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KINETIC CHARACTERIZATION OF NITRIFYING PURE CULTURES IN CHEMOSTATE

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ABSTRACT

The process of nitrification in wastewater treatment is widely accepted as a two-step process. In the first step the ammonia is oxidized to nitrite, a process considered to be carried out mainly by the *Nitrosomonas sp.*, while in the second step the *Nitrobacter sp.* oxidizes the nitrite to nitrate. Both species are autotrophic (chemolithotrophic) and they use CO_2 as the only carbon source for growth and maintenance, as well as, inorganic reduced nitrogen compounds to satisfy their basic needs for energy.

In the present work, experiments were carried out in a chemostate reactor, using a specific synthetic medium, in order to study the kinetic characteristics of nitrifying pure cultures. In the first set of experiments, the nitrosifying bacteria *Ns. europaea* were used, while in the second set we employed the nitrifying bacteria *Nb. winogradkyi*. Subsequently, a specially prepared mixed culture, consisting of the two above mentioned species was studied, in order to evaluate the possible interactions between them.

In order to determine the influence of the pH on the growth rate of pure cultures and to determine the optimum pH value, a series of chemostate experiments was conducted with gradual changes of the pH. The optimum pH was determined at 7.5-7.6.

The nitrosifying bacteria oxidize ammonia to nitrous acid, whereas the nitrifying bacteria oxidize nitrous acid to nitrate. Their growth rate and kinetic behavior depend on the concentration of the energy source and also on the concentration of the dissolved oxygen and CO_2. Therefore, the kinetics can be described by means of the Monod equation.

The half-saturation coefficient for the energy source was determined by non-linear regression of the steady state data, which provided the corresponding values of $K_{m,NH3}$ = 0.62 mg NH_3 l^{-1} and $K_{m,HNO2}$ = 21.8 μg HNO_2 l^{-1} for each pure culture, on the actual substrates for the specific species.

The influence of the dissolved oxygen concentration on the microbial activity was studied under controlled conditions in the chemostate, i.e. pH=7.6, T=30°C and HRT=14 h. The results for several steady state conditions and for different dissolved oxygen concentrations provided the value K_{m,O_2} = 0.408 mg O_2 l^{-1} for *Ns. europaea*. Under similar conditions for the culture of *Nb. winogradskyi*, altering only the retention time in the chemostate i.e. pH=7.6, T=30°C and HRT=28 h, the results provided the value K_{m,O_2} = 1.657 mg O_2 l^{-1}.

The influence of the CO_2 concentration and its limiting role on the bacterial growth was also investigated, under steady state conditions, as it is important for the synthesis of new cells by the autotrophic bacteria. The values of K_{m,CO_2} for *Ns. europaea* and *Nb. winogradskyi* were calculated to 3.8μmol l^{-1} and 0.37μmol l^{-1} respectively, which indicates that the substrate affinity for the *Ns. europaea* is higher by one order of magnitude than the one for the *Nb. winogradskyi*.

KEYWORDS: nitrification kinetics, ammonia oxidation, nitrite oxidation.

1. INTRODUCTION

The half-saturation constants for NH_3, HNO_2, dissolved oxygen and CO_2 were determined, independently for each parameter, in chemostate experiments, i.e. under saturation conditions of any two parameters the limiting affect of the third was investigated. Accordingly, for the determination of the K_m values for the terminal electron acceptor the energy substrate and the carbon source were available in excess.

2. NITRIFICATION KINETICS

Monod kinetics is frequently used to describe microbial kinetics in general and can be also used to describe the growth of ammonia and nitrite oxidizing bacteria. Incorporating the requirements of the microbial cells for maintenance, the Monod equation can be expressed as follows:

$$\mu = \mu_{max} \frac{S}{K_m + S} - mY_{max}$$

where Y_{max}: maximum yield constant, $[g_X/g_{\Delta S}]$,
m: substrate consumption rate for cell maintenance, $[g_{\Delta S}/(g_X \cdot d)]$,
μ_{max}: maximum specific growth rate, $[d^{-1}]$,
K_m: half-saturation constant, $[g_{\Delta S}\, l^{-1}]$, and
S: substrate concentration, $[g_{\Delta S}\, l^{-1}]$.

Taking into consideration two other important factors, that is the carbon source and the oxygen concentration, the applied bacterial growth kinetics for the ammonia oxidation, is described by:

$$\mu_{AO} = \mu_{max,AO} \frac{S_{NH_3}}{K_{m,NH_3} + S_{NH_3}} \cdot \frac{S_{O_2}}{K_{m,O_2} + S_{O_2}} \cdot \frac{S_{CO_2}}{K_{m,CO_2} + S_{CO_2}} - m_{AO} \cdot Y_{max,AO}$$

Regarding that the NH_3 is the limiting substrate and that the oxygen and CO_2 concentrations are considered to be in excess, we accept that the terms $\frac{S_{O_2}}{K_{m,O_2} + S_{O_2}}$ and $\frac{S_{CO_2}}{K_{m,CO_2} + S_{CO_2}}$ converge to 1 and therefore the simplified expressions of the growth rate for nitrifying bacteria are:

Nitrosifiers (NH_3 oxidizing bacteria)

$$\mu_{AO} = \mu_{max,AO} \frac{S_{NH_3}}{K_{m,NH_3} + S_{NH_3}} - m_{AO} \cdot Y_{max,AO}$$

Nitrifiers (HNO_2 oxidizing bacteria)

$$\mu_{NO} = \mu_{max,NO} \frac{S_{HNO_2}}{K_{m,HNO_2} + S_{HNO_2}} - m_{NO} \cdot Y_{max,NO}$$

3. EXPERIMENTAL SETUP

3.1. Culture media and growth conditions

Ammonia-oxidizing bacteria: Sampling, enrichment and isolation of the nitrifiers were conducted already in our laboratory and are described in another publication (in preparation). A suitable culture medium was prepared for the chemostate (CSTR) which contained 40mmol NH_4Cl l^{-1}. A trace elements solution was used to provide the bacteria with the necessary nutrients. The pH in the bioreactor was adjusted to 7.6 by means of a base solution (10% $NaHCO_3$). A color indicator was added to observe the bacterial activity through the pH variations *(Table 1).*

Nitrite-oxidizing bacteria: For the experiments with nitrite-oxidizing bacteria two different media were used containing KNO_2 (*Table 2*). The concentration of KNO_2 in the medium was increased stepwise to 40mmol l^{-1}. The pH was kept constant by the addition of a biological buffer solution (N-2-Hydroxyethylpiperazine-2-ethansulfonacid). The same solution of trace elements was used for the continuous culture of the nitrite-oxidizing bacteria.

Table 1. Composition of the culture medium and trace elements solution

A. culture medium			B. trace elements solution		
components	**mg l^{-1}**	**mmol l^{-1}**	**components**	**mg l^{-1}**	**mmol l^{-1}**
NH_4Cl	2140	40.0	$MgSO_4 \cdot 7H_2O$	178.4	0.8
KH_2PO_4	54	0.4	H_3BO_3	197.6	3.3
KCl	74	0.9	$ZnSO_4 \cdot 7H_2O$	172.4	0.6
$MgSO_4 \cdot 7H_2O$	49	0.1	$FeSO_4 \cdot 7H_2O$	3892.4	14.0
NaCl	584	10.0	$CuSO_4 \cdot 5H_2O$	250	1.0
$CaCl_2 \cdot 2H_2O$	147	1.0	$Na_2MoO_4 \cdot 2H_2O$	238.6	0.8
			$Na_2SeO_3 \cdot 5H_2O$	263	1.0
color indicator (cresol red)		1 ml l^{-1}	HCl (1N)	25	
trace elements solution		1 ml l^{-1}			
EDTA		5 ml l^{-1}			

Table 2. Culture media for the nitrifiers (nitrite oxidizers)

culture medium	**A**		**B**	
components	**mg l^{-1}**	**mmol l^{-1}**	**mg l^{-1}**	**mmol l^{-1}**
KNO_2	3400	40.0	2550	30.0
$KH_2PO_4 \cdot 7H_2O$	150	1.1	54	0.4
KCl	-	-	74	0.9
$MgSO_4 \cdot 7H_2O$	50	0.2	49	0.1
NaCl	500	8.6	584	10.0
$CaCl_2$	-	-	147	1.3
$CaCO_3$	3	0.03	-	-
trace elements solution	1 ml l^{-1} (**Table 1B**)		1 ml $^{-1}$l	
EDTA	5 mg l^{-1}		5 mg l^{-1}	

For the experiments of the influence of CO_2 concentration on the bacteria various base solutions were used containing different concentrations of Na_2CO_3 or $NaHCO_3$ and NaOH (*Table 3*).

3.2. Design and operation of chemostate (on-line measurements)

A chemostate Biostat-V (CSTR) of a total volume 10l was used for the experiments. All components of the experimental system were sterilized. The operational volume during all experimental runs was maintained at 5l by a level controller and an effluent peristaltic pump. The medium was introduced by a peristaltic pump (Watson-Marlow) through a sterile filter (Sartorius). A pH controller was used for the adequate supply of the sterilized alkaline solution (10% $NaHCO_3$ or 10% $NaCO_3$). The temperature and pH were monitored on-line and they were kept constant at 30°C and 7.6 respectively. The dissolved oxygen in the reactor and the CO_2 concentration in the gas outlet stream were monitored on-line. In addition, the concentrations of NH_4^+, NO_2^- and NO_3^- anions were measured intermittently.

The air supply was controlled by a flow meter at 70–140 l h^{-1} and was pumped through a filter for the retention of microorganisms (Gelman Sciences), before its introduction to the bioreactor. The reactor content was continuously stirred for full suspension of the biomass, and for proper diffusion of air and alkaline solution (*Figure 1*).

4. RESULTS OF KINETIC EXPERIMENTS

The course of the experiments in the continuous culture of *Ns. europaea* is shown in **Figure 2**. For a residence time (μ) of 0.64–1.85 d^{-1}, the entire NH_4^+-N concentration was converted to nitrite. Before adjusting the residence time to 2.18 d^{-1}, an amount of 100mg NH_4^+-N l^{-1} was added. Further increase of the residence time led to a decrease of the consumption rate, thus by the end of the experiments more than 500 mg NH_4^+-N l^{-1} were detected for a value of μ=3.7 d^{-1}.

Table 3. Base solutions for CO_2 influence experiments

stage	Na_2CO_3 [g l^{-1}]	[mol l^{-1}]	NaOH [g l^{-1}]	[mol l^{-1}]
A	100	0.94		
	$NaHCO_3$			
B	99.96	1.19	46.4	1.16
C	100	1.19	29	0.73
D	100	1.19	20	0.50
E	10	0.12	55	1.38
F	100	1.19	0	0
G	50.4	0.60	55	1.38
H	40	0.48	55	1.38
I	30.24	0.36	55	1.38
J	20	0.24	55	1.38
K	10	0.12	55	1.38
L	5	0.06	55	1.38
M	0	0	55	1.38

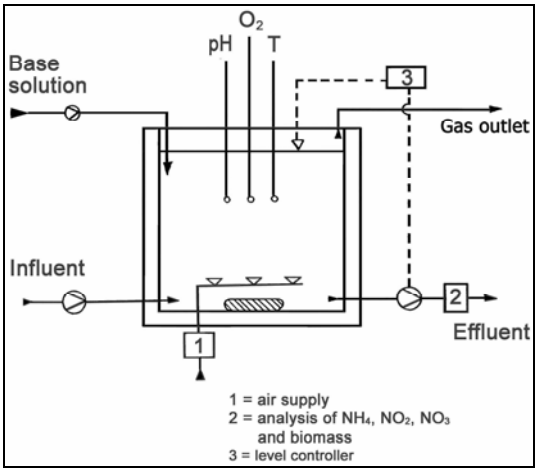

Figure 1. Chemostate Biostat-V (Braun, Melsungen)

The biomass concentration was continuously increasing up to a value of μ=1.1 d^{-1}. The increase of the volumetric loading rate (R_{RN}) was proportional to the residence time up to a value of R_{RN}=0.848 $g_{\Delta N}$ $l^{-1}d^{-1}$. The corresponding parameters are also depicted for *Nb. winogradskyi* (*Figures 3, 4*).

The specific substrate utilization rate (q_N) was evaluated from the NH_3 and HNO_2 concentration in the reactor. The half-saturation constants were calculated from the graphical representation of the q_N to the substrate concentration, by means of linear regression, at $K_{m,NH3}$ = 0.62 mg NH_3 l^{-1} for *Nitrosomonas* and $K_{m,HNO2}$ = 21.8 µg HNO_2 l^{-1} for *Nitrobacter*. Respectively, the $q_{Nmax,AO}$ = 13.5 $mg_{\Delta N}$ $mg_{X,AO}^{-1}$ d^{-1} and the $q_{Nmax,NO}$=42.1 $mg_{\Delta N}$ $mg_{X,NO}^{-1}d^{-1}$ were estimated and are depicted in *Figures 5, 6.*

In *Figures 7, 8* the specific growth rates (μ) are depicted in relation to the specific substrate utilization rates (q_N). The maximum yield coefficient was derived from the linear correlation, $Y_{Nmax,AO}$ = 0.177 $g_{X,AO}/g_{\Delta N}$. Thus, the maintenance factor was calculated to m_{AO}= 3.42 $g_{\Delta N}$ $g_{X,AO}^{-1}$ d^{-1}). The corresponding parameters for *Nb. winogradkyi* were estimated at $Y_{N,max,NO}$= 0.058 $g_{X,NO}$ /$g_{\Delta N}$ and m_{NO} = 6.1 $g_{\Delta N}$ $g_{X,NO}^{-1}$ d^{-1}). Furthermore, the values for the maximum specific growth rate (μ_{max}) was derived from the correlation of the specific growth rate (μ) to the corresponding substrate (*Table 4*).

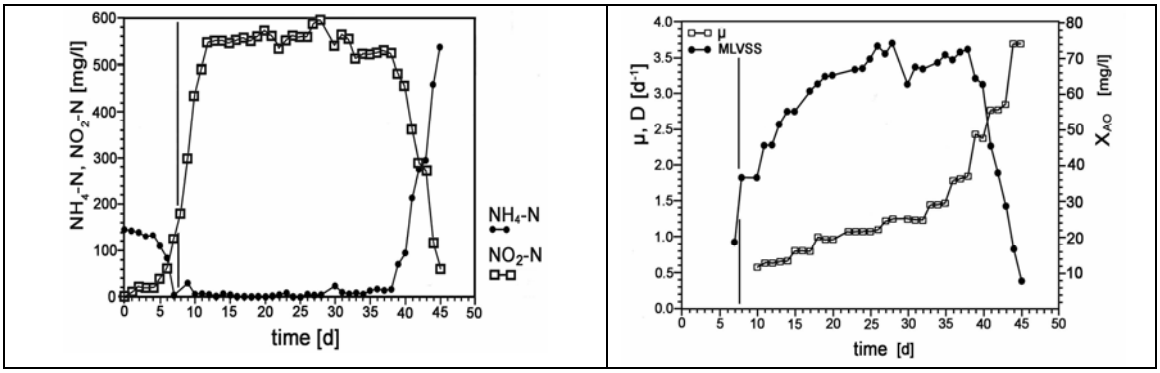

Figure 2. Course of the experiments on the continuous culture of *Ns. europaea*

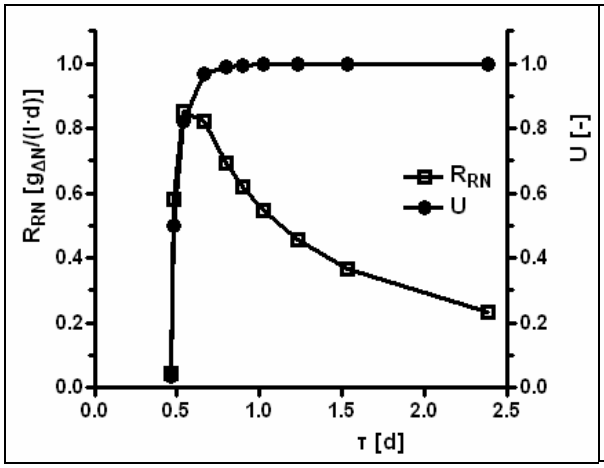

Figure 3. Correlation of the volumetric loading rate (R_{RN} [gN removed $l^{-1}\cdot d^{-1}$)]) and the conversion rate (U) to the residence time (τ) for *Ns. europaea*

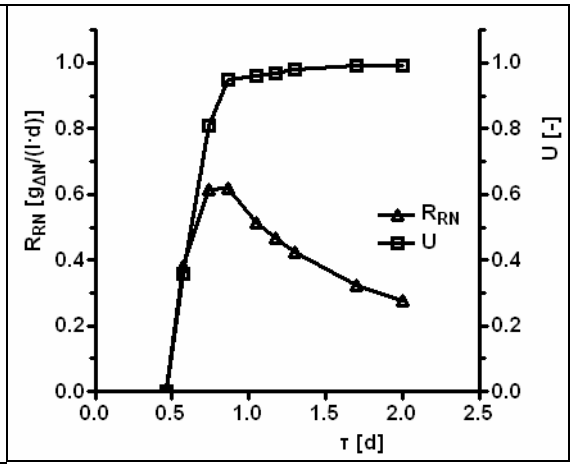

Figure 4. Correlation of the volumetric loading rate (R_{RN} [gN removed $l^{-1}\cdot d^{-1}$)]) and the conversion rate (U) to the resi-dence time (τ) for *Nb. winogradskyi*

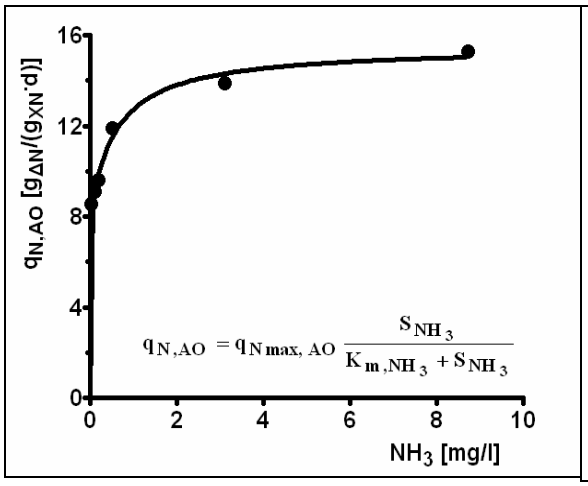

Figure 5. Specific substrate utilization rate ($q_{N,AO}$) to the NH_3 concentration for *Nitrosomonas*

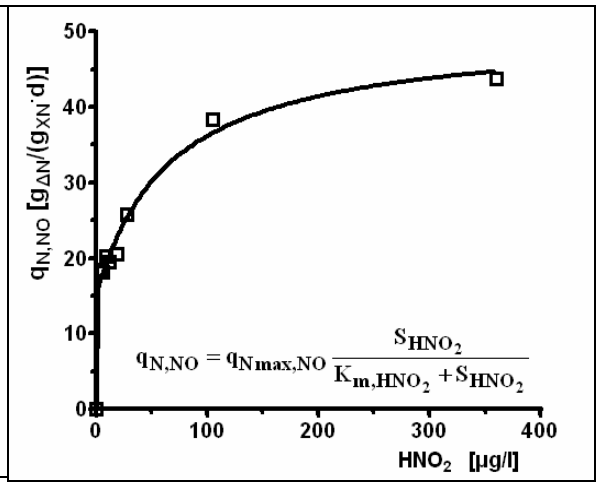

Figure 6. Specific substrate utilization rate ($q_{N,NO}$) to the HNO_2 concentration for *Nitrobacter*

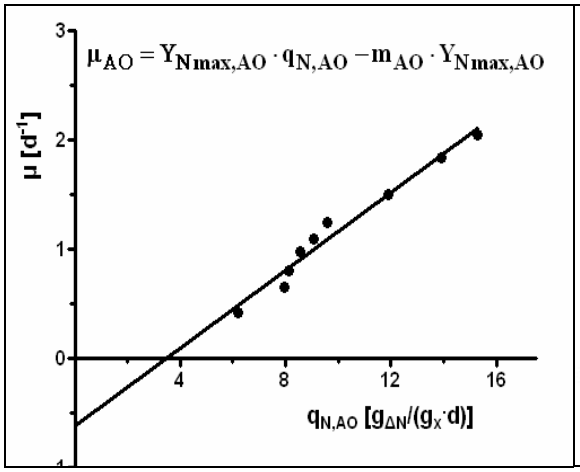

Figure 7. Correlation of the specific substrate utilization rate ($q_{N,AO}$) to the specific growth rate (μ) for *Ns. europaea*

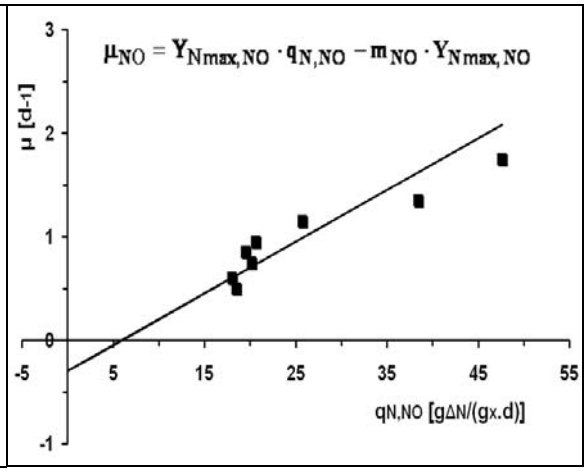

Figure 8. Correlation of the specific substrate utilization rate ($q_{N,NO}$) to the specific growth rate (μ) for *Nb. winogradsky*

4.1. Kinetics of oxygen in nitrifiers

Experiments were conducted in the bioreactor under controlled conditions in order to study the oxygen kinetics (40 mmol N l^{-1}, T=30°C, pH 7.6). The residence time was set to 0.58d for *Ns. europaea* and 1.17 d for *Nb. winogradskyi*. The oxygen flow was set to 7.5 $mgO_2\ l^{-1}$. In *Figures 9, 10* the correlation of the specific substrate utilization rate (q_N) to the oxygen concentration is depicted for the two pure cultures. Using non-linear regression the K_{m,O_2} value is calculated to 0.408 $mgO_2\ l^{-1}$ for *Ns. europaea* and 1.657 $mgO_2\ l^{-1}$ for *Nb. winogradskyi* respectively.

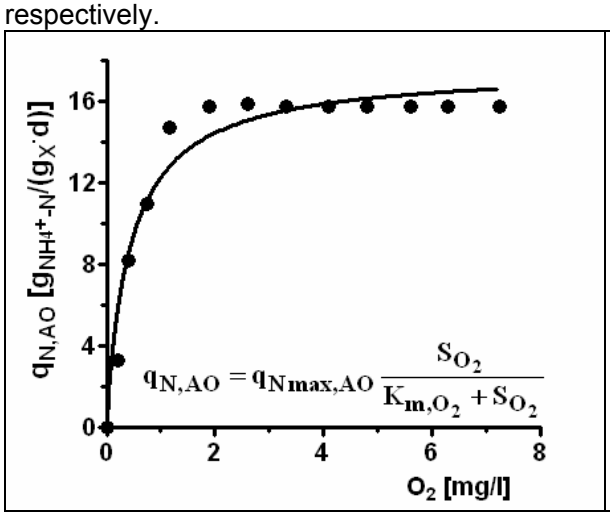

Figure 9. Correlation of the specific substrate utilization rate ($q_{N,AO}$) to the oxygen concentration for *Ns. europaea*

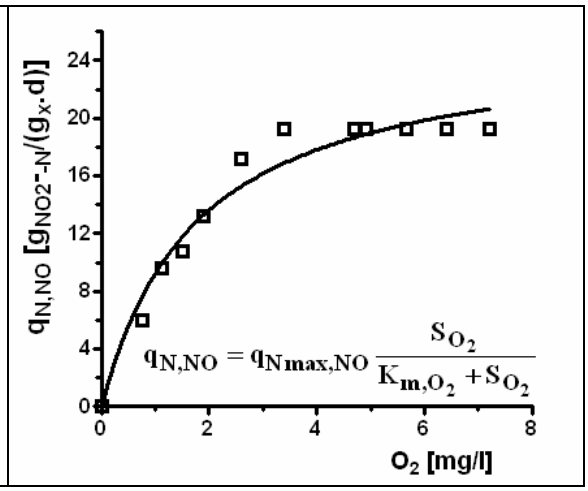

Figure 10. Correlation of the specific substrate utilization rate ($q_{N,NO}$) to the oxygen concentration for *Nb. winogradskyi*

4.2. The CO_2 kinetics for nitrifiers

The CO_2 is used by the autotrophic nitrifiers as a carbon source for biomass growth according to the Calvin cycle. In the present study a novel attempt was made in order to study the influence of the CO_2 concentration on the nitrosifying cultures. During the experiments the following conditions were kept constant: pH=7.6, T=30°C, oxygen saturation=70% with volumetric air supply of 2.17 l min^{-1}, and constant base solution flow with τ=0.66 d. Various base solutions are used containing different concentrations of Na_2CO_3 or $NaHCO_3$ and NaOH (*Table 3*).

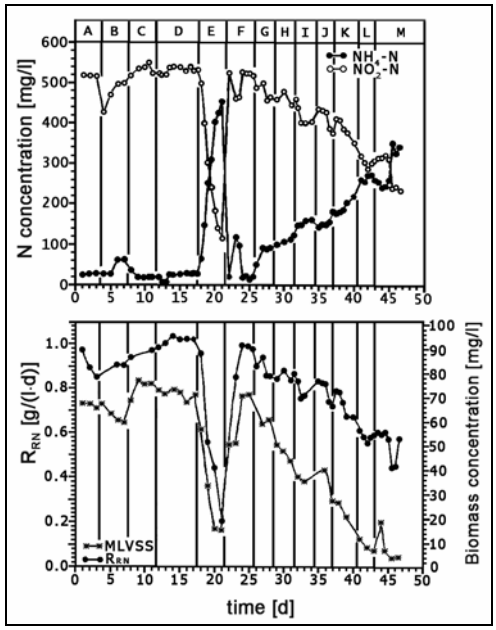

Figure 11. The influence of various CO_2 concentrations on a continuous culture of *Ns. europaea*

The estimated values of K_{m,CO_2} are 3.8 μmol l^{-1} for *Nitrosomonas* and 0.37 μmol l^{-1} for *Nitrobacter*. The correlation of specific substrate utilization rate (q_N) to the concentration of CO_2 is depicted in *Figures 12, 13*.

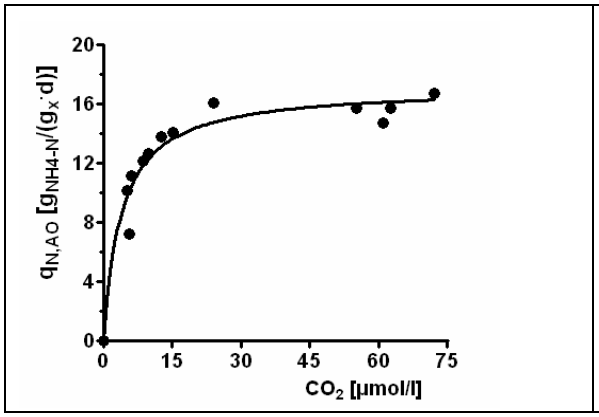

Figure 12. Correlation of the specific substrate utilization rate ($q_{N,AO}$) to the CO_2 concentration for *Ns. Europaea*

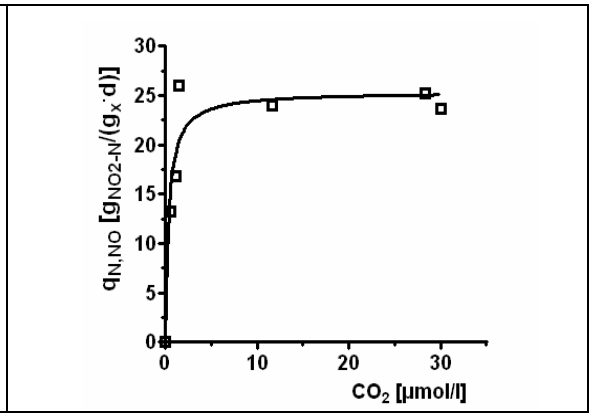

Figure 13. Correlation of the specific substrate utilization rate ($q_{N,NO}$) to the CO_2 concentration for *Nb. Winogradskyi*

4.3. The influence of pH on nitrification kinetics

The pH plays an important role on the nitrification kinetics as it affects several different parameters which in turn affect the growth and activity of the bacteria involved. In order to establish the influence of the pH, a series of experiments was conducted with gradual changes of the pH. The experiments were carried out at a residence time of 0.073 d^{-1}, at a temperature of 30°C, starting from pH=7.9 and gradually decreasing. At pH=7.9 full conversion of the substrate was observed. Decreasing to 7.6 and then to 7.3 the conversion percentage was reduced and at pH 7.6 an amount of 20mg NH_4-N l^{-1} was measured. At pH 7.0 a washout of the MLVSS was observed and furthermore at 6.7 no measurement was possible. During the second phase (pH increase), starting from a value of 7.9 the pH was raised gradually up to 9.1. Ascending to pH 8.5, the substrate was almost completely consumed, although at pH 8.8 an increase to the NH_4^+ concentration was observed with a parallel decrease to the biomass concentration from 70 mg l^{-1} to 2 mg l^{-1}. At pH 9.1 no further conversion was noticed.

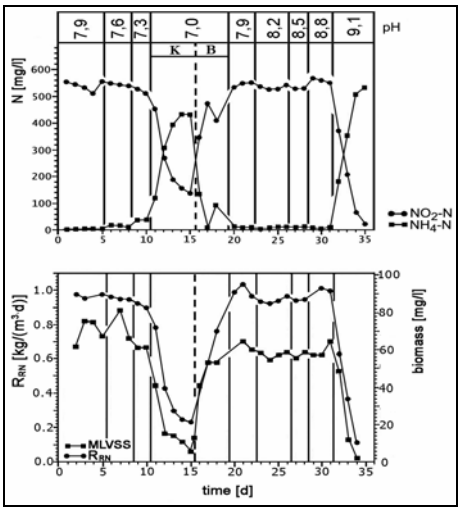

Figure 14. The influence of various pH values on a continuous culture of *Ns. europaea*

5. CONCLUSIONS

The present study provides values for the most important kinetic constants describing the growth of pure bacteria cultures in a chemostate. The half-saturation coefficient for the energy source was determined and it provided the value $K_{m,NH3}$ = 0.62 mg NH_3 l^{-1} for the ammonia oxidizers and $K_{m,HNO2}$ = 21.8 μg HNO_2 l^{-1} for the nitrite oxidizers. The results on the influence of the dissolved oxygen concentration on the microbial activity for several steady state conditions provided the values K_{m,O_2} = 0.408 mg O_2 l^{-1} for *Ns. europaea* and K_{m,O_2} = 1.657 mg O_2 l^{-1} for *Nb. winogradskyi*.

A novel aspect of this study is that it focuses on the influence of CO_2 concentration and its limiting role on the growth of autotrophic bacteria. The resulted values of K_{m,CO_2} = 3.8 μmol l^{-1} for *Ns. europaea* and K_{m,CO_2} =0.37μmol l^{-1} for *Nb. winogradskyi*, indicate approximately one magnitude of order higher substrate affinity for *Ns. europaea* compared to the *Nb. winogradskyi*.

The most important results of this study are summarized in the *Table 4* below.

Table 4. Calculated kinetic constants for the ammonia and nitrous acid oxidizing bacteria**.**

	Ns. europaea	***Nb. Winogradskyi***
Michaelis-Menten constant	$\mathbf{K_{m,NH3}}$ = 0.62 mg NH_3 l^{-1}	$\mathbf{K_{m,HNO2}}$ = 21.8 μg HNO_2 l^{-1}
maximum specific growth rate	$\mathbf{\mu_{max,AO}}$ = 1.94 d^{-1}	$\mathbf{\mu_{max,NO}}$ = 1.9 d^{-1}
maintenance factor	$\mathbf{m_{AO}}$ = 3.42 $g_{\Delta N}$ $g_{X,AO}^{-1} \cdot d^{-1}$)	$\mathbf{m_{NO}}$ = 6.1 $g_{\Delta N}$ $g_{X,NO}^{-1} \cdot d^{-1}$
maximum yield coefficient	$\mathbf{Y_{max,AO}}$ = 0.177 $g_{X,AO}/g_{\Delta N}$	$\mathbf{Y_{max,NO}}$ = 0.058 $g_{X,NO}/g_{\Delta N}$
half-saturation constant for O_2	$\mathbf{K_{m,O2}}$ = 0.408 mg O_2 l^{-1}	$\mathbf{K_{m,O2}}$ = 1.657 mg O_2 l^{-1}
half-saturation constant for CO_2	$\mathbf{K_{m,CO2}}$ = 3.8 μmol CO_2 l^{-1}	$\mathbf{K_{m,CO2}}$ = 0.37 μmol CO_2 l^{-1}

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