The Influence of nitrogen to the development of biofilms

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Abstract
The effects of different nitrogen concentrations in a submerge medium to the development of biofilms have been evaluated. Nitrogen was added in eight several concentrations. The inoculation was done with a natural mix population. We used a biofilm-reactor arrangement with a synthetic medium.

Multiple parameters were monitored to characterize changes in bacterial biofilm (water- oxygen- hydrogen- nitrogen- carbon- carbohydrate- protein- colonized forming units (cfu)- calcium- phosphate- matters).

The results showed effects of different nitrogen availableness in synthetic media to the composition of produced biofilms. The water- nitrogen- carbohydrate- protein- cfu- and calcium-contents in biofilms are depended from the available nitrogen. The contents of oxygen, hydrogen, carbon and phosphate are not influenced by the available nitrogen.
The composition of EPS (extracellular polymeric substances) has been measured too – the data will be present later.

Keywords: Biofilmreactor; Biofilm; Biological degradation; Nutrient nitrogen, phosphate; Water content; Carbohydrate; Proteins; CFU in Biofilms

Introduction:
During the last decades much information has been gathered and published on the effect of nutrients to building up of biofilms (Stoodley et al., 1999; Møller et al., 1997) - but the effect of nitrogen was neglected. Nitrogen is important for proteins and different carbohydrates – in biomass and in EPS of Biofilms. Thus, the amount of nitrogen available for microorganism in the surrounded environmental effected the composition of the harvested biofilm.

Material and methods

Biofilm reactor system
The reactor system consists of three principal components (Scheen, 1998). Reactor one is necessary to constant the experimental conditions (see below). The excess pressure squeezes the gas/liquid mixture in reactor two (s. fig 1).
Reactor two consists out of 19 glass-tubes. The tubes are connected in series. In the glass-tubes are in all 700 immobilization units (Polyethylene, high density). The units obtain a total surface of 0,8 m^2 present itself for biofilm development.
The gas/liquid mixture reaches reactor three. Reactor three closed the batch-circle and supplied reactor one again with the gas/liquid mixture.

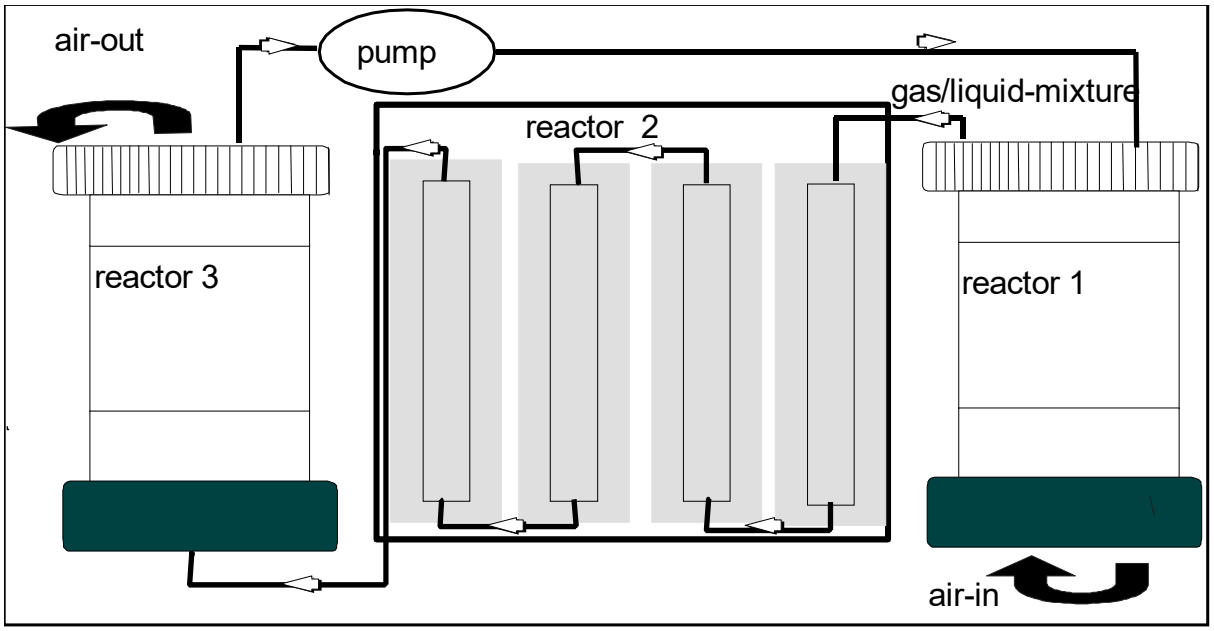

Fig. 1. Schematic diagram of the biofilm reactor system

Synthetic medium

The synthetic medium consist of following components: Sucrose, 4,37 mmol, Merck 1.07651; Ethanol, 1,05 mL/L, p.A. 99,8%, AppliChem A 1613; Ammonium chloride, see table 1, Merck 1145; Dipotassium hydrogenphosphate, 0,43 mmol, Merck 5104.1000; Iron (II)sulphate, 0,036 mmol, Merck 3965; Magnesium sulphate, 0,051 mmol, Merck 5886; Calcium chloride, 0,099 mmol, Merck 2382; Trace-element stock-solution, 0,2 mL/L.

Table 1. Dosage Ammonium chloride per liter and C:N:P-ratio:

C:N:P	100:1:1	100:5:1	100:8:1	100:10:1	100:12:1	100:14:1	100:18:1	100:20:1
mmol	0,7	3,5	5,6	7,0	8,4	9,8	12,6	14,0

Biofilm-production

The biofilm reactor system started constantly under following experimental conditions: constant water volume 20 L of synthetic medium (evaporated water was filled up daily); temperature 30°C; stirring speed 400 upm; pH-value 7; frozen inoculums; oxygen content 9,2-10 mg/L (measured with Oxymeter WTW GmbH, Weilheim, Germany, type Oxi 330/set); flow rate 4,8 L/h; hydraulic retention time of reactor one: 64 min; excess pressure 0,35-0,6 bar; air supply 6 L/min; inoculation density $4 \bullet 10^3$ cfu/mL in reactor one.

The DOC (Dissolved organic carbon, measured with Dimatoc 100, Fa. Dimatec, Essen, Germany) in synthetic media started with 1.040 mg/L. The DOC was measured daily. After receiving a DOC value <50 mg/L the appropriate run was finished and reactor two was opened to harvest the evolved biofilm from the immobilization units.

Inoculation

In advance a 2-liter sample was taken from a activated-sludge tank of a biological treatment plant (Ölbach municipal sewage treatment, Bochum, Germany). The sample was centrifuged (15 min, 4°C, 12.000 ´g). The supernatant was decanted off and the pellet was resuspended in sodium pyrophosphate (Merck 6591; 1,6 g/L). The resuspension was once more centrifuged. The same procedure took place three

times. The pellet was taken off in sterilized ¼ conc. ringer solution. The solution was portioned (2 mL) and frozen in (-18°C).
The water phase of the biofilm reactor system was inoculated with this stock-population (10^7 cfu/mL) in reactor one. These procedure meets the requirements to inoculate all runs with the same inoculums out of natural environment.

Biofilm isolation
Biofilms were harvested by removing the immobilization units from reactor two. Afterwards the units were treated with high shear forces (Ultra Turrax) in deionized water to divide the biofilm from the units. After separation the biofilm were centrifuged (12.000 ´g, 4°C, 15 min). The separated biofilm was sampled in advance weighted glass dishes. The glass dishes with biofilm were weighted to obtain the biofilm yield with water contents – explained in the following as biofilm-moisture (abbreviation biofilm-M).

Water contents in harvested biofilms
The harvested biofilm was dried (105°C) in glass dishes, over night, till constant weight achieved. The difference of weight agrees with the water contents in the biofilm. The results are diagrammed in percent of biofilm weight or mg/g. The dried biofilm (abbreviation biofilm-D) served for further measurements (see below).

Cfu
The biofilm-M was diluted in sterilized ¼ conc. ringer solution. Cfu was measured by plating dilution on standard I nutrient agar (Merck 7881). The agar plates were stored in incubator (30°C) till stable colonizes number achieves. The colonizes were counted out and calculated to mg of biofilm-M.

Determination of oxygen, hydrogen, nitrogen and carbon in the biofilm-D
The biofilm-D was carefully homogenized in a mortar. The concentrations of oxygen, hydrogen, nitrogen and carbon of the homogenate was measured with an elemental analyzer (LECO, type: CHNS-932).

Determination of calcium and phosphorus in the biofilm-D
The concentration of calcium and phosphorus in biofilm-D were measured with an ICP (Inductively coupled plasma, Liberty 200, Fa. Varian).

Protein and carbohydrate determination in biofilm-D
A sample of biofilm-D was resuspended in deionized water (sterile filtrated, 0,2 µm) with the help of a sonication-stick (Sonifier, 80%, on ice, 3 min). Humic acid has not been established – thus the Lowry et al. (1951) method was applied for protein determination in biofilm-D. The same suspension served for the measuring of carbohydrates to apply the method of Dubois et al. (1956).

Results

Water contents in biofilm-M
Figure shows the results.

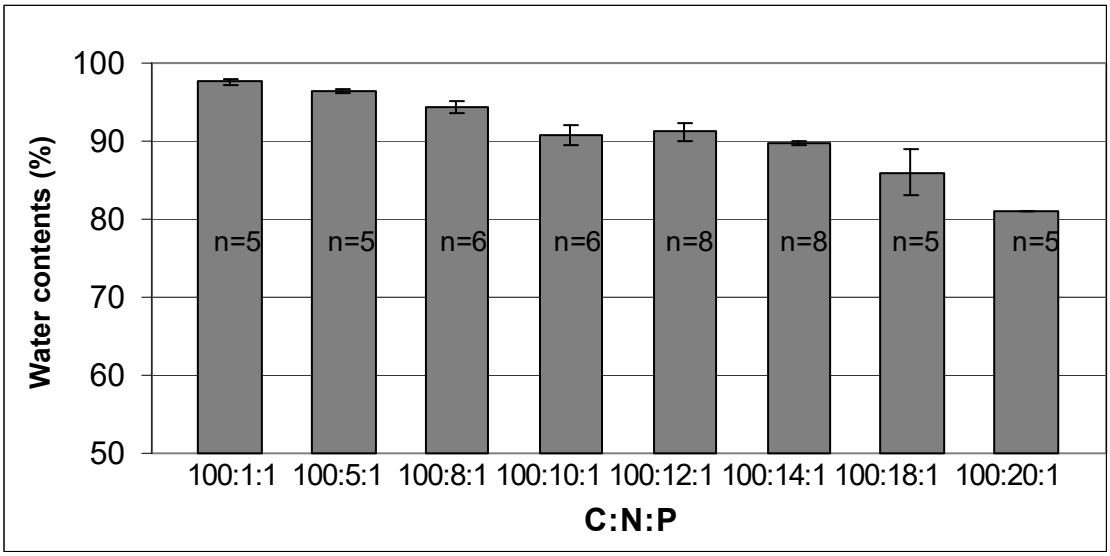

Fig. 2. Water contents in biofilms (w/w)

The contents of water in the sampled biofilms exhibit a decrease of water dye to the increase of nitrogen in the synthetic media. The contents of water in biofilm is depended of the available nitrogen in synthetic media.

Cfu
Figure 3. shows the results of cfu (as alive bacterial count) in biofilm-M.

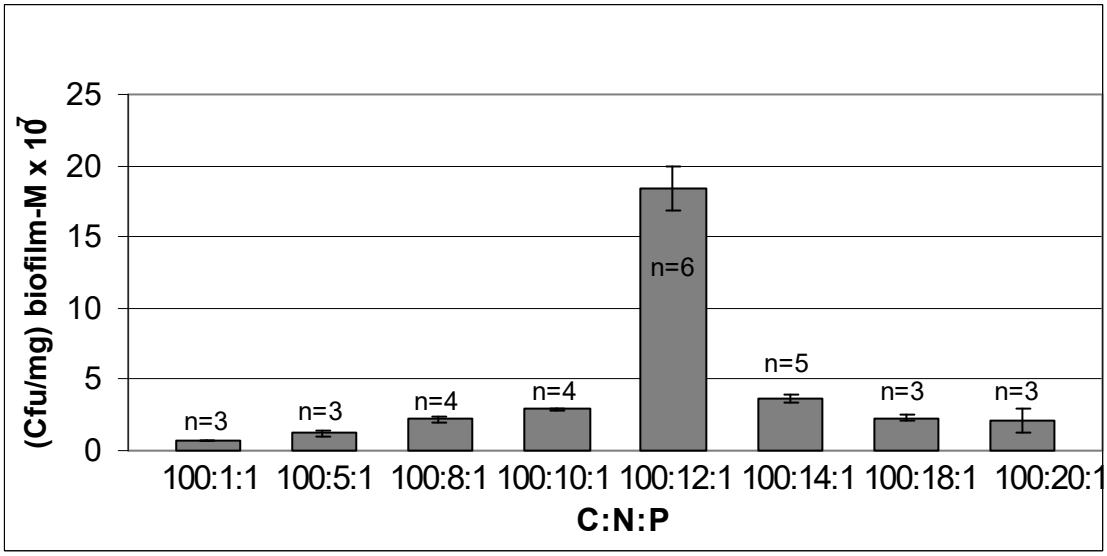

Fig. 3 Density of cfu in biofilm-M

The distribution of cfu in biofilm-M exhibit a dependence of the contents of nitrogen in the synthetic medium. The C:N:P-ratio of 100:12:1 indicate an especially high density of cfu in biofilm-M. It can be assumed that this C:N:P-ratio supported the fertility of

microorganism and the ability of microorganism to respond due to different C:N:P-ratios.
The density of microorganism, measured with fluorescent-microscopy (stained with acridinorange), announced amazingly no higher values.

Contents of oxygen, hydrogen, carbon and nitrogen in the biofilm-D
The contents of oxygen acquired in biofilm–D of different C:N:P-ratios an average of 35,1%±1,1, for hydrogen 7%±0,2 and for carbon 46,9%±0,7. The results are approximate evenness and are independent from the available nitrogen in the synthetic media. If the results are calculated to biofilm-M, the average matter of oxygen, hydrogen and carbon shown an ascending elution in the ascending C:N:P-ratios. The enhancement effects based on the lower water contents in biofilm due to higher availability of nitrogen in the synthetic media.

Figure 4. shows the contents of nitrogen in biofilm-D.

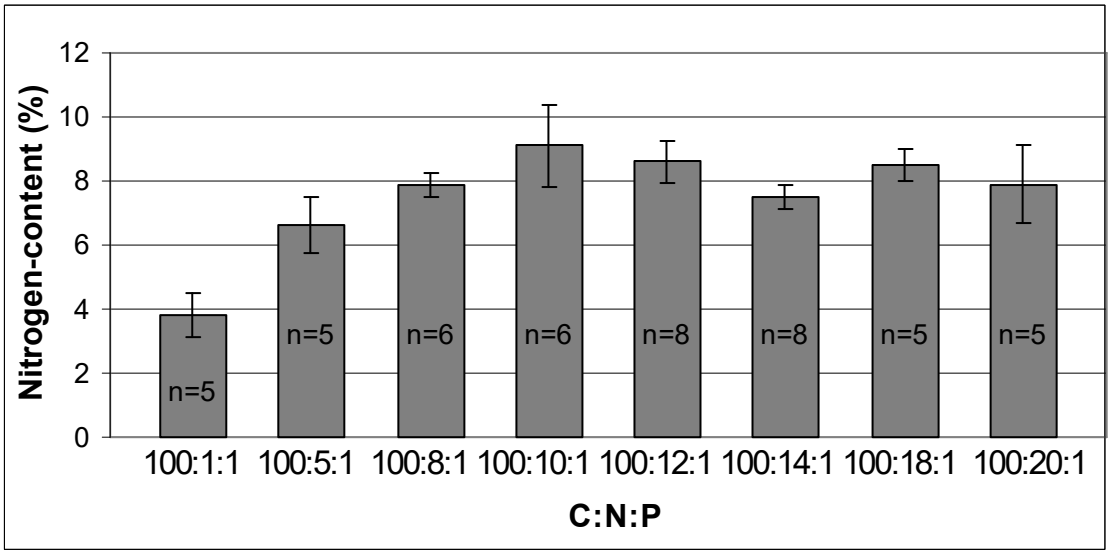

Fig. 4 Contents of nitrogen in biofilm-D (w/w)

The results shown an increase of nitrogen in biofilm-D unless the C:N:P-ratio of 100:10:1. In subsequent higher C:N:P-ratios the contents of nitrogen doesn't increased and reached an approximately content. These development correlate with the values of NH_4-N, measured in the rest synthetic media – after determining the single runs. Table… comprised the results of NH_4-N in the synthetic media after determining the single runs.

Table 2: Contents of NH_4-N in synthetic media – start, end and differences as adsorption in biofilm

C:N:P	100:1:1	100:5:1	100:8:1	100:10:1	100:12:1	100:14:1	100:18:1	100:20:1
NH_4-N_{start} mg/L	10,3	51,5	82,4	103	123,6	144,2	185,4	206
NH_4-N_{end} mg/L	0	1,8	0	4	22	47	92	103

NH_4-N difference mg/L = adsorp-tion	10,3	49,7	82,4	99	101,6	97,2	93,4	103

Table 2 indicated that the provided nitrogen out of the synthetic media has been adsorbed completely only ad the C:N:P-ratio of 100:10:1. The nitrogen beyond the C:N:P-ratio of 100:10:1 remains in the rest liquid phase of the appropriate run. Although the uptake of nitrogen was finished after higher-levels of C:N:P-ratios than 100:10:1 – but the composite of the biofilm in higher C:N:P-ratios has been changed furthermore.

Contents of phosphorus and calcium in the harvested biofilm-D
All different C:N:P-ratios has equal start-conditions concerning the contents of calcium and phosphorus in synthetic medium.
The contents of phosphorus in biofilm-D achieved an average of 1,05%±0,14 (w/w, weight/weight) in all different C:N:P-ratios. Thus, phosphorus was adsorbed overall.

Figure 5 shows the contents of calcium in biofilm-D. The calcium concentrations in biofilm-D decreased with increasing of nitrogen in the C:N:P-ratios in the synthetic medium.

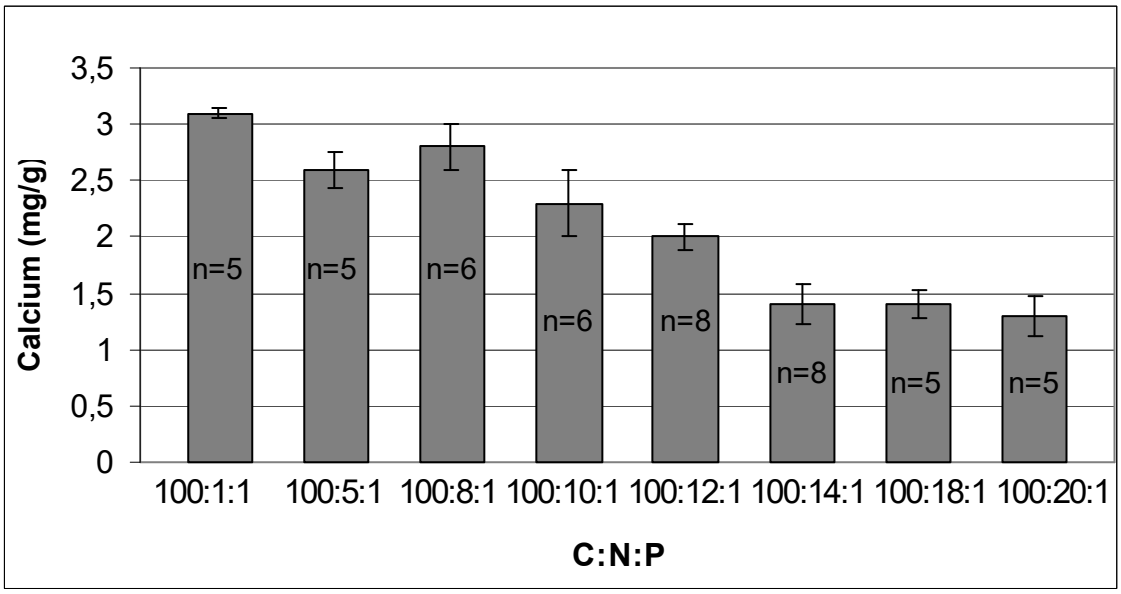

Fig. 5. Contents of calcium in biofilm-D (w/w)

Contents of protein and carbohydrate in biofilm-D

Figure 6. shows the different matters of proteins and carbohydrates in different C:N:P-ratios. Astonishingly the amount of protein and carbohydrate in each biofilm-D reaches an average of 70,8%±3,6.

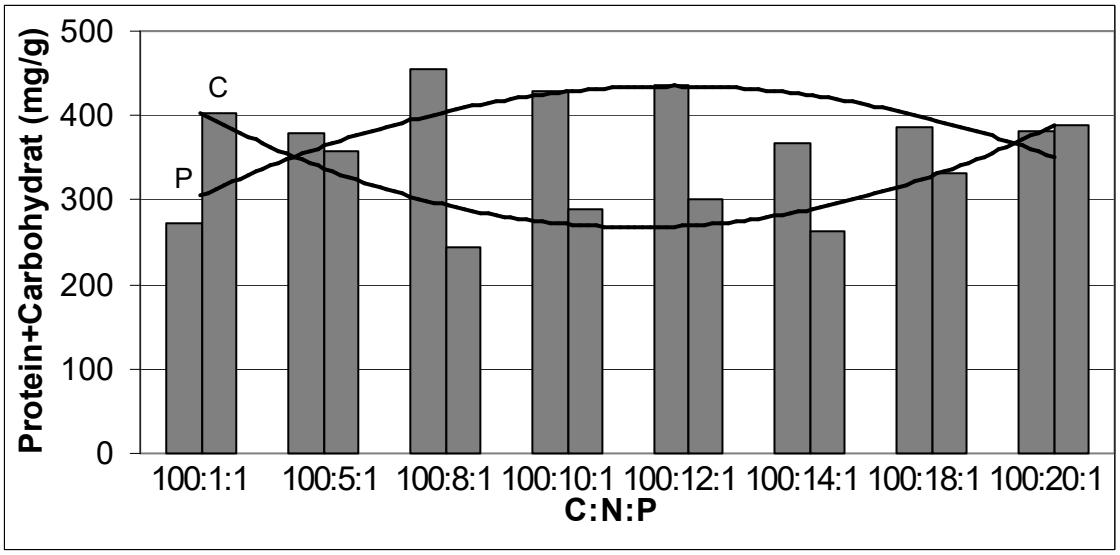

Fig. 6. Contents of protein (P) and carbohydrate (C) in biofilm-D (polynomial)

The above results can be assumed that the availability of nitrogen in the synthetic media has an applicable influence on a quantity basis of the production of proteins and carbohydrates in biofilm.

As has been shown in Figure 6. stopped the adsorption of nitrogen in the biofilms from the C:N:P-ratio of 100:10:1. But the contents of proteins and carbohydrates alter furthermore – with an constant summation (s.o.). These results protect the assumption that the rest of nitrogen in the synthetic media has a controlling influence to the microbial creation of proteins and carbohydrates in the biofilms.

Discussion

Varied matters of nitrogen in the synthetic media caused diverse biofilms.

The excess supply of nitrogen remains in the synthetic media after the C:N:P-ratio of 100:10:1. But the composition of biofilm changes furthermore in higher C:N:P-ratios. These results shown an influence of the nitrogen in synthetic media to the organisation of the biofilm. How far these influences are indicated by genetic control may be the contents of further investigations.

A toxic influence of nitrogen may be excluded by the measuring of BOD (Biological oxygen demand) with different C:N:P-ratios (data not shown). It can be assumed that the more uptake of nitrogen was bordered through the limitation of organic carbon in synthetic media.

The differences are observable in water contents in the harvested biofilms. The more of nitrogen in synthetic media the lower of water in biofilm-M.

The differences are also observable in the density of microbes in biofilm-M – measured as CFU. The CFU are not directly depended from the contents of nitrogen in the synthetic medium. They achieved highest values in the C:N:P-ratio of 100:12:1. It can be assumed that this ratio gives the best conditions for replication of microorganism dependent on nitrogen availability.

On the other hand - the contents of oxygen, carbon (total-organic), hydrogen and phosphorus reached average values in all biofilm-D resulted out of different C:N:P-ratios. The ahead parameters demonstrated other values if the calculation was obtained to biofilm-M. It must be always accentuated to show the differences between the contents of biofilm-D and biofilm-M.

The contents of calcium in biofilm-D decreases with the increase of nitrogen in the synthetic media. Figure… shows the explicit influence of nitrogen in the synthetic media to the resulted contents of calcium in the biofilm-D. The decrease of calcium took place also in higher C:N:P-ratios than 100:10:1. The contents of nitrogen in synthetic media has an influence to the contents of calcium in the resulted biofilm-D.

We previously stated that the content of carbohydrates and proteins reached in addition in all biofilm-D an average of 70,8%±3,6. But the several amount of carbohydrates and proteins alternate in the different C:N:P-ratios. The amount of nitrogen has an effect to the content of carbohydrates and proteins in biofilm-D.

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