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A method for investigating the heterotrophic assimilation of ammonium and nitrate through planktonic organisms

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ABSTRACT. The kinetics of assimilation of ammonium and nitrate is investigated for various predetermined concentrations of water samples extracted from the upper subsurface of the littoral zone of a small reservoir (Lake IAG) in São Paulo, Brazil (23º39' S and 46º37' W). Five concentrations were predetermined for added ammonium: 300; 370; 440; 650; 1280 μg.N.L^{-1} and nitrate: 340; 590; 890; 1090; 1590 μg.N.L^{-1}. The samples were maintained under dark and oxygenated at a controlled temperature of 20ºC ± 1ºC, for the period of 9 days for ammonium and 11 days for nitrate. Once a day the concentrations of ammonium and nitrate in the flasks were analyzed colorimetric method. The highest uptake rate occurred at the initial concentrations of 300 μg.N.L^{-1} (k = 0,23 day^{-1}) and 340 μg.N.L^{-1} (k = 0,25 day^{-1}) for ammonium and nitrate respectively. These results indicate that the latter concentrations are close to the ideal conditions for assimilation of ammonium and nitrate by the microorganisms in the system. It is suggested therefore that these are the concentrations at which incubation processes should be started. In addition, concentrations above 500 μg.N.L^{-1} for ammonium and 600 μg.N.L^{-1} for nitrate are likely to cause inhibition of the assimilation process. The data do not follow a simple Michaelis-Menten equation, probably because some inhibition in the assimilation occurred.

Key words: kinetics, uptake rate, assimilation, ammonium and nitrate.

RESUMO. Um método de investigação da assimilação heterotrófica do amônio e nitrato por organismos planctônicos. O presente estudo tem por objetivo descrever os processos de assimilação do amônio e nitrato através de ajustes cinéticos de assimilação em função de concentrações preestabelecidas. Para a execução dos experimentos foram coletadas amostras de água da subsuperfície da região litorânea do reservatório denominado "Lago do IAG". Este reservatório situa-se na área do Parque Estadual das Fontes do Ipiranga (PEFI), 23º39'S e 46º37'W, São Paulo, Brasil. Estabeleceram-se para os experimentos cinco diferentes concentrações para o amônio: 300; 370; 440; 650 e 1280 μg.L^{-1} de N-NH_4^+ e para o nitrato: 340; 590; 890; 1090 e 1590 μg.L^{-1} de N-NO_3^-. Os experimentos foram mantidos no escuro, oxigenados e à temperatura de 20ºC num período de 9 dias para o amônio e 11 dias para o nitrato. A cada dia, foram determinadas as concentrações do amônio e do nitrato por colorimetria. Os resultados mostraram que o maior coeficiente de decaimento (k) para o amônio ocorreu sob a concentração inicial de 300 μg.N.L^{-1} (k = 0,23 dia^{-1}) e para o nitrato o maior coeficiente foi obtido na concentração inicial de 340 μg.N.L^{-1} (k = 0,25 dia^{-1}). Estes resultados fazem supor que tais concentrações estejam próximas das condições ideais de assimilação dos referidos compostos pelos microrganismos deste sistema, propondo-se, portanto, que, no caso da realização de incubações dessa natureza, os experimentos devem ser iniciados a partir das concentrações próximas às descritas e que valores acima de 500 μg.N.L^{-1} de amônio e 600 μg.N.L^{-1} de nitrato poderiam ocasionar uma inibição dos processos de assimilação dos respectivos substratos. É importante notar que as descrições das variações dos coeficientes de decaimento em função das concentrações iniciais de amônio e nitrato não foram ajustados ao modelo proposto por Michaelis-Menten devido ao provável processo de inibição observado.

Palavras-chave: cinética, assimilação, amônio e nitrato.

The study of biogeochemical cycles is of great importance for the understanding of the metabolism of aquatic ecosystems. These processes can supply basic information about the many ways of production, transformation and recycling of organic matter (Carmouze, 1994). In such processes autothrophic as well as heterotrophic microorganisms play an important role (Rheinheimer, 1992). According to Wetzel (1981), the cycle of nitrogen in lakes is of a microbial nature, in which the oxidation and reduction of nitrogenated compounds by bacteria are associated with the photosynthetic assimilation by algae and aquatic macrophytes. Besides these organisms, aquatic fungi also have great importance in the decomposition of organic matter of allochthonous origin. They represent a complex group, able to turn into soluble substances such as starch, cellulose, chitin, pectin and queratin; there activities play the main role in the recycling of aquatic ecosystems (Margalef, 1983).

Most models of water quality are generally based on the kinetics of Monod or Michaelis-Menten (EPA, 1985). McCarthy (1981) has evaluated works on the kinetics of assimilation, starting with the studies by Dugdale (1967) and Eppley and Coastsworth (1968). Dugdale (1967) proposed that the assimilation of nitrate and ammonium, through marine phytoplankton, could be described by the Michaelis-Menten expression, predicting the speed of assimilation from the concentration of the substratum. Dugdale *et al*. (1981) explained that the kinetics of assimilation may be modified as a function of changes in concentration of nutrients, irradiance and temperature, also assuming that organisms may adapt in many ways to the stress imposed by the environment as a function of the nutrients that come into the system. Dugdale *et al.* (1981) also pointed out that such an adaptation is the final result of a chain reaction of events occurring in a span of time scales. To know these time scales and their relationships with the marine environments is essential for the understanding of the meaning of the adaptation phenomena in such ecosystems. According to Wetzel (1981), independently of the intracellular concentration of nutrients, the specific rate of assimilation is tied to the concentrations in the medium, following the Michaelis-Menten function, by which the rate of assimilation increases with the concentration of nutrients in the external environment before reaching a concentration where the addition of nutrients has no further effect on the rate.

Almost all the researches on nutrient limiting in freshwaters in Brazil were carried out in the eighties; they were primarily centered on N and P deficiencies, mostly to the phytoplankton community in its great majority *in situ* experiments (Arcifa *et al*. 1995).

The present study is aimed at describing the kinetics of assimilation of ammonium ($N\text{-}NH_4$) and nitrate ($N\text{-}NO_3$) using preset concentrations, determining their respective coefficients of decay (assimilation) over the heterotrophic microorganisms found in samples of water from a small, shallow reservoir.

Material and methods

The "Lago do IAG" (IAG Lake) is an artificial body of water (reservoir), held in a sub-basin located at the Parque Estadual das Fontes do Ipiranga – PEFI (Ipiranga Springs State Park), geographic position 23º39'S and 46º37'W. There are the springs of the historic Ipiranga Creek, distributed in sub-basins that integrate the great Tietê River Basin. The IAG Lake is under jurisdiction of the Institute of Astronomy and Geophysics of the USP, São Paulo. According to results obtained by the integrated project: "Typology, surveillance and recuperation of the reservoirs in the Biological Station of the Ipiranga Springs State Park" (1998), the "IAG Lake" is characterized as an oligotrophic system, with a high NT/PT ratio, indicating conditions of limitation by phosphorus; during most of the year the concentrations of PT and $P\text{-}PO_4$ were below the limit of the method of 10 $\mu g.L^{-1}$.

To perform the experiments of assimilation of ammonium and nitrate, samples of water (~10L) were collected from the subsurface of the shoreward area of the reservoir, filtered beforehand through glass wool to exclude detritus and zooplankton. The experiments were carried out at the Laboratories of Bioassays of the CEBH (Centro de Estudos de Bacias Hidrográficas), of the Instituto de Pesca (São Paulo, Brazil). For the experiments with ammonium, quantities of 1 L of lake water with an initial ammonium content of 93 $\mu g.L^{-1}$ were transferred to 5 containers. A solution of NH_4Cl (stock solution) was added to each flask; the final (nominal) concentrations being: flask 1 = 300 $\mu g.L^{-1}$, flask 2 = 370 $\mu g.L^{-1}$, flask 3 = 440 $\mu g.L^{-1}$, flask 4 = 650 $\mu g.L^{-1}$ and flask 5 = 1280 $\mu g.L^{-1}$ of $N\text{-}NH_4^+$. To all flasks a solution of K_2HPO_4 was added to avoid limitation by phosphorus in the processes of decay staying a relationship N/P ideal for the growth of the organisms (Reynolds, 1984). For 9 days (December, 1997) the flasks were kept permanently oxygenated, in darkness and at a temperature of 20ºC. The same procedure was used

for the experiments of nitrate assimilation. In this case, the stock solution of KNO_3 was used, and the following final (nominal) concentrations established were: (blank) = 90 $\mu g.L^{-1}$; flask 1 = 340 $\mu g.L^{-1}$; flask 2 = 590 $\mu g.L^{-1}$; flask 3 = 890 $\mu g.L^{-1}$; flask 4 = 1090 $\mu g.L^{-1}$ and flask 5 = 1590 $\mu g.L^{-1}$ of $N\text{-}NO_3$. In this experiment the flasks were kept in incubation for 11 days (January, 1998). Each day a quantity of water was taken from each flask, and filtered by a vacuum pump, using a Millipore GS filter in cellulose ester, 0.22 μm pore. Once a day the concentrations of ammonium and nitrate in the flasks were analyzed for colorimetric method by spectrophotometry. The sample filtered was immediately analyzed according to Solorzano (1969), for ammonium and FEEMA (1979) for nitrate. The initial pH of the lake was of 6.5 and in the end of the experiments it stayed constant, showing a buffer system. To evaluate the coefficients of decay (k), a nonlinear regression (by software Origin for Windows) was used through the interactive method, based on the "Levenberg-Marquardt" algorithm, assuming that the assimilation processes of nitrogenated compounds follow a first order kinetics.

Results and discussion

The temporal variations of ammonium concentration in the incubations are shown in Table 1. Overall, the concentration dropped for all conditions investigated, which also applies to the nitrate concentration, as shown in Table 2. Figure 1 shows that the detected concentration, obtained from chemical analyses, is consistently lower than the nominal concentration at the beginning of the experiments, for both ammonium and nitrate. This indicates that chemical interactions probably took place (adsorption and complexing agents) between the ions added and the substances present in the samples, such as clay, particles of organic matter, dissolved organic compounds and aquatic humic substances. In this context, from the linear fits, it was verified that the interactions involved about 28% of the added ammonium and 42% of the added nitrate (Figure 1).

The speeds of decay were intensified as a function of the initial quantities of added ammonium and nitrate, up to 300 $\mu g.L^{-1}$ for ammonium and 340 $\mu g.L^{-1}$ for nitrate as shown in Table 1 and 2. For higher concentrations, the speed of decay tended to decrease. From the kinetics fittings, for ammonium the highest coefficient of decay (assimilation) occurred under the nominal concentration of 300 $\mu g.L^{-1}$ (K = 0,23 day^{-1}). This coefficient shows that the global decay of ammonium under the most favorable conditions has a half-life of 3.1 days. In a less favorable concentration (1280 $\mu g.L^{-1}$) this process showed a half-life of about 17 days. As for nitrate, the highest coefficient of decay was estimated from the flask with nominal concentration of 340 $\mu g.L^{-1}$ (K = 0.25 day^{-1}). It should be mentioned that in the kinetics fittings, the predetermined (nominal) concentrations were used as the initial concentrations. It was assumed therefore that while ions of ammonium and nitrate have undergone chemical interactions, they were still available for the biological processes (assimilations).

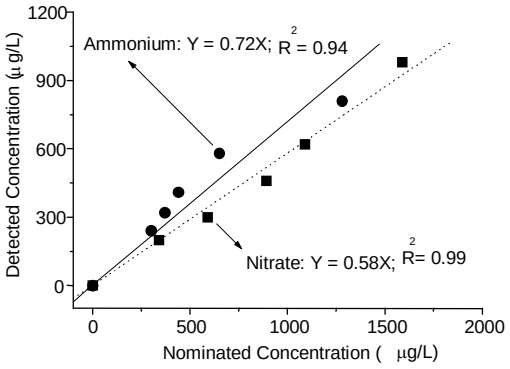

Figure 1. Relationships between the nominal concentrations and those detected at the beginning of the experiment (day zero)

Table 1. Temporal variations in the concentrations of ammonium ($N\text{-}NH_4$) from the incubations of samples of water from the IAG reservoir ($ug.L^{-1}$)

days	Blank	Flask 1	Flask 2	Flask 3	Flask 4	Flask 5
nominal	93,00	300,00	370,00	440,00	650,00	1280,00
0	93,00	241,00	320,50	410,00	580,00	810,00
1	89,13	208,11	294,50	364,35	525,52	734,61
2	111,54	205,47	256,56	368,29	445,09	734,61
3	70,01	182,72	277,66	412,79	491,57	752,42
4	130,98	184,70	273,70	353,13	488,60	756,37
5	126,79	150,76	229,87	345,88	482,34	685,18
6	122,75	233,82	332,70	405,21	541,01	788,01
7	128,67	196,25	331,71	371,59	520,24	744,51
8	168,55	187,67	354,12	427,62	546,94	744,51
9	192,82	165,79	263,02	309,30	507,92	749,55
10	160,86	54,51	188,01	209,76	330,06	527,39
11	63,41	0,00	176,14	52,87	169,88	376,87

The coefficients of assimilation are shown in Figures 2 and 3 as a function of the nominal concentration for ammonium and nitrate respectively. These data could not be fitted with the Michaelis-Menten (hyperbolic function) model because assimilation was apparently inhibited for concentrations above 500 and 600 $\mu g.L^{-1}$ for ammonium and nitrate respectively. The hyperbolic behavior is probably applicable only to concentrations lower than 300 $\mu g.L^{-1}$ for ammonium

and 340 $\mu g.L^{-1}$ for nitrate. To support this supposition, an additional experiment was conducted for ammonium, where the following concentrations were used: 66 $\mu g.L^{-1}$; 103 $\mu g.L^{-1}$ and 163 $\mu g.L^{-1}$. The rates of assimilation were 0.43 day^{-1}; 0.53 day^{-1} and 0.45 day^{-1} respectively. Upon analyzing these results, it is observed that for ammonium concentrations under 300 $\mu g.L^{-1}$ higher rates are obtained, which also suggests that the linear dependence between reaction rates and ammonium concentration should occur under concentrations lower than 103 $\mu g.L^{-1}$. However, at IAG Lake the concentrations are usually higher, making it difficult, for this environment, to recommend the use of these levels of concentrations. Also, we presume that such inhibitions happened due to an excess of added nutrients, since in a natural environment (IAG Lake), the concentrations of ammonium and nitrate were respectively 93 $\mu g.L^{-1}$ and 90 $\mu g.L^{-1}$. If these values are compared with the flasks 3, 4 and 5, we note that the concentrations of both nutrients were increased by about 5, 8 and 16 times.

Table 2. Temporal variations in the concentrations of Nitrate ($N\text{-}NO_3$) from the incubations of samples of water from the IAG reservoir ($ug.L^{-1}$)

days	Blank	Flask 1	Flask 2	Flask 3	Flask 4	Flask 5
nominal	90	340	590	890	1090	1590
0	90	200	300	460	620	980
1	100	160	260	530	620	1.070
2	77	180	280	650	640	1.200
3	80	140	230	380	440	950
4	77	115	159	356	380	860
5	80	100	150	340	370	860
6	77	75	92	224	276	668
7	80	100	160	460	510	680
8	100	100	130	210	250	290
9	77	110	90	250	180	320

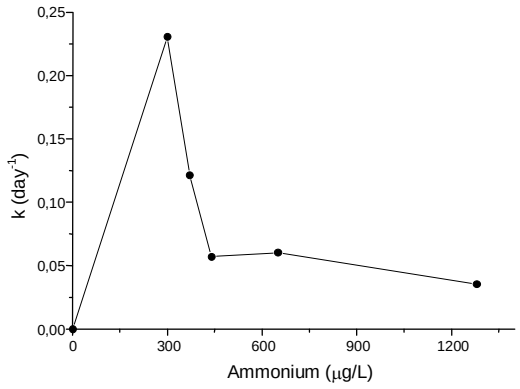

Figure 2. Variation in the coefficients of assimilation (day^{-1}) of Ammonium ($N\text{-}NH_4$) relative to the initial concentrations that were preestablished (300; 370; 440; 650 and 1280 μg/L)

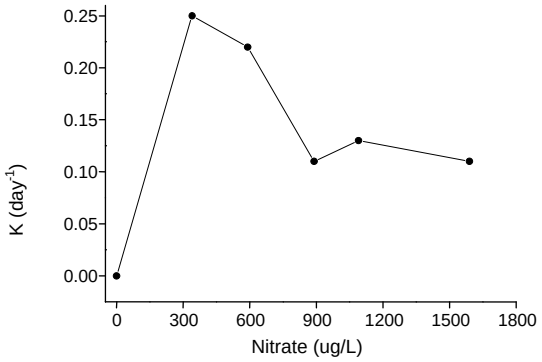

Figure 3. Variation in the coefficients of assimilation (day^{-1}) of Nitrate ($N\text{-}NO_3$) relative to the initial concentrations that were preestablished (340; 590; 890; 1090 and 1590 μg/L)

The growth of organisms may be limited, saturated, or, in some cases, inhibited by the availability of nutrients (Reynolds, 1984). According to that author, to keep the internal reserves when excessive demand occurs, an immediate accumulation may happen while the substratum is still available in the environment; this process is called luxury uptake. Collos and Slawyk (1980) hold that the parameters used by the equation of MONOD (particularly V_{max}) depend in a nonlinear fashion on the cellular allotment of nitrogen. The rate of assimilation may also vary nonlinearly with the concentration of NH_4 and NO_3. According to Conway *et al.* (1976), the rate of assimilation increases with the increase of NH_4 concentration, up to a value where the rate suddenly decreases because higher concentrations do not generate the same effect anymore. These authors used cultures with algae of the *Skeletonema* kind, limited by nitrogen (N), having observed that the rate of ammonium assimilation follows the kinetics of Michaelis-Menten, but only up to the point where an internal factor imposes a limitation to the rate of assimilation. Syrrett (1981) emphasizes that we are still far from understanding the inner control regulating the metabolical adjustments that take place during the depletion of N. Margalef (1983) suggests that the Michaelis-Menten equation is based on the growth curve of monospecific cultures, thus excluding the interactions between different kinds of culture and with the medium. The work of Antia *et al.* (1975) showed that some species of chlorophytes and diatoms (ex: *Tetraselmis maculata* and *Nitzschia acicularis*) are able to use a wide range of sources of organic N, while other species are incapable of it (ex: *Nannochloris oculata* and *Cyclotella eryptica*). Possibly as Naylor (1970) suggested, such

ability depends on small genetic differences, but further studies about the evolutive relationships among the species would be needed to reach any firm conclusions. According to Syrret (1981), an algae cell would convert the assimilated nitrogen. All sources of nitrogen going through the cellular walls are considered, where there are indications of the presence of an active system of assimilation, dependent on the metabolism. Although there can be passive intake of nitrogen by free diffusion. Overall, the methodological approach proposed intended to establish the connections between the heterotrophic activity of the planktonic organisms (algae, bacteria and fungi) and the environmental drive functions.

The results indicate that, in the case of incubations performed to seek a quantification of the processes of assimilation of inorganic forms of nitrogen, the experiments must be initiated at near 300 μg.N.L^{-1} for ammonium and 340 μg.N.L^{-1} for nitrate. To obtain the highest rates of decay, i.e. fastest assimilation, the experiments should be started at concentrations lower than those indicated. However, in such instances care must be taken with the sensitivity of the analytic methods adopted, and the ranges of variation of concentrations of ammonium and nitrate in the environment. It also must be considered that the assimilation coefficients vary in space and time, as a function of the organisms and their densities.

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