Nitrogen cycle and blue green algae (1)

1. Introductory

Unlike the other minor elements or compounds such as phosphate and silicate nitrogen occurs in molecules at several different oxidation states in the lake water. The following forms are quantitatively of importance.

a. N_2, dissolved in the water in equilibrium with the nitrogen in the atmosphere (about 15 mg per litre at 20 °C);
b. NH_3 in equilibrium with NH_4^+ and NH_4OH being produced from organically bound nitrogen, either from allochthonous or autochthonous sources;
c. NO_2^-, small quantities of nitrite are a normal constituent of most lakewaters. Larger quantities occur under certain conditions. They are formed during the oxidation of ammonia, but will normally be oxidised to NO_3^-, exept in anaerobic conditions.
d. NO_3^- the only stable form of nitrogen, unless taken up by algae and bacteria;
e. Organic N, either in dissolved state or as particulate nitrogen.

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Stages in the oxidation-reduction series are: (reductions as shown)

$$\underset{\text{Oxidation state of N:}}{} \quad \overset{}{NO_3^-} \xrightarrow{2e} \overset{}{NO_2^-}$$
$$\text{Electric charge of N-atom:} \quad +5 \qquad +3$$

$$\xrightarrow{3e} \underset{0}{N} \xrightarrow{3e} \underset{-3}{NH_3} \rightarrow \underset{-3}{\text{organic-N}}$$

Several species of bacteria which can convert one form into another are known. The oxidation steps produce energy for the bacteria; Ammonia and nitrate may occur up to 2 mg $\cdot l^{-1}$ in Dutch polderwater; normal concentrations are 0.1 mg $\cdot l^{-1}$; in polluted rivers nitrate may reach 10 mg $\cdot l^{-1}$ of NO_3—N.

2. Interconversion of the different nitrogen forms

2.1. *Oxidation of ammonia*

As early as in 1890 Winogradsky isolated two species of bacteria which oxidise NH_3 in a 2-stage process:

$$NH_3 \xrightarrow{\text{Nitrosomonas sp}} NO_2^- \xrightarrow{\text{Nitrobacter sp}} NO_3^-$$

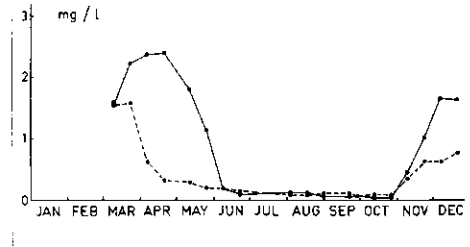
Both of these stages yield energy which is used by the bacteria for the reduction of CO_2. If ammonium salts are added to lake water several days usually elapse before the bacterial populations are sufficiently large to produce a measurable oxidation rate. At low temperatures and in the presence of organic compounds this rate is often low as can be seen from the changes of the concentrations of NH_3 and NO_3^- in Dutch polder lakes (see fig. 1). During the autumm and early part of the winter, water entering those lakes has ammonia concentrations up to 2 mg/l of NH_3—N. The concentration remains high until the temperature begins to rise.
The polder water contains much organic matter which probably inhibits oxidation of the NH_3; nevertheless if the lake water temperature is raised to 10 °C either in the field or in the laboratory the oxidation begins after a lag of a few days indicating that the low temperature in the lake is partly responsible for the low rate of ammonia oxidation. It is possible that the early 'lag phase' before oxidation of NH_4^+ begins, may be due to the oxidation of certain inhibitory substances by other bacterial species.
It can be seen from fig. 1. that not all NH_3 is recovered as NO_3^-. Similar losses are also described from deeper lakes and from artificially managed fish ponds. At least under anaerobic circumstances losses may be caused by bacterial denitrification, when nitrogen is lost as the gas N_2.

2.2. *Synthesis of cellular nitrogen*

Most algae have a relatively high protein content, often exceeding 50 %. Their nitrogen requirements are therefore quite considerable and normally supplied by NO_3^- and NH_3 both of which forms are commonly available. Assuming a mean algal composition $C_5H_7NO_2$ the following reactions describe the conversion of soluble inorganic nitrogen compounds into cellular matter.

$$5CO_2 + 2H_2O + NH_3 \rightarrow C_5H_7NO_2 + 5\,O_2$$
$$5CO_2 + 3H_2O + HNO_3 \rightarrow C_5H_7NO_2 + 7O_2$$

Fig. 1 - NH_3-N (- - - -) and NO_3-N (——) concentrations in Tjeukemeer, 1970 (Golterman, 1973).



The assimilation coefficient reflects the oxidation state of the nitrogen source

$$(\text{A.Q.} = \frac{O_2}{CO_2}).$$

Thus it is approximately 1.0 during NH_3 and 1.4 during NO_3^- uptake. If algae are given both NH_3 and NO_3^-, they appear to prefer NH_3 as source of N.
(Fogg, 1966, Golterman, 1967).
When an algal culture is given NH_4NO_3, the pH falls at first, caused by the NH_4^+ uptake, but it later returns to its original value due to the NO_3^- uptake. Cultures growing on KNO_3 become alkaline.

The preference for NH_3 over NO_3^- as nitrogen source can be attributed to the inhibition of the enzyme nitrate reductase by NH_3 (Syrett and Morris, 1963, Thacker and Syrett, 1972 a + b and Herrera et al 1972) Thacker and Syrett demonstrated that *Chlamydomonas reinhardi* — like *Chlorella* in the earlier studies — did not assimilate nitrate or ammonia unless a suitable source of carbon was provided, either by photosynthesis or in darkness by acetate or accumulated carbon reserves in nitrogen starved cells. The ammonium inhibition seems to be caused more by products of ammonium assimilation or depletion of carbon sources than by the ammonium itself. After depletion of the NH_3 the nitrate reductase activity was resumed again after a lag phase of about 2 hours. Herrera et al showed that ammonium inactivates the nitrate reductase primarily by raising the cellular level of reducing power (—NADH.) which in turn determines the rate of reversible reduction of the enzyme. The inactivation by NADH in cell free extracts could be prevented by NO_3^-.
The formula $C_5H_7NO_2$ does not mean that there are sufficient data to suggest that the mean 'global' algal composition can be calculated. It merely describes the empirical observation that the C/N ratio is often 5 and that protein-content is about 50 %. The formula implies 13 % for the total N content. Allowing 3 % for non amino acid nitrogen the remaining 10 % or so must represent aminoacid-nitrogen. With the commonly used conversion factor 6.2 this 10 % means roughly 60 % protein for algal cells. If the algae contain more fat than normal (certain diatoms in a state of starvation and for synchronised algae during certain stages of their life cycle) the hydrogen content may be increased. The formula is useful inso far as it enables rough calculations to be made concerning nutrient budgets, turn over times of different algal nutrients etc.

2.3. *Denitrification*

Some micro-organisms which can grow aerobically can also grow anaerobically when they use substances other than O_2 as the final electron acceptor. Some *Bacillus* spp metabolise anaerobically, converting nitrate to ammonia, although their presence is not yet reported for many lakes or other surface waters. Baier (1936) however demonstrated reduction of NO_3^- to NH_3 in various ponds in Kiel in some experiments, while in other experiments N_2 and NO_2^- was produced. Baier reported the presence of bacteria comparable to *Thiobacillus denitrificans,* which would suggest the occurrence of the reaction:

$5S + 6NO_3^- + 2H_2O \rightarrow 5SO_4^= + 3\ N_2 + 4\ H^+$

Strains of *Pseudomonas* spp which can convert NO_3^- to N_2 are well known and they are used in sewage-water treatment for removing nitrogen. The same process occurs in anaerobic hypolimnia even before oxygen is completely removed.

Chen et al (1972) showed in a field investigation that when NO_3-N added to lake Mendota sediments was converted to organic and NH_4-N fractions only 37 % was recovered after 4 days while the remaining 63 % was thought to be lost through denitrification. These processes must be taken therefore into account in nutrient budgets especially in seepage lakes receiving nitrate in ground waters. Brezonik and Lee (1968) found a denitrification loss of 11 % of the annual nitrogen input in lake Mendota.

For a long time denitrification was considered not to occur in well oxygenated shallow lakes since the process is anaerobic. Jannasch (1973), however found that if these *Pseudomonas* bacteria occur in water containing much suspended material, then denitrification may take place, probably because a micro-anaerobic zone may develop around the particles.

3. Mineralization of cellular nitrogen

The first extensive studies concerning the mineralization of cellular nitrogen were carried out by Waksmann (1937) (marine algae) and Von Brand (1937-42) · (fresh water algae). Waksmann killed diatoms by suspending them in seawater in the dark and concluded from the resultant bacterial growth that nitrogen was liberated from the diatoms. Von Brand using the same technique found a 100 % liberation of NH_3, which subsequently oxidised to NO_2^- followed by NO_3^-.

Von Brand described his results as showing a quantitative conversion, but in most of his experiments an important increase in total inorganic nitrogen was

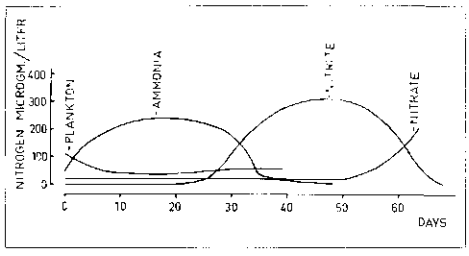


Fig. 2 - The decomposition of nitrogenous organic matter in mixed plankton, showing the appearance of soluble nitrogen compounds in the water in which it is suspended (See v. Brand).

found compared with the amounts of organic cellular nitrogen originally present. Von Brand assumed that this increase was due to oxidation of organic dissolved nitrogen compounds but this seems unlikely as in his later experiments this fraction increased rather than decreased. It is perhaps more likely that the determination of Particulate Nitrogen at zero time did not give a 100 % estimate. His experiments are nevertheless important as they gave the first real evidence for the occurence of rapid bacterial mineralization.

The time scale which Von Brand used in static laboratory experiments should not however be extrapolated to a natural situation. The rate of nitrite formation in his experiments was quite slow, which might be attributable to the long period required to build up a sufficiently large population of relevant bacteria but may also be partly due to a possible inhibition by organic matter.

In natural conditions NH_3 is released every day, so the *Nitrosomonas* population is constantly active and the non-cellular organic matter concentration is much lower than in cultures where read algae are introduced suddenly. The real situation should be studied in a continuous culture. Golterman (1960, 1964, 1968, 1973) could never detect a 100 % conversion of Part. N to NH_3 but found values between 50 and 75 % after 10 - 15 days. Later only traces of nitrite were found and about the same amount of nitrate as ammonia (partly unpublished results). He suggested that the incomplete conversion to ammonia could be due to the fact that the bacteria use one part of the algal protein to build up their own proteins, while a second part is used for the energy demands of the bacteria, the nitrogen only of this latter part being released as NH_3. Furthermore the bacterial nitrogen cannot be separated from the residual algal nitrogen (not 100 % is digestible) so that Von Brand's values, indicating a complete disappearance of part-N, seem unlikely.

Some of the Part-N cannot be hydrolysed or used by bacteria and this will sediment. Very little is known about the processes in the sediments. In some cases N_2 formation has been demonstrated — it is often present in natural gas — but probably the main product formed is NH_3. This might be liberated directly (as seen in hypolimnetic waters) or adsorbed physically and released later. Part of the refractory nitrogen will be in humic compounds.

4. Nitrogen fixation and blue-green algae

4.1. *Occurrence of N_2 fixation*

A review of the earliest demonstrations of the occurence of N_2 fixation by free living organisms in water is given by Fogg and Horne (1967), from whose paper the following is taken. Mortimer (1939) compared the in- and output of nitrogen in Windermere which he found to be 326 and 318 ton respectively per year. A similar budget for lake Mendota gave an input of 156 tons and an output of 40 tons per year (Rohlich and Lea 1949).

The loss of nitrogen is probably caused by sedimentation and subsequent denitrification. Goering and Ness demonstrated the occurence N_2 fixation in L. Mendota. Hutchinson (1941) using bottles of Linsley Pond water found an increase of 0.5 $g \cdot m^{-3}$ of N in 10 days at a time when there was a dense population of *Anabaena circinalis.*

Real progress was made when Neess, Dugdale, Dugdale and Goering (1962) developed a method in which $^{15}N_2$ is used, not unlike the ^{14}C method for measuring primary production. The difference is that ^{15}N is not radioactive and so an expensive mass-spectrometer is essential. The water sample to be analysed is placed in a special 1 l bottle leaving 40 ml of air above the water. The N_2 in the sample is then removed by flushing with a mixture of oxygen (25 %), argon (74,96 %) and carbon dioxide (0.04 %). The bottle is then replaced in the water to be studied, quite often for a period of 24 hrs. $^{15}N/^{14}N$ (atom excess) ratios are then determined from which the amount of N fixed can be calculated if the amount of particulate N is known. Stewart (1966) Neess et al (1962) found fixation for sea-water under normal conditions in the range of 0.05 - 0.15 mg · N_2 per m^3 per hour, with maximal values of 2 mg, these being an order of magnitude greater than those for ammonia uptake.

Using the same technique Horne and Fogg (1970) found total fixation for Windermere to be 10 tons per year. Fogg pointed out that although this quantity is probably not important as a significant factor of the

N-budget for the whole lake it may be important for some waterlayers at certain moments. The fixation appeared to be dependent on both light and the presence of blue green algae.
In 1967 Stewart, Fitzgerald and Burris studied blue-green algal activity both in lake water and culture, using a technique based on the fact that acetylene can be reduced by the algal N-fixing enzyme complex; the ethylene formed is then measured by gas-chromatography. It has been demonstrated that the reaction

$$\underset{\text{acetylene}}{CH \equiv CH} + H_2 \rightarrow \underset{\text{ethylene}}{CH_2 = CH_2}$$

can be used as a quantitative measurement for the reaction since in N-fixing cells: $N \equiv N + 3H_2 \rightarrow 2HN_3$ similarities between the two reactions are shown by the following facts:

1. reduction of acetylene like that of N_2 needs ATP and a reducing agent.
2. Purification of the N-fixing enzyme enhances the acetylene reducing capacity.
3. N-fixation and acetylene reduction are both inhibited or inactivated at the same rate by the same agents.
4. Acetylene (C_2H_2) inhibits N_2 reduction and probably acts as a competetive H-acceptor.

Its sensitivity and cheapness make this method more suitable than the N^{15} method for a wide range of investigations and especially for cultures and cell-free extracts. Comparison between the older biochemical work using anaerobic bacteria and experiments with blue green algae is now possible.
It is wellknown that the facultative anaerobic bacteria *Azotobacter* and *Clostridium* can fix N_2, but with a low efficiency. Stewart (1969) found a fixation of 10 - 20 mg N_2 for *Azotobacter* and 2.27 mg of N_2 for *Clostridium* both per gram carbohydrate oxidised. For lakes this quantity would be of no importance.
The fixation efficiency increases with decreasing O_2 concentration reaching a maximum at 0.001 atm. Apparently there is competition between O_2 and N_2 as final hydrogen acceptor. As early as 1937 N fixation was demonstrated to occur in Myxophyceae (Cyanophyceae) commonly called blue-green algae especially in the filamentous heterocyst forming orders Stigonematales and Nostocales. The following genera from the order Nostocales are of importance in aquatic habitats, especially in eutrophic waters:

a. *Anabaena (flos aquae* and *spiroides).*
b. *Nostoc.*
c. *Aphanizomenon.*
d. *Oscillatoria* (e.g. agardhii)
e. *Anacystis* (or *Aphanocapsa*).

The blue green algae have a nitrogenase enzyme very similar to that occurring in the N-fixing bacteria, with Fe and Mo in the prosthetic group. Nitrogenase activity has been shown to be positively correlated with the presence of heterocysts. Fixation is (of the order of) 0.4 — 10 mg of N_2 per mg of particulate nitrogen per day.
For Tjeukemeer Horne found a fixation of 5 μg N_2 per mg of N per day in the autumn, a considerable contribution to the N balance. For Lake George a value of 33 % is given (Viner and Horne 1971).
It seems likely that in eutrophic lakes N_2-fixation makes an important contribution especially in certain layers when the inorganic nitrogen compounds are exhausted.
If we assume a 10 % N and a 1½ % chlorophyll content for algal cells the N_2 fixation can be expressed as 2.8 - 70 μg of N per mg of chlorophyll per day.
Assuming a respiration rate of 24 mg of O_2 expressed per mg of chlorophyll per day. we can calculate that N_2 is fixed with an efficiency of 3 mg of N_2 per g carbohydrate which compares favourably with the lower efficiences in the N-fixing anaerobic bacteria, especially if it is realised that the protein-nitrogen component usually includes non blue-green algal protein as well.

4.2. *Site of the N_2 fixation in blue green algal cells*

Stewart (1969) suggested that since nitrogenase activity is correlated with the number of heterocysts a count of those could perhaps be used as a rough estimate of N_2 fixation in field studies. The heterocysts are large pale green cells with thick cell walls and are formed by division from vegetative cells. The normal blue green cells are different from those of other algae in that as they contain no visible nucleus or chloroplasts. The chlorophyll is homogeneously 'dissolved' through the whole cell in a manner similar to that found in many bacteria (prokaryotic cell structure).
In addition to chlorophyll and yellow pigments they contain phycocyanine, a pigment that is photosynthetically active.
The arguments that the heterocyst is the site of N_2 fixation are summarised by Fay, Stewart, Walsby and Fogg (1968, Nature) as follows:

1. Only heterocyst containing blue green algae are able to fix N_2, the others are unable to do so at least under aerobic conditions.
2. NH_3 or NO^-_3 inhibits both N-fixation and heterocyst formation.
3. New heterocysts are formed in filaments between two old ones, which might be due to a NH_3 gradient.
4. Spore formation is induced by NH_3 or near heterocysts if no NH_3 is available, but N_2 fixation does take place.
5. In isolated heterocysts respiration creates anaerobic conditions which are essential for the nitrogenase activity.

Furthermore Bone (1971) has shown that nitrate decreases the amount of nitrogenase activity per heterocyst in chemostat culture of *Anabaena flos aquae,* probably as the result of NH_3 formation which may inhibit N-fixation. The activity of the nitrogenase is decreased by a factor of 6 or 9 so that counting heterocysts in a natural algal population gives no indication of N-fixing capacity in the field if NO_3^- is present.
Ohmori and Hattori however found that neither ammonia, nor nitrate suppressed nitrogenase activity in *A.cylindrica* immediately.
When ammonia was added to the culture medium nitrogenase activity decreased rapidly becoming negligible within 24 hours. Variation in enzyme activity during growth in the presence of nitrate was relatively small. The concentrations used are however from ecological point of view rather high (10^{-3} — 10^{-2} M).
A.cylindrica (and *A.flos aquae*) can take up nitrogen concurrently from both N_2 and nitrate, provided that the two are simultaneously supplied.
Fay (1969) has shown that although heterocysts do contain chlorophyll, they, unlike the normal cells do not contain phycocyanine, which is part of photosystem (II), the part that produces the O_2. Furthermore Fay (1970) showed that the action spectrum of acetylene reduction correspond with the light absorption of chlorophyll a indicating the involvement of photosystem I only.
As this is the only system present in the heterocysts and it performs cyclic photophosphorylation without O_2 production it can be explained that in the heterocyst anaerobic conditions may occur while ATP formation is possible through photosystem I.
Furthermore Cox and Fay showed that even when CO_2 uptake was inhibited by CMU (CMU: Chlorophenyl dimethyl urea) N_2-fixation continued being especially great in N starved cells. CMU inhibits specifically photosystem II which is not present in heterocysts.

Bothe and Loos (1972) using uncouplers of the photosynthetic electron transport found that the direct role of photosynthesis in nitrogen fixation is restricted to supply energy by the cyclic protophosphorylation, thus without

oxygen production. The reductant probably ferredoxin — is reduced indepedently from photosynthetic electron transport utilizing probably pyruvate as the reducing substrate through a pyruvate clastic reaction and not the glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, NADPH-ferredoxin oxidoreductase pathway.
Thomas and David (1972) also showed the heterocyst to be the site of the N-fixation at last during active aerobic growth.
Photosystem II pigments reappear in old heterocysts and N fixation capacity is then greatly reduced. Heterocyst development in *Anabaena cylindrica* was studied by Kulasooriya, Lang and Fay (1972) who found that it correlated with N-fixing capacity. Both reached a steady state if the C : N ratio increased from 4.5 : 1 to 8 : 1 if the cells were originally cultured with NH_3. Anaerobic incubation enhanced heterocyst production as well as nitrogenase activity.
Oxygen production by the normal blue green cells inhibits the nitrogenase activity although this enzyme was found to be present in these cells (Haystead et al 1970).
Van Gorkum and Donze (1971) reported that in *Anabaena* under aerobic conditions nitrogen fixation is confined to the heterocysts, while in anaerobic conditions the vegetative cells fix nitrogen as well.
Under normal conditions therefore the nitrogenase of the vegetative cells is presumably not active.
Nitrogen fixation does occur in non-heterocyst containing filamentous algae and in unicellular blue green algae, but only under micro-aerophilic conditions and not under air.

(the second part of this article will be published in the next issue of H_2O)

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