

AUSTRALIA

Patents Act

DECLARATION FOR A PATENT APPLICATION

INSTRUCTIONS

(a) Insert "Convention" if applicable
 (b) Insert FULL name(s) of applicant(s)

In support of the (a) Convention application made by
 (b) GIST - BROCADES N.V., Wateringseweg 1, 2611 XT DELFT,
 the Netherlands

(c) Insert "of addition" if applicable
 (d) Insert TITLE of invention

(hereinafter called "applicant(s) for a patent" (c) for an
 invention entitled (d)
 Anoxic ammonia oxidation

(e) Insert FULL name(s) AND address(es) of declarant(s)
 (See headnote*)

I/XXX (e) Hans Walter RAVEN of Everocken 21, Mijnsheerenland, the Netherlands,
 Director of Patents, Trade Marks and Regulatory Affairs of
 GIST - BROCADES N.V., A Dutch Body Corporate

do solemnly and sincerely declare as follows:

1. XXXXXXXXXXXXXXXXXXXXXXXX

(or, in the case of an application by a body corporate)

1. I am/We XXXX authorized to make this declaration on behalf of the applicant(s).

XX
 XXX

(f) Insert FULL name(s) AND address(es) of actual inventor(s)

2. (f) Arnold Mulder of Kluutring 27, Delft, the Netherlands

(g) Recite how applicant(s) derives title from actual inventor(s)
 (See headnote*)

is/are the actual inventor(s) of the invention and the facts upon which the applicant(s)
 is/are entitled to make the application are as follows:

(g) The applicant is the assignee of the said invention from the actual inventor.

(Note: Paragraphs 3 and 4 apply only to Convention applications)

(h) Insert country, filing date, and basic applicant(s) for the/each basic application

3. The basic application(s) for patent or similar protection on which the application is based is/are identified by country, filing date, and basic applicant(s) as follows:

(h) European patent application No. 88200204.1, filed on February 5, 1988 designating the Netherlands

4. The basic application(s) referred to in paragraph 3 hereof was/were the first application(s) made in a Convention country in respect of the invention the subject of the application.

(k) Insert PLACE of signing

Declared at (k) Delft, the Netherlands

(l) Insert DATE of signing

Dated (l) August 28, 1989

(m) Signature(s) of declarant(s)

(m) GIST - BROCADES N.v.

Note: No legalization or other witness required

To: The Commissioner of Patents

Hans Walter RAVEN

(12) PATENT ABRIDGMENT (11) Document No. AU-B-30502/89
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 614580

(54) Title
ANOXIC AMMONIA OXIDATION

International Patent Classification(s)
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(56) Prior Art Documents
GB 2099807
US 4552663
FR 2434122

(57) Claim

1. A biological denitrification process comprising the use of a microorganism or a combination of microorganisms, capable of bringing about the biological anaerobic denitrification in which ammonium ion is used as electron donor under conditions of pH of 5 to 9, temperature of 5 to 60°C and an initial ammonium concentration of 5-5000 mg/l NH_4^+-N .

PCT

OPI DATE 25/08/89

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w AOJP DATE 28/09/89

PCT NUMBER PCT/NL89/00004

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(21) International Application Number: PCT/NL89/00004**(22) International Filing Date:** 2 February 1989 (02.02.89)**(31) Priority Application Number:** 88200204.1 (EP)**(32) Priority Date:** 5 February 1988 (05.02.88)**(33) Priority Countries:** NL, et al.**(71) Applicant (for all designated States except US):** GIST - BROCADES N.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).**(72) Inventor; and****(75) Inventor/Applicant (for US only):** MULDER, Arnold [NL/NL]; Kluutring 27, NL-2623 NM Delft (NL).**(74) Agents:** HUYGENS, Arthur, Victor et al.; Gist - Brocades N.V., Patents and Trademarks Department, Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).**(81) Designated States:** AU, BB, BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CM (OAPI patent), DK, FI, GA (OAPI patent), HU, JP, KP, KR, LK, MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NO, RO, SD, SE, SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.**Published***With international search report.***(54) Title:** ANOXIC AMMONIA OXIDATION**(57) Abstract**

A biological denitrification is provided in which ammonium ion is used as an electron donor in the denitrification. The process may advantageously be used under conditions of high ammonium ion concentrations in waste water or to remove ammonium ion from manure, soil or surface water. Microorganisms are described which may be used in these processes.

Anoxic ammonia oxidation.

The present invention relates to a process for biological denitrification combined with the oxidation of ammonium ions.

The denitrification process is based on the
5 reduction of nitrate or nitrite ions into nitrogen gas, via the intermediate nitrogen oxides NO and N₂O, by, in essence, aerobic bacteria, see for example R. Knowles, Denitrification, Microbiol. Rev., 46, 43 (1982). The above
10 mentioned nitrogen oxides often act as terminal electron acceptors in the absence of oxygen. Under anoxic conditions, the overall reaction will be (R.K. Thauer et al., Energy conservation in chemotrophic anaerobic bacteria, Bacteriol. Rev. 41, 100 (1977)):

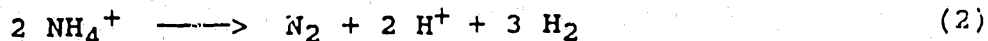


$$\Delta G^{01} = -1120.5 \text{ kJ/reaction}$$

20 In respect of the electron donor, distinction can be made between heterotrophic denitrification, with organic compounds used as electron donor, and autotrophic denitrification with sulphide used as electron donor (see U.S. patent 4,384,956).

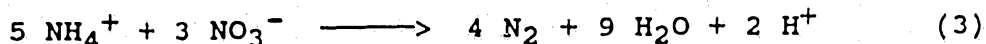
25 E. Broda (Two kinds of lithotrophs missing in nature, Z. Allg. Microbiol. 17, 491 (1977)) suggested that, in theory, ammonium ion can be used as inorganic electron donor as well:

- 2 -



$$\Delta G^{01} = + 79 \text{ kJ/reaction}$$

The overall denitrification reaction (reaction scheme
5 3) is then rather exergonic.



$$\begin{aligned} \Delta G^{01} &= -1483.5 \text{ kJ/reaction} \\ &= - 297 \text{ kJ/M.NH}_4^+ \end{aligned}$$

10

Therefore E. Broda suggested that denitrifying
microorganisms which use ammonium ion as electron donor,
theoretically may exist. However, the existence of these
15 microorganisms has never been demonstrated, as is indicated
in the title of the article by E. Broda "lithotrophs missing
in nature".

As a result of extensive research and experimentation
we have now surprisingly found a process for biological
20 denitrification in which ammonium ion is used as electron
donor in the denitrification.

The advantages of the process are enormous, much
less oxygen is required for the ammonium ion oxidation and
no extra carbon-source is necessary to achieve
25 denitrification.

The process may advantageously be applied under
conditions of high ammonium ion concentrations in waste
water or in cases in which conventional nitrification will
result in high concentrations of nitrate ion in the
30 effluent. For example the process may be applied in the
purification of fish ponds, swimming pools or manure.

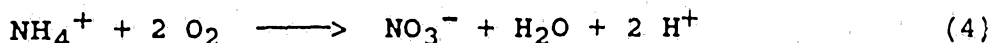
Generally, the denitrification process is preferably
carried out at pH of 5-9, more preferably 6-8 and preferably
at a temperature of 5 to 60°C, more preferably 15 to 40°C.

35 The process is preferably used under conditions resulting in

- 3 -

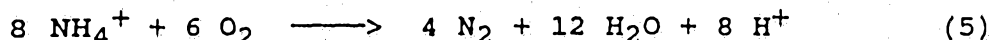
5-5000 mg/l $\text{NH}_4^+\text{-N}$ and 0-5000 mg/l $\text{NO}_3^-\text{-N}$. This gives an overall efficiency of at least 80-90% nitrate removal according to reaction scheme 3.

According to another advantageous aspect of the invention a process is carried out in which the combination of reactions 3 and 4 takes place resulting in the overall reaction scheme 5



10

$$\Delta G^0 = -348.9 \text{ kJ/reaction}$$



15

In this process part of the ammonium ion is oxidized into nitrate ion by nitrifying bacteria. This nitrate ion will be treated further with the remaining ammonium ion, according to reaction scheme 3. This process may be applied where substantially no nitrate is present or when the ratio of nitrate and ammonia is not in proportion to reaction scheme 3.

20

A person skilled in the art will easily find optimal microbiological conditions or will be able to design appropriate reactor(s) in which the process of the invention is to be carried out. For example, when using the process in a waste water purification plant, in which reaction 5 will take place, it is possible to denitrify in more than one reactor, in each part of this the process occurs. One reactor in which the whole process takes place is also possible, for example, by using zones having different reaction conditions or by immobilising all necessary microorganisms on a particulate solid phase e.g. on solid particles.

25

30

The process of the invention is advantageously carried out, for example when treating waste water in an

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activated sludge reactor, fluidized-bed reactor or fixed film reactor.

The ammonium ion oxidation into nitrogen gas using nitrate ion (according to reaction scheme 3) appears from the difference in ammonium ion concentration in the influent and effluent of the process of the invention. Other indications for the presence of ammonium ion oxidation appear from:

- Increase of the use of nitrate ion. At substantially equal nitrate ion supply the nitrate ion concentration in the effluent will decrease proportional with the capacity of the ammonium oxidation.
- Increase of gas production. This partly coincides with the increased conversion of nitrate ion, but because of the conversion of ammonium ion into nitrogen gas (reaction scheme 3), extra gas is produced as well.
- Redox balance. This balance is in equilibrium and makes allowance for the oxidation of the ammonium ion.
- Decrease in pH. During the ammonium ion oxidation (see reaction scheme 3), acid is formed. This may result in a decrease of pH of for example 0.1-0.5 pH unit.

Another aspect of the invention provides the microorganisms which are capable of bringing about the above described process. Preferably these microorganisms are bacteria. The microorganisms may be obtained by natural selection (see for example Example 1) and may be cultivated further in order to use them as inoculation material. They may be present in isolated culture form or may be present as a sludge, preferably a granular sludge, or immobilized sludge. An example of suitable sludge is deposited with the CBS (Centraal Bureau voor Schimmelcultures, Oosterstraat 1, 3742 SK Baarn, The Netherlands) under the accession number CBS 949,87 on December 12, 1987.

The microorganisms may be enriched by inoculation of an anaerobic chemostat feed with mixtures (in e.g. a molar

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ratio of 5:3) of NH_4^+ and NO_3^- as electron donor and electron acceptor. The above mentioned sludge may be used as inoculum. After enrichment the microorganisms may be isolated using standard isolation techniques for ammonium ion oxidizing bacteria as described by B. Sorriano and M. Walker (J. Applied Bacteriology 31: 493-497 (1968)).

Alternatively, number dilution series of chemostat cultures will permit the isolation of the microorganisms by procedures and techniques well understood by persons skilled in the art.

According to another embodiment of the invention (waste) material containing ammonium ion such as manure is inoculated with cultures of the anaerobic ammonium ion oxidizing microorganisms. The cultures may be added in isolated culture form, or as sludge, optionally obtained from processes in which these microorganisms may be present or cultivated.

When, for example, liquid manure is treated according to the invention, an electron acceptor such as nitrate ion may be added to the liquid manure in addition to the inoculated microorganisms.

The liquid manure is often dispersed as such across farming land. Such distribution of the manure on the land is one of the known sources of the so-called acid rain.

Ammonium ion originating from, for example, manure, distributed on farming land is taken up in the air in NH_4^+ form. During rainfall, the NH_4^+ enters the soil or surface water. The nitrifying bacteria then produce the acid according to reaction scheme 4. When NO_3^- is denitrified, part of the acid is removed according to reaction scheme 1. There is a net gain of one H^+ produced per NH_4^+ denitrified. Because the denitrification does not usually take place completely, part of the nitrate ion will accumulate in surface water.

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Addition of the microorganisms described above, to a farming land on which manure is spread, will reduce the volatilisation of ammonia which consequently leads to a reduction of stench emission and contributes to reduce the acid rain problem.

When the ammonia is added to the land, part of this ammonia will be oxidized into nitrate ion by nitrifying bacteria already present in the soil.

The added anaerobic ammonium ion oxidizing microorganisms will be able to convert the ammonia and nitrate ion into odourless gaseous nitrogen, subsequently. Although still acid is formed, now only one H^+ is produced per $2\frac{1}{2} NH_4^+$.

Moreover according to this aspect of the invention less nitrate ion will accumulate in the surface water since part of the nitrate will be denitrified. The microorganisms of the invention may be applied as well on the farming land which is treated with manure as well as on other soil, water (fish ponds, swimming pools, lakes, etc.), which are or will be exposed to acid rain.

Brief description of the Drawings.

Figure 1 shows a fluidized-bed reactor in which the process take place.

Figure 2 shows the concentrations of ammonium in influent and effluent of a fluidized-bed reactor process.

Figure 3 shows the efficiency of the anoxic ammonium removal in this fluidized-bed reactor process.

Figure 4 shows the ammonium and nitrate concentrations during a batch experiment.

Figure 5 shows the gas production during this batch experiment.

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The present invention will be illustrated by the following examples, however, without restricting the scope of the invention.

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Example 1

On top of a fluidized-bed reactor (1) of 17 l capacity and a diameter of 10 cm, a three-phase separator
5 with a content of 6 l was situated (see Figure 1).

The fluidized bed was inoculated with carrier overgrown with biomass originating from a denitrification process. During steady state process conditions, the carrier concentration was 225-275 g/l (3.8-4.7 kg/17 l)
10 corresponding with 150-300 mg o.s./g carrier (o.s. = organic solids) and a mean biomass concentration of 14 g o.s./l. The terminal velocity of the carrier material (sand, 0.3-0.6 mm in diameter) was 175 m/h determined at 30°C in H₂O. The terminal velocity of the overgrown carrier material was 95-
15 145 m/h.

The superficial liquid velocity in the column was maintained during the experiment at 30-34 m/h.

The liquid leaving the reactor via pipe (6) was partly recirculated via pipe (8), the remaining part was
20 discharged via pipe (7).

Waste water (4) was introduced together with the recirculated part of the effluent.

The gas formed was collected in chamber (3) and discharged via pipe (5).

25 The liquid in the reactor was kept at 36°C. The pH was 6.9-7.2.

To obtain the conversion rates the following parameters were measured

- the liquid and gas flows, gas composition
- 30 - influent: NH_4^+ , NO_3^- , NO_2^- and SO_4^{2-} contents
- effluent: NH_4^+ , NO_3^- , NO_2^- and SO_4^{2-} contents.

Via pipe (4) a mixture of NaNO_3 (400-500 ml/h of a 75 g/l solution) and waste water (5-6 l/h) was introduced.

35 The mean composition of the waste water was:

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COD_{centrifuged} 500-800 mg/l, sulfide 125-130 mg S/l, volatile fatty acids about 50 mg COD/l, ammonium 100-140mg N/l. The hydraulic residence time was kept at 3.8-4.6 h. After about 2 months the ammonium ion oxidation started (see Figures 2 and 3). At steady state conditions, the following data were obtained:

Denitrification

specific conversion rate of nitrate ion: 0.5-0.6 kg NO₃⁻-
10 N/m³.d
(nitrogen removed via ammonium ion oxidation is excluded)
conversion rate on basis of sludge: 0.05-0.07 kg NO₃⁻-
N/kg.o.s.d
15 (nitrogen removed via ammonium ion oxidation is excluded).

Sulphide ion oxidation

specific conversion rate of sulphide ion: 0.7-0.8 kg S/m³.d
20 conversion rate on basis of sludge: 0.07-0.08 kg S/kg o.s.d.

Ammonium ion oxidation

specific conversion rate of ammonium ion: 0.4 kg NH₄⁺-N/m³.d
conversion rate on basis of sludge: 0.04 kg NH₄⁺-N/kg o.s.d.
25

In Figure 2, the NH₄⁺-N concentrations of the influent and effluent are given as a function of time. As mentioned above, after about 2 months the ammonium ion oxidation process starts and the ammonium ion concentration
30 in the effluent becomes lower than in the influent. Figure 3 shows the efficiency increases after 2 months up to at least 80%.

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Example 2

A stirred batch reactor (2.4 l) was inoculated with 4.0 g sludge (CBS 949.87) on the carrier (160 mg o.s./g carrier) originating from the earlier described fluidized-bed reactor in Example 1.

The temperature was maintained at 36°C and the pH was 7.0 at the beginning and 7.5 at the end of the experiment (after 700 h).

The concentrations of the ammonium ion and the nitrate ion in the reaction liquid are shown in Figure 4. At time zero, ammonium ion as well as nitrate ion was added. When the nitrate ion concentration became low or no longer detectable, another quantity of nitrate ion was added.

50 mM $\text{NH}_4^+\text{-N}$ and 178 mM $\text{NO}_3^-\text{-N}$ were added in total during the experiment. Theoretically this corresponds with 114 mM N_2 . During the experiment the amount of gas was measured (Figure 5) and the percentage of N_2 in this gas was determined. The amount of N_2 produced was 124 mM which corresponds rather well with the quantity theoretically expected.

Figure 5 shows that during the experiment when after some time the nitrate ion concentration became zero the removal of ammonium ion stopped. The conversion of ammonium ion started again when nitrate ion was added.

Figure 6 shows that when the removal of ammonium ion stopped, the gas production stopped as well.

The overall conversion rate of ammonium ion was 0.7 mg $\text{NH}_4^+\text{-N/g}$ o.s.d and the nitrate ion conversion rate was 2.0 mg $\text{NO}_3^-\text{-N/g}$ o.s.d.

The claims defining the invention are as follows:

1. A biological denitrification process comprising the use of a microorganism or a combination of microorganisms, capable of bringing about the biological anaerobic denitrification in which ammonium ion is used as electron donor under conditions of pH of 5 to 9, temperature of 5 to 60°C and an initial ammonium concentration of 5-5000 mg/l NH_4^+-N .
2. Process according to claim 1 characterized in that part of the ammonium ion is oxidized into the nitrate to be denitrified.
3. Process according to claim 1 or 2 characterized in that the denitrification is carried out in a waste water purification process.
4. Process according to claim 1 characterized in that the denitrification is used to remove ammonium ion from manure.
5. Process according to claim 1 characterized in that the denitrification is used to remove ammonium ion from soil or surface water.
6. Process according to claim 5 characterized in that the ammonium ion originates from manure.
7. Process according to claim 5 characterized in that the ammonium ion originates from acid rain.
8. Microorganisms or combination of microorganisms capable of bringing about a biological denitrification in which ammonium ion is used as an electron donor and deposited as CBS 949.87.
9. Microorganism(s) according to claim 8 which are bacteria.



10. Inoculation material, applicable for a process according to any one of claims 1-7, which at least partly consists of microorganisms according to any one of claims ~~8-10~~^{8 or 9}.

11. A method of biological denitrification comprising utilizing microorganisms according to any one of claims 8 or 9.

12. A process according to any one of claims 1-7 utilising in the denitrification, a microorganism according to any one of claims 8 or 9.

13. A process as claimed in claim 1 substantially as hereinbefore described with reference to any one of the examples.

DATED: 16 January 1991

PHILLIPS ORMONDE & FITZPATRICK

Attorneys for:

GIST-BROCADES NV

David B Fitzpatrick



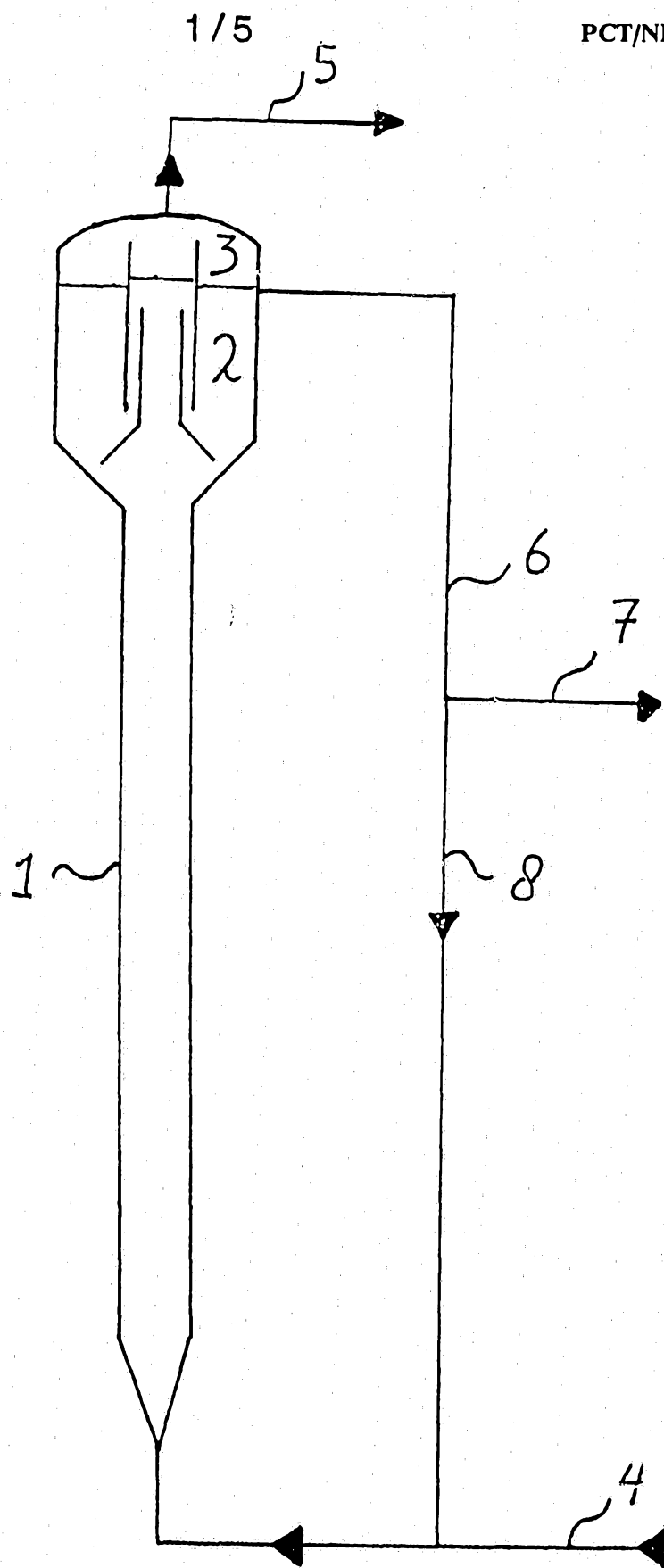
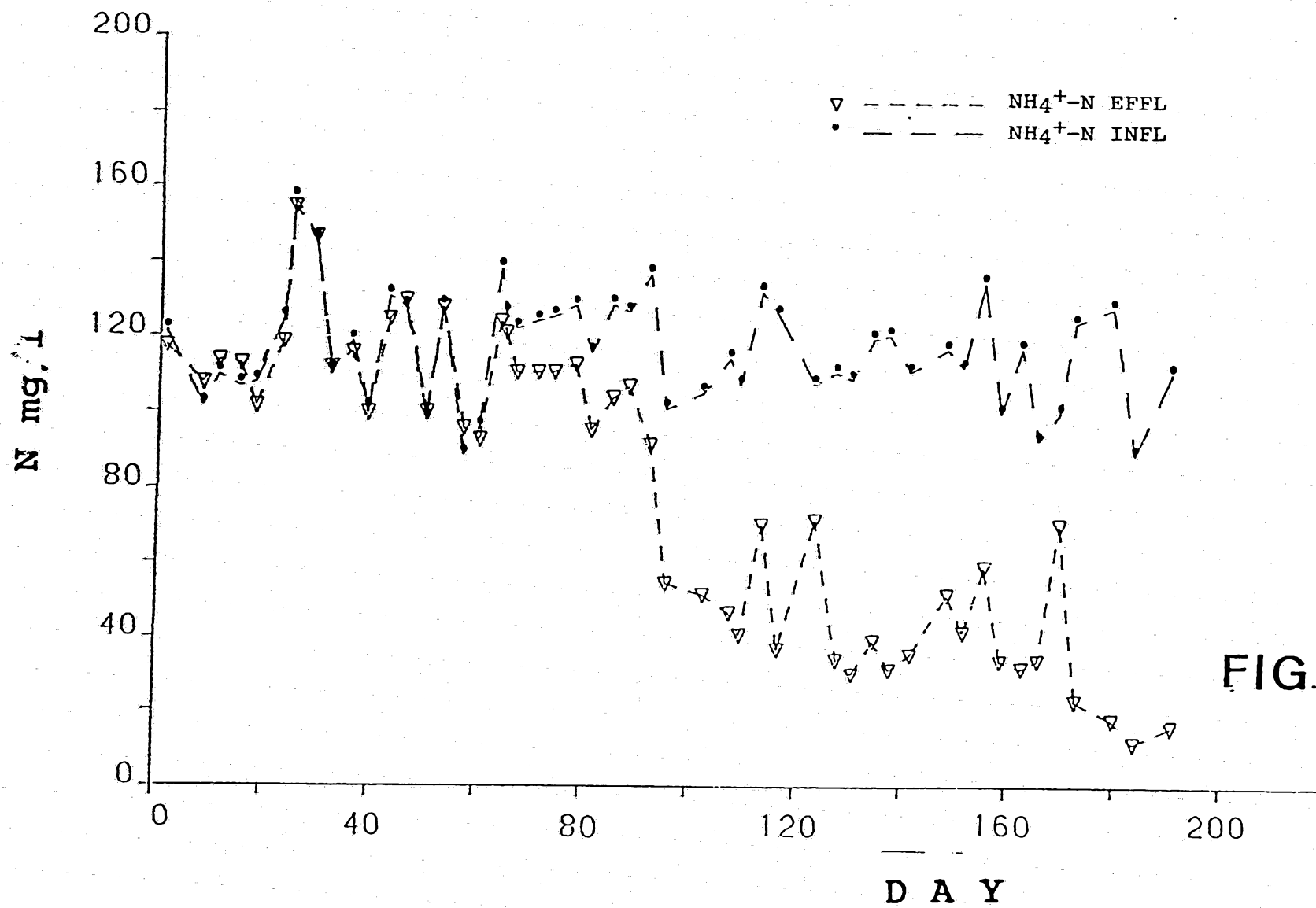
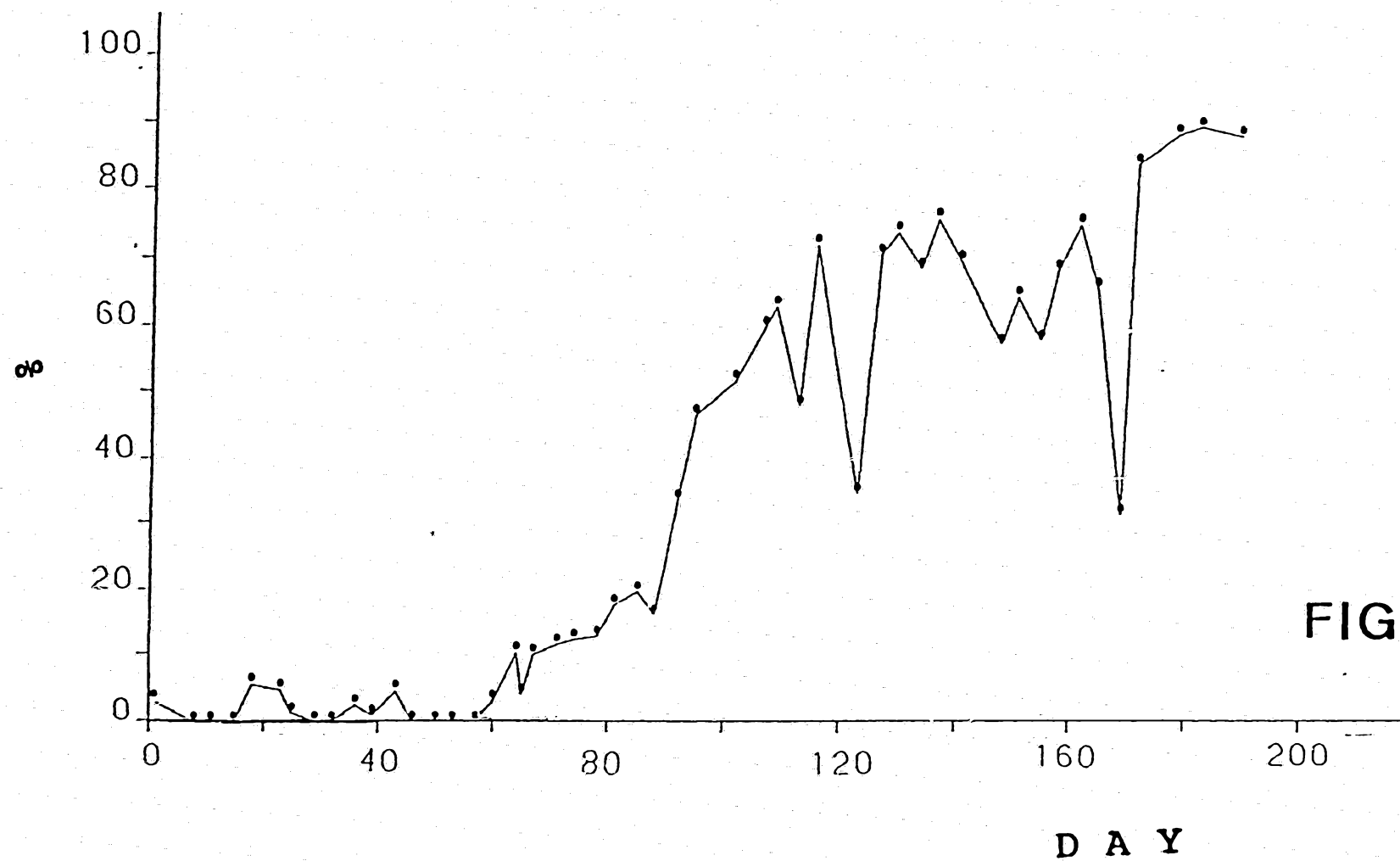


FIG. 1

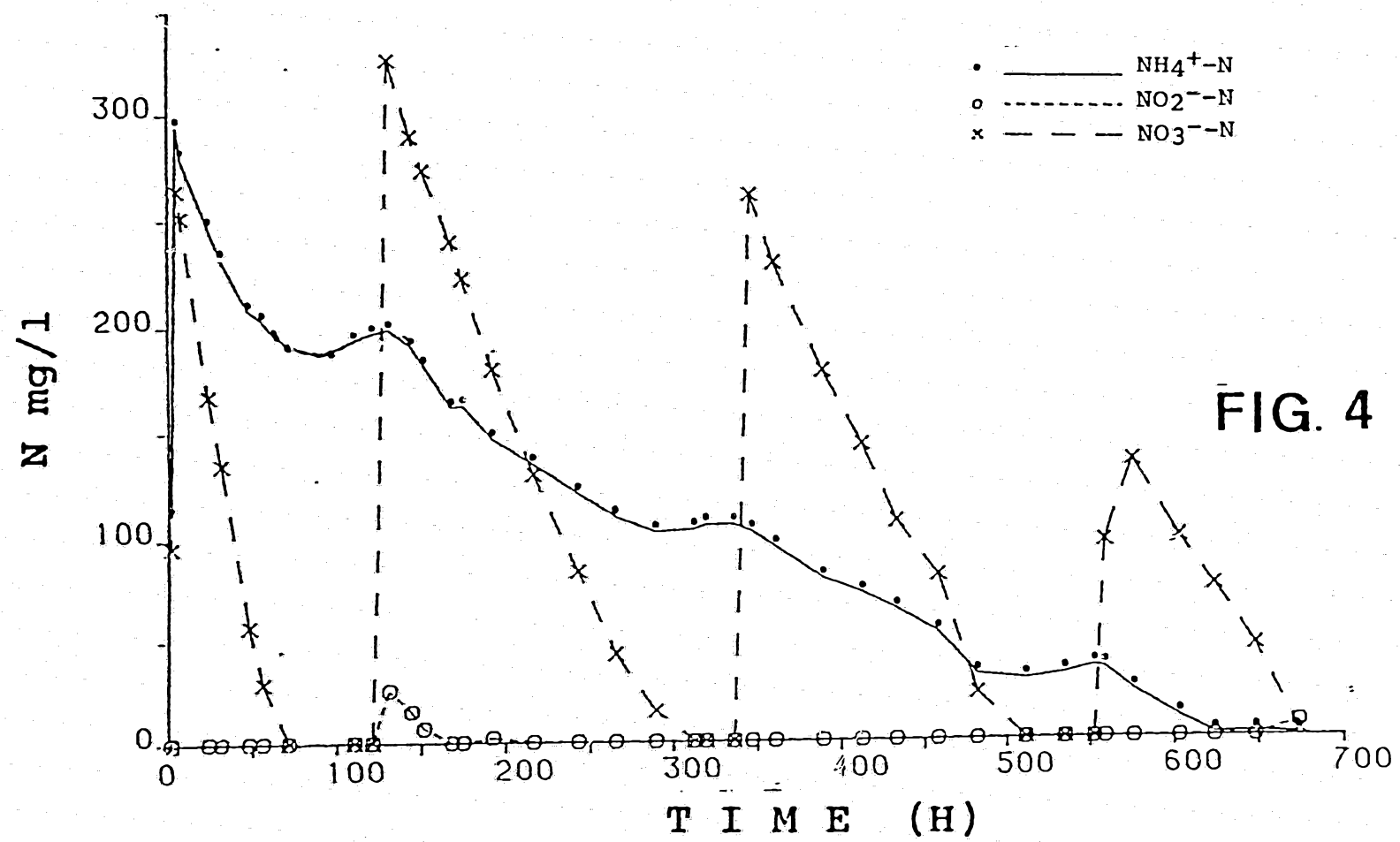
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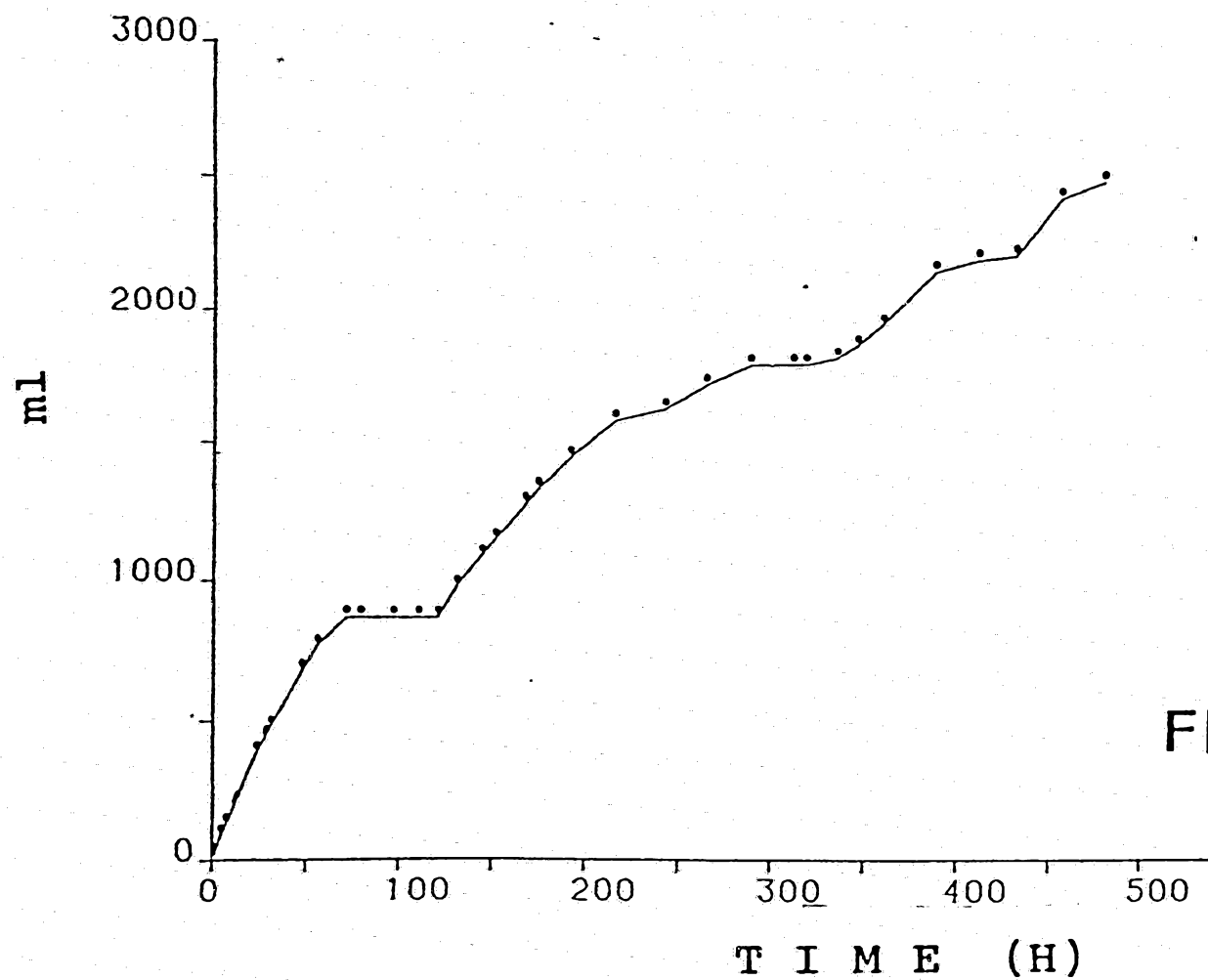


FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 89/00004

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁴ : C 02 F 3/30; C 02 F 3/34		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁴	C 02 F	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ⁶	Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	FR, A, 2434122 (GUBERNATOROVA et al.) 21 March 1980, see page 9, claim 1; page 2, lines 13-20; page 4, line 2 - page 5, line 21	1-3
X	EP, A, 0004304 (BAYER) 3 October 1979, see page 22, claims 1,4; page 24, claim 10; page 3, line 20 - page 6, line 24	1,3,8,9, 11-13
X	US, A, 4552663 (AIR PRODUCTS AND CHEMICALS) 12 November 1985, see column 1, line 31 - column 2, line 49; column 3, line 1 - column 5, line 57	1-3
A	GB, A, 2099807 (KUE TA) 15 December 1982, see page 1, line 38 - page 2, line 8	1,3

* Special categories of cited documents: ¹⁰ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the International Filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the International filing date but later than the priority date claimed "T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "A" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
18th April 1989	- 3 MAY 1989	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	L. ROSSI	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

NL 8900004
SA 26785

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 27/04/89. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-A- 2434122	21-03-80	None	
EP-A- 0004304	03-10-79	DE-A- 2811719	27-09-79
		JP-A- 54127151	02-10-79
US-A- 4552663	12-11-85	EP-A- 0170991	12-02-86
		AU-A- 4556985	06-02-86
		JP-A- 61064394	02-04-86
		AU-B- 558209	22-01-87
GB-A- 2099807	15-12-82	JP-A- 57204294	14-12-82
		AU-A- 8197982	16-12-82
		AU-B- 544855	13-06-85