known at present (DeJong et al., 2013; Haouzi and Courcelles, 2018).

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21 66 The major reason of limitation for practical applications of the biogrouts is environmental
22
23 67 harm of the most popular biogrouting process, which often is called as “microbially-induced
24
25 68 calcite (or carbonate) precipitation” (MICP). This process has disadvantage, which is formation
26
27 69 and release of ammonia to atmosphere, as well as ammonium and hydroxide ions to water. It is
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29 70 performing according to Equations 1-3:
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41 74 Theoretical molar ratio urea:Ca in biogrout is 1:1. However, the real molar ratio urea:Ca in
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43 75 biogrout in majority of experiments was from 1.5 to 2 (Ivanov and Stabnikov, 2017) because a
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45 76 significant portion of urea must be hydrolyzed to increase the pH *in situ* to 8.5 - 9.5 for effective
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48 77 crystallization of calcium carbonate. This increase of the pH also shifts equilibrium between
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50 78 ammonium and ammonia to formation of gaseous toxic ammonia (Equation 4):
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55 80 The major enzyme involved in the biogrouting process is urease of bacterial (Ivanov and
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58 81 Stabnikov, 2017; Krajewska, 2017) or plant origin, usually from jack bean (*Canavalia*
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4 82 *ensiformis*) (Dilrukshi et al. 2018). Because of high cost of pure enzyme urease, it is replaced by
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7 83 either biomass of urease-producing bacteria of pure and identified culture (Ivanov and
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9 84 Stabnikov, 2017) or potentially unsafe enrichment culture of non-identified indigenous soil
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12 85 bacteria (Burbank et al., 2011; Gomez et al., 2017; Rajasekar et al., 2017).

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14 86 To avoid environmental harm during biogrouting, an addition of the zeolite to the
15
16 87 conventional grout to adsorb ammonium ions (Keykha et al., 2017), retention of ammonium ions
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18 88 near cathode during electro-biostabilization of soil (Keykha and Asadi, 2017), or absorbance of
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21 89 released ammonia by sulphuric acid (Ivanov and Stabnikov, 2017) were proposed.

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24 90 Alternative solution is biogrouting due to precipitation of calcium phosphate compounds
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26 91 from inorganic phosphates (Akiyama and Kawasaki, 2012; Dilrukshi et al., 2018) or
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28 92 organophosphates (Roeselers and van Loosdrecht, 2010) to produce insoluble and strong crystals
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31 93 of hydroxyapatite (Akiyama and Kawasaki, 2012; Yu and Jiang, 2018).

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34 94 Therefore, one goal of this study was evaluation of the low cost, environmentally friendly
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36 95 biogrout based on hydroxyapatite precipitation (see Table 1, Equation 11). It is well known that
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38 96 at pH 4.5 calcium concentration of dissolved hydroxyapatite is about 0.25 g L⁻¹, while at pH 7.0
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41 97 it is about 0.001 g Ca L⁻¹ (see Table 1). Precipitation in this method could be due to enzymatic
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43 98 hydrolysis of urea that increases the pH from 4.5 to 7.

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46 99 Another new environmentally friendly biogrout with similar pH-dependent mechanism of
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48 100 precipitation could be soluble calcium bicarbonate that is transforming *in situ* to insoluble
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51 101 calcium carbonate (Equation 5) due to increase of the pH from 6.0 to 8.0 during hydrolysis of
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53 102 urea:



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104 Theoretical molar ratio urea:Ca for this biogrout is 0.5, which is 3 - 4 times lower than
105 usual urea:Ca ratio in the conventional MICP. Therefore, solution of calcium bicarbonate was
106 also evaluated in this study as environmentally friendly biogrout. Increase of the pH from 6 to 8
107 that is required for decay of calcium bicarbonate can be performed by addition of urease-
108 producing bacteria and urea.

109 The aim of the research was testing of new environmentally friendly biogrouts based on the
110 precipitation of either hydroxyapatite or calcium carbonate.

111

112 **2. Materials and Methods**

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114 Production of hydroxyapatite grout was from the acidified bone powder. Production of
115 calcium bicarbonate biogrout was from limestone and carbon dioxide. Their use for the sealing
116 of the porous materials requires an addition of biomass of acidotolerant urease-active bacteria
117 and urea.

118

119 *2.1. Production and use of hydroxyapatite biogrout*

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121 To produce this biogrout, 10 g of commercial feed bone meal and 15 mL of concentrated
122 HCl were added to 1 L of tap water. Tap water was used because the concentrations of the
123 biogrout components exceed concentrations of inorganics in tap water for one or two orders of
124 magnitude. So, theoretically there will be no effect of tap water on the results of biogrouting.
125 Additionally, parallel experiments on biogrouting/biocementation made in Kyiv, Ukraine, or in
126 Singapore, showed that the results, for example specific growth rate of bacteria, are the same

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127 using tap or deionized water in these cities because concentrations of medium components are
128 too high in comparison with the concentrations of water components. So, there is no need to
129 account properties of tap water for geotechnical experiments on biogrouting. The bottle was
130 rolled for 5 h at 20°C, then the pH of extract was adjusted to pH 4.5 and solution of
131 hydroxyapatite was decanted.

132 Hydroxyapatite biogrout contained two liquid components: 1) 1 L of hydroxyapatite
133 solution with about 0.25 g Ca L⁻¹ and 0.1 g urea L⁻¹ at pH 4.5; 2) 0.1 L of suspension of
134 acidotolerant urease-producing bacteria. The biogrouting process was performed in two steps: 1)
135 addition of bacterial suspension to the cracked concrete or stone cylinder with 10 cm diameter
136 and 10 cm height, coated and bound by the plastic tape, with the rate of 10 mL min⁻¹ for
137 adhesion of bacterial cells on the surface of the cracks; 2) the solution of hydroxyapatite with
138 urea was supplied and recycled by the peristaltic pump with the rate about 10 mL min⁻¹ ensuring
139 clogging of the cracks after two days of the treatment. Effluent from the cracked cylinder was
140 collected to measure flow rate, pH and concentration of ammonium.

141
142 *2.2. Production and use of calcium bicarbonate biogrout*

143
144 To produce this biogrout, 1 g of calcium carbonate powder was added to 1.5 L plastic
145 bottle with 1 L of tap water and 0.5 L CO₂ at atmospheric pressure. The bottle was rolled for 5 h
146 at 20°C. After 1 h of sedimentation, the solution of calcium bicarbonate was decanted.

147 Calcium bicarbonate biogrout contained two components: 1) 1 L of calcium bicarbonate
148 solution with pH 6.0 - 6.3, containing 0.40 g Ca L⁻¹ and 0.2 g urea L⁻¹; 2) 0.1 L of suspension of
149 urease-producing bacteria *Bacillus sp. (Sporosarcina pasteurii)* VS1 (Ivanov and Stabnikov,

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4 150 2017). The biogrouting process was performed in two steps: 1) addition of bacterial suspension
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7 151 *Sporosarcina pasterii* VS1 with the rate of 10 mL min⁻¹ to the cracked concrete or stone cylinder
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9 152 with 10 cm diameter and 10 cm height, confined by the plastic tape. It was performed for
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11 153 adhesion of bacterial cells on the surface of the cracks; 2) the solution of calcium bicarbonate
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14 154 with urea was supplied and recycled with the rate about 10 mL min⁻¹. It was performed to clog
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16 155 the cracks after two days of the treatment. Effluent from the cracked cylinder was collected to
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19 156 measure flow rate, pH and concentration of ammonium.
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23 158 2.3. Selection of acidotolerant urease-producing bacteria

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29 160 Acid urease is needed for the precipitation of hydroxyapatite due to increase of the pH
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31 161 from 4.5 to 7. Several samples of acidic soil were taken in the park with the pine trees and lawns
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33 162 fertilized with carbamide and used for preparation of the enrichment culture of acidotolerant
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35 163 urease-producing microorganisms. Growth medium for the enrichment culture was of the
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38 164 following content: Tryptic Soya Broth, 30 g L⁻¹; urea, 20 g L⁻¹; NaCl, 15 g L⁻¹, , NiCl₂, 24 mg L⁻¹,
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40 165 pH indicator bromocresol purple, 20 mg L⁻¹, 0.1 N HCl to adjust the pH to 4.5, soil sample, 10
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43 166 g L⁻¹, distilled water, 1 L. Cultivation of soil inoculum was in 70 mL of this medium in 300 mL
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45 167 flasks on the shaker at 200 rpm at 25°C for 72 hours. Change of medium color from yellow-
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48 168 green to violet indicated urease activity that increased pH from 4.5 to 6.8. Obtained enrichment
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50 169 culture was used for pure culture isolation. Aliquots of 0.1 mL from ten-fold dilutions in sterile
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53 170 1.5% NaCl solution were spread on Petri dishes with growth medium shown above and solidified
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55 171 with 2.0% Bacto Agar (Difco). Petri dishes were incubated after inoculation at the room
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58 172 temperature for 5 days. The urease-positive colonies changed color of the surrounding medium
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173 from yellow-green at pH 4.5 to violet at pH 6.8 due to urease activity and increase of the pH.
174 Pure culture, isolated from one colony, marked as strain AU1, was grown in the liquid medium
175 shown above, for three days at 25°C on the shaker for aeration and then was used for biogrouting
176 as described above.

178 *2.4. Measurements*

180 The seepage through the cracked stone or concrete was used to evaluate effects of the
181 grouting. The seepage was measured at the constant head 5-10 cm.

182 The surface of the treated material was observed using a scanning electron microscope
183 Quanta 250 FEG (FEI Corporation, Oregon, USA) with a light-element X-ray detector and an
184 Indium X-ray Fluorescence (IXRF) Systems EDS analysis system.

185 Calcium concentrations in the liquid before and after biogrouting were determined using
186 standard ethylene diaminetetraacetate (EDTA) titrimetric method 2340C with Eriochrome Black
187 T indicator (APHA, 1999). Liquid sample after filtration through 0.2 µm membrane, 50 mL, was
188 placed in a 100 mL conical flask and 1 mL of a buffer solution was added to maintain the pH of
189 10.0, then few drops of Eriochrome Black T indicator were added as an indicator. The sample
190 was titrated with 0.01 M solution of EDTA until the color was changed from purple to blue.

191 Concentration of ammonium in effluent after treatment was measured by the APHA
192 standard 4500-NH₃ F Phenate method (APHA, 1999). An intensely blue compound, indophenol,
193 is formed by the reaction of ammonia, hypochlorite, and phenol catalyzed by sodium
194 nitroprusside. To measure ammonium, add 1 mL of 10% (v v⁻¹) phenol solution in ethanol, 1 mL
195 of 5% (w v⁻¹) sodium nitroprusside solution, and 2.5 mL oxidizing solution (a mixture of 25 mL

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4 196 5% ($w v^{-1}$) sodium hypochlorite with 20 g of trisodium citrate, 1 g of sodium hydroxide and 100
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7 197 mL of deionized water) to a 25 mL sample in a 50 mL Erlenmeyer flask, let color develop at
8
9 198 room temperature (22 to 27°C) for 1 h, and measure light absorbance at 640 nm.

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11 All control and experiments, as well as chemical analysis have been done in triplicate.
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14 200 Mean values \pm standard deviations are shown for the data comparisons.

15 16 201 17 18 19 202 **3. Results and Discussion**

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23 204 To produce and apply new biogrouts, the strain of acidotolerant urease-producing
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25 205 microorganism was selected and a choice of inorganic component for environmentally friendly
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27 206 biogrout based on calcium phosphate precipitation has been done. The testing of environmentally
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29 207 friendly biogrouts for the sealing of the solid materials has been done at laboratory conditions.

30 31 208 32 33 209 *3.1. Selection of acidotolerant urease-producing microorganism*

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37 211 The strain AU1 was identified using PCR and sequencing of 16S rRNA gene (Ivanov and
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39 212 Stabnikov, 2017). The full-length sequence was compared with all other sequences available in
40
41 213 the NCBI Genbank database using BLAST (<http://blast.ncbi.nlm.nih.gov>). Strain AU1 that
42
43 214 produced acid urease was identified as *Staphylococcus saprophyticus* with 100% identity with
44
45 215 the 16S rRNA gene sequences of four other strains of this species.

46
47 216 Biosafety is important question in soil biogrouting and soil bioremediation. Prokaryotes are
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49 217 classified into four risk groups (RG) according to German Technical Rules for Biological Agents
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51 218 on classification of prokaryotes into risk groups (TBRA 466, 2010). Microorganisms from RG 1

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219 are unlikely to cause human disease, and microorganisms from RG 2 can cause human disease
220 but unlikely to spread to the community.

221 There are known safe and unsafe strains of *Staphylococcus saprophyticus*. For example,
222 *Staphylococcus saprophyticus subsp. bovis* belongs to RG1, while *Staphylococcus saprophyticus*
223 *subsp. saprophyticus* belongs to RG2. The strain of *Staphylococcus saprophyticus* was selected
224 not only in our study on biogrouting but also was selected by urease activity among numerous
225 isolates (Ghezelbash and Haddadi, 2018).

226 Generally, according to the main Koch's postulates the pathogenic microorganism must be
227 found in abundance in all organisms suffering from the disease, but should not be found in
228 healthy organisms, and the cultured microorganism should cause disease when introduced into a
229 healthy organism. However, *Staphylococcus saprophyticus* is a representative of common
230 gastrointestinal flora in pigs and cows. It is also is a part of the normal human flora that
231 colonizes the perineum, rectum, urethra, cervix, and gastrointestinal tract. Over 40% of all young
232 women contain *S. saprophyticus* as part of their normal genitourinary flora. However, at the
233 same time there are known strains of this species that were isolated from patients with urinary
234 tract infections associated with *S. saprophyticus* (Ehlers and Merrill, 2018; Martins et al., 2018).
235 So, *Staphylococcus saprophyticus* could probably cause disease in a host whose immune system
236 is not functioning properly or after accident.

237 Most active bacteria that are currently used in soil bioremediation and municipal
238 wastewater treatment are similar microorganisms, i.e. they are representatives of normal human
239 microflora but cause disease occasionally or in the immune-compromised patients. Therefore, it
240 is a common environmental engineering practice to use representatives of normal human
241 microflora. However, it could be safer to replace any live bacteria with dead but urease-active

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242 cells (Ivanov and Stabnikov, 2017). It is safer also to use enzyme urease instead of urease-
243 producing bacteria, but this is limited by the high cost of enzyme. However, future industrial-
244 scale production of crude acid or neutral urease could diminish cost of enzyme to the practically
245 acceptable level.

246 There are also known other acidotolerant urease-producing bacteria such as *Arthrobacter*
247 *mobilis*, which produce acid urease that has been used to remove urea from sake and wine
248 (Miyagawa et al., 1999; Panesar and Marwaha, 2010), fish pathogen *Edwardsiella ictaluri*
249 (Booth et al., 2009), as well as lactic acid bacteria that are using in yogurt, probiotics, fermented
250 milk, or cheese production such as *Lactobacillus reuteri*, *Streptococcus salivarius*, *S.*
251 *thermophilus* (Mora and Arioli, 2014). Application of lactic acid bacteria as a source of acid
252 urease could be most interesting approach to replace *Staphylococcus saprophyticus* or other
253 sources of acid urease.

254
255 *3.2. Choice of inorganic component for environmentally friendly biogrout based on calcium*
256 *phosphate precipitation*

257
258 The main condition for precipitation of hydroxyapatite from different calcium phosphates
259 (see Table 1) is increase of the pH, which is caused by enzymatic hydrolysis of urea (Eq. 1).

260
261 **Table 1.** Precipitation of hydroxyapatite due to transformation of calcium phosphate compounds
262 shown in Equations 6 - 12 (modified from Tung, 1998; Pan and Darvell, 2010; Akiyama and
263 Kawasaki, 2012; Kuroda and Okido, 2012)

Compound	Ca/P	Urea/Ca	Solubility at 25°C, g L ⁻¹	Reaction of precipitation
Monocalcium phosphate	0.5	2.10	20	$5 \text{ Ca}(\text{H}_2\text{PO}_4)_2 + 21 \text{ OH}^- \rightarrow \text{Ca}_5(\text{PO}_4)_3\text{OH}\downarrow + 7 \text{ PO}_4^{3-} + 21 \text{ H}_2\text{O} \quad (6)$
Dicalcium phosphate	1.0	0.60	0.2	$5 \text{ CaHPO}_4 + 6 \text{ OH}^- \rightarrow \text{Ca}_5(\text{PO}_4)_3\text{OH}\downarrow + 6 \text{ PO}_4^{3-} + 6 \text{ H}_2\text{O} \quad (7)$
Octacalcium phosphate	1.33	0.22	0.28 - 0.56	$5 \text{ Ca}_8(\text{PO}_4)_6\text{H}_2 + 18 \text{ OH}^- \rightarrow 8 \text{ Ca}_5(\text{PO}_4)_3\text{OH}\downarrow + 6 \text{ PO}_4^{3-} + 18 \text{ H}_2\text{O} \quad (8)$
Tricalcium phosphate	1.5	0.10	0.64 g CaL ⁻¹ at pH 4.5	$5 \text{ Ca}_3(\text{PO}_4)_2 + 3 \text{ OH}^- \rightarrow 3 \text{ Ca}_5(\text{PO}_4)_3\text{OH}\downarrow + \text{ PO}_4^{3-} + 3 \text{ H}_2\text{O} \quad (9)$
			0.008 g CaL ⁻¹ at pH 7.0	
Hydroxyapatite	1.67	0.40	0.25 g CaL ⁻¹ at pH 4.5	<p>Dissolution of hydroxyapatite at low pH due to formation of slightly soluble dicalcium phosphate:</p> $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 8 \text{ H}^+ \rightarrow 4 \text{ Ca}^{2+} + 6 \text{ CaHPO}_4 + 2 \text{ H}_2\text{O} \quad (10)$ <p>Crystallization of hydroxyapatite <i>in situ</i> due to increase of the pH by urease:</p>

Compound	Ca/P	Urea/Ca	Solubility at 25°C, g L ⁻¹	Reaction of precipitation
			0.001 g CaL ⁻¹ at pH 7.0	$4 \text{ Ca}^{2+} + 6 \text{ CaHPO}_4 + 8 \text{ OH}^- \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2\downarrow + 6 \text{ H}_2\text{O} \quad (11)$
Tetracalcium phosphate	2.0	0.17	0.02	$3 \text{ Ca}_4(\text{PO}_4)_2\text{O} + 3 \text{ H}_2\text{O} + 4 \text{ OH}^- \rightarrow 2 \text{ Ca}_5(\text{PO}_4)_3\text{OH}\downarrow + 2 \text{ Ca}(\text{OH})_2 \quad (12)$

As can be seen from the Table 1, dissolved hydroxyapatite at pH 4.5 could be the best component for grouting among other calcium phosphate compounds. The molar ratio urea:Ca for this biogrout is 1:1, which is 30% - 50% lower than urea:Ca ratio in conventional MICP. Due to neutral pH, the main harmful product of this grouting will be ammonium. The release of ammonia will be low, so it could be relatively safe to use this biogrout in the confined space of the tunnels or buildings.

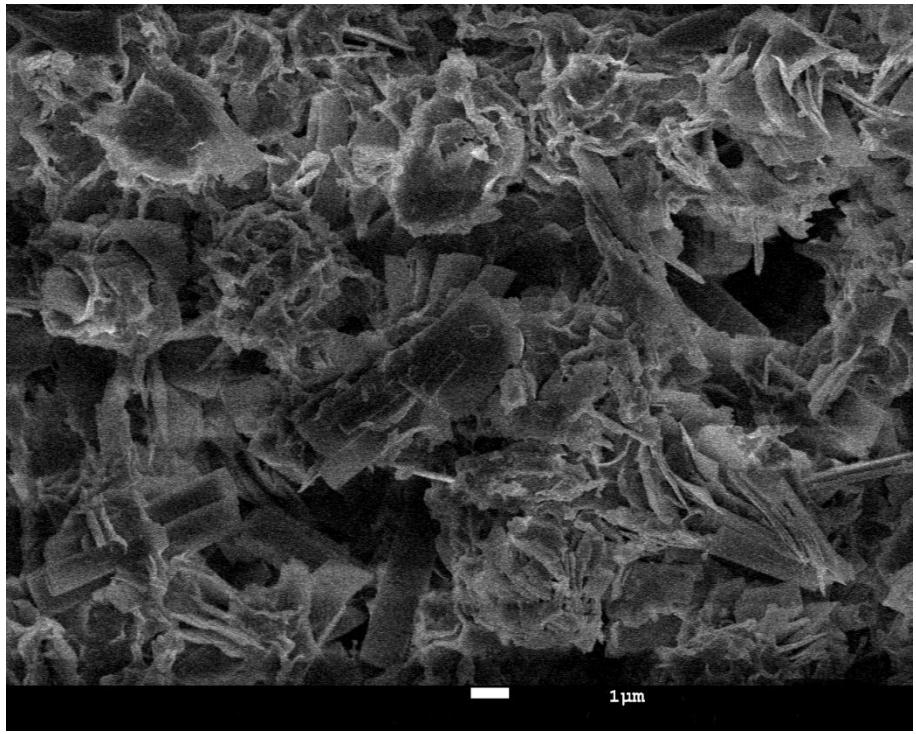
3.3. Environmentally friendly biogrout based on hydroxyapatite and its use for biogrouting

Stability of the hydroxyapatite biogrout depends on the pH only, so the solution of hydroxyapatite components will be stable at the pH 4.5. The rate of the biogrouting based on precipitation of hydroxyapatite can vary in the wide range because it depends on many factors such as: 1) porosity of the material; 2) the number of bacterial cells adhered to the surfaces of the cracks; 3) the urease activity of these bacterial cells; and 4) the retention time for the biogrout in

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279 the pores. For the contents, dosages, and flow rates shown in the Materials and Methods section
280 the clogging of the cracked concrete or stone typically can be done for 2 days of the treatment.
281 Seepage through the cracked specimen depended on the quantity of precipitated calcium
282 phosphate. To diminish the seepage through the specimen of the cracked concrete from 1×10^{-4}
283 $\text{m}^3 \times \text{m}^{-2}$ of the cracked surface $\times \text{s}^{-1}$ by three orders of magnitude it was necessary to precipitate
284 about 20 g of Ca in form of hydroxyapatite per 1 kg of the cracked concrete. Crystals formed in
285 the grouting space were rod-like and plate-like microparticles (Fig.1).

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288 **Figure 1.** Crystals formed in the grouting space.

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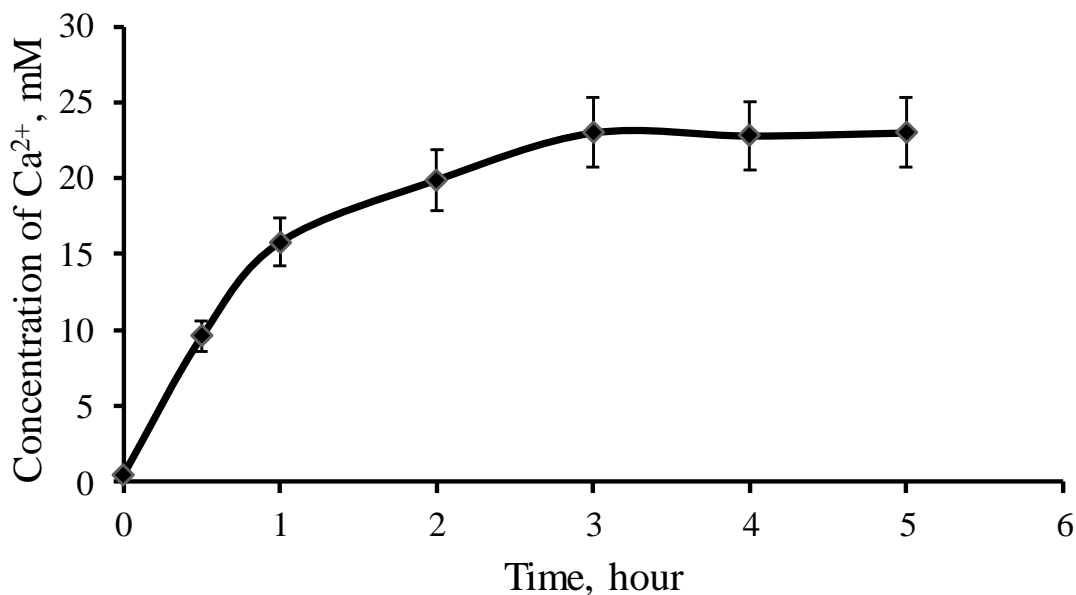
290 Concentration of ammonium in effluent was below $50 \text{ mg N-NH}_3 \text{ L}^{-1}$, pH was in the range
291 6.8 - 7.4. Therefore, it was no smell of ammonia from the treated cylinder of the cracked

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292 concrete. So, the biogrout for the sealing of porous materials can produced from the bone
293 powder, urea and a source of acid urease.

3.4. Environmentally-friendly biogrout based on calcium bicarbonate

297 Solution of calcium bicarbonate from calcium carbonate in atmosphere of CO₂ at 1 bar was
298 produced for 3 hours (Fig. 2) with maximum concentration of 0.9 g Ca L⁻¹.

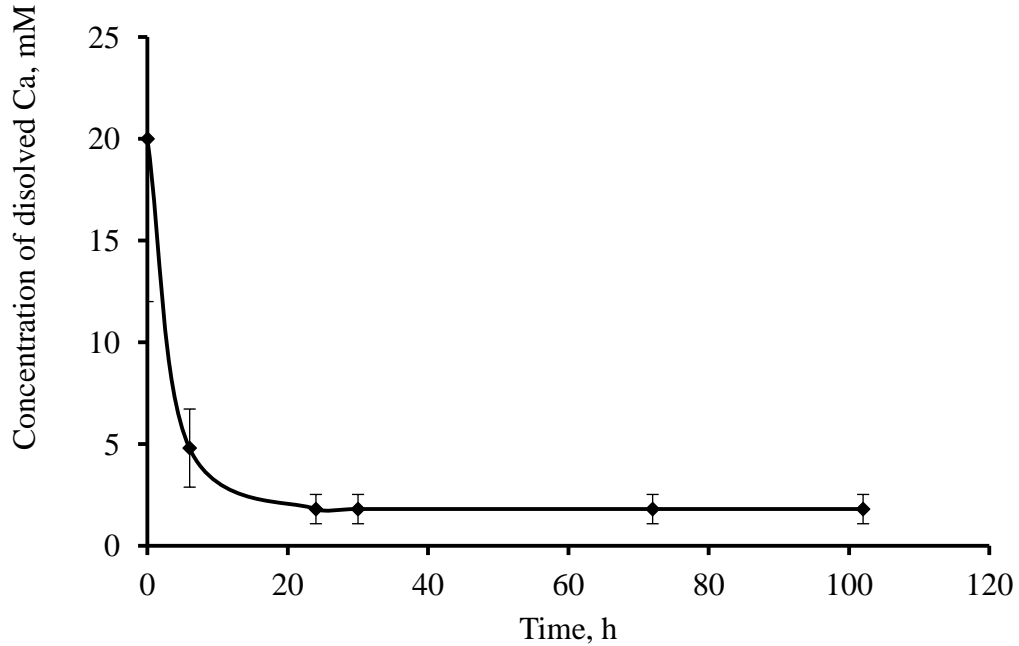


301
302 **Figure 2.** Production of calcium bicarbonate from CaCO₃ and CO₂.

304 The solution of calcium bicarbonate in the atmosphere of CO₂ was stable for at least one
305 week at the pH 6.0 - 6.3. However, calcium bicarbonate decayed with the production of calcium
306 carbonate either under urease-initiated increase of the pH to 8 or even at a low partial pressure of

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307 CO₂, without addition of urea and urease. The average rate of self-decay on air, i.e. the rate of
308 the biogrouting, was 0.84 g Ca×m⁻³ of solution×h⁻¹. The decay of calcium bicarbonate on air is
309 shown in Fig. 3.



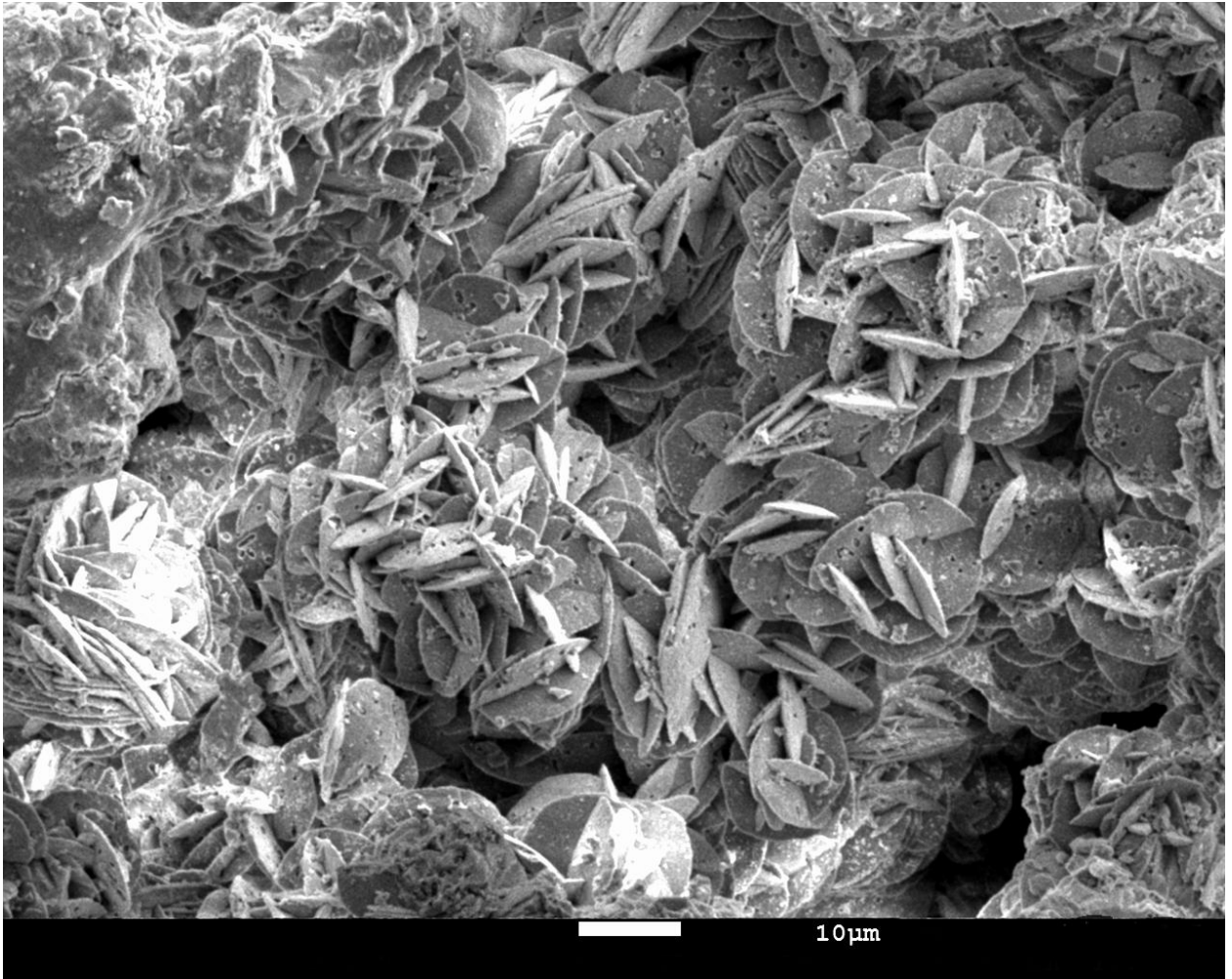
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311 **Figure 3.** Self-decay of calcium bicarbonate on air.

312
313 The biogrouting process was performed by addition of bacterial suspension *Sporosarcina*
314 *pasterii* VS1 to the cracked concrete or stone cylinder with the rate about 10 mL min⁻¹ and then
315 by addition and recycle of the calcium bicarbonate solution with urea with the rate about 10 mL
316 min⁻¹ that ensured clogging of the cracks after two days of the treatment.

317 Generally, the rate of this biogrouting depends on: 1) the partial pressure of CO₂; 2) the
318 pH; 3) the material porosity; 4) the urease activity of bacterial cells on the surface of the cracks;
319 5) the retention time for the biogrout inside the pores. In our experiments, the clogging of the

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320 cracked concrete or stone can be made for two days of the treatment. Calcium bicarbonate
321 solution produced during clogging the plate-shaped crystals (Fig.4).



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323 **Figure 4.** Crystals produced after treatment of the fissures with calcium bicarbonate grout.

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325 These crystals clog the fissures in stone (Fig.5) in linear correlation with the volume of the
326 used grout (Fig.6).

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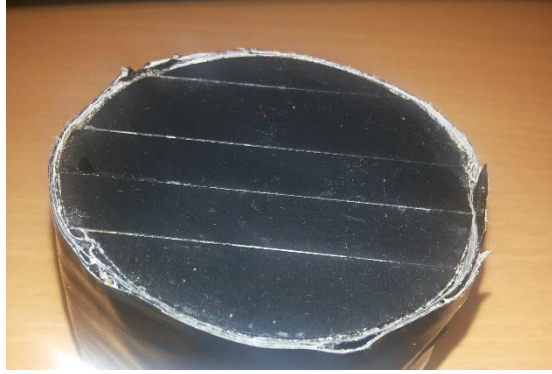


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27 (b)

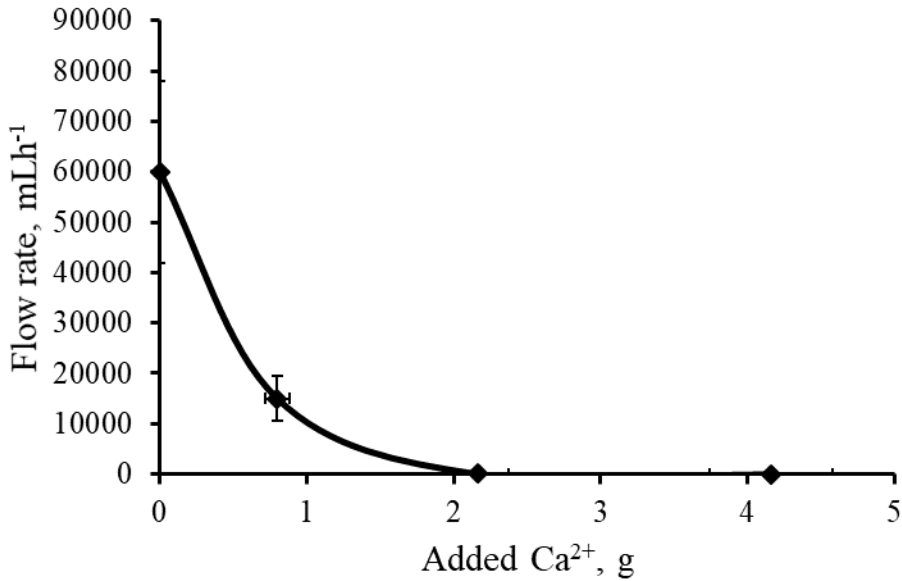


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330 **Figure 5a-c.** Cracked stone before (a) and after treatment (b), and the treated stone cylinder with
331 the cuts along the axis (c).



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347 **Figure 6.** Correlation of seepage (flow rate) through the cracked stone cylinder with the
348 content of precipitated calcium during grouting with calcium bicarbonate solution.
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337 To diminish the seepage of the specimen of the cracked stone by three orders of magnitude
338 it was necessary to use about 2 g of precipitated calcium carbonate per 1 kg of the cracked stone.
339 Self-decay of calcium bicarbonate at low partial pressure of CO₂ is the most environmentally

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340 friendly grouting process. Concentration of ammonium in effluent was below 100 mg N-NH₃ L⁻¹, pH was about 8.0, therefore it was no any ammonia smell from the treated cracked stone cylinder. So, the biogrout for the sealing of porous materials can produced from the limestone powder, carbon dioxide, urea and a source of acid urease.

345 *3.5. Industrial aspects of the hydroxyapatite biogrout production*

346
347 Economic advantage of hydroxyapatite biogrout is an opportunity to produce it from such sustainable material as the bones of livestock. The bone meal powder is used as a feed component and partially as the fertilizer but about half of the bones that remain after meat processing are incinerated, usually in the cement kilns (Moller, 2015). So, there available million tons of the bone wastes for biogrout production. The calculated cost of calcium from the bone meal powder is US \$525 – 1250 per metric ton. This is comparable with the cost of calcium from calcium chloride, US \$416 – 832 per metric ton, which is the most popular component for the conventional MICP biogrout. Additionally, the supply of urea is four times lower than in conventional MICP grouting. The cost of calcium from the bone meal was calculated for the feed grade bone meal powder with cost of US \$125-250 per metric ton and the content of Ca about 20% (w w⁻¹). The cost of calcium from calcium chloride was calculated for technical grade anhydrous calcium chloride with cost of US\$150 - 300 per metric ton and the content of Ca 36% (w w⁻¹).

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359 Production of hydroxyapatite liquid biogrout should include the following stages: grinding of the row bones, extraction of soluble hydroxyapatite from the grinded raw bones or dry bone meal powder by 1M hydrochloric acid to the concentration of dissolved calcium about 0.25 gL⁻¹ and pH 4.5, and decantation of acid solution. The precipitating protein part of the bones after

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363 extraction of calcium phosphates could be used as a feed or food product. Concentrated solution
364 of urea and acid urease or suspension of urease-containing bacteria should be added to the acid
365 solution of hydroxyapatite components before the biogrouting procedure. This biogrout is
366 sustainable due to the production from meat-processing waste and more environmentally-friendly
367 than conventional MICP biogrout.

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369 *3.6. Industrial aspects of the calcium bicarbonate biogrout production*

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371 An economic advantage of calcium bicarbonate biogrout is an opportunity to produce it
372 from cheap limestone powder, chalk powder, or even from the waste cement powder. The
373 calculated cost of calcium from calcium carbonate powder, US \$20 - 32 per metric ton, is
374 significantly lower than the cost of calcium from calcium chloride, US \$416 – 832 metric ton,
375 which is the most popular component for the conventional MICP biogrout. Additionally, the
376 supply of urea is 3 - 4 times lower than in conventional MICP grouting.

377 The cost of calcium from the calcium carbonate powder was calculated for the calcium
378 carbonate powder with price of US \$ 50-80 per metric ton and the content of Ca 40% (w w⁻¹). The
379 cost of calcium from calcium chloride was calculated for technical grade anhydrous calcium
380 chloride with its price of US \$150 - 300 per metric ton and the content of Ca 36% (w w⁻¹).

381 Production of the liquid calcium bicarbonate grout should include the following stages:
382 grinding of the limestone, stirring of limestone in the rotating reactor under atmosphere of CO₂ to
383 concentration of dissolved calcium about 0.4 g L⁻¹, and decantation of the calcium bicarbonate
384 solution. The concentrated solution of urea and acid urease or suspension of urease-containing
385 bacteria should be added to this solution before the biogrouting procedure.

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386 In case of production of the calcium bicarbonate solution from the waste cement powder and
387 carbon dioxide released during cement production the cost of this grout will be significantly lower
388 than the cost of the conventional MICP biogROUT using calcium chloride. So, the best way of the
389 calcium bicarbonate biogROUT production could be the use of the waste cement powder and CO₂ of
390 produced on the cement plant.

391

4. Conclusions

393

394 The experiments outlined that environmentally friendly biogROUTS can be produced from
395 the cheap sources like bone powder, limestone powder, and carbon dioxide. These biogROUTS
396 release to environment 2 or 4 times less toxic ammonium or ammonia than MICP biogROUT. The
397 obtained results are important for the sustainable development and cleaner production of the
398 biogROUTING materials for civil and environmental engineering. New biogROUTS help to overcome
399 the problem of environmental safety of the biogROUTS. So, the consequences of the obtained
400 results will be industrial sustainable production and the large-scale use of new environmentally
401 friendly biogROUTS.

402 However, these new biogROUTS are not universal ones. Concentration of dissolved calcium
403 in these liquid biogROUTS is low, from 200 to 400 mg L⁻¹, so they could be used only under the
404 following conditions: 1) when the release of toxic gaseous ammonia is a big concern due to the
405 confined space in the tunnels or buildings; 2) when porosity of the grouted material is lower than
406 approximately 2%, so low quantity of grout is needed for the sealing; 3) the geotechnical
407 application site is not far from the biogROUT production site. For example, the best use of these
408 grouts could be the sealing of the fractured rocks in the tunnels and other underground facilities,

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409 nuclear waste storages, repair of the cracks in concrete and stone, soil dust control, and
410 confinement of the chemical, biological, and radiological agents in soil crust.

411

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413

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504
505 **Figure captions**

- 506 **Fig. 1.** Crystals formed in the grouting space.
- 507 **Fig. 2.** Production of calcium bicarbonate from CaCO_3 and CO_2 .
- 508 **Fig. 3.** Self-decay of calcium bicarbonate on air.
- 509 **Fig. 4.** Crystals produced after treatment of the fissures with calcium bicarbonate grout.
- 510 **Fig. 5a-c.** The cracked stone cylinder before (Fig.5a) and after treatment (Fig.5b), and the treated
511 stone cylinder with the cuts along the axis (Fig.5c).
- 512 **Fig. 6.** Correlation of seepage through the cracked stone cylinder with the content of precipitated
513 calcium during grouting with calcium bicarbonate solution.

To: Dr. Giorgio Besagni,
Associate Editor
Journal of Cleaner Production

27th of January, 2019

Cover letter

Dear Dr. Besagni,

The reviewers have suggested the acceptance of our submission, but you mentioned in your letter that the manuscript contains some issues to be solved before final acceptance.

On behalf of the authors, I am pleased to submit to the Journal of Cleaner Production the manuscript “Ecofriendly calcium phosphate and calcium bicarbonate biogrouts” revised according to your comments. A list of the answers and actions to all your comments is given in the separate file.

Thank you for your kind and very useful consideration of our manuscript.

Yours sincerely,

Volodymyr Ivanov

Emeritus Professor, Doctor of Biological Sciences: Microbiology and Biotechnology

Deputy Rector on International Biotechnological Projects

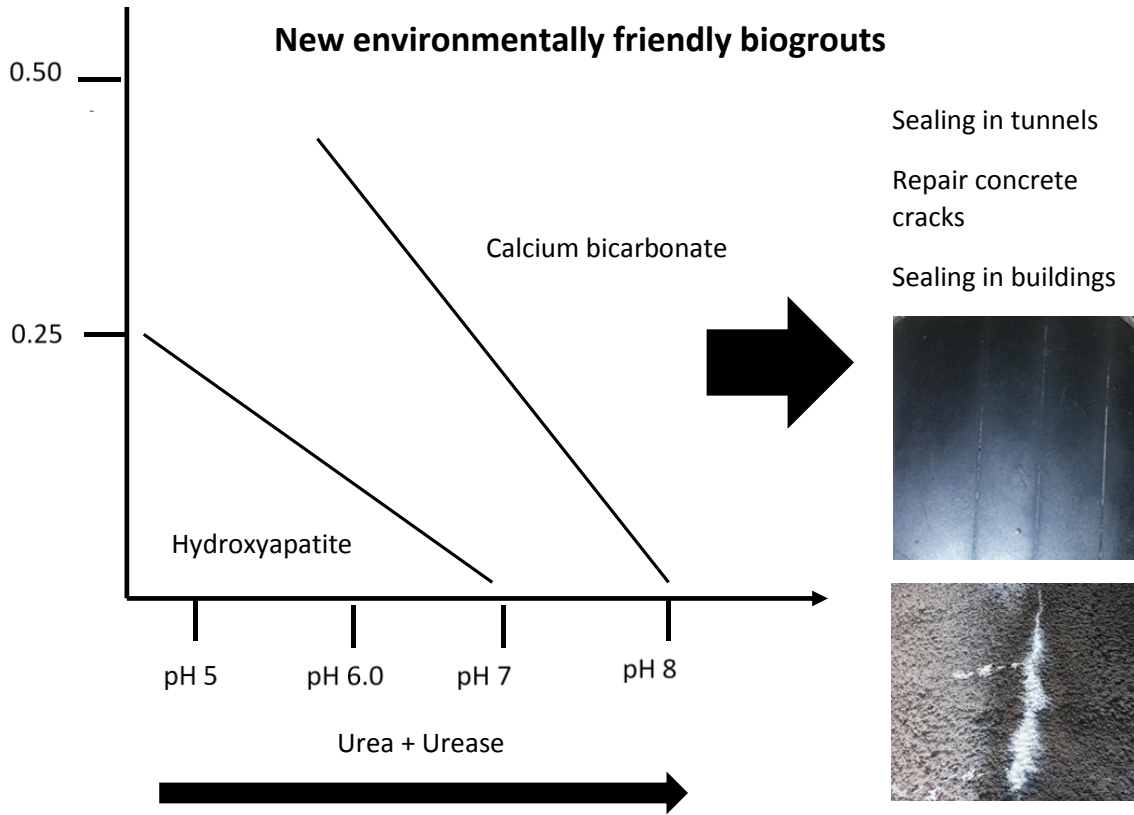
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Concentration of dissolved calcium, gL^{-1}



Highlights

- environmentally friendly biogrout can be produced from hydroxyapatite of the bones;
- environmentally friendly biogrout can be produced from limestone and carbon dioxide;
- new biogrouts can be used for the sealing of the fractured rocks and cracked concrete.

Figure 1
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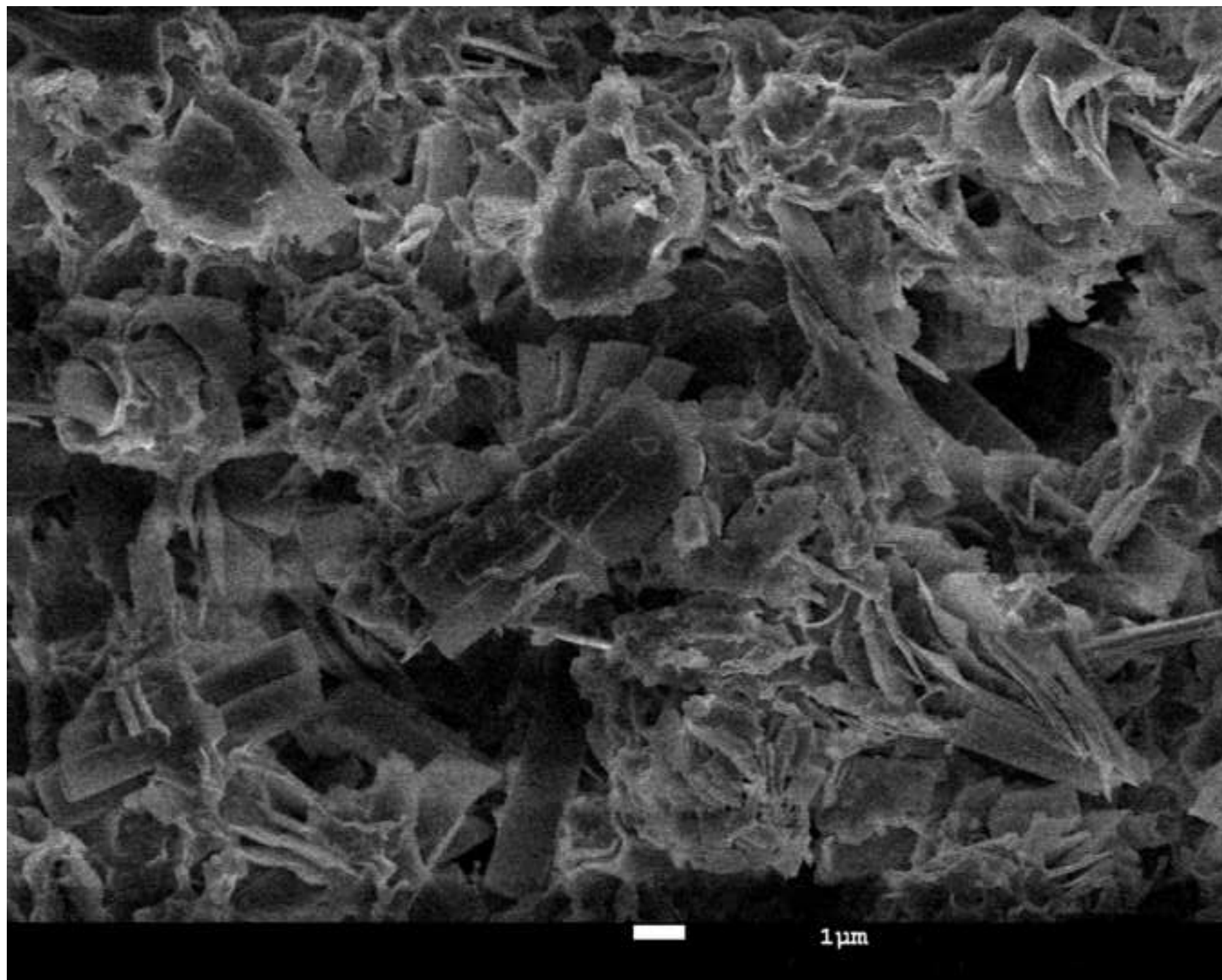


Figure 2

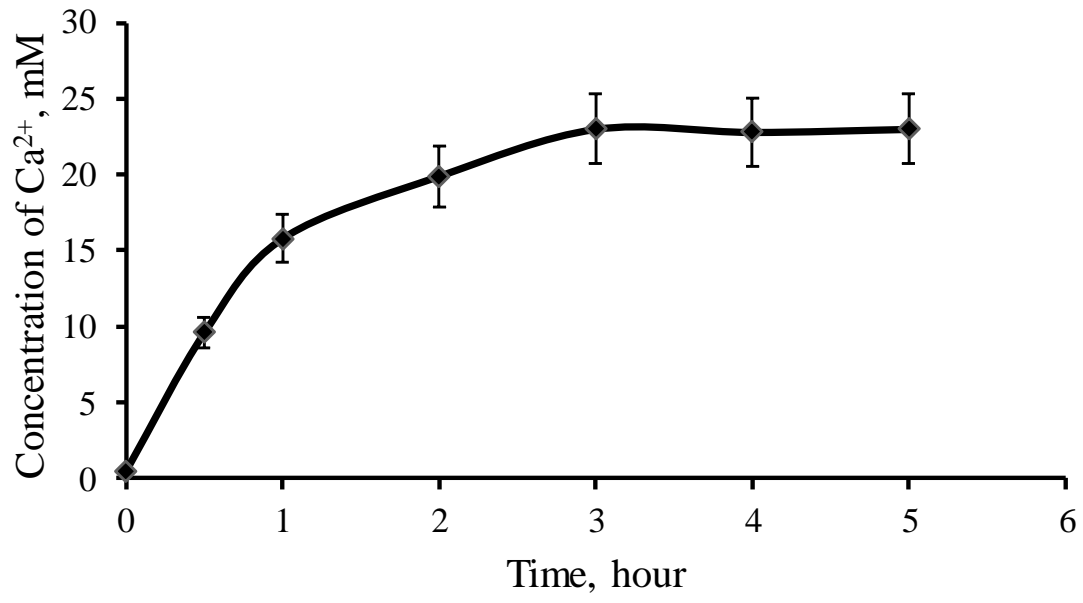


Figure 3

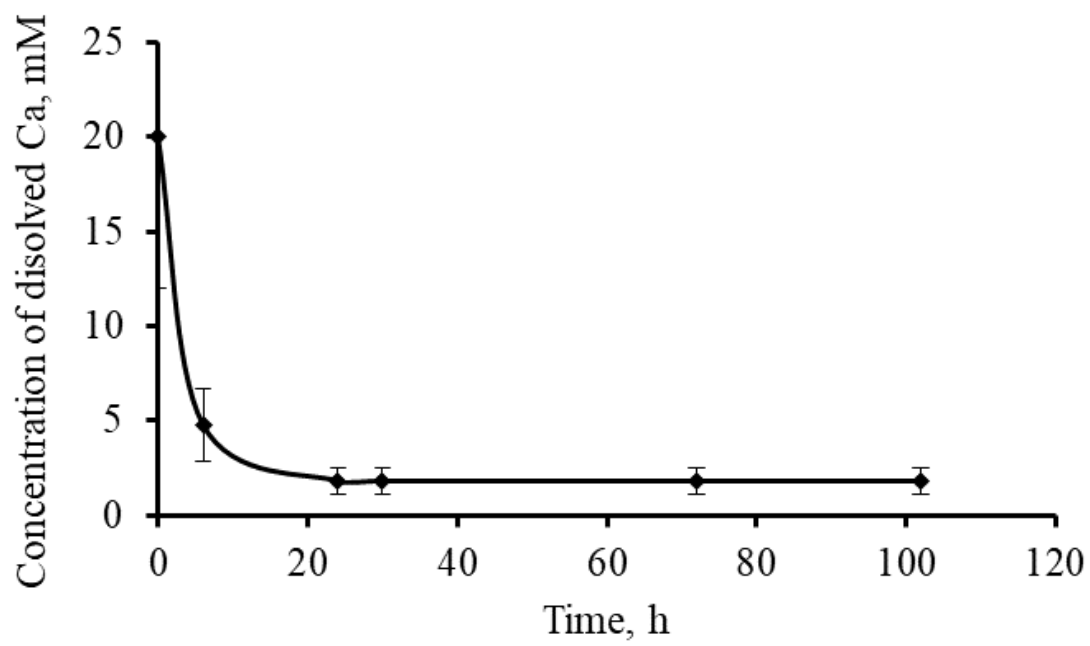


Figure 4
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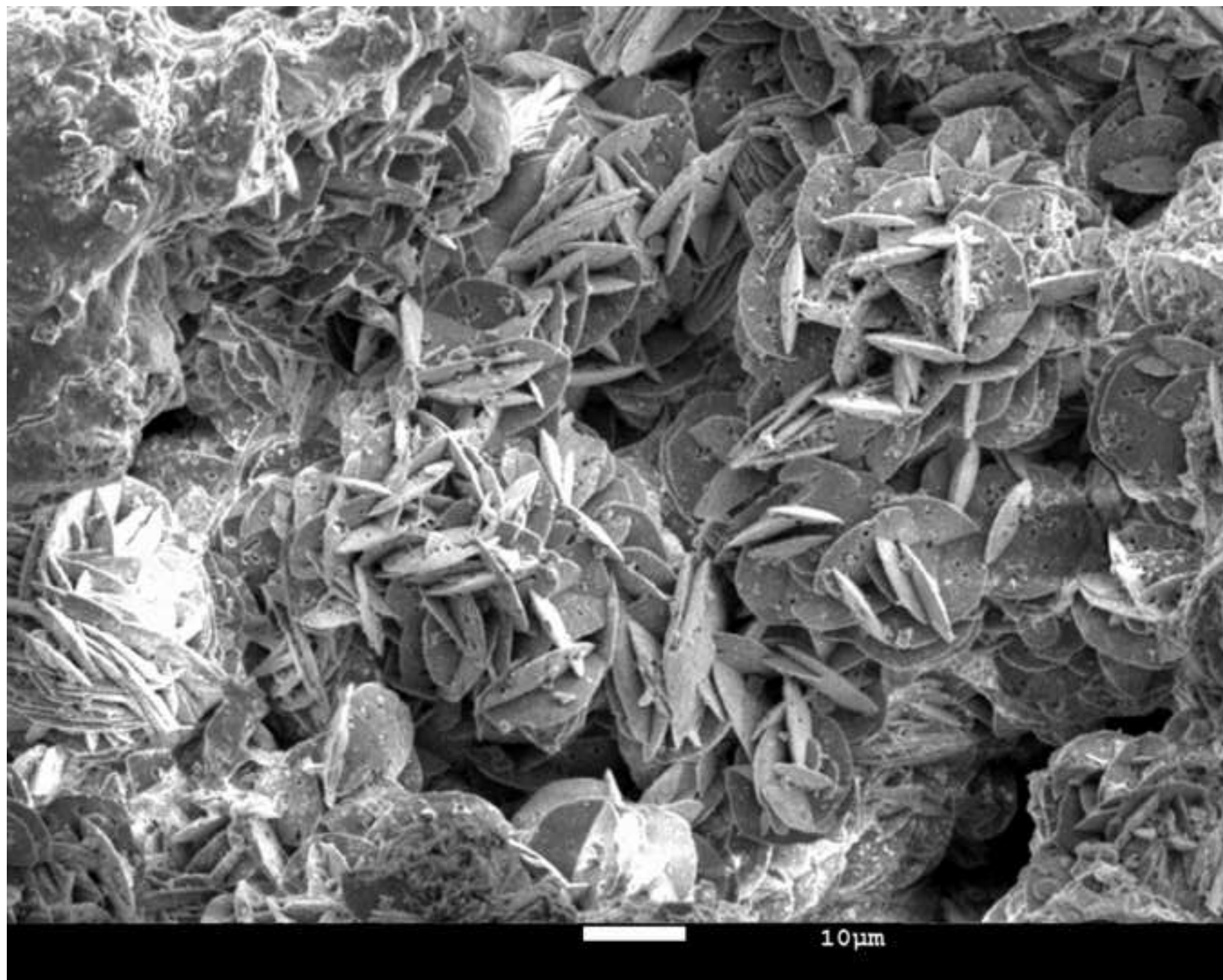


Figure 5

(a)

(b)



(c)

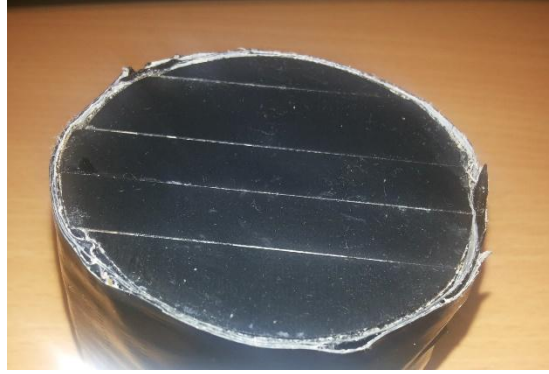


Figure 6

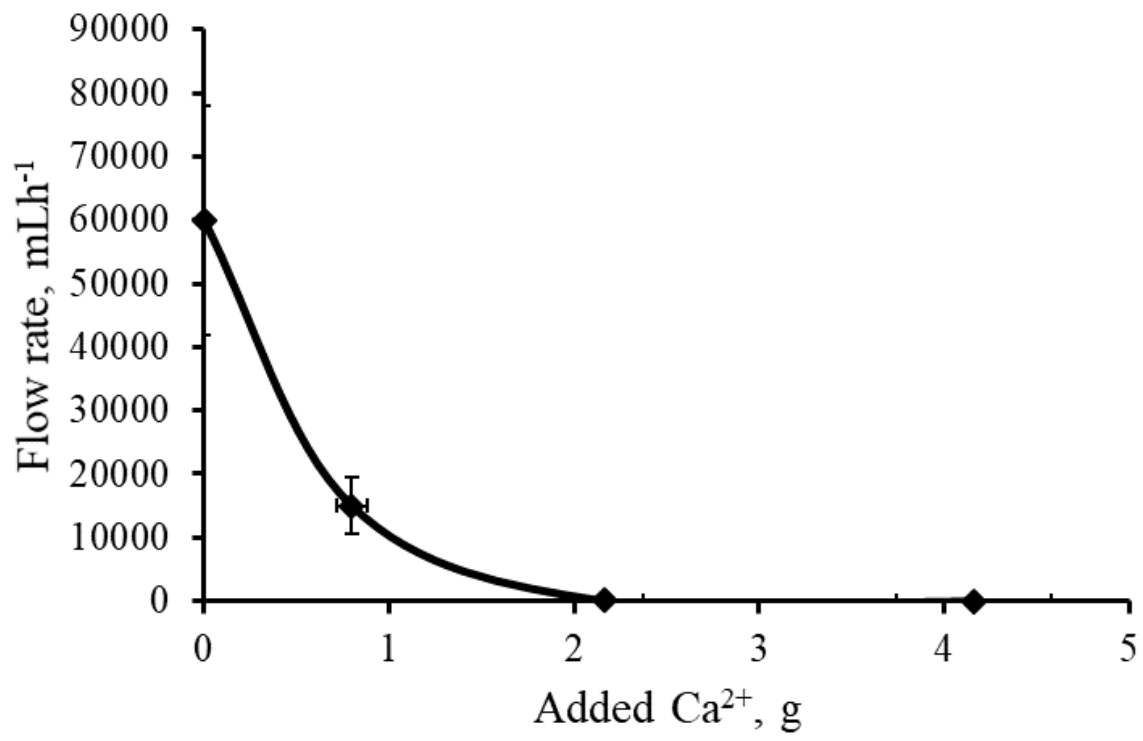


Table 1. Precipitation of hydroxyapatite due to transformation of calcium phosphate compounds shown in Equations 6 - 12 (modified from Tung, 1998; Pan and Darvell, 2010; Akiyama and Kawasaki, 2012; Kuroda and Okido, 2012)

Compound	Ca/P	Urea/Ca	Solubility at 25°C, g L ⁻¹	Reaction of precipitation
Monocalcium phosphate	0.5	2.10	20	$5 \text{ Ca}(\text{H}_2\text{PO}_4)_2 + 21 \text{ OH}^- \rightarrow \text{Ca}_5(\text{PO}_4)_3\text{OH}\downarrow + 7 \text{ PO}_4^{3-} + 21 \text{ H}_2\text{O} \quad (6)$
Dicalcium phosphate	1.0	0.60	0.2	$5 \text{ CaHPO}_4 + 6 \text{ OH}^- \rightarrow \text{Ca}_5(\text{PO}_4)_3\text{OH}\downarrow + 6 \text{ PO}_4^{3-} + 6 \text{ H}_2\text{O} \quad (7)$
Octacalcium phosphate	1.33	0.22	0.28 - 0.56	$5 \text{ Ca}_8(\text{PO}_4)_6\text{H}_2 + 18 \text{ OH}^- \rightarrow 8 \text{ Ca}_5(\text{PO}_4)_3\text{OH}\downarrow + 6 \text{ PO}_4^{3-} + 18 \text{ H}_2\text{O} \quad (8)$
Tricalcium phosphate	1.5	0.10	0.64 g CaL ⁻¹ at pH 4.5	$5 \text{ Ca}_3(\text{PO}_4)_2 + 3 \text{ OH}^- \rightarrow 3 \text{ Ca}_5(\text{PO}_4)_3\text{OH}\downarrow + \text{ PO}_4^{3-} + 3 \text{ H}_2\text{O} \quad (9)$
Hydroxyapatite	1.67	0.40	0.008 g CaL ⁻¹ at pH 7.0 0.25 g CaL ⁻¹ at pH 4.5	Dissolution of hydroxyapatite at low pH due to formation of slightly soluble dicalcium phosphate: $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 8 \text{ H}^+ \rightarrow 4 \text{ Ca}^{2+} + 6$

Compound	Ca/P	Urea/Ca	Solubility at 25°C, g L ⁻¹	Reaction of precipitation
				$\text{CaHPO}_4 + 2 \text{H}_2\text{O} \quad (10)$ <p>Crystallization of hydroxyapatite <i>in situ</i> due to increase of the pH by urease:</p> $0.001 \text{ g CaL}^{-1} \quad 4 \text{ Ca}^{2+} + 6 \text{ CaHPO}_4 + 8 \text{ OH}^- \rightarrow$ <p>at pH 7.0 $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2\downarrow + 6 \text{H}_2\text{O} \quad (11)$</p>
Tetracalcium phosphate	2.0	0.17	0.02	$3 \text{ Ca}_4(\text{PO}_4)_2\text{O} + 3 \text{H}_2\text{O} + 4 \text{ OH}^- \rightarrow 2$ $\text{Ca}_5(\text{PO}_4)_3\text{OH}\downarrow + 2 \text{Ca}(\text{OH})_2 \quad (12)$

Abstract

An important direction of construction biotechnology is production and application of the biogrouts to decrease the hydraulic conductivity of soil or fractured rocks. The most popular biogrout is a mixture of calcium chloride, urea, and either ureolytic bacteria or enzyme urease. A disadvantage of this biogrout is the release of a large quantity of toxic ammonia to air, as well as harmful ammonium and hydroxide ions to water. The aim of this study was evaluation of two new biogrouts that release less of harmful by-products to environment. First biogrout was a mixture of acidified hydroxyapatite solution from the bone powder with an addition of two times less urea that is used in conventional biogrout. Final pH was 7. Second biogrout was a solution of calcium bicarbonate produced from chalk powder and carbon dioxide with an addition of urea and acidotolerant ureolytic bacteria. This biogrout released to environment four times less ammonium and ammonia than conventional biogrout. Final pH was 8. Precipitation of about 2 g of calcium carbonate per kg of the cracked stone diminished the water seepage through the specimen by three orders of magnitude. Geotechnical or environmental engineering applications of these environmentally friendly biogrouts are limited by the consumption of a large volume of the grouting solution. It is due to low concentration of dissolved calcium, about 200 - 400 g m⁻³, in these liquid biogrouts. So, these biogrouts could be most effective for the sealing and strengthening of the materials with low porosity such as the fractured rocks or cracked concrete.