Colonization strategies to establish microscopic biofilm reactors for simultaneous nitrification/denitrification

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Abstract
In biological wastewater treatment technologies the majority of systems are based on activated sludge technology. In these systems the population structure and dynamics of the activated sludge microbial community is largely governed by indirect regulatory mechanisms, such as aerated/non-aerated reactors, reactor configurations and sludge recycling ratios. The build-up of activated sludge flocs (bacterial aggregates) that are the key elements of the wastewater treatment process remains uncontrolled. The project started to overcome these problems by the introduction of artificial microstructures (microscopic carrier structures) to which the activated sludge bacteria adhere and meet technological objectives. In this paper the results of simultaneous nitrification and denitrification technology is presented on the basis of laboratory scale results. Application of two inoculation techniques (in consecutive steps) made possible the coexistence of ammonium oxidizer (nitrification) and nitrate reducer (denitrification) bacteria in the same bioreactor volume. In spite of the contradictory environmental requirements of the two bacterial consortia (i.e., dissolved oxygen concentration) the developed experimental set up enabled the simultaneous functioning of the two groups showing high efficiency in terms of nitrogen removal.

Keywords
Simultaneous nitrification/denitrification, Wastewater treatment, Colonization procedure

INTRODUCTION

The attachment of the bacteria to the surface and the establishment of biofilm are considered as survival strategy, which the bacteria have been using for ages. One advantage of this process, is that bacteria immobilized in a nutrient rich microenvironment. The process of bacterial adhesion is divided to different phases. The primary one is adhesion, the reversible attachment on the surface, and the second step is a more or less irreversible process and development of complex biofilm structure. In the microenvironment the biofilms might develop extraordinary complex structures, where the bacterial cells and the microorganism are located within the matrix of exracellular material (glycocalix, or ESP structures). The flux of the electron acceptors and donors and the flow of the terminal product are governed by the channels and open spaces within the glycocalix structures. The biofilm ensures safe environment for the bacterial cells and the microorganism and protects the bacterium from the predation, dehydratation and the extreme environmental changes (pH, temperature). Generally bacteria preferentially exist in biofilms contrary to free, suspended life forms. This fact was well known since the late thirties of the past century [1; 2; 3]. In the aquatic environment the number of the immobilized bacterial cells are much higher than the free living suspended cells.

The process of the bacterial adhesion

The bacterial adhesion and the biofilm formation could be considered and described by either complex or simple way. Bacterial adhesion and the biofilm formation are modified by many environmental factors (the properties of the surface, type of the bacterium, shear forces, etc.). Generally the attachment onto the abiotic solid surfaces is modified by non-specific factors (i.e., hydrophobic forces) and adhesion on biotic surfaces is modulated by specific mechanism. The process of bacterial adhesion is divided into two phases the primary adhesion which are called docking phase and the second adhesion step (locking phase). According to some author [4; 5] a

third phase (condition phase) also exists when the modification of the surface is achieved.

The physico-chemical interaction between the bacterial cells and the surfaces (docking phase)
The bacterial adhesion starts by the attraction of the cells and continues the adsorption and attachment of the cells on the surface [6]. Some of the bacteria are able to move actively to reach the target surface with flagella or other locomotoric cellular organs or the bacterial cells are allocated on the surfaces by the Brown movement, van der Waals interaction, gravitation settlement, electrostatic and hydrophobic interaction [7]. According to Kirov [8] chemotaxis might also play a role in attraction process near to solid surfaces. The adsorption of the bacterial cells on the surface is led by the concentration gradient (chemotaxis) and the immobilised molecules (amino acids, carbohydrates and oligolipids) on the surface (haptotaxis). The chemotaxis known to exists in many types of bacteria. Materials that governs this process in the meantime also accelerate adhesion processes and enhance the further interaction of cells-surface and cell-cell coupling mechanisms [9]. Furthermore the physical interactions are divided to long-range and short-range connections. The long-range connection is not specific and the distance between the surface and the bacterial cell is typically >50 nm. The importance of the short-range connections become more apparent when the distance of the bacterial cell and the surface is <5 nm. Long-range connections are usually controlled by physical forces. The short-range connections are managed by the chemical bonds (hydrogen bridges) and ionic and dipole-dipole connections as well as hydrophobic interaction [10]. In summary, the bacterial adhesion starts with the long-range connection which is the responsible to allocate the bacterial cell close to the surface and immobilisation of the bacterial cells occurs by short-range connection (docking phase).

Molecular and cellular interactions of the surface and the bacterial cell (locking phase)
During the locking phase the bacterial adhesion is achieved by specific molecular interactions. The bacteria synthesise different types of polymers which bond the bacterial cells onto the solid surfaces. These polymers could be in forms of capsules, pili, fimbria and different types of slime materials (ESP). Indeed these different morphological type materials in any cases have the same functional component called as adhesins [11; 12; 13]. In case of *S. epidermidis* several materials were identified as adhesin: PS/A (galactose rich capsular polysaccharide adhesin, consisting of β-1,6-N-acetilglucose amins, és β-linked succinates, phosphates and acetate and also of SAA), SAA (*slime-associated antigen composed of N-acetyl-glucosamine*), PIA (*polysaccharide composed of* β-*1,6-linked N-acetylglucosamines*), AAP (*accumulation-associated protein*). The PS/A and the SAA are responsible for the connection of the surface-bacterial cell, the PIA and the AAP are the responsible for the cell-cell interaction. The clumping factors (different type of proteins and teichoacids) also are significant agents of the biofilm morphology, these factors could modify the viscosity of the biofilm.

Environmental factors determining adhesion process in the experimental set up
Hereinafter listed environmental factors are able to modify the bacterial adhesion and the optimal biofilm formation on the surface structure of applied carrier material. Hence some of these factors were optimized during the colonization and experimental phase.

- *The substances supporting the attachment on the biofilm.* In our research, we aimed to establish the biofilm in the possible simplest way, without using any chemical substances that could have contributed to the attachment. We have accomplished some studies concerning the effect of some materials but the results didn't verify their necessity.
- *The porosity of the surface.* The increased porosity of the surface is evidently favourable for the attachment of suspended cells and small flocks. This consideration was essential in the course of our colonisation process. Hence, we enhanced the porosity of the starch gel PVA-PAA (polyvinyl alcohol - polyacrylic acid) during the preparation treatment. We have also studied the

attachment on this gel without this treatment, and as a result, the suspended cells have constituted sludge flocks in the reactor, and their attachment on the surface was negligible.

- *The surface charge.* Referring to previous studies, we could observe that, as the surface of the bacteria has a negative specific surface charge, they attach much more easily if the surface used to the colonization has a positive specific charge. Influencing the attachment, this factor caused a number of problems as the surface of the gel we used has a negative charge. We could not modify of the surface charge of the gels, so we had to ameliorate the other factors to reduce this negative effect.
- *The viscosity of the surface.* This property of the gel was not considered in the research.
- *Shear forces in the reactor.* During the docking phase, the contact frequency between the gels and the bacteria is low, so the attachment on and detachment off the gel surface is highly influenced by the hydraulic circumstances and the shear forces in the reactor. The development of the convenient hydraulic conditions was essential in the course of the colonisation and also later on, as these circumstances influence the development of the biofilm structure. This will be discussed in details later.
- *Other surfaces susceptible of attachment.* Due to its surface charge, the gel isn't favourable for the attachment of bacteria, thus the other surfaces susceptible of attachment had to be reduced in the reactor. Note, that the concentration of the suspended cells is also an important factor of the attachment. If the concentration of the suspended cells is too high in the reactor, the development of the flocks is too intensive which leads to the decrease of the attachment on the gel surface. If, during the colonisation there are the bacterial flocks in the reactor as well, then these will attach the suspended cells on their surface, so the attachment on the gel surface will be negligible.
- *The size proportion between the flocs and the bacteria* The size of the suspended bacterial flocs must be optimized to adhesion, in case of the flocs are larger than 50 μm the adhesion of the bacterial cells wasn't occurred on the surfaces of the gels beads. Inasmuch as the concentration of the bacterial cells is not optimal in the reactor the suspended flocs are constructed by bacterial cells. Hence the bacterial cells prefer to build suspended flocs (spontaneous flocs) rather than the adhesion on the gel surface. Aforementioned cases the inoculation process was optimised for the adhesion and the biofilm formation on the surfaces of the gel beads.

METHODS

Simultaneous nitrification and denitrification

Simultaneous nitrification and denitrification was achieved by the establishment of multiple strata protobiofilm formed by wastewater bacterial culture. The biofilm was attached onto the surface of the PVA-PAA hydrogels beads embedded with starch. Suspended growth activated sludge flocs or in the experiments the biofilm on hydrogel surfaces have several hundreds μm in diameter. On microscopic scale this would involve the presence of steep concentration gradients, at a given dissolved oxygen concentration in bulk solution the deeper layers of the biofilm could develop anoxic or anaerobic conditions inside the flocs or the biofilm. The upper layer of the protobiofilm was dominated by the autotrophic nitrifyer bacteria as this layer was well aerated, nearest to bulk solution. The deeper laying layers of the established biofilm were composed predominantly by the heterotrophic bacteria (facultative anaerobic microorganisms) hence denitrification was expected to occur in this zone. Establishment of heterogeneous layers in biofilm developing on the PVA-PAA carriers was the most difficult step in the simultaneous reactor. The structure of the biofilm depends on many environmental conditions and biotic factors (inoculation of the gels, composition of influent (raw) wastewater, diurnal changes of influent wastewater, flow conditions and apparent shearing forces in the reactor, the continuously changing density and average thickness of the biofilm, texture and 3D structure of the biofilm. Numerous papers report various effects of presence or absence of microscopic channels and voids, dosing of the methanol, buffer solutions on biofilm

structure [14], [15]. The texture of the double layers biofilm could be controlled indirectly by the concentration levels of various electron acceptors and donors in influent wastewater (such as NH_4-N, NO_2-N, NO_3-N) and aeration intensity. Environmental conditions in bulk solution (i.e., the reactor space) can be finely tuned by regulating aeration intensity (fine and coarse bubble aeration), methanol dosing that indirectly effect dissolved oxygen concentration etc. Conditions favouring the growth of heterotrophic biomass that has higher growth rate than autotrophic counterparts could be altered by organic carbon source (methanol) dosage as evidenced by microscopic observation during the experiments. Wastewater characteristics during the inoculation period is shown in Table 1.

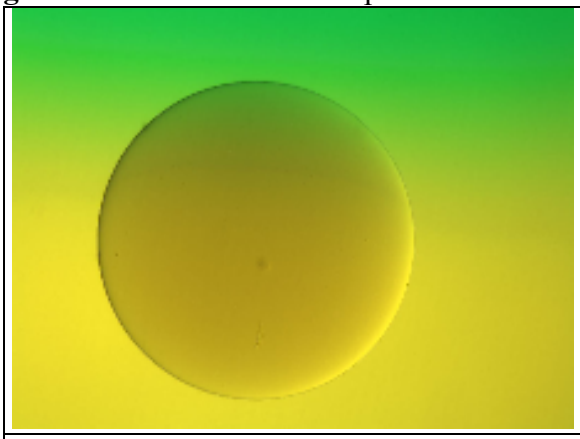
Table 1. Influent wastewater quality during the multiple step inoculation.

		Heterotrophic biofilm			**Double layered biofilm**		
Parameter	Unit	Minimum	Maximum	Average	Minimum	Maximum	Average
NH4-N	mg/l	13.7	24	21	23.8	52.2	30
NO2-N	mg/l	0.1	3.9	1.8	0.05	0.7	0.14
NO3-N	mg/l	21	63	44	17	52	28.5
pH		7.8	8.2	8.1	7.86	8.26	8.04
Conductivity	μS/cm	1765	1862	1800	2110	2120	2110
Temperature	^oC	20	21	20.5	20	21	20.5

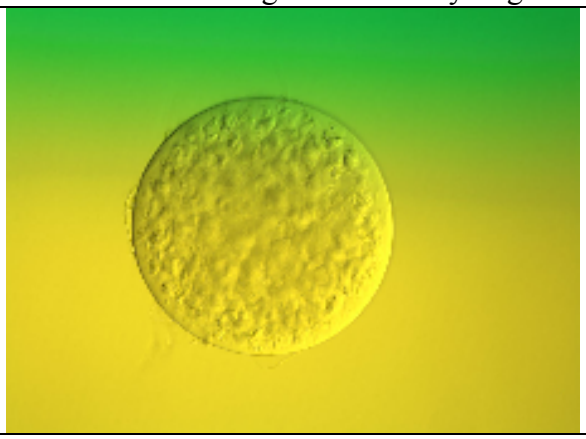
Colonization process

The colonization process and the inoculation technique were developed for three years. The inoculation bacterial suspension was made from different type of bacteria. In case of heterotrophic denitrifying biofilm the basic bacterial suspension was the cleaning water of the denitrifying biofilter of a communal wastewater treatment plant and the basic suspension of the autotrophic bacterial suspension was the cleaning water of the nitrifying biofilter of the same plant at Budapest. The preparation techniques of the inoculated suspension were the same in case of both types of the biofilm. Prior to inoculation the suspended biomass was treated by ultrasonic treatment for disaggregating the flocs followed by a filtration step on 50 μm glass fiber filter to avoid any aforementioned spontaneous clogging phenomena. To avoid the high concentration of the bacterial cells and flocs in the reactor the bacterial suspension was dosed slowly for 24 hours at the reactor start up period. The quantity of the inoculated suspension was optimized according to the amount of the gel beads in the reactor and the size of the reactor. In case of the simultaneous nitrification and denitrification a multiple layer biofilm was established on the surface of the gels by double step inoculation process. As an initial step time suspension of heterotrophic bacterial cells was dosed to the reactor twice at 24 hrs intervals. In the second step of inoculation autotrophic nitrifying bacteria were dosed into the reactor on the 18th day of the inoculation process. The presence of spontaneously suspended flocs (truly “planktonic” biomass portion) in the bioreactor might cause false results as well as structural damage in biofilm. Removal of spontaneously formed suspended planktonic flocs from the bioreactor was maintained on a continuous basis complemented by thorough washing and withdrawal of the not gel-bound biomass at every second day in wastewater. Environmental conditions such as temperature, exposure time, concentration of suspended bacteria (initial inoculum concentration) and most notably reactor hydraulics effect bacterial adhesion and the ultimate structure of formed biofilm. The Figures 1a-1f show the development of the biofilm on the surfaces of the gels beads.

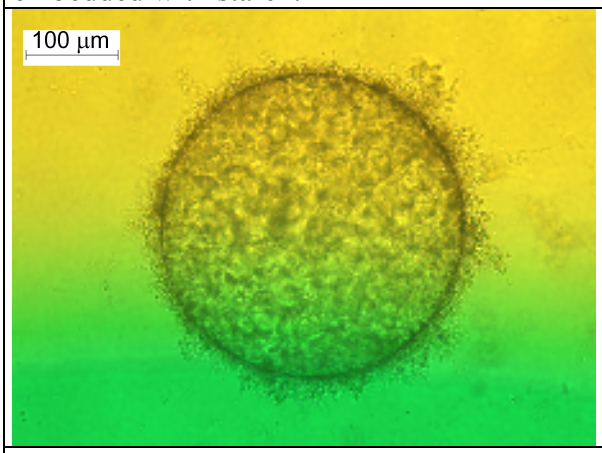
Figures 1a-1f. Biofilm development on NaOH treated starch containing PVA-PAA hydrogel beads.



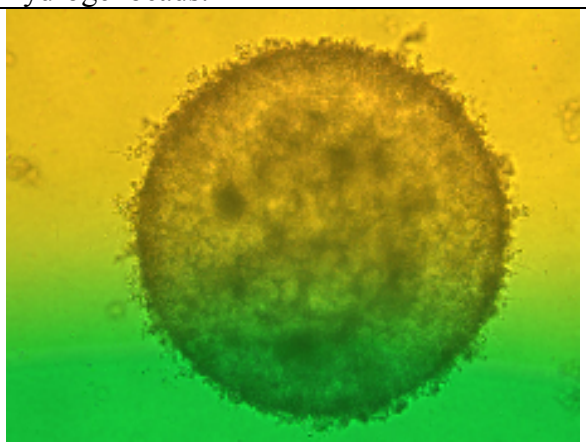
1a Raw PVA-PAA hydrogels beads embedded with starch.



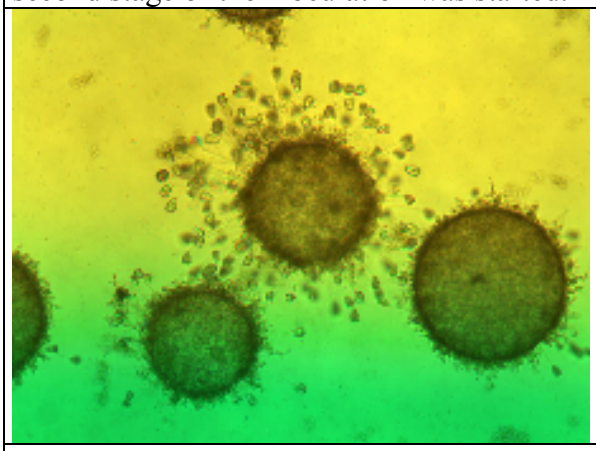
1b NaOH treated starch containing PVA-PAA hydrogel beads.



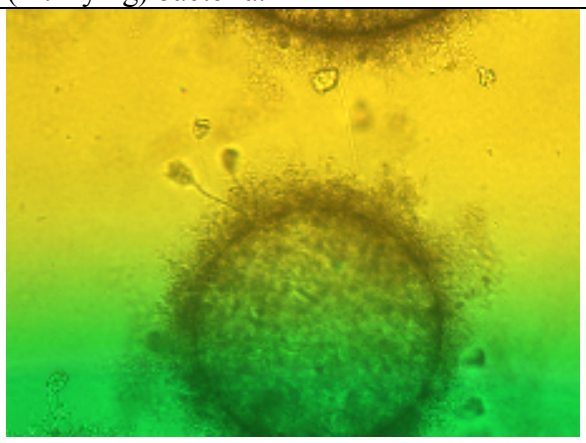
1c. State of heterotrophic biofilm development on the 18th day. At this time the second stage of the inoculation was started.



1d. Multiple layered biofilm on the 22nd day. 3 days after the inoculation with autotrophic (nitrifying) bacteria.



1e. Fully developed multiple layer mature biofilm on the 25th day. *Epistylis spp*. and *Vorticella spp*. started to grow on the surface of the autotrophic biofilm indicating non-toxic environmental conditions and high sludge retention time.

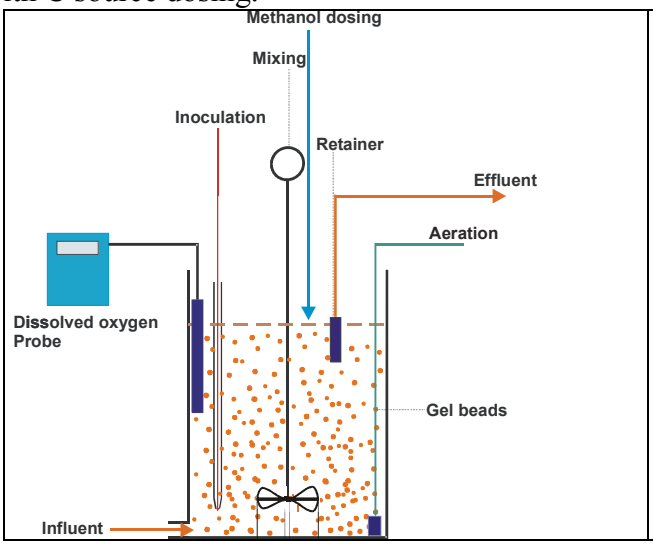


1f. Heterotrophic biomass from deeper biofilm layer starts to overgrow the upper autotrophic biofilm.

Control of dissolved oxygen concentration
The efficiency of denitrification in the deeper biofilm layers depends on the bulk dissolved oxygen concentration and the diffusion through the upper layers of fully grown biofilm. Structural changes of biofilm were monitored at daily intervals, while technological performance of the biomass was checked continuously. Should nitrification efficiency drop throughout the experimental period adjustment of dissolved oxygen concentration in the bioreactor was adjusted to favour autotrophic growth (i.e., stepwise increase of bulk oxygen concentration).

Dosage of the methanol (organic carbon source for denitrification)
Growth rate of heterotrophic denitrifying bacteria had to be controlled to maintain the depth and texture of the deeper layers of biofilm. During development period of the biofilm the growth of heterotrophic bacteria was always limited by concentration of organic carbon source (methanol). The background COD concentration of methanol dosed influent wastewater was kept constant throughout the experimental period.

Figure 2. Laboratory scale experimental system for simultaneous nitrification and denitrification with C source dosing.

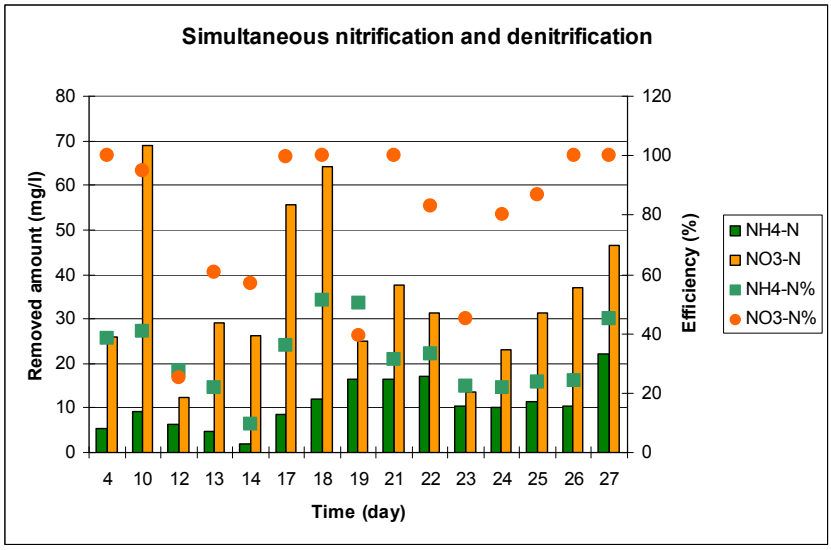


Hydraulic reactor volume: 6.3-7 liters
Volume of the PVA-PAA gels: 0.6 liter
Hydraulic loading: 0.5-0.7 liter/hour
Methanol dosing: 0.3 g/liter of influent wastewater (corresponding to 415 mg/l COD value)

Dissolved oxygen level was measured at every 15 minutes (see also Fig 6a and 6b).
The intensity of the aeration was controlled according to the level of DO.

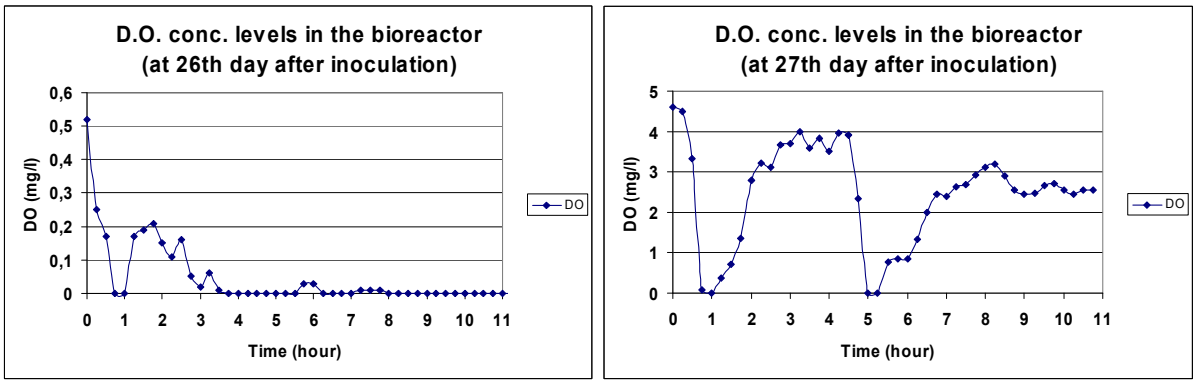
RESULTS AND DISCUSSION
Influent and effluent wastewater quality of the laboratory system was measured daily to obtain information on the technological capabilities of the system and its temporal changes. Fig 3 depicts the daily removal efficiencies for various N compounds and removed amount of a given component.

Figure 3. Removal efficiency and removed NH_4-N and NO_3-N in the simultaneous nitrification and denitrification system.



Upon the completion of biofilm development we achieved to develop a double layered biofilm; the upper layer consisted of autotrophic bacteria able to oxidize ammonium in the presence of oxygen (nitrifying) and the lower layer consisting of heterotrophic bacteria able to reduce nitrate to di-nitrogen gas in the absence of oxygen and by utilizing methanol as electron donor for the reduction process (denitrification). As the oxygen requirement for these two processes is contradicting it is of interest to monitor closely the DO level across the experiments as depicted on Figures 4a and 4b. Considering the low, to very low dissolved oxygen concentration on Fig 4a nitrification would not be expected to occur under these conditions, however, the correspondent results on Fig 3 clearly indicate the opposite. Nitrification activity of autotrophic bacteria was clearly observed at DO level below 0.2 mg/l on the 26th day while removal efficiency of denitrification was similar to rates observed around 0.2 mg/l and remained at bulk oxygen concentration levels higher then 2 mg/l. It is noted that efficiency of nitrification increased slightly by elevated DO concentration levels.

Figure 4a and 4b. Dissolved oxygen concentration profiles in the bulk solution during simultaneous nitrification/denitrification experiments



CONCLUSION

The most widely used method in biological wastewater treatment is the activated sludge process. The biomass used by this technology is multiple species, complex microbial community occurring in suspended form. Hence the biological wastewater treatment is to a large extent depends on the texture of the activated sludge flocs. Number of technological problems of the biological wastewater treatment is emanated from the textural properties of the biofilm or activated sludge

flocs. The solution of the problems (i.e., diffuse floc structure, filamentous growth etc.) could only controlled by indirect methods with additional hardware elements and system modification. The biofilm ensures safe environment for the bacterial cells and the microorganism and protects the bacterium from the predation, dehydratation and the extreme environmental changes (pH, temperature). The removal efficiency of the biological wastewater treatment strongly depends on the microscopic texture, morphology, density and thickness of the activated sludge flocs and biofilm. By the selected carrier hydrogel material the following key technological problems were addressed:

- Biofilm could be used in the activated sludge reactor;
- Regulated artificial carrier size and settleability.

Simultaneous removal of nitrogen forms (NH_4^+, NO_2^- and NO_3^-) in a single, aerated biological reactor (controlled aeration and carbon surce) Application of two inoculation techniques (in consecutive steps) made possible the coexistence of ammonia oxidizer (nitrification) and nitrate reducer (denitrification) bacteria in the same bioreactor volume. In spite of the contradictory environmental requirements of the two bacterial consortia (i.e., dissolved oxygen concentration) this experimental set up enabled the simultaneous functioning of the two groups showing high efficiency values in terms of nitrogen removal.

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