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Production and compatibility assessment of denitrifying biogranules tailored for self-healing concrete applications

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ABSTRACT

Microbial granules have been mostly used for wastewater treatment. Recently, biogranules consisting nitrate-reducing microorganisms have appeared as a unique healing agent providing simultaneous self-healing of cracks and corrosion inhibition of rebar in concrete. Yet, information about the production process and microbial activity of these biogranules as well as their compatibility with cementitious materials remains unknown. This study presents the biogranule production procedure in detail and evaluates the compatibility of the produced biogranules with the cementitious composites. In the form of biogranules, bacteria doses varying between 0.25% and 3.00% w/w cement were incorporated into mortar and the variations in fresh and hardened properties of mortars were evaluated with respect to abiotic mortars. Biogranules were also tested for their compatibility with concrete at minimum and the defined maximum tolerable doses. Biogranules with a NO_x-N reduction activity of 0.10 g NO_x-N.g^{-1} bacteria.d^{-1} and organic carbon oxidation activity of 1.50 g $HCOO^-$.g^{-1} bacteria.d^{-1} were produced successfully by using minimal medium. It was found out that biogranules enable bacteria incorporation into mortar up to a dose of 2.50% w/w cement without compromising fresh and hardened properties of cementitious composites. It was revealed that the compatibility of the biogranules was due to the mineral layer surrounding the biogranules which prevented interaction between the cement matrix and the microbial content. The thickness of the protective mineral layer around the granules was varying between 50 and 300 μm depending on the granule size. Net yield for concrete compatible biogranule production was determined as 0.05 g biogranule.g^{-1} $HCOO^-$.

1. Introduction

Exploiting microbes and microbial induced calcium carbonate precipitation (MICP) can be mentioned among the popular self-healing concrete development strategies. Metabolic by-products (i.e. CO_2) produced by heterotrophic microbes has appeared to be useful for creating an autonomous repair system in concrete, as microbially produced CO_2 turns into CO_3^{2-} (carbonate) in the alkaline environment of concrete and precipitates as $CaCO_3$ (calcite) inside the cracks.

Various microbes have already been tested for the proof of the concept and several useful strains were identified for the development of microbial self-healing concrete (Table 1). In the vast majority of the conducted studies, the nutrient ratio of the mix and the amount of bacterial healing agent were arbitrarily chosen without a solid rationale behind because the major objective was to reveal that the type of the isolated (or in-house developed) and encapsulated culture were functional, and the defined nutrients were suitable precursors for microbial induced self-healing of concrete cracks.

The state of the art about the "proof-of-concept" studies is close to saturation and new set of challenges started to appear such as cost-efficiency, public perception, standardization and practicality. In these new set of challenges, cost-efficiency, practicality in application and the delicacy of the microbial healing agent should be evaluated carefully. Optimization of nutrient and bacteria contents of microbial self-healing concrete should also be considered since such information will pave the way for providing a more standardized and cost-efficient recipe for self-healing concrete. Content optimization can be conducted in two consecutive steps; (i) by defining the constraints for the doses of typical components of microbial self-healing concrete (nutrients, protective carriers and microbial healing agents) based on their influence on

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Table 1
Various bacteria, protective carriers and doses tested for development of microbial self-healing concrete.

Study	Bacterial strain - pathway	Protective carrier	Amount of bacterial agent[a]
[1]	*Bacillus alkalinitrilicus* – aerobic oxidation of organic carbon	Light weight aggregates	~1.3×10^6 spores/g cement (0.15% w/w cement)
[2,3]	*Bacillus sphaericus* – urea hydrolysis	Hydrogels	~50×10^6 spores/g cement (7.3% w/w cement)
		Microcapsules	30×10^6 and 50×10^6 spores/g cement (4.3% and 7.3% w/w cement)
[4,5]	*Bacillus sphaericus* – urea hydrolysis	Diatomaceous earth	1.0% w/w cement
	Cyclic EnRiched Ureolytic Powder (CERUP) - urea hydrolysis	Self-protected culture	0.5% w/w cement
[6,7]	*Diaphorobacter nitroreducens* – anoxic oxidation of organic carbon (nitrate reduction metabolism)	Granular activated carbon	0.5% w/w cement
	Activated compact denitrifying core (ACDC) - anoxic oxidation of organic carbon (nitrate reduction metabolism)	Self-protected granular culture	0.5% w/w cement
[8]	*Bacillus halmapalus* – aerobic oxidation of organic carbon	Calcium alginate beads	0.3% w/w cement
[9]	*Bacillus cohnii* – aerobic oxidation of organic carbon	Expanded clay	0.03% w/w cement
[10]	*Bacillus subtilis* – aerobic oxidation of organic carbon	Light weight aggregates	0.7% w/w cement
[11]	*Bacillus pseudofirmus* – aerobic oxidation of organic carbon	Expanded perlite	2.6% - 13.3% w/w cement
[12]	Mixture of activated sludge, garden soil and *Bacillus cohnii* – aerobic and anaerobic oxidation of organic carbon	Expanded perlite	1.2% w/w cement
[13]	*Bacillus cohnii* – aerobic oxidation of organic carbon	Polyhydroxy-alkanoates	0.01% w/w cement

[a] In calculations of the spore content in terms of "% w/w cement", average dry weight of *Bacillus* spores were taken as 1.44 ng based on Tisa et al. [14]. Defining the microbial healing agent dose in terms of "% w/w cement" instead of spores/mL, cell/cm^3 or CFU/cm^3 is better for on-site practicality.

concrete properties, (ii) by revealing the relationship between healing agent content and self-healing efficiency. For the latter, one must define the compatibility of the self-healing agent, nutrients and, if applicable, the protective carriers with the cementitious composites. In almost all "proof-of-concept" studies, for the investigated bacteria doses, the influence on mechanical properties were shown [1–13]. However, these dose specific results do not picture a comprehensive compatibility assessment and most of the time limit the use of the proposed healing agent to a single dose. When currently used commercial products in the construction sector, such as air entraining agents, superplasticizers, set accelerators, etc., are considered, their effects on fresh and hardened concrete properties are well presented for a range of doses rather than a single dose. Therefore, it is also necessary to reveal how a proposed microbial healing agent affects fresh and hardened concrete properties when incorporated at different doses.

In recent studies, it was revealed that non-axenic cultures can also be an alternative microbial healing agent and they might even outperform axenic cultures in terms of the provided self-healing capacity [5,7,12,15,16]. Self-protected nitrate-reducing biogranules can be mentioned among the proposed non-axenic cultures for development of microbial self-healing concrete [7]. One of the advantages of this new generation microbial healing agent is its layered structure that avoids the need for protective carriers such as porous aggregates or microcapsules [17]. Nitrate reducing biogranules were defined to have a core community composed of a highly active nitrate reducing bacteria that were surrounded by extracellular polymeric substances (EPS) and calcium-based minerals [7]. The layers surrounding the core community ensure the resilience of the biogranules under harsh conditions [17], which make biogranules advantageous over axenic cultures in terms of practicality, shelf-life and cost-efficiency. Yet, previous studies did not evaluate these properties in detail. Nitrate reducing biogranules, specifically granulated for concrete application were determined to be compatible with cementitious matrix and useful for crack healing in concrete at a bacteria dose of 0.50% w/w cement [7,15,18]. For the same mixture exposed to marine similar conditions (0.5 M Cl^- solution), simultaneous corrosion inhibition and healing of a 300-μm-wide crack were also reported [16]. Such an added corrosion inhibition function of the proposed biogranules might also be advantageous to accelerate the transition of the microbial self-healing concrete technology from concept to market. The aforementioned advantages make biogranules worth investigating further in detail for up-scaling of microbial self-healing concrete technology, and the details of this healing agent should be well defined. Yet, all of these biogranule studies were limited to the tests on mortar and the tests were conducted only for a single bacteria dose (e.g. 0.50% w/w cement) similar to the other "proof-of-concept" self-healing studies [1–13]. Therefore, compatibility of the biogranules with the cementitious matrix (both in mortar and in concrete) at various biogranule doses still remains unknown. Moreover, in previous studies proposing biogranules as healing agent, some essential details about the biogranules such as production yield, production cost, detailed composition of the biogranule layers, water absorption, specific activity of the denitrifying core etc. were not thoroughly presented. Therefore, this study addresses the aforementioned needs by revealing important biogranule properties in detail and defines the dose constraints for the use of nitrate reducing biogranules in development of microbial self-healing concrete by evaluating the fresh and hardened properties of various cementitious composites (mortar and concrete) containing 0.35% to 4.30% of biogranules w/w cement (0.25%–3.00% of bacteria w/w cement).

2. Materials and methods

2.1. Preparation of the seed for biogranule production

Beet-sugar processing water has alkali pH (pH > 11) and high temperature (>80 °C) so that it can serve as a source for selection of alkali resistant spore forming bacteria. Therefore, in this study beet-sugar processing water was used as a liquid medium for preparation of the seed mixture for biogranule cultivation. The seed mixture was composed of beet-sugar processing water and old dry biogranules which had been cultivated in our previous studies [15,16]. In order to prepare the mixture, beet-sugar processing water was concentrated by gravity settling of the suspended solid content and removing the supernatant. Concentrated mixture was further pasteurized at 80 °C for 30 min to remove the vegetative cells. Then, old dry biogranules were ground to a size of less than 0.212 mm (minimum floc size to be considered as a granule) and added to the pasteurized concentrated mixture. This new mixture was used as a seed for production of fresh nitrate reducing biogranules in a sequencing batch reactor.

2.2. Reactor configuration and the operational conditions for production of nitrate reducing biogranules

A cylindrical sequencing batch reactor (SBR) with an effective height of 600 mm and internal diameter of 124 mm ($V_{effective}$ = 7.25 L) was used to produce fresh biogranules (Fig. 1). Volume exchange ratio of the SBR was set to 50% with a hydraulic retention time of 12 h. The

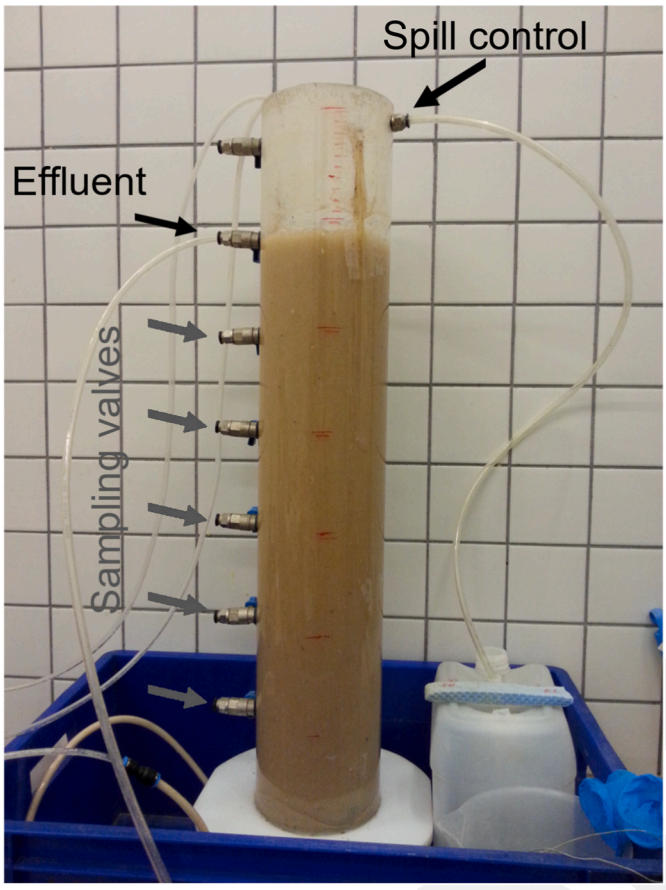

Fig. 1. A 7.25 L size biogranule production reactor.

bioreactor was operated with four cycles/day. Each cycle consisted of a simultaneous fill/draw period, sole anoxic period, aerobic period and a settling period. Simultaneous fill/draw was run under anoxic conditions with an upflow influent rate of 0.5 mL s^{-1} and lasted for 120 min in each cycle. After the fill/draw period, the reactor was operated under anoxic conditions for another 60 min to complete the overall anoxic period duration to 180 min. At the end of the first 15 min of the sole anoxic period, the reactor content was completely mixed for 1 min to enable a better contact of nutrients and the biogranules. Anoxic period was followed by an aerobic period with a duration varying between 150 and 180 min depending on the duration of the settling period. The aerobic period was obtained by aeration with an upflow air velocity of 8 mm s^{-1}. Duration of the settling period was gradually decreased from 30 min to 2 min by following the granulation performance of the reactor, and upon full granulation (granule content >%85) settling period was completely removed from the operation. SBR was operated for 232 days and the variation of operational parameters were given in Table 2.

Table 2
Operational parameters for concrete compatible nitrate reducing biogranule production.

Days	OLR[a] (g $HCOO^-$. L^{-1}.d^{-1})	NLR (g NO_3–N. L^{-1}.d^{-1})	Anoxic period[b] (min)	Aerobic period (min)	Settling period (min)
1–20	2.29	0.08	180	150	30
21–34	2.29	0.08	180	165	15
35–39	2.29	0.08	180	168	12
40–44	2.86	0.12	180	172	8
45–51	2.86	0.12	180	175	5
52–88	3.43	0.16	180	178	2
89–232	4.29	0.20	180	180	–

[a] OLR: Organic loading rate; NLR: Nitrogen loading rate.
[b] The first 120 min of the anoxic period was simultaneous fill/draw.

The alkaline minimal nutrient medium given in Table 3, was used in cultivation process to facilitate formation of biogranules resilient enough to survive the alkaline and micronutrient deficient concrete environment. The pH of the feed solution was set to 10.2 by using 10 M NaOH solution.

2.3. Control parameters for granulation and microbial activity

Agglomerated microbial cultures up to a size of 200 μm are considered as floccular culture while the ones larger than 200 μm are considered as granular culture [19]. Moreover, Liu et al. [20] defined a threshold sludge volume index (SVI_{30}) value of 50 mL g^{-1} to determine if a system is granular or not and also revealed that SVI_{30}/SVI_5 ratio is a useful parameter for determination of granular biomass percentage in a bioreactor. Accordingly, in this study, granulation of bacteria was followed by monitoring SVI_5, SVI_{30}, and the particle size distribution of the microbial culture inside the bioreactor. Granulation process was considered to be completed when more than 85% of the suspended solid content was composed of biogranules and SVI_{30} value decreased below 50 mL g^{-1}. Granule percentage of the suspended solid content was followed based on SVI_{30}/SVI_5 ratio and wet particle size distribution analyses.

The particle size distribution of the microbial culture was determined by sampling the bioreactor under completely mixed conditions. A 750 mL sample was taken from the reactor and the mixture was poured through a series of sieves. The sieve sizes were arranged to decrease from top to bottom in the following order; 2.00 mm, 1,00 mm, 0.85 mm, 0.45 mm and 0.210 mm. Granules staying on sieves were collected from the sieves into separate beakers. Total suspended solids (TSS) and volatile suspended solids (VSS) analyses were conducted for the collected samples and the size distribution was determined in terms of weight percent. Total granule percentage inside the bioreactor was determined by using Eq. (1).

$$Granule\ percentage\% = \frac{m_{d>0.210mm}}{m_T} \times 100 \quad (1)$$

- $m_{d\ >\ 0{,}210mm}$: Dry weight of the suspended solid content of the microbial agglomerates caught on 0.210 mm and larger sieves.
- m_T: Dry weight of the suspended solid content of the sample taken from the bioreactor

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2.4. Confirmation of the denitrifying core community in biogranules by means of kinetic analysis

After achieving a granular-biomass-dominant bioreactor (biogranules >85% of VSS content), specific NO_3–N reduction and carbon oxidation activities in anoxic and aerobic periods were determined by conducting kinetic analysis in a single cycle. These tests were particularly important to confirm the presence of a denitrifying core

Table 3
Nutrient solution for concrete compatible nitrate reducing biogranule production.

Substrate	Concentration (mg.L^{-1})
NaHCOO	3239
$NaNO_3$	331
$Ca(NO_3)_2$	267
$MgSO_4.7H_2O$	89
KH_2PO_4	13

community in biogranules as nitrate reduction under aerobic conditions could only occur at the core of the biogranules where oxygen diffusion is limited with the first 200–400 μm of the granules [21]. During kinetic analysis, throughout the cycle, samples were collected every 15 min from the sampling ports located at different levels (every 10 cm) of the bioreactor. Collection of samples at different levels were essential to determine the availability of nutrients for the biogranules during the feeding and anoxic periods occurring under non-mixing conditions.

2.5. Granule harvesting and the characterization of dry biogranules

Different from the previous studies [7,16], harvesting intervals were kept shorter in this study. After achieving a granular-biomass-dominant bioreactor, biogranules were harvested about every 15 days.

Harvested biogranules were physically and chemically characterized by conducting TSS and VSS analyses, Fourier-transform infrared spectroscopy (FTIR), energy-dispersive X-ray spectroscopy (EDS) coupled scanning electron microscopy (SEM), elemental mapping of a granule cross section, water absorption (1 h and 24 h) and dry granule size analysis. VSS offers an approximation of the amount of organic matter and can be used as the bacteria content if the measurements are conducted for a well-controlled bacteria cultivation reactor. As the biogranule cultivation reactor and the nutrients described in this study were well-controlled compared to a wastewater treatment plant, VSS content of the harvested biogranules represented the bacteria content. The difference between TSS and VSS content of biogranules represented the inorganic fraction, mostly minerals.

In order to conduct FTIR analysis, dried granules were ground to very fine powder. Obtained powder was analysed, and the spectra for 32 scans with resolution of 4 cm^{-1} in the range of 4000–500 cm^{-1} was obtained and interpreted.

EDS-coupled SEM analysis were conducted under an accelerating voltage of 15 kV and a working distance of 7.5–9.0 mm by using either a secondary electron (SE) detector or a backscatter electron (BSE) detector. One of the dry granules was split into two halves by using a stainless-steel lancet and elemental mapping was conducted for Ca, P and O elements on one of the cross sections to determine the presence and the distribution of $CaCO_3$ minerals, $Ca_3(PO_4)_2$ minerals and spores.

As water absorption is one of the important parameters for compatibility of the admixtures with the cementitious matrix, dried granules were tested for their water absorption. Water absorption test was conducted for dried granules soaked in water for 1 h to mimic the possible water absorption before the initial setting time. The test was also conducted for dried granules soaked in water for 24 h to determine the possible water absorption before demoulding. Water absorption tests were conducted by using a vacuum filtration setup as shown in Fig. 2a. After the defined soaking period, granules were filtered through a cellulose filter paper (Whatman Grade 42) by using a vacuum pump (Fig. 2a). The sample size for each test was 1 g and for each duration the tests were conducted in triplicates. Appearance of surface saturated dry biogranules on the filter paper are given in Fig. 2b. The results were presented as mean and standard deviation of the mean. The water absorption was calculated by applying Eq. (2) where w_{SSD} indicates saturated surface dry weight of biogranules and w_{dry} is the dry weight of the sample (1 g in this study).

$$water\ absorption\ \% = \frac{w_{SSD} - w_{dry}}{w_{dry}} \times 100 \quad (2)$$

Dried granules that were ready for mortar incorporation were visualised under light microscope. In addition to that an arbitrarily chosen dry granule was split into two and the difference between the outer and inner layers of the biogranules was visualised under light microscope.

2.6. Analytical methods

In most of the experiments, fresh biogranules were tested immediately after the sampling. Unless it was possible to test immediately, samples were stored at +4 °C until the testing time. SVI measurements, TSS and VSS measurements were conducted according to the standard methods [22].

Liquid samples taken from the biogranule reactor were filter sterilised immediately after the sampling and stored at +4 °C until the tests were conducted. Liquid samples were tested for $HCOO^-$, NO_3–N and NO_2–N. $HCOO^-$ concentrations were measured via high-performance liquid chromatography (HPLC) and in some cases by using standard COD kits (Hach Lange LCK 314 and LCK 400). The COD equivalent of $HCOO^-$ was used as 0.23 g $COD.g^{-1}$ NaHCOO. NO_3–N and NO_2–N analyses were conducted by using Metrohm 930 Compact IC (Metrohm, Switzerland). Except kinetic analysis, all analyses were conducted in triplicates and the results were presented as mean and a standard deviation.

2.7. Compatibility assessment of biogranules with fresh and hardened mortar

The biogranules investigated in this study were composed of an active denitrifying core mostly surrounded by EPS and calcium salts. Therefore, the application of these biogranules for development of microbial self-healing concrete requires two major chemical admixtures; (i) an organic carbon source as electron donor, (ii) a nitrate source as an electron acceptor. Based on the previous studies that nitrate reducing cultures were tested for development of microbial self-healing concrete, in this study, calcium formate (CF) and calcium nitrate (CN) were used as nutrient sources. Throughout the experiments, the nutrient contents of different biomortars were kept identical to clearly distinguish the effect of biogranule dose on fresh and hardened mortar properties. Accordingly, except the plain mortar mixture, in all the other mix designs, 5.00% calcium formate w/w cement and 2.00% calcium nitrate w/w cement were included as nutrient sources.

Plain control specimens, abiotic control specimens and self-healing

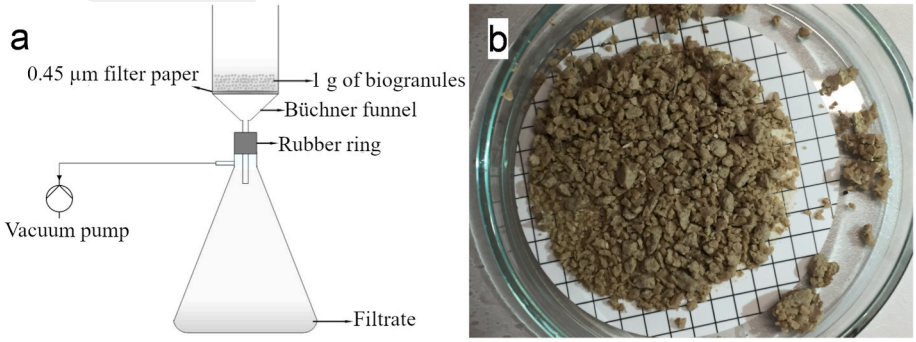

Fig. 2. Vacuum filtration test setup to determine water absorption of dry biogranules; (a) vacuum filtration setup; (b) surface saturated dry biogranules.

biomortar specimens were prepared and tested. Plain mortar specimens were used as reference. Abiotic control specimens were used to distinguish the effect of chemical admixtures. Biomortar specimens were used to determine the influence of varying biogranule content. All the mortar specimens were prepared by following the standard procedure described in EN 196-1. Plain control mixture was composed of DIN EN 196-1 standard sand (1350 g), CEM I 42.5R cement (450 g) and, tap water (225 g) with a weight ratio of 3:1:0.5. Abiotic control mixture contained 5.00% CF w/w cement (22.5 g) and 2.00% CN w/w cement (9.00 g), which was determined based on a previous study [23], in addition to the typical aforementioned standard ingredients (i.e. sand, cement, water). In biomortar specimens, various amounts of biogranules (0.35%–4.30% w/w cement; 0.25% to 3.00% bacteria w/w cement, respectively) were tested together with the fixed amount of chemical admixtures CF (5.00% w/w cement) and CN (2.00% w/w cement). It should be noted that 70% of the tested biogranule content was bacteria and the rest was minerals which corresponded to a bacteria incorporation at doses of 0.25% w/w cement to 3.00% w/w cement. In the manuscript, biomortar specimens were presented with their bacteria content with the following notation "Bio-##" where "##" represented the bacteria content (i.e. 0.25% w/w cement to 3.00% w/w cement). In each biomortar mix, 50% of the total biogranule content were between 1.00 and 2.00 mm in size, 35% of the total biogranule content were between 0.85 and 1.00 mm in size and 15% of the total biogranule content were between 0.45 and 0.85 mm in size. Tested mixtures were summarized in Table 4.

Fresh mortar properties of various mixtures were determined by means of setting test and flow table test. Strength development and compressive strength were used to assess the hardened mortar properties.

Setting tests were conducted in accordance with the ASTM807 standard by using an automatic vicat equipment (Matest E−449 N Vicatronic, Italy). Obtained initial and final setting results were evaluated based on the criteria defined in ASTM C191-04 and NBN EN 1008. Accordingly, the minimum acceptable initial and final setting limits for the tests conducted by a single operator were defined as 60 ± 12 min and 90 ± 20 min, respectively.

The flow table tests were conducted in accordance with the NBN EN 1015-3 standard. Obtained flow values were evaluated based on the criteria given in NBN EN 1015-3. Accordingly, to confirm a workability and consistency difference between two mixtures the flow values should deviate from each other by at least 10%. The results obtained in this study were interpreted by comparing the flow values of biomortar specimens with flow values of both abiotic control and plain mortar mixtures.

Compressive strength tests were conducted upon 3-, 7-, 28- and 56-days curing periods in accordance with the ASTM C109 standard. Tests were conducted by using 50 mm × 50 mm × 50 mm cubic specimens. After demoulding, specimens were cured in a tightly sealed bag to prevent evaporation. The presence of humidity in the bags was confirmed by placing a small container filled with water and monitoring possible evaporation. Compressive strength tests were conducted by using a test equipment (UTC-5700, Turkey) with a 300 kN capacity. In tests, loading rate was 2 kN s^{-1}.

Table 4
Mixing proportion of the tested mortar and biomortar specimens.

Mortar mixtures	Biogranule content (g)			CF (g)	CN (g)
	0.45–0.85 mm	0.85–1.00 mm	1.00–2.00 mm		
Plain mortar	–	–	–	–	–
Abiotic control	–	–	–	22.50	9.00
Bio-0.25%	0.24	0.56	0.81	22.50	9.00
Bio-0.50%	0.48	1.12	1.61	22.50	9.00
Bio-0.75%	0.72	1.69	2.41	22.50	9.00
Bio-1.00%	0.97	2.25	3.21	22.50	9.00
Bio-1.50%	1.45	3.37	4.82	22.50	9.00
Bio-2.00%	1.93	4.50	6.43	22.50	9.00
Bio-2.50%	2.41	5.62	8.04	22.50	9.00
Bio-3.00%	2.89	6.75	9.64	22.50	9.00

2.8. Compatibility assessment of biogranules with fresh and hardened concrete

Concrete compatibility of biogranules was also tested at two biogranule doses (BioC 0.25% and BioC 2.50). The tested doses were determined based on the initial results obtained from mortar compatibility. According to the mortar tests, the maximum allowable bacteria content in biogranule form was determined as 2.50% w/w cement and thus the dose was also tested for concrete compatibility. Additionally, BioC-0.25% was prepared to assess the minimum dose. Concrete mix was designed by modifying the ACI 211 concrete mix design procedure. Accordingly, concrete specimens consist of 966.0 kg m^{-3} coarse aggregate, 870.0 kg m^{-3} fine aggregate, 394.0 kg m^{-3} CEM I 42.5R cement and 236.4 kg m^{-3} water (water cement ratio 0.6) were prepared. In concrete mix design C25/C30 concrete properties were targeted. Coarse aggregate d_{max} was chosen as 16 mm and the particle sizes of fine aggregates varied between 4 mm and 12 mm. The ideal granulometry and the deviation of fine and coarse aggregates were given in Fig. 3.

Similar to the mortar mixtures, plain concrete, abiotic concrete and bioconcrete specimens were prepared. In each preparation 10 L of concrete was prepared, and in accordance with TS-EN-12390-1, cubic specimens of 100 mm × 100 mm × 100 mm were casted for tests. In abiotic control 5.00% CF w/w cement (19.70 kg m^{-3}) and 2.00% CN w/w cement (7.88 kg m^{-3}) was used. In bioconcrete specimens 0.25% bacteria w/w cement (1.4 kg m^{-3} biogranule) and 2.50% bacteria w/w cement (14.1 kg m^{-3} biogranule) were added in the form of biogranules together with 5.00% CF w/w cement and 2.00% CN w/w cement. Fresh concrete specimens were compacted by using a vibration table with 50 Hz frequency (at 3000 rpm) and stored in a nylon bag for 24 h. Upon demoulding they were cured in a water tank until the test day.

2.9. Statistical analysis

Results obtained for bio-based specimens were compared with both abiotic control specimens and plain control specimens by applying one-way ANOVA analysis and Holm-Šídák's multiple comparison method ($p = 0.05$). Bio-based specimens were also compared among each other and with the plain concrete by applying one-way ANOVA and Fisher's LSD 5%.

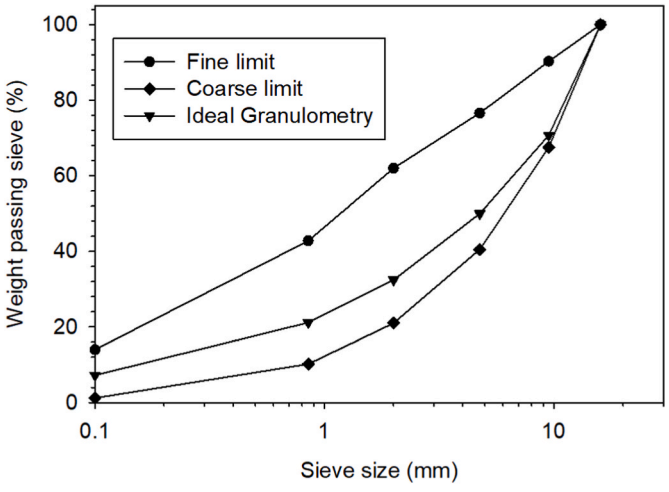

Fig. 3. Granulometry of the used fine and coarse aggregates compared to ideal granulometry curve.

3. Results

3.1. Biogranulation and characterization of the produced biogranules

Compact biogranules with an activated denitrifying core were produced and they were characterized in detail. During the operation of the biogranulation reactor, parameters such as $HCOO^-$, NO_3–N, NO_2–N, and pH were regularly monitored. Their evolution and the [C]:[N] consumption ratio during anoxic period were given in Fig. 4.

In order to acclimate the seed culture to the operational conditions and facilitate reproduction of the resilient and relatively faster settling bacteria retained in the reactor, a low strength nutrient solution was used in the first 40 days. As seen in Fig. 4c, although the organic substrate was completely consumed in the first 30 days, the consumption was mostly due to aerobic activity and NO_3–N reduction in the system was limited (48 ± 19% in anoxic period, 0% in aerobic period). In addition to that the consumed [C]:[N] ratio in anoxic period was recorded as around 0.4 (Fig. 4b). Between days 30 and 40, both nitrate reduction and anoxic organic consumption increased rapidly (95% anoxic NO_3–N reduction on day 37) which indicated successful enrichment of nitrate reducing organisms in the system. Therefore, on day 40, organic and nitrogen loading rates were further increased to 2.86 g $HCOO^-.L^{-1}.d^{-1}$ and 0.12 g NO_3–$N.L^{-1}.d^{-1}$, and kept constant until day 52. The increase in loading rates led to a rapid decrease in microbial activity in the following days (26% anoxic NO_3–N reduction on day 41) and nitrite accumulation was observed. Consumed C:N ratio increased to around 9 which indicated growth of microorganisms on nitrate. Nonetheless, these changes in operational conditions were necessary to trigger biogranulation, nitrite accumulation and to select the microorganisms of interest. Recovery in the system performance was achieved in the second half of this phase and 70% anoxic NO_3–N reduction with significant nitrite accumulation (75% of the total NO_3–N reduced turned into NO_2–N) was recorded (Fig. 4d). After the recovery of the microbial activity, on Day 52, organic and nitrogen loading rates were increased to 3.43 g $HCOO^-.L^{-1}.d^{-1}$ and 0.16 g NO_3–$N.L^{-1}.d^{-1}$, respectively and the reactor was operated for another 35 days. The full granulation of the reactor content could be achieved in this period, on day 69, and sustained throughout the rest of the operation. Consumed [C]:[N] ratio also dropped below 6 in the rest of the operational period which indicated that the most of the consumed C was due to nitrate reduction. In biogranule-dominant reactor (after day 69), 75% of the NO_3–N was reduced in anoxic period, and 50% of the remaining NO_3–N was consumed in aerobic period which was the first indication of a denitrifying core. On Day 88, the nutrient loading rate was slightly increased to increase the mass production rate. The reactor was further operated for 143 days with these operational conditions (Fig. 4).

Apart from modifying the nutrient loading rates in the first 88 days, another operational parameter of SBR, the settling period, was gradually changed to increase selective pressure on microorganisms and favour the ones that can form biofilm on each other and agglomerate. In the first 40 days, duration of the settling period in SBR was gradually decreased from 30 min to 8 min in order to enrich floc-forming bacteria. This decrease in settling period was done by carefully monitoring the VSS concentration inside the bioreactor as such interference to the settling period would lead to wash out of the relatively slow floc-formers from the system. The effect of wash-out could be seen in Fig. 5a as TSS and VSS concentrations in the reactor did not increase and even a slight decrease occurred despite all the efforts to keep it as constant as possible. Moreover, VSS/TSS ratio dropped below 65% which was a sign that microorganisms that cannot form flocs were washed out and the ones attached on rapidly settling solids such as minerals retained inside the reactor. In order to further increase the selective pressure on the microorganisms inside the reactor and trigger a more compact agglomeration, settling period was gradually decreased from 8 min to 2 min between days 40–52. As a result, loose flocs were continuously washed out from the system which favoured the competition on nutrients towards the granule-forming bacteria. Due to the enrichment of granule forming bacteria which are self-immobilizing, VSS/TSS ratio

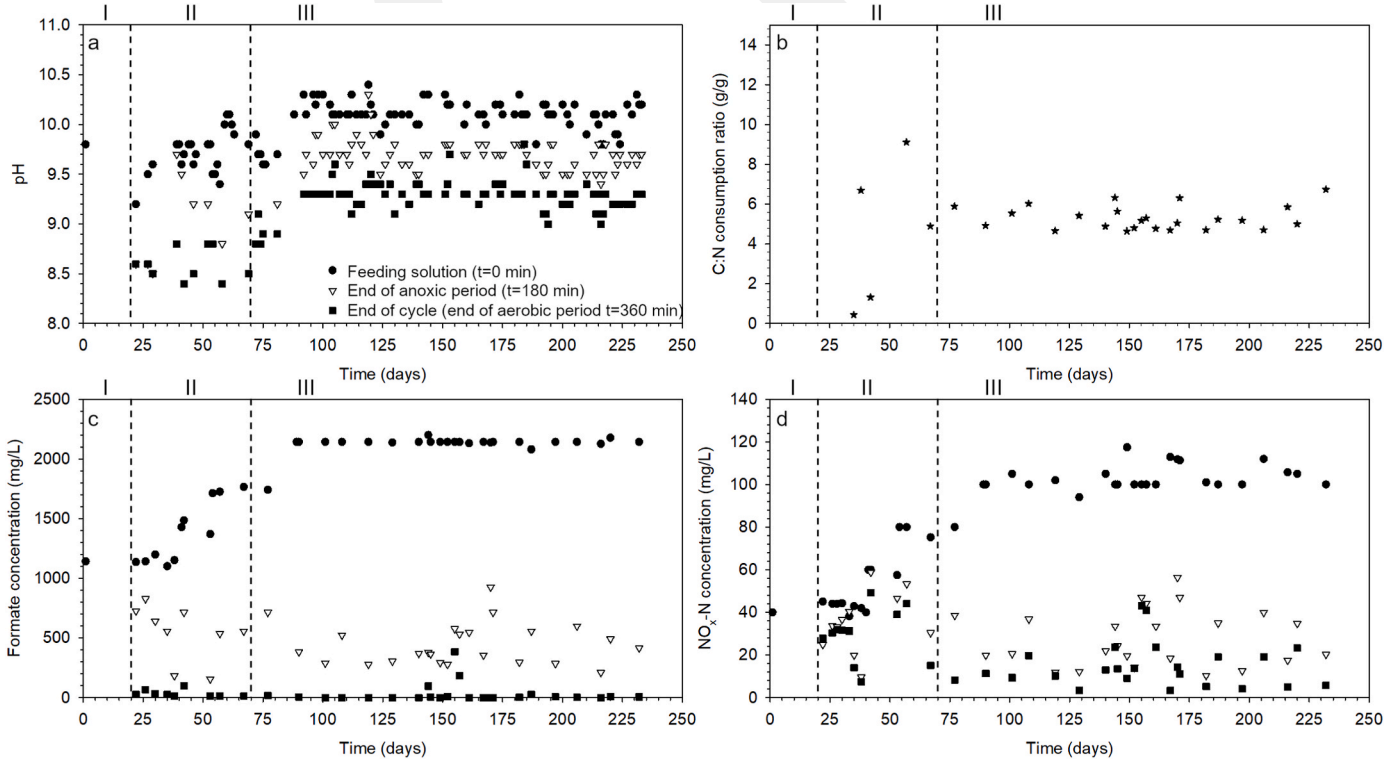

Fig. 4. Monitored parameters throughout the acclimation (I), granulation (II) and biogranule production (III) process; (a) pH; (b) [C]:[N] consumption ratio in anoxic period; (c) cycle specific $HCOO^-$ consumption; (d) cycle specific NO_x-N consumption.

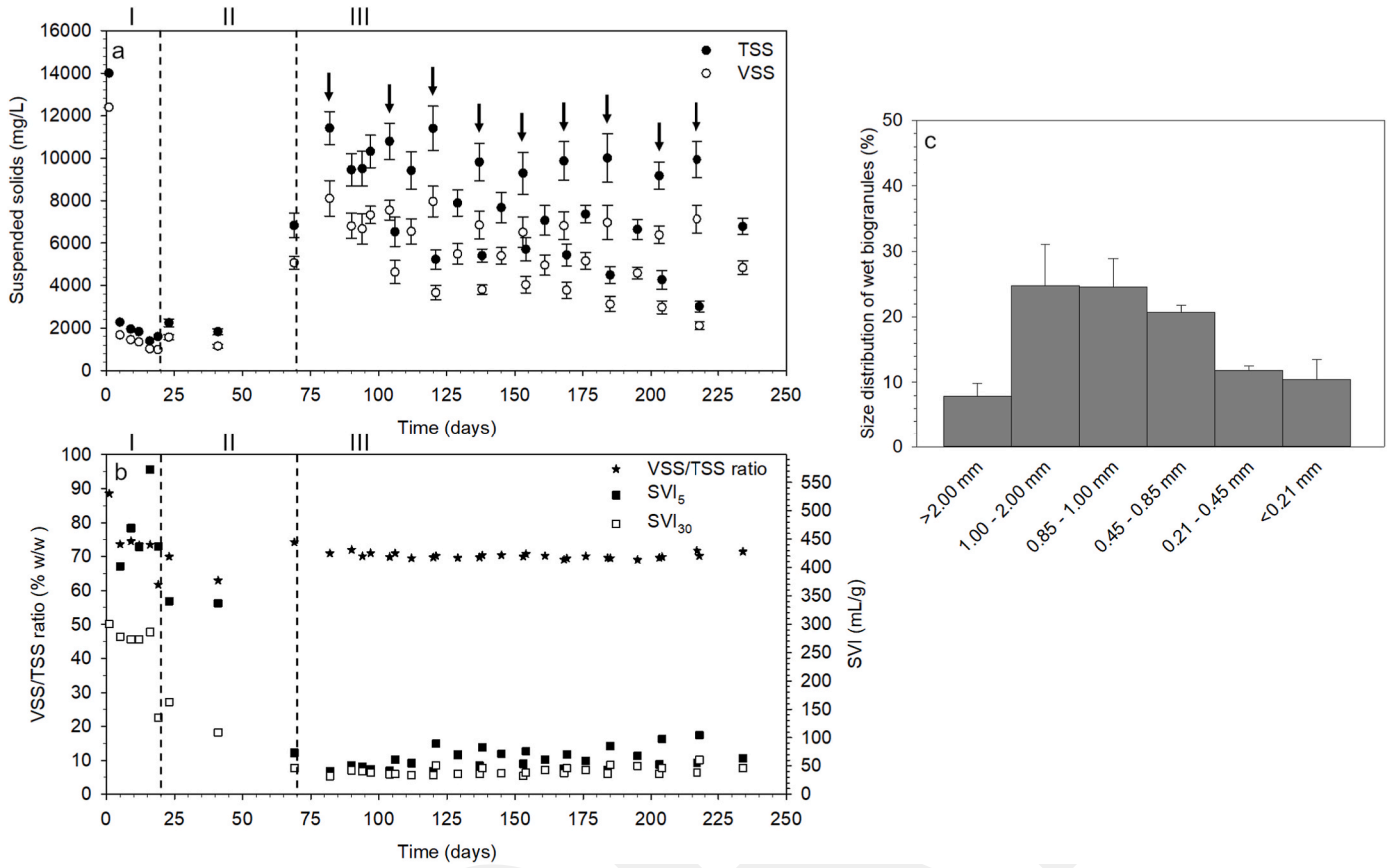

Fig. 5. Monitored granulation parameters throughout the acclimation (I), granulation (II) and biogranule production (III) process. (a) suspended solid content (n = 3); (b) SVI and bacteria/biogranule ratio; (c) average biogranule size distribution in reactor (n = 5).

was recovered back to more than 70% on Day 69, and the reactor content became fully granular with a recorded SVI_{30} value of 46 mL g^{-1} (Fig. 5b). On Day 82, the first batch of biogranules could be harvested to conduct some quality tests.

The granule production rate of the bioreactor was 0.07 g biogranule. g^{-1} $HCOO^-$. Accordingly, biogranules could be harvested almost every two weeks without significantly affecting the production rate (Fig. 5a). Each harvest was indicated with an arrow in Fig. 5a which also indicated the significant drop in biogranule content of the bioreactor due to harvesting. Harvested granules were always tested for their bacteria content which was shown as VSS/TSS ratio in Fig. 5b. As can be seen, the production of granules and their bacteria content were stable under the defined operational conditions. Although the biogranule reactor had an SVI_{30} value below 50 mL g^{-1} before each harvest, granule harvesting from the reactor also caused a slight decrease in settling properties of the remaining biogranules, particularly on their SVI_5 (Fig. 5b), because in granule harvesting mostly large granules were taken from the reactor and smaller fraction was either retained in the reactor or returned back to the reactor after sorting based on their dry particle size.

Granule harvesting also negatively affected the nitrate consumption and organic matter consumption in the reactor. The recovery of the performances could be achieved when VSS concentration in the reactor increased to ~5000 mg L^{-1} (Fig. 4c and d, Fig. 5a).

After achieving the full granulation, granule harvesting and granule characterization analyses were started. Granule size distribution inside the reactor was conducted at 5 different times (On days 82, 104, 137, 168, 203). As seen in Fig. 5c, ~90% of the reactor was composed of biogranules (size >0.21 mm). Similarly, SVI_{30}/SVI_5 ratio for same time periods were recorded as ~%85 (Fig. 5b). More than 90% of the biogranules were found to be less than 2.00 mm in size and around 55% of the biogranules were between 0.85 and 2.00 mm in size (Fig. 5c).

Kinetic analysis was conducted in a single SBR cycle (120 min feed, 60 min sole anoxic, 180 min aerobic) by collecting samples from each 10 cm height every 15 min to picture the distribution of the nutrients inside the reactor and to confirm the presence of a denitrifying core in biogranules. The results indicated that the nutrient feeding rate was higher than the consumption rate, and accumulation of nutrients occurred during simultaneous fill/draw period. Nonetheless, the available nutrients were mostly concentrated around the granular biomass (first 10 cm of the reactor) (Fig. 6a and b). After the mixing (for 1 min) in sole anoxic period (at 135 min), the remaining nutrients above the biogranules were equally distributed inside the reactor and get in contact with the biogranules which provided rapid consumption (Fig. 6a,b, c). The denitrifying core in biogranules could be confirmed by the ongoing consumption of NO_x-N in the first hour of the aerobic period during which the DO concentration varied between 1 mg L^{-1} and 6 mg L^{-1} (Fig. 6d). The consumption occurred in the presence of a little soluble formate (between 180 and 195 min), and continued even after the soluble formate was depleted. This observation also revealed that the biogranules stored some internal polymers during the feast period (rich in nutrients) to consume under famine conditions (one of the nutrients is limited or depleted). Microbial activity of biogranules under anoxic conditions were determined as 0.10 g NO_x-N.g^{-1} bacteria.d^{-1} and 1.50 g $HCOO^-$.g^{-1} bacteria.d^{-1} (Fig. 6c). The kinetic analysis also enabled determination of the [C]:[N] consumption ratio under anoxic conditions. Accordingly, the C:N ratio was fluctuating between 3.9 and 6.5 with an average value of 5.3 ± 1.0 g g^{-1} (Fig. 6e).

Harvested biogranules were inspected for their physical and chemical properties. First of all, size distribution analyses were conducted on the dried biogranules and the fractions larger than 2.00 mm and smaller than 0.45 mm were returned back to the bioreactor. The average size distribution of the biogranules harvested at different time periods (n =

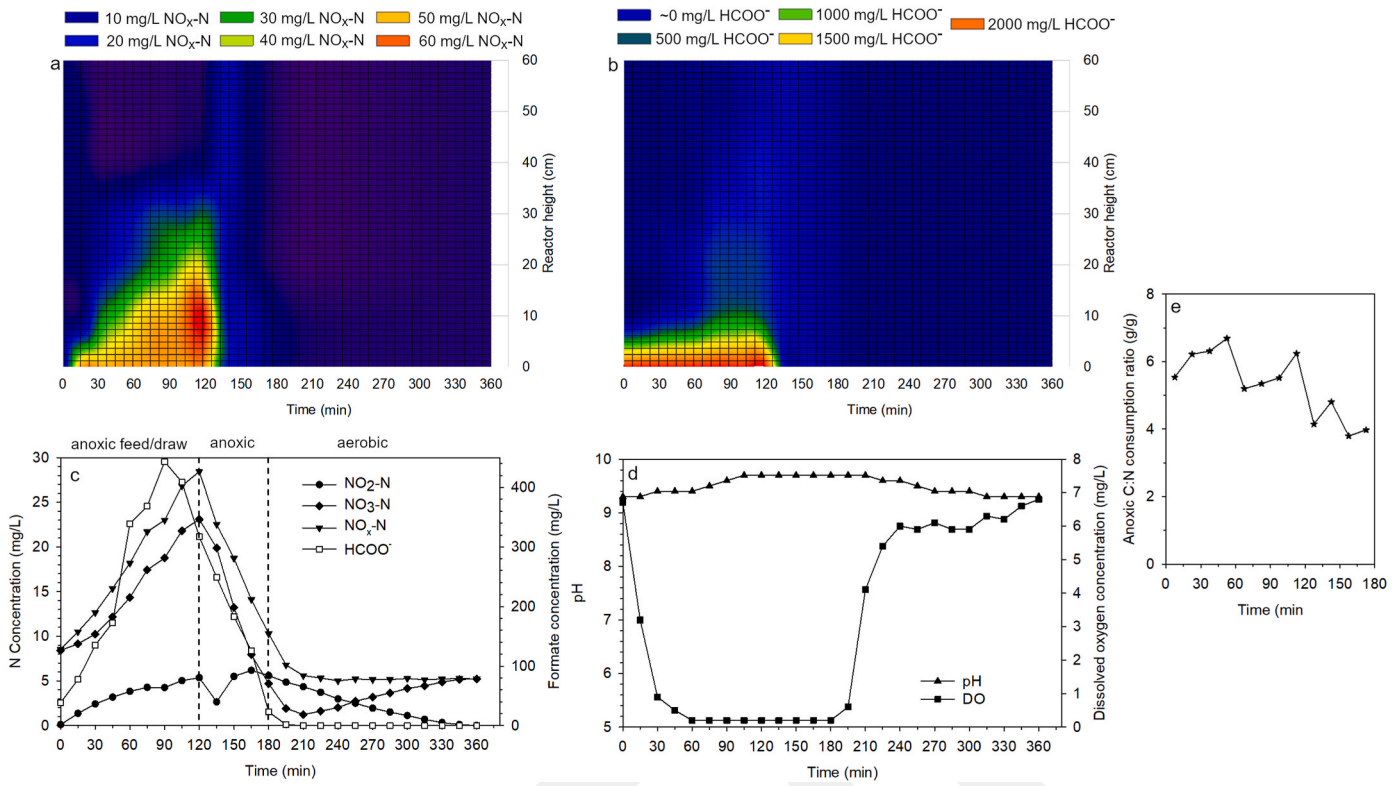

Fig. 6. Kinetic observations of changes in (a) NO_x-N distribution; (b) $HCOO^-$ distribution; (c) average nutrient concentrations due to biogranule activity; (d) pH and DO; (e) anoxic [C]:[N] consumption ratio.

10) was presented in Fig. 7a. The dry particle size distribution of different harvests did not deviate much from each other. In total about 34% of the harvested biogranules were returned back to the biogranule production reactor as they were either larger than 2.00 mm or smaller than 0.45 mm. The highest fraction was the ones with dry sizes between 1.00 and 2.00 mm which corresponded to about half of the biogranules selected for mortar tests.

As water absorption is one of the important parameters for compatibility of the admixtures with the cementitious matrix, dried granules were tested for their water absorption. Water absorption test was conducted for dried granules soaked in water for 1 h to mimic the possible water absorption before the initial setting time. The test was also conducted for dried granules soaked in water for 24 h to determine the possible water absorption before demoulding. It was found that the water absorptions of dry biogranules were 16 ± 8% and 20 ± 8% at the end of 1 h and 24 h of soaking under water, respectively (Fig. 7b). These results showed that the biogranules mostly absorb water in the first hour of their contact with water and it does not change significantly afterwards. According to ASTM C191-04 standard, the minimum allowed initial setting time of mortar is 60 ± 12 min. Therefore, before the initial setting time biogranules' water absorption would be almost completed. Accordingly, considering the biogranule content in tested biomortar specimens, about 0.06–3.80 g of water might be absorbed by biogranules which would not affect the water:cement ratio of the mortar specimens.

The biogranule production reactor was operated to induce $CaCO_3$ and $Ca_3(PO_4)_2$ precipitates around the biogranules to create a mineral layer that can prevent the interaction between the organic matter and the cementitious matrix and may function against the shrinkage forces occurring on biogranules during hydration of the cementitious materials. Moreover, produced biogranules should also contain some EPS which plays a significant role in self-protection capability of the biogranules. In order to confirm these properties of biogranules, FTIR analyses were conducted after each harvest and one of them was presented in Fig. 7c. FTIR results showed that the biogranules were composed of water, amine groups (3271–3600 cm^{-1} OH and NH peaks), proteins (1683 cm^{-1} amide in secondary proteins) that provide rigidity and polysaccharides (1730.82 cm^{-1} carbonyl ester bond stretching peak corresponds to internal PHA and 1027 cm^{-1} carbohydrates peak corresponds to EPS) as well as some inorganic matter including calcium carbonate (1404 cm^{-1}, 871 cm^{-1}, carbonate peaks of calcite and 699 cm^{-1} carbonate peak of aragonite) and calcium phosphate (583.50 cm^{-1} phosphate peak). The FTIR results conducted on different harvests did not differ much from each other which was another indicator of stable production of biogranules with similar properties. The presence of proteins and polysaccharides were the major indicator of the polymeric substances in the harvested biogranules. The significant protein peak was also an indirect indicator of the presence of spores in biogranules, as spores are composed of a thick protein layer that protects the genetic information of the cells.

The location of the EPS and inorganic mineral layers were as important as their presence in the biogranules. Therefore, wet and dry biogranules were initially visualised under light microscope. Afterwards EDS-coupled SEM analyses were conducted to visualize harvested dry biogranules and the cross section of a biogranule. Elemental mapping was also conducted on the cross section of a biogranule to reveal the distribution of the compounds, spores and minerals. Fig. 8 reveals the micrographs of wet and dry biogranules obtained under light microscope. As can be seen in micrographs, both wet and dry biogranules had an oval shape. Moreover, in micrographs of the wet biogranules, light penetration through the biogranules was higher at the sides compared to the core which was related with the presence of crystals at the outer layer while at the core a compact bacterial community was present (Fig. 8a and b). Moreover, in dried biogranules a white layer of precipitates surrounding the creamy colour bacterial granule could be visualised (Fig. 8c). When a dry biogranule was split into two, the inner colour was getting even darker from side to centre, while outside of the biogranule was almost completely covered with precipitates (Fig. 8d).

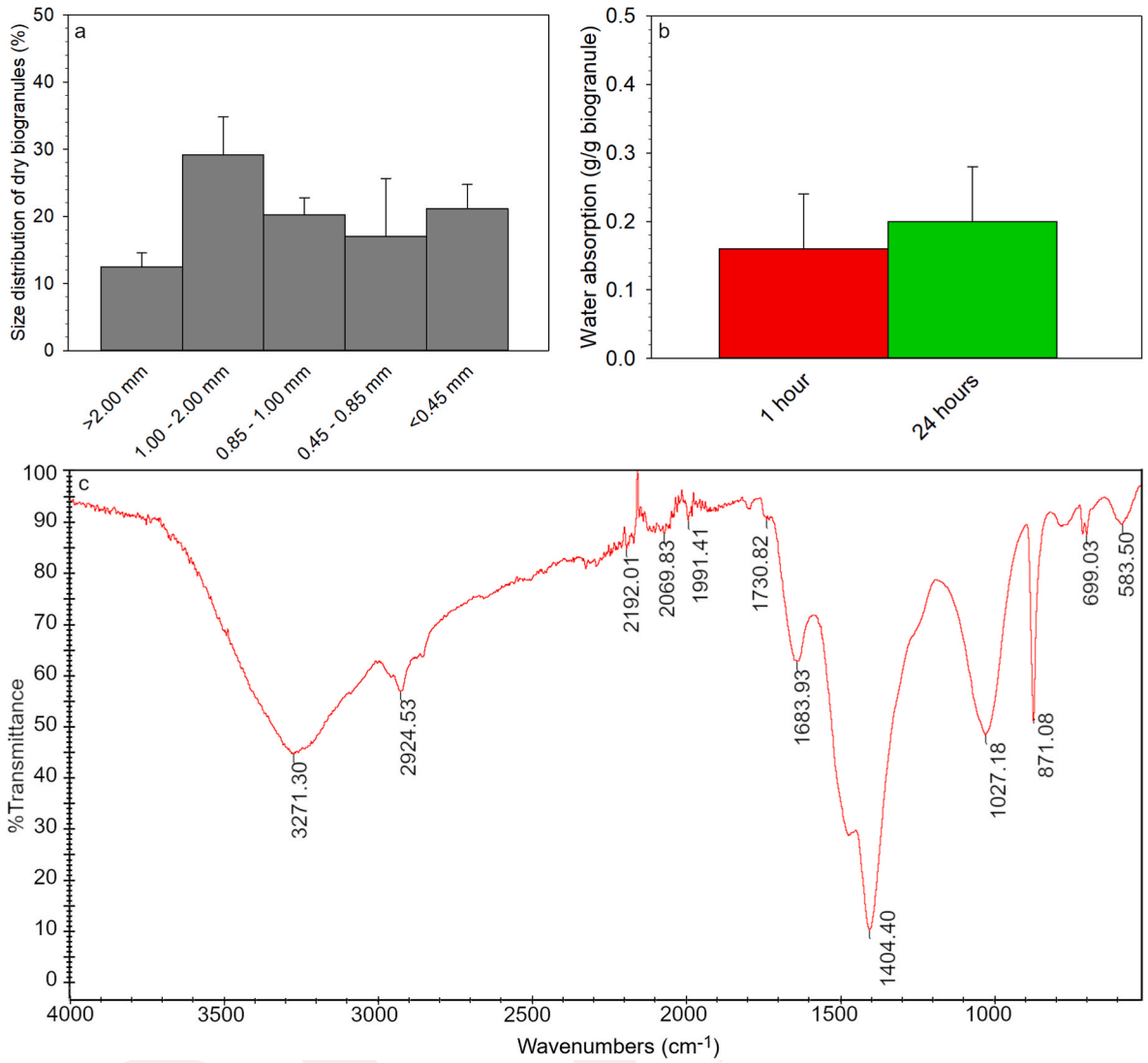

Fig. 7. Some physical and chemical properties of dry biogranules; (a) average particle size distribution of different harvests (n = 10); (b) water absorption of biogranules (n = 3); (c) FTIR spectrum of an individual harvest.

Micrographs and the elemental mapping obtained under EDS-coupled SEM confirmed the hypothesis derived from the micrographs obtained under light microscope. Fig. 9 presents the elemental mapping of a cross section of a biogranule. On the one hand, inside the biogranules P which is a common element in proteins and thus spores, was the abundant element (Fig. 9). Moreover, the presence of O together with P indicated that the nitrate reducing bacteria enriched in this study might be polyphosphate accumulating nitrate reducing bacteria. On the other hand, the outermost layer of the biogranules were mostly composed of Ca and O which were indicators of the presence of $CaCO_3$ crystals. Absence of Ca inside the biogranule structure indicated that significant amount of EPS were released during biogranule formation and thus the negative charge on cell surfaces decreased. The latter avoided accumulation of Ca ions and the MICP around the active cells. As MICP was avoided, a possible decrease in the microbial activity and the hindrance in biogranulation process that might occur due to the occlusion of bacteria could also be avoided. The folded porous structure was the dehydrated EPS surrounding the spores inside the biogranules (Figs. 9a and 10a,b).

The spores embedded in the EPS layer could also be visualised (Fig. 10a and b). Elemental mapping of the area also showed that the abundance of P was consistent with the presence of spores in the area (Fig. 10c and d). No vegetative bacteria were detected in dried biogranules which indicated that produced dry biogranules were composed of bacterial spores. As most of the pathogenic bacteria are gram negatives and cannot form spores, the absence of vegetative bacteria in dry biogranules was noteworthy in terms of revealing that contamination with other, possibly pathogenic bacteria was unlikely.

Since the produced biogranules were successfully passed through a comprehensive set of quality tests, they were separated based on their dry particle size. Afterwards the granule packs with an identical particle size distribution were prepared and stored until their incorporation into cementitious composites for compatibility tests (Fig. 11).

3.2. *Determination of the maximum allowable biogranule dose in cementitious composites based on fresh mortar properties*

Fresh mortar properties were determined based on ASTM C191–04a, NBN EN 1008 and NBN EN 1015-3 standards. The minimum acceptable initial and final setting times were defined as 60 min and 90 min, respectively. Setting results of different mixtures were given in Fig. 12.

As mentioned earlier, in the manuscript, biomortar specimens were presented based on their bacteria content by using the following notation "Bio-##". It should be noted that the bacteria content corresponds to the 70% of the biogranule content.

Initial and final setting values for plain mortar were determined as

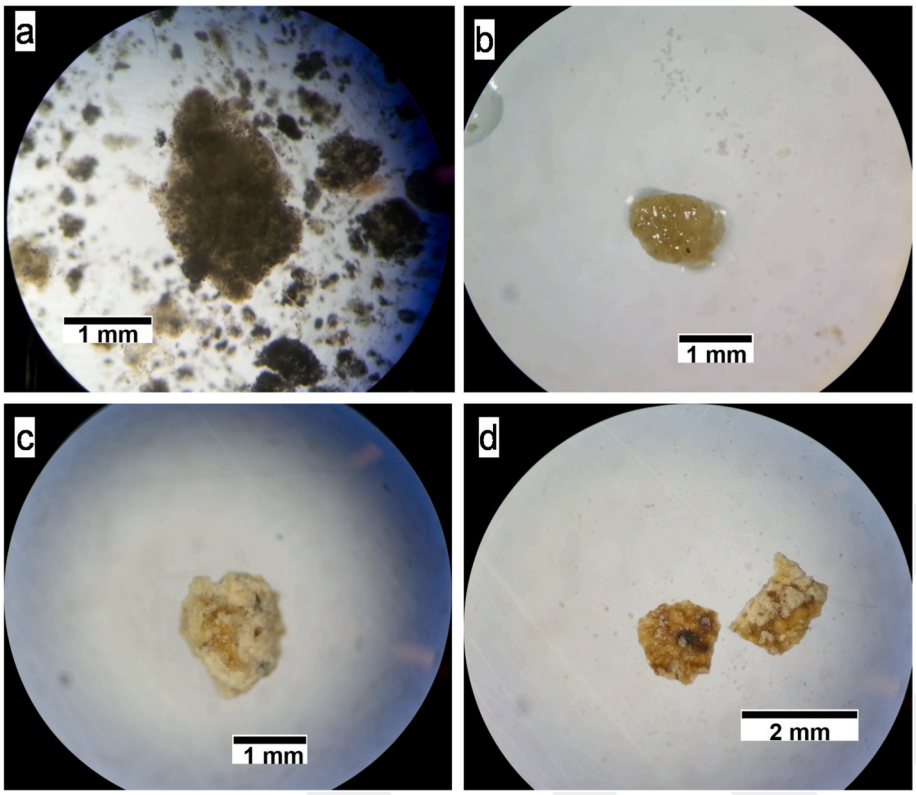

Fig. 8. Micrographs of (a,b) wet biogranules inside the reactor; (c) harvested dry biogranule, (d) cross sectional view of a split biogranule.

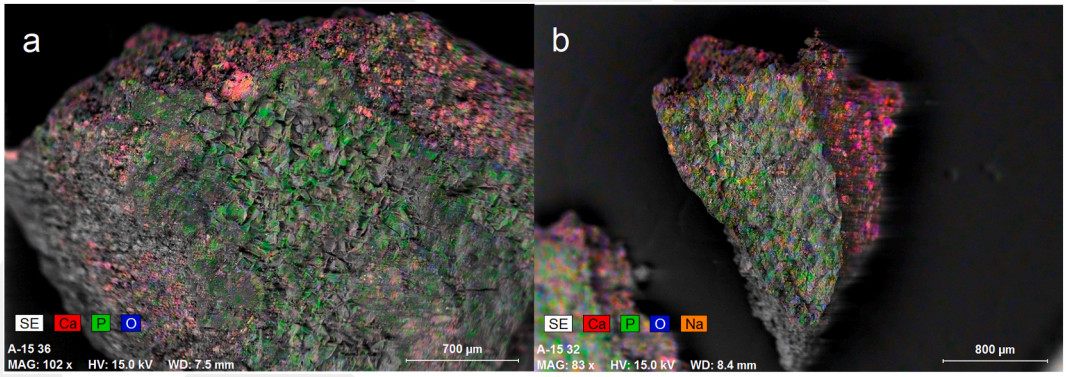

Fig. 9. EDS-coupled SEM micrograph and elemental map (Ca, P, O) of a cross section of a dry biogranule (a-b) two split surfaces.

164 ± 12 min and 322 ± 20 min, respectively. Setting times of abiotic control mixture and either of the biomortar mixtures were significantly different than the plain mortar mixture ($p = 0.05$). The initial and final setting times of abiotic control mixture were recorded as 55 ± 12 min and 172 ± 12 min, respectively. Despite the drastic decrease due to the nutrient incorporation, both of the setting times of abiotic control mixture were in the acceptable zone based on the limit values described in ASTM C191-04a (60 min for initial setting) and NBN EN-1008 (90 min for final setting).

The effect of biogranules on fresh mortar properties were investigated for the dose range of 0.25% bacteria w/w cement (0.36% biogranules w/w cement) to 3.00% bacteria w/w cement (4.30% biogranules w/w cement). Results were compared with both plain mortar and abiotic control mortar. However, to distinguish the sole impact of addition of biogranules, deviations from the abiotic control were considered. Accordingly, addition of biogranules up to a bacteria dose of 2.50% w/w cement (3.60% biogranules w/w cement) did not cause any significant change in initial setting time of the fresh cement mortar. When biogranules were added up to a bacteria dose of 3.00% w/w cement (4.30% biogranules w/w cement), initial setting time decreased significantly and was recorded as 34 ± 12 min. Therefore, in terms of defined setting properties in ASTM C191-04a standard, Bio-3.00% was labelled as inadequate for in situ applications. All the biomortar mixtures revealed a decent performance in terms of final setting times which varied between 160 and 185 min. Therefore, the maximum allowable biogranule dose could be defined as 3.60% w/w cement (2.50% bacteria w/w cement in the form of biogranules) unless any set retarders are used.

Flow table test is essential to evaluate the consistency and workability of different mixtures. The percent deviation of each mixture from the plain mortar and the abiotic control flow values were presented in Table 5. Similar to the interpretation of setting results, the sole effect of

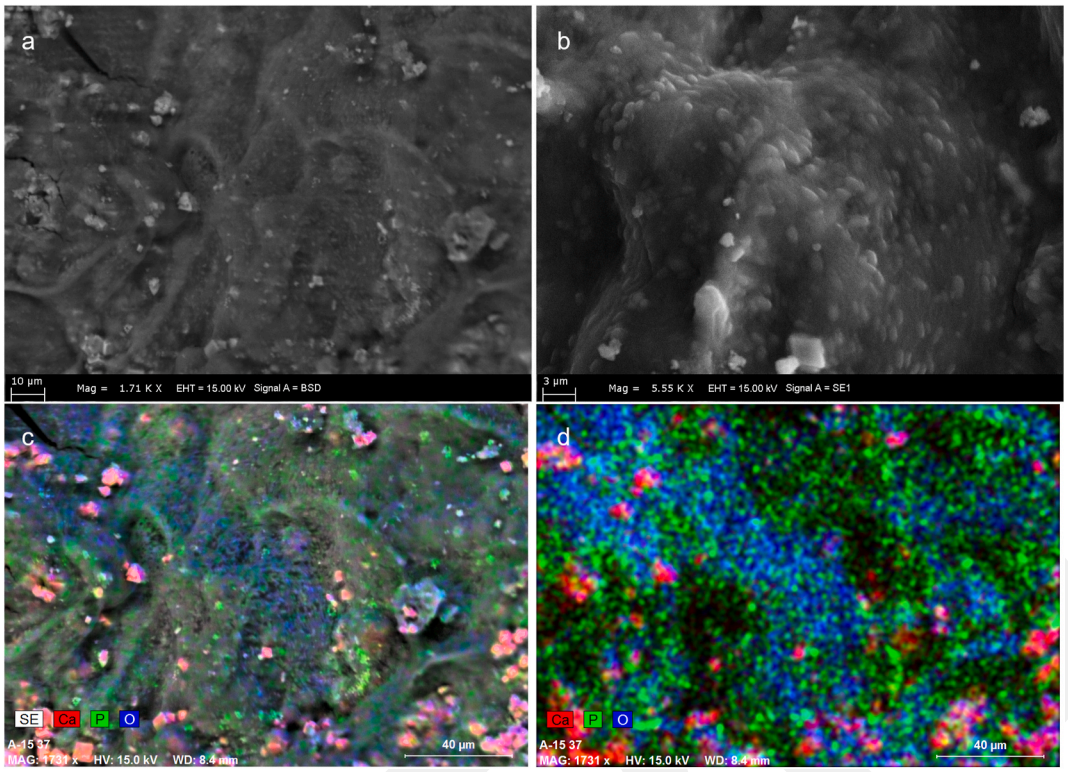

Fig. 10. EDS-coupled SEM micrographs of biogranules revealing (a) part of the granule core; (b) spores embedded in EPS layer at the core; (c-d) elemental mapping (Ca, P and O) of the core with and without the base micrograph.

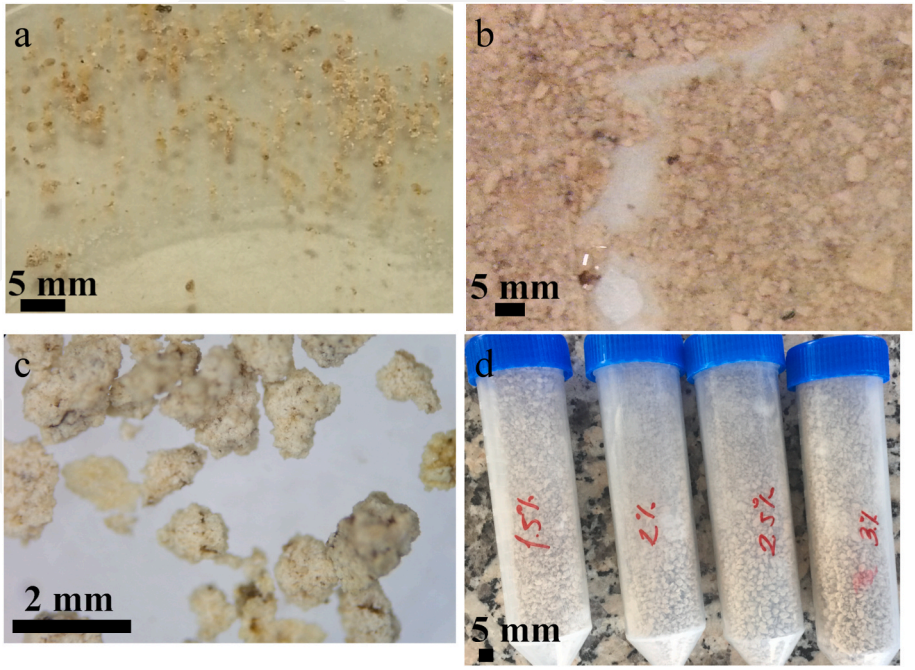

Fig. 11. The view of the biogranules used in mortar and concrete specimens. (a) dried biogranules; (b) harvested biogranules; (c) micrograph of dry biogranules; (d) biogranules prepared with identical particle size distribution for further tests.

biogranule dose on flow values was determined by comparing the bio-mortar mixtures with the abiotic control mixture. Accordingly, Bio-3.00% mixture appeared to deviate significantly from the abiotic control mixture (89%) based on the constraints defined in NBN EN 1015-3. The same mixture also showed poor compaction and thus the workability of the mixture was considered as inadequate.

3.3. Determination of the maximum allowable biogranule dose in cementitious composites based on hardened mortar properties

The strength development and the ultimate compressive strength values of biomortar mixtures were evaluated based on the compressive strength tests performed on Day 3, Day 7, Day 28 and Day 56.

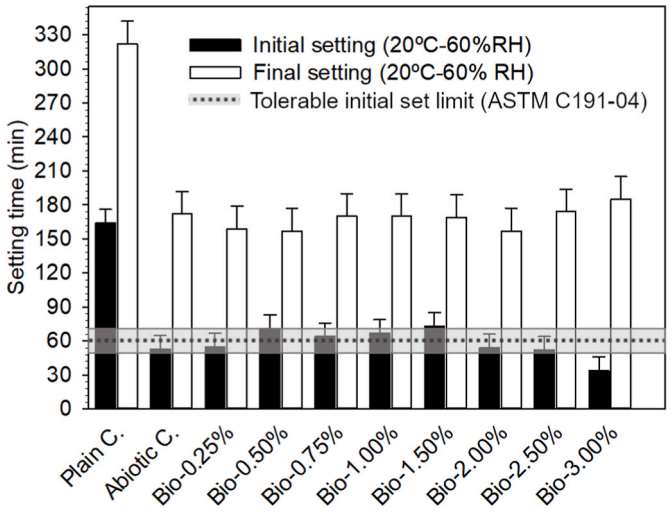

Fig. 12. Initial and final setting times of the tested control and biomortar mixtures. The bars represent standard deviation values of ±12 min for the initial setting time and ±20 min for the final setting time according to ASTM C191–04a and NBN EN 1008.

Table 5
Flow table results of different mixtures with respect to reference and control mortars.

Mortar mix	Flow[a] (mm)	Flow (% vs Plain C.)	Flow (% vs Abiotic C.)
Plain control	118	100	98
Abiotic control	121	103	100
Bio-0.25%	116	98	96
Bio-0.50%	124	105	103
Bio-0.75%	125	106	103
Bio-1.00%	123	104	102
Bio-1.50%	123	104	102
Bio-2.00%	121	103	100
Bio-2.50%	113	96	93
Bio-3.00%	108	91	89

[a] Based on NBN EN 1015-3, the highest acceptable deviation between subjected specimen flow and the reference flow is 10% for significance.

Compressive strength results obtained upon different curing periods were demonstrated in Fig. 13. The early age strength values of abiotic control mixture ($\sigma = 30 \pm 2$ MPa) and plain control mixture ($\sigma = 29 \pm 4$ MPa) were not significantly different from each other. The compressive strength of biomortar specimens up to Bio-2.00% mixture revealed similar 3-day strength values with plain and abiotic control mixtures. Bio-2.50% and Bio-3.00% mortars showed around 25% decrease in the compressive strength in the first 3 days (Fig. 13a). At the end of 7 days, all mortar mixtures, even Bio-2.50% and Bio-3.00% revealed similar strength performances varying around 40 MPa (Fig. 13b). The major difference between the plain mortar and the other mixtures appeared on 28-day strength. Compressive strength values of the abiotic control mixture and all the biomortar mixtures were distributed around 56 MPa which was 30% higher than the plain mortar mixture (Fig. 13c). Plain mortar mixture reached to a compressive strength of 52 ± 2 MPa at the end of 56 days curing. Except Bio-3.00%, all the other mixtures had 15% to 25% better strength values than the plain mortar mixture and they did not show significant differences among each other ($p = 0.05$). The strength of Bio-3.00% was similar to the reference mixture ($p = 0.05$) (Fig. 13d).

3.4. Bioconcrete properties at minimum and maximum tolerable doses

Compatibility of the biogranules with the cementitious matrix was also investigated by casting concrete samples. As concrete preparation required large volumes and thus large amount of biogranules (varying between 1.4 kg m^{-3} to 14.1 kg m^{-3} biogranule), concrete compatibility was only tested by checking the concrete properties at minimum (0.25% bacteria w/w cement) and maximum tolerable (2.50% bacteria w/w cement) biogranule doses determined based on previous tests on mortar. Workability of bioconcrete was evaluated based on the slump values and EN 12350-2 standard. The slump values of the tested mixtures were given in Table 6.

The slump value of plain control mixture was in the allowable range defined in EN 12350-2 (50 ± 25 mm to 80 ± 25 mm). Although abiotic control had a slightly better workability, plain control and abiotic control were not significantly different from each other ($p = 0.05$). BioC-0.25% was found to be more workable compared to plain and abiotic control but it should be noted that the slump value was above the defined range in EN 12350-2. Among the specimens, BioC-2.50% showed the lowest slump value and the poorest workability, but it was still acceptable by considering the minimum slump value defined for a medium workability mix in EN 12350-2 (50 ± 25 mm).

The influence of biogranule content on strength of concrete was also investigated. Compressive strength values of different mixtures were given in Fig. 14. The mix design of plain concrete was C25/30. Based on the 28-day results, it can be said that the mix design was successful in achieving the target strength. Bioconcrete specimens were not significantly different from the control specimens (i.e plain control and abiotic control) based on their 28-day and 56-day compressive strength values (tested for $p = 0.05$).

3.5. Economic feasibility

The production cost for the concrete compatible nitrate reducing biogranules can be calculated by considering the 7.25 L production setup used in this study. As mentioned earlier, biogranules could be harvested in every two weeks (Fig. 5a) without disrupting the biogranulation process and microbial activity in the reactor. In every harvest, about 34% of the harvested biogranules were returned back to the biogranule production reactor as they were either larger than 2.00 mm or smaller than 0.45 mm. The gross biomass yield of the bioreactor was 0.07 g biogranule.g^{-1} $HCOO^-$ (0.05 g bacteria.g^{-1} $HCOO^-$). Considering the portion that was returned to the reactor, the net useful biogranule production yield of the bioreactor was determined as 0.05 g biogranule.g^{-1} $HCOO^-$ (~0.03 g bacteria.g^{-1} $HCOO^-$). As the biogranule production at 7.25 L scale reactor was achieved at an organic loading rate of 4.29 g $HCOO^-$.L^{-1}.d^{-1}, the net useful biogranule production in this reactor could be calculated as ~0.22 g biogranule.L^{-1}.d^{-1}. At 1 m^3 scale, daily production would be 220 g biogranule.d^{-1} which corresponds to production of 1.5 kg biogranules per week. Table 7 presents the essential parameters to consider whilst calculation of operational costs for production of 1 kg of biogranules. Accordingly, the total operational cost for production of biogranules was determined as ~39 €.kg^{-1} biogranule and 73% of this cost was the labour cost.

4. Discussion

Biogranules were successfully developed in about two months by using minimal nutrient medium lacking of micronutrients, trace elements and vitamins. Beun et al. [19] reported that the microbial agglomerates can be considered as biogranules if they are larger than 0.2 mm. The particle size distribution of the reactor content revealed that 90% of the microbial culture was composed of biogranules (Fig. 5c). SVI_{30}/SVI_5 ratio was defined as another indicator of granular biomass abundance [20]. Comparing these two values for the exact same day in this study, Day 104, revealed that both particle size distribution test (89%) and SVI_{30}/SVI_5 ratio (85%) can be used interchangeably to monitor the granular biomass inside the biogranule reactor. One of the major advantages of biogranules is their easy separation from the liquid with their advanced settling properties. It was reported that the SVI_{30}

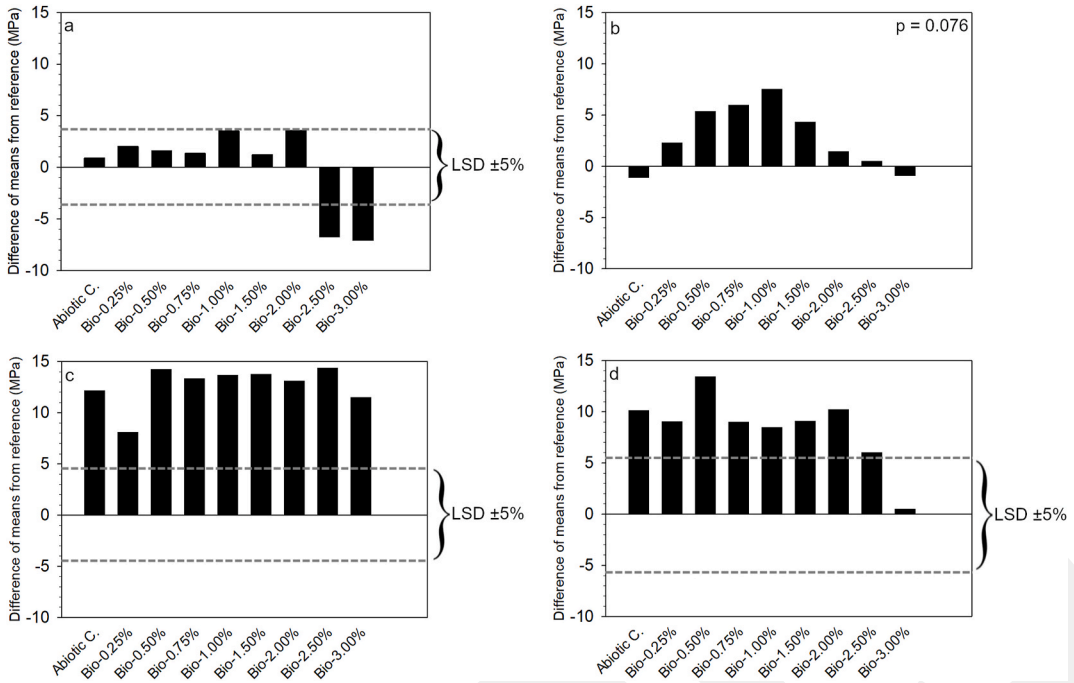

Fig. 13. Difference of the mean compressive strength values of tested mixtures from plain control specimen after curing at 20 °C and RH > 90% for (a) 3 days; (b) 7 days; (c) 28 days; (d) 56 days. LSD (3 days) = 3.6 MPa, LSD (7 days) = NA as p = 0.076, LSD (28 days) = 4.5 MPa, LSD (56 days) = 5.7 MPa. Plain control strength values on 3, 7, 28 and 56 days were 29 ± 4 MPa, 37 ± 2 MPa, 43 ± 3 MPa and 52 ± 2 MPa, respectively.

Table 6
Slump values of the tested mixtures.

Mix	Slump (mm)
Plain concrete	80
Abiotic control	105
BioC-0.25%	147
BioC-2.50%	40

value is an important indicator of granular sludge reactor and the bacterial culture should be as compact as 50 mL g^{-1} [20]. As SVI_{30} was continuously monitored throughout the granulation and granule production process in this study, it was seen that immediately after each granule harvest, settling properties disrupted significantly. The disruption was mainly attributed to the harvest of larger granules from the system. Nonetheless, the recovery could be achieved in a relatively short period of 2 weeks which indicated that in granular biomass abundant reactor nitrate reducing biogranules can be harvested every two weeks. Upon harvesting, dry biogranules that were either smaller than 0.45 mm or larger than 2.00 mm, which corresponded to 34% of the harvested biogranules, were returned back to the bioreactor. Therefore, the net useful biogranule production yield of the bioreactor was determined as 0.05 g biogranule.g^{-1} $HCOO^-$ (~0.03 g CDW.g^{-1} $HCOO^-$). The gross biomass yield of the bioreactor was 0.07 g biogranule.g^{-1} $HCOO^-$ (0.05 g CDW.g^{-1} $HCOO^-$). The mean granule size inside the reactor was 0.95 ± 0.20 mm (Fig. 5c). Obtained production yields and abundant granule size inside the reactor were comparable to those previously reported for granular sludge reactor operating with 4 h cycle time and 8 h hydraulic retention time [24,25]. Achieving such yield under minimal nutrient conditions is promising for further consideration of a cost-efficient healing agent production at a higher scale. There is a study reporting a 5-fold higher biomass yield in a full scale aerobic granular sludge reactor, but the abundant granule size in that study was varying between 0.12 and 0.35 mm [26]. It is known that the compactness of biogranules is important for stability and easy separation from the liquid media, and compactness of granules is mainly related with the growth rate of the

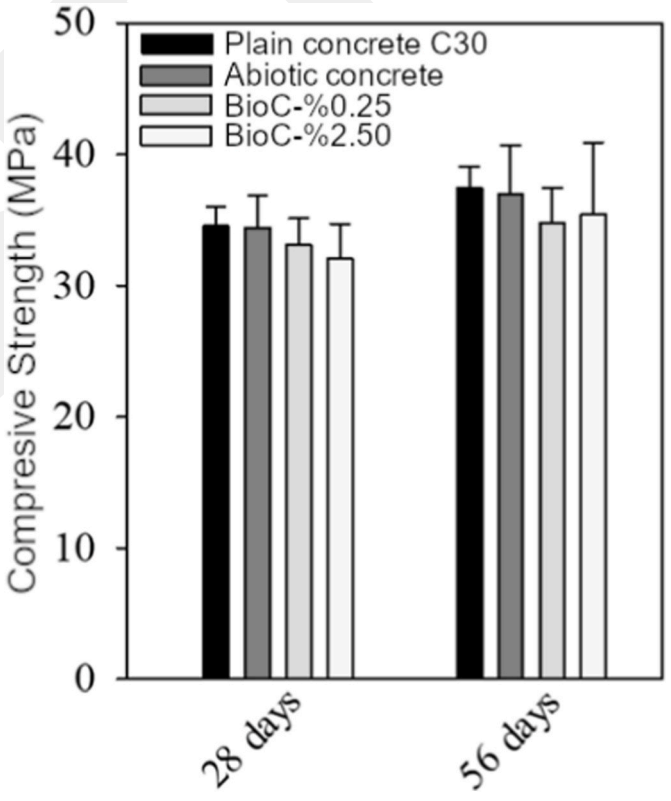

Fig. 14. Similar compressive strength values of the plain concrete and bio-concrete mixtures.

biomass that higher biomass yields lead to either flocculant abundant granular sludge or large fluffy granules [25,27–29]. Therefore, biogranule production rate can be enhanced in accordance with the sectoral demand if one considers to achieve smaller size biogranules. On the

Table 7
Operational costs for biogranule production (operational data based on 7.25 L scale setup).

Item	Relevant values	Unit costs	Cost (€.kg^{-1} biogranule)
Feeding solution[a]	2.00 m^3 d^{-1}	0.85 €.m^{-3}	7.70
Labour work[b]	1.50 h week^{-1}	28.50 €.h^{-1}	28.50
Energy for pumps[c] (aeration + feed)	0.48 kWh.d^{-1}	0.12 €. kWh^{-1}	1.30
Harvested wet mix	SVI: 40.00 mL g^{-1} Water content (w/w): 0.96	NA	NA
Drying[d]	0.04 m^3 water	30.00 €.m^{-3} of water evaporated	1.20
Biogranule production	Yield: 0.22 kg m^{-3} d^{-1}	NA	38.7

[a] Average commercial market prices of the constituents for orders of Mt scale.
[b] Average labour costs in EU in 2020.
[c] Average energy prices for non-household energy consumers in EU in 2020.
[d] Process cost reported in Ref. [5].

contrary, obtaining large biogranules (>2 mm) similar to those reported in several other granulation studies are not recommended for concrete application, because they are reported to be fluffy, weaker against shear stress and easily disintegrate due to the nutrient limitation at the core [29–33].

In this study, kinetic analysis revealed that the biogranule sizes were small enough to enable nutrient access to the core, while it was large enough to prevent oxygen diffusion to the core. The oxygen diffusion depth in microbial flocs were defined as 0.2–0.4 mm from the surface [21]. In this study the particle size distribution revealed that 78% of the biogranules were larger than 0.45 mm and the mean granule size was 0.95 ± 0.20 mm which indicated that the core of the produced biogranules were anoxic. The kinetic analyses also confirmed the compact nitrate reducing core of the biogranules as NO_x-N consumed while DO levels in the media was varying between 1 mg L^{-1} to 6 mg L^{-1}. Similar observations were previously reported even for aerobically grown granular consortium and the process was named as simultaneous nitrification and denitrification [34,35]. Another indicator of a denitrifying core was based on the visual observations. It is known that biogranules cultivated under anaerobic conditions are dark in colour, even black [36], while biogranules cultivated under aerobic conditions are lighter in colour [37]. Obtained micrographs of the biogranules produced in this study, particularly the cross-sectional view (Fig. 8d), revealed that when moved from sides of a biogranule to the centre, the colour became darker. The colour gradient was an indication of a completely anoxic zone at the core which enabled enrichment of a denitrifying core community. Therefore, it was safe to claim that a compact denitrifying core could be achieved in the biogranules. The zone surrounding the core was also darker in colour compared to the biogranules grown under aerobic conditions [20,37] which might indicate that the surrounding culture was mostly composed of a facultative community rather than strictly aerobic. Considering that almost all $HCOO^-$ was consumed during the anoxic period (Fig. 6c), the growth of strictly aerobic heterotrophic bacteria seems unlikely which also supported the aforementioned hypothesis.

The kinetic analyses showed that there was NO_x-N reduction even in the absence of $HCOO^-$, although in anoxic period, the average [C]:[N] consumption ratio was found as 5.3 (Fig. 6e). The observed behaviour was attributed to the feast/famine operation of the biogranule production reactor which forced microorganisms to convert some of the nutrients ($HCOO^-$ in this case) into polymers to store inside the microbial cells as internal polymers (polyhydroxyalkanoates-PHA) during the feast period for further consumption in famine conditions [34]. In many studies, the carbonyl peaks observed at 1730-1740 cm^{-1} in FTIR analyses were referred to PHA [38,39] and similar peaks were also observed for the harvested dry biogranules in this study. Such metabolism can be followed by either glycogen or polyphosphate accumulating organisms [34]. Although no further experiments were conducted to identify the microbial species inside the produced biogranules, the elemental mapping of a cross section of a biogranule showed significant amount of P and O at the core of the biogranules. Based on these observations we suspect that a fraction of the microorganisms in the core could be polyphosphate accumulating nitrate reducing bacteria. Moreover, in our previous studies, we revealed that dry biogranules could resuscitate, grow and reduce nitrate in the absence of external PO_4–P supply [17,23] which might also indicate that the core of the produced biogranules possesses some polyphosphate, most probably due to the presence of phosphate accumulating nitrate reducing bacteria. However, the amount of NO_x-N reduction in the absence of $HCOO^-$ was limited with 5.2 mg L^{-1} NO_x-N which indicated that the internal polymer (PHA) storage was limited. Similar observations such as decrease in denitrification rate of bacteria and limited denitrification in aerobic period were also determined in previous studies reporting denitrification in aerobic periods [34,35]. The P uptake rate and thus the available PO_4–P in the medium was determined to be one of the governing parameters of PHA storage [34]. Considering that the PO_4–P concentration in the feed solution was 3 mg L^{-1} which was a P-limited nutrient solution (C:N:P – 190:33:1 compared to recommended 100:5:1 [40]), the observed limitations in PHA storage and nitrate reduction in aerobic periods were meaningful.

This study has shown consistent results with the previous study investigating a mortar mixture similar to Bio-0.50% [18]. In that study, addition of 0.50% bacteria w/w cement (0.71% biogranule w/w cement) together with 3.00% CN and 2.00% CF did not cause any significant change in setting time of the mortar mixture which was noted as an advantage of this culture when compared to axenic cultures. However, the overall recorded setting times in this study were significantly shorter than the previously reported results. Such decrease in setting times were attributed to the higher nutrient content of the newly designed mix which was based on an optimum nutrient content study conducted by Kardogan et al. [23]. In that study, 5.00% CF and 2.00% CN was abiotically tested for fresh mortar properties and the reported initial (65 ± 12 min) and final (146 ± 20 min) setting times were almost the same with the ones reported in this study. These similarities indicated that the difference between the plain mortar and all the other tested mortar mixes were due to the nutrients; namely calcium nitrate and calcium formate. The used nutrients are commercially available concrete admixtures which are used to increase freeze-thaw resistance and accelerate setting [41,42]. Previous studies using only 2.00% CN w/w cement showed similar initial setting times varying around 70 min [43] and those using only 2.00% CF w/w cement reported initial setting time of 80 min [42]. Considering that combination of the two set accelerators were used in this study, significant decrease in the initial setting time became meaningful. Although decreasing the CF and CN content of the mix may extend the initial and final setting times up to 110 min and 170 min, respectively, one should concede that such reduction in nutrient content results in a considerable decrease in nutrient availability for microbial crack healing [23].

Obtained fresh mortar properties were also meaningful to evaluate the self-protection capability of biogranules, or at least the interaction between the spores and the cementitious matrix. In previous studies investigating direct bacteria/spore incorporation into mortar mixtures, it was reported that set retardation is eventual due to the contact between the bacteria, simply an organic matter, and the cementitious matrix [18,44]. Basically, when bacteria died at highly alkaline pH environment of the concrete, their organic content was released into the fresh mortar mixture and resulted in set retardation. Jonkers et al. [45], stated that during mixing, hardening and subsequent shrinkage of pores, spores can be totally or partially lost. Upon the loss the protein coating of the spores is released and causes foaming and interferes with the rate of hydration reactions which delay initial setting significantly. Moreover,

in our previous study, we revealed that when unprotected denitrifying microbial culture (i.e. *Diaphorobacter nitroreducens*) was added into mortar with a dose of 0.50% bacteria w/w cement, both initial and final setting times increased about 40 min [18]. On the contrary, the setting results of this study indicated that incorporation of bacteria in the form of biogranules (i.e. with EPS and mineral layer), avoided possible interaction between the cement matrix and the organic matter, and thus even at high bacteria doses (i.e. Bio-3.00%) no set retardation occurred. The observed set acceleration was only due to the nutrients CF and CN which were already proven as set accelerators mainly increasing the rate of belite hydration [41,42].

The influence of biogranule addition on hardened properties were investigated through strength development of various mixtures. Obtained plain control and abiotic control mixtures were consistent with our previous findings where CF and CN positively contributed to the long-term strength development. Similar positive influence of CF and CN were also reported in other studies [18,41–43]. Both CF and CN accelerated the setting, yet they did not influence the early age strength development in any of the tested mixtures. This was due to the fact that the acceleration effect of CF and CN were defined in several studies as set acceleration rather than hardening acceleration [41–43]. According to the EN 934-2 standard set accelerators do not necessarily increase the early age strength. Consistently, it was previously reported that CF and CN increase the rate of belite hydration and thus contribute to the long-term strength development rather than early age strength development [41,42]. It was also reported for CF that it could contribute to the early age strength only if C_3A/SO_3 ratio of the cement was higher than 4 [42], which was not the case for the cement used (CEM I 42.5R) in this study. Therefore, in this study, the positive effect of CF on strength development was also limited to the long-term strength.

Incorporation of biogranules negatively affected the early age strength only at doses of 3.60% and 4.30% w/w cement (Bio-2.50% and Bio-3.00%). It should be noted that the negative influence was not related to the hydration reactions, but mostly due to poor compaction of the relevant mixtures as the workability of these two mixtures were considerably worse than the others. Since the observed positive effect in strength development of abiotic control and biomortars was only due to the chemical admixtures (i.e CF and CN) and consistent among the specimens, the relatively lower 56 days strength of Bio-3.00% was attributed to its poor compaction and related poor strength development in the first 3 days (Fig. 13a). The strength development in early ages is highly dependent on the physical conditions of the cementitious composites and compaction issues due to poor workability can cause severe loss of strength similar to the one observed in this study. Nonetheless, the ultimate strength of all of the biogranule containing mortars were equal to or higher than plain control that none of the tested biomortar mixtures would lead to a service issue in terms of strength.

In several bacteria incorporation studies, it is stated that bacteria addition into the mortar causes increase in early age strength due to microbial induced calcium carbonate precipitation [46–48]. However, biogranules in this study were produced at pH value around 10 and they neither germinate nor use the nutrients at actual concrete pH which makes MICP during hydration unlikely. Therefore, in this study, bacteria incorporation did not cause any significant improvement. Additionally, biogranules produced in this study were not adequate for such applications as they had a coating layer composed of $CaCO_3$– $Ca_3(PO_4)_2$ which minimized the interaction between the core bacteria and the mortar matrix.

Tests conducted with concrete specimens confirmed the compatibility of biogranules with the cementitious matrix. Among the specimens, BioC-2.50% had relatively lower workability with a slump value of 40 mm. Lyons [49] categorized workability of mixtures based on their slump values as follows; 0–25 mm for very dry mix, 10–40 mm for low workability mix, 50–90 mm for medium workability mix and >100 mm for high workability mix. Accordingly, BioC-2.50% could be categorized as a low workability mixture. Abiotic control and BioC-0.25% could be categorized as high workability mixtures.

In concrete experiments, different mixtures revealed similar compressive strengths. Compared to the mortar tests, the use of a higher water:cement ratio in concrete tests might slightly improve the workability of BioC-2.50% and avoided possible compaction issues, thus all specimens could reach similar strength values in the same curing period. Therefore, one may consider using superplasticizers together with the biogranules if it is necessary to use a bacteria dose of more than %2.50 w/w cement.

Since functionality of biogranules as a microbial healing agent have already been proven in some previous studies [7,15,16], their functionality as a microbial healing agent was not a matter of some concern. Yet, changes in the dose, especially the increments, were a matter of concern as the compatibility of biogranules with the cementitious matrix was unknown before this study. As mentioned earlier, content optimization of microbial self-healing concrete requires two consecutive steps: (i) defining the constraints for the doses of typical constituents of microbial self-healing concrete (nutrients, protective carriers and microbial healing agents) based on their compatibility with the cementitious materials, (ii) revealing the relationship between healing agent content and self-healing efficiency. A previous study focusing on biogranule-based self-healing concrete reported the influence of initial nutrient dose on the following three subjects: (i) properties of cementitious materials, (ii) the bioavailability of nutrients upon crack formation, (iii) the MICP performance of biogranules [23]. Subsequently, based on the results of the compatibility assessment conducted in this study, we defined the quantity constraints for direct incorporation of biogranules into cementitious materials and thus proposed an upper dose limit for further healing tests. Overall, significant progress has been achieved on completing the initial requirements before embarking on further recipe optimization tests for biogranule containing microbial self-healing concrete. Therefore, this study paves the way for further studies aiming to reveal the self-healing and corrosion inhibition performance of biogranule containing self-healing concrete at varying doses up to 2.50% bacteria w/w cement.

The operational cost for production of 1 kg of biogranules was determined as 39 €.kg^{-1} which was consistent with the previously reported cost of 40 €.kg^{-1} biogranule produced at 3.2 L scale [7]. The operational costs for production of commonly investigated *Bacillus cohnii*, *Bacillus sphaericus* cultures and a novel non-axenic culture called cyclic enriched ureolytic powder (CERUP) were reported as 560 €.kg^{-1} bacteria, 266 €.kg^{-1} bacteria and 25.2 €.kg^{-1} CERUP, respectively [4,5]. It should be noted that the axenic cultures (*Bacillus cohnii*, *Bacillus sphaericus*) would also require protective carriers during their incorporation into the cementitious materials which would bring extra costs. Therefore, the use of non-axenic concrete compatible biogranules appear as an economically feasible alternative compared to the use of axenic cultures for development of microbial self-healing concrete. Yet, the production cost of biogranules is still higher than that of the previously reported non-axenic culture, CERUP, as the production yield reported for CERUP (1.21 kg m^{-3} d^{-1}) is about 6 times more than the biogranule production yield (0.22 kg m^{-3} d^{-1}) achieved in this study. Nonetheless, biogranules have an advantage over CERUP in terms of the functions provided in concrete. In a previous study, it was revealed that biogranule containing microbial self-healing mortar could simultaneously inhibit rebar corrosion and seal the cracks while CERUP containing microbial self-healing mortar could only seal the cracks which was not enough to stop the corrosion [16]. Owing to this added benefit, the relatively higher cost of biogranules might still be tolerated. It should also be noted that 73% of the production cost was due to the labour work. If the cultivation reactor and the quality of the harvested biogranules can be monitored by using automated systems, the production cost can be decreased significantly.

Overall, the main objective of this study was to present the production procedure of non-axenic nitrate reducing biogranules tailored for self-healing concrete application and assess their compatibility with the

cementitious matrix at different application doses. Defined operational conditions led to production of wet biogranules with a mean size of 0.95 ± 0.20 mm. Under anoxic conditions the microbial activity of biogranules were determined as 0.10 g NOx-N.g^{-1} bacteria.d^{-1} and 1.50 g $HCOO^-$.g^{-1} bacteria.d^{-1}. [C]:[N] consumption ratio in anoxic period became stable around 5:1 after granulation was completed. In biogranule-dominant reactor 75% of the NO_3–N was reduced in anoxic period and 50% of the remaining NO_3–N was consumed in aerobic period which indicated the presence of nitrate reducing bacteria at the core. It was revealed that in dry biogranules the bacterial spores at the core were embedded in an EPS matrix composed of proteins and polysaccharides. The outermost layer with a thickness varying between 50 and 300 μm of the dry biogranules was composed of $Ca(PO_4)_3$ and $CaCO_3$ which minimized the interaction between the microbial content and the cementitious matrix. Owing to their minimum interaction with the cementitious matrix, produced biogranules were found to be compatible with both mortar and concrete up to a dose of 2.50% bacteria w/w cement (3.60% biogranules w/w cement). The highest tolerable dose enabled incorporation of bacteria as high as 2.50% w/w cement without affecting the setting time, workability and compressive strength development. Water absorption of dry biogranules were found as 16 ± 8% which avoided adjustment in water:cement ratio. Net (useful for concrete application) and gross (observed) biogranule production yields in a 7.25 L bioreactor were determined as 0.05 g biogranule.g^{-1} $HCOO^-$ and 0.07 g biogranule.g^{-1} $HCOO^-$, respectively. Achieving such yield under minimal nutrient conditions is promising for a cost-efficient healing agent production at a higher scale. In this study, the operational cost for production of biogranules was determined as 39 €.kg^{-1} which could be further decreased at larger scales by using automated systems. Further work should be conducted to optimize the biogranule content based on the variation in self-healing properties of biomortars at different biogranule doses.

Author's contributions

MS and YCE conceived and designed the experiments. MS conducted the experiments and analyses. MS and YCE wrote the manuscript.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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