

CHEMICAL-BIOLOGICAL TREATMENT METHOD FOR NITROGEN POLLUTANTS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority of Singapore Patent Application No. 10202403153X filed on October 9, 2024, the contents of it being hereby incorporated by reference in its entirety for all purposes.

TECHNICAL FIELD

[0002] The present disclosure generally relates to a chemical-biological treatment method for nitrogen pollutants. In particular, the present disclosure relates to a chemical-biological treatment method for removing nitrogen pollutants from wastewater.

BACKGROUND

[0003] Nitrogen pollution is one of the environmental challenges that we face today. Both organic and inorganic nitrogen pollutants are produced by human beings as plastics and fertilizers, but these products often end up in the environments as pollutants. Biological processes may have long been considered the appropriate and economic way to remove nitrogen pollutants such as ammonia and nitrate in wastewater. For example, in the nitrification/denitrification process, ammonia-nitrogen is oxidized to nitrite, nitrate, and eventually become N_2 . However, the process is too slow for high-strength industrial wastewater. The process is also hampered by toxic substances such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and other compounds such as surfactants.

[0004] For chemical treatment processes, advanced oxidation processes (AOPs) are generally used to treat organic nitrogen pollutants such as amines, nitrosamines, and nitriles. In AOPs, oxidant such as ozone is used to oxidize the pollutants into ammonia or nitrate. Although

AOPs are effective methods for treating nitrogen pollutant, the cost of running AOPs is high due to the cost of reagents and energy. During the AOPs, some undesired by-products which are more toxic than the original pollutants may also form.

[0005] It is therefore desirable to provide an alternative treatment method for nitrogen pollutants in wastewater which seeks to address at least one of the problems described hereinabove, or at least to provide an alternative solution.

SUMMARY

[0006] In one aspect, a chemical-biological treatment method for removing nitrogen pollutants from nitrogen-containing wastewater is provided. The method comprises pre-treating the nitrogen-containing wastewater with an oxidizing agent in the presence of a catalyst to form an oligomer mixture; precipitating the oligomer mixture by further addition of the oxidizing agent to obtain a solid precipitate and a supernatant, wherein the supernatant comprises one or more inorganic nitrogen compounds; separating the supernatant from the solid precipitate; and contacting the supernatant with a microbial culture to convert the one or more inorganic nitrogen compounds into nitrogen gas, wherein the catalyst comprises iron-tetraamido macrocyclic ligand (Fe-TAML) or peroxidase; and wherein the microbial culture comprises denitrifying bacteria and anammox bacteria.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] In the drawings, like reference characters generally refer to the same parts throughout the different views. The drawings are not necessarily drawn to scale; emphasis instead generally being placed upon illustrating the principles of various embodiments. In the following description, various embodiments of the invention are described with reference to the following drawings.

FIGs. **1A** and **1B** depict the effect of a catalyst, Fe-TAML on the oxidation of 1 g/L aniline with 1 g/L of hydrogen peroxide (H_2O_2) at 60°C. FIG. **1A** shows that when in the presence of 3 mg/L

Fe-TAML and H_2O_2 , the solution containing aniline turned dark after 1 h. After 24 h, the solution remained dark in color and a layer of solid precipitate formed at the bottom. FIG. **1B** is a control experiment which shows that when the solution containing aniline was subjected to oxidation without Fe-TAML, only a slight color change was observed after 24 h.

FIG. **1C** is a graph illustrating conversion of aniline after 24 h as a function of Fe-TAML dosages.

FIGs. **2A** to **2C** are bar graphs illustrating total dry weights of precipitates (w_s) and suspended solids (w_p) formed with different reaction times at (FIG. **2A**) 20°C, (FIG. **2B**) 40°C and (FIG. **2C**) 60°C, respectively. The H_2O_2 concentration was fixed at 1 g/L.

FIG. **3** illustrates the effect of H_2O_2 concentration and temperature on the formation of solid precipitates.

FIGs. **4A** and **4B** illustrate a two-step chemical process for the pretreatment of aniline wastewater in accordance with some embodiments of the present disclosure.

FIG. **5** is a graph illustrating the removal of ammonium and nitrite using a highly enriched bacterial culture in accordance with some embodiments of the present disclosure.

DESCRIPTION

[0008] The following description sets forth exemplary methods, parameters, and the like. The embodiments are described in sufficient detail to enable those skilled in the art to practise the invention. Other embodiments may be utilized, and structural and logical changes may be made without departing from the scope of the invention. The various embodiments are not necessarily mutually exclusive, as some embodiments can be combined with one or more other embodiments to form new embodiments.

[0009] Features that are described in the context of an embodiment may correspondingly be applicable to the same or similar features in the other embodiments. Features that are described in the context of an embodiment may correspondingly be applicable to the other embodiments,

even if not explicitly described in these other embodiments. Furthermore, additions and/or combinations and/or alternatives as described for a feature in the context of an embodiment may correspondingly be applicable to the same or similar feature in the other embodiments.

[0010] In the context of various embodiments, the articles “a”, “an” and “the” as used with regard to a feature or element include a reference to one or more of the features or elements.

[0011] In the context of various embodiments, the term “about” or “approximately” as applied to a numeric value encompasses the exact value and a reasonable variance, e.g. within 10% of the specified value.

[0012] As used herein, the term “and/or” includes any and all combinations of one or more of the associated listed items.

[0013] By “comprising” it is meant including, but not limited to, whatever follows the word “comprising”. Thus, use of the term “comprising” indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present.

[0014] By “consisting of” is meant including, and limited to, whatever follows the phrase “consisting of”. Thus, the phrase “consisting of” indicates that the listed elements are required or mandatory, and that no other elements may be present.

[0015] In one aspect, the present disclosure relates to a chemical-biological treatment method for removing nitrogen pollutants from nitrogen-containing wastewater. The method comprises pre-treating the nitrogen-containing wastewater with an oxidizing agent in the presence of a catalyst to form an oligomer mixture; precipitating the oligomer mixture by further addition of the oxidizing agent to obtain a solid precipitate and a supernatant, wherein the supernatant comprises at least one or more inorganic nitrogen compounds; separating the supernatant from the solid precipitate; and contacting the supernatant with a microbial culture to convert the one or more inorganic nitrogen compounds into nitrogen gas, wherein the catalyst

comprises iron-tetraamido macrocyclic ligand (Fe-TAML) or peroxidase; wherein the microbial culture comprises denitrifying bacteria and anammox bacteria.

[0016] The “nitrogen-containing wastewater” as used herein refers to wastewater streams that may contain nitrogen pollutants in various chemical forms. The nitrogen-containing wastewater may comprise organic nitrogen pollutants such as amines including aniline, nitrosamine, nitriles, and other nitrogenous organics, and/or inorganic nitrogen pollutants or compounds such as ammonium, ammonia, nitrite, and nitrate. The nitrogen-containing wastewater may be obtained from any source, including industrial, agriculture, aquaculture or domestic sources. Aniline is one of the amines generally found in industrial wastewater. In an exemplary embodiment, the nitrogen-containing wastewater may comprise aniline having a concentration of 1 g/L.

[0017] The oxidizing agent may be any suitable oxidizing agent for use in the treatment of nitrogen-containing wastewater. Such oxidizing agent may include, but not limited to, hydrogen peroxide, oxygen, ozone, sodium percarbonate, and hypochlorite. In various embodiments, the oxidizing agent comprises hydrogen peroxide (H_2O_2). In various embodiments, the oxidizing agent employed in the pre-treating step has a concentration of 1 g/L.

[0018] The pre-treating step is performed in the presence of a catalyst. Iron-tetraamido macrocyclic ligand (Fe-TAML) and enzyme peroxidase are considered as green catalysts, which do not produce or contribute to the formation of toxic by-products during the oxidation of nitrogen-containing wastewater. For example, the metal center of Fe-TAML can be activated by hydrogen peroxide to form a highly active reagent, which may then react with amino groups of the nitrogen pollutants (such as aniline) to initiate an oligomerization reaction. In some embodiments, the catalyst comprises Fe-TAML. In other embodiments, the catalyst comprises an enzyme peroxidase. The catalyst plays an important role in the oxidation reaction. For example, oxidation of aniline in the nitrogen-containing wastewater can be completed within

an hour in the presence of the Fe-TAML, while no oxidation takes place in the absence of Fe-TAML even after 24 hours. The extent of conversion of, for example, aniline may vary depending on the amount of the catalyst used. In various embodiments, the catalyst employed in the method of the present disclosure has a concentration of 3 mg/L.

[0019] In various embodiments, the step of pre-treating is conducted under ambient temperature under a neutral or alkaline condition.

[0020] In various embodiments where the nitrogen-containing wastewater contains aniline, the pre-treating step produces oligomers with 3-6 units of monomers and some inorganic nitrogen compounds including ammonium and nitrate. In conventional methods, oligomers form suspension and cannot be separated easily without additional membrane filtration or centrifugation process. This problem is addressed in the method of the present disclosure by having a treatment process which forms solid precipitate at the end of the reaction such that the pollutants in the form of solid precipitate can be separated easily.

[0021] In various embodiments, the precipitating of the oligomer mixture is conducted by the addition of additional oxidizing agent to continue the oxidation reaction but under a higher temperature. In various embodiments, the precipitating step may be conducted at a temperature ranging from 60°C to 80°C. In some embodiments, the precipitating step may be conducted at a lower temperature such as 20°C to 40°C for a longer period, with decreased precipitation yield. The precipitation duration may be 24 hours or longer.

[0022] In the precipitating step, raising the temperature or heating to 60°C to 80°C or 80°C may promote a cyclization reaction. Products such as N-phenylphenazine may be formed, and they can be separated from the solution as solid precipitates.

[0023] In some embodiments, the concentration of the oxidizing agent added in the precipitating step ranges from 1 g/L to 10 g/L. In an exemplary embodiment, 1 g/L of H₂O₂ may be added to continue the oxidation reaction.

[0024] The supernatant obtained from the precipitating step comprises one or more inorganic nitrogen compounds. In various embodiments, the inorganic nitrogen compounds are selected from ammonium, nitrate and nitrite. In some embodiments, after pre-treating the nitrogen-containing wastewater, nitrate and ammonium are detected as side products of the oxidation reaction. In other embodiments, after pre-treating the nitrogen-containing wastewater, nitrate and ammonium are detected as side products of the oxidation reaction, including traces of nitrites.

[0025] In some embodiments, nitrite is produced by the microbial culture during denitrification process. In particular, the supernatant has a carbon-to-nitrogen ratio of less than 0.5. This allows the denitrifying bacteria and the anammox bacteria to convert the nitrate in the supernatant into nitrite.

[0026] In some embodiments, the method further comprises adding a carbon source prior to or during the step of contacting the supernatant with the microbial culture to adjust the carbon-to-nitrogen ratio of the supernatant to less than 0.5, to allow the denitrifying bacteria and the anammox bacteria to convert the nitrate in the supernatant into nitrite. This applies to situations where the supernatant may not contain carbon. In some exemplary embodiments, the carbon source may include sodium acetate, lactate, or glycerol.

[0027] After the conversion of nitrate into nitrite, the supernatant comprises primarily ammonium and nitrite. Both the ammonium and nitrite are converted simultaneously into nitrogen gas by the microbial culture under an anaerobic condition, in the absence of carbon source, and without any side products. In various embodiments, the ammonium and nitrite in the supernatant are converted into nitrogen gas by bacteria belonging to the *Brocadia* sp. under anaerobic conditions.

[0028] In various embodiments, the denitrifying bacteria are of the genus *Thauera* and the anammox bacteria are of the genus *Brocadia*. Specifically, the denitrifying bacteria is *Thauera*

sp. N101 and the anammox bacteria is *Brocadia* sp. G56. In some embodiments, a highly enriched microbial culture containing two species *Thauera* sp. N101 and *Brocadia* sp. G56 is employed to treat the inorganic nitrogen compounds in the nitrogen-containing wastewater.

[0029] Certain bacteria require a minimal amount of carbon source to sustain growth. For example, *Brocadia* sp. G56 requires a minimal amount of carbon source to maintain its growth and to prevent further reaction of nitrite. In such embodiments, the carbon-to-nitrogen (C/N) ratio may be in the range of less than 0.5, depending on the carbon requirement of the microbial culture. Other suitable ranges of C/N ratio may be employed for denitrification with other types of denitrifying bacteria and anammox bacteria.

[0030] Any suitable types of separating method may be employed to separate the solid precipitate from the supernatant. Examples of separating methods include, but are not limited to, filtration, centrifugation, decantation, sedimentation and combinations thereof. The choice of separating method may depend on factors such as the particle size of the solids, the concentration of the solids in the liquid phase and cost considerations. In some embodiments, the separation is carried out by decanting the supernatant after the solid particles have settled to form the solid precipitate.

[0031] Advantageously, the present disclosure provides a method to overcome challenges in advanced oxidation processes such as Fenton reaction. Generally, Fenton reaction is used for the treatment of non-biodegradable, high-strength industrial wastewaters. Although Fenton reaction is effective, the running cost is high due to the nonspecific nature of the chemical reactions. Moreover, Fenton reaction only happens in a very narrow pH range (2.8-3.2). Hence, pH adjustment is needed prior to the treatment. At the end of the reaction, ferrous ions are oxidized to ferric ions and precipitated as iron sludge together with remaining pollutants. Because the sludge is hazardous, incineration is the only solution, but it is costly and heavily regulated by governments due to air pollution.

[0032] Compared to conventional biological process of treating inorganic nitrogen, the method as disclosed in the present disclosure is more versatile and energy efficient. Only hydrogen peroxide, bacteria and a small amount of metal catalyst are used, making it a green and environmentally friendly process. Hydrogen peroxide is considered green because it decomposes into water and oxygen after usage. As for the nitrogen pollutants, the end products are oligomers and nitrogen gas. The former can be collected and used as a supercapacitor. The latter can be released into the air. The method is suitable for the removal of both organic and inorganic pollutants, and the combined chemical and biological process leads to high treatment efficiency and low cost.

[0033] Furthermore, the method of the present disclosure has a removal efficiency of 96.8% for aniline, indicating its effectiveness in treating industrial wastewater with nitrogen pollutants, making it a cost-effective solution for industrial applications. The highly enriched microbial culture shows a high activity against inorganic nitrogen compounds. It is capable of converting ammonium, nitrite and nitrate to nitrogen gas simultaneously without any inhibition or forming any undesirable side products. The formation of solid precipitates after the chemical treatment allows the reaction products to be separated from the water easily. This feature simplifies the downstream separation process, reducing the need for complex and costly separation techniques.

[0034] To facilitate a better understanding of the present disclosure, the following examples of specific embodiments are given. In no way should the following examples be read to limit or define the entire scope of the disclosure. One skilled in the art will recognize that the examples set out below are not an exhaustive list of the embodiments of this disclosure.

EXAMPLES

Example 1: Chemical process for the treatment of aniline wastewater

[0035] 1. 1 Effect of catalyst on the oxidation of aniline

[0036] The effect of catalyst on the oxidation of aniline is studied. As high-strength wastewater containing organic nitrogen pollutants is too toxic for biological treatments, a chemical process was introduced to reduce its toxicity before the biological treatment. Herein, a synthetic wastewater containing 1 g/L of aniline was used as a model system.

[0037] Firstly, 1 g/L of H₂O₂ was added to the solution, and the initial pH was 6.9. The reaction was carried out at 60°C for 24 h. To study the effect of catalyst, 3 mg/L of Fe-TAML was also added to the solution. Conversion of aniline was defined as:

$$Conversion (\%) = \frac{c_0 - c}{c_0} \times 100\% \quad (1)$$

where c_0 is the initial aniline concentration, and c is the aniline concentration at time t .

[0038] FIG. 1A shows oxidation of aniline using H₂O₂ in the presence of Fe-TAML (3 mg/L). After 1 h, the solution turned black. After extending the reaction time to 24 h, the color of the solution became darker, and a layer of solid precipitates formed at the bottom. However, in the absence of Fe-TAML, there was only a slight color change after 24 h (FIG. 1B). This experiment demonstrated the important role of catalyst in this reaction.

[0039] Next, the effect of Fe-TAML dosages on the aniline conversion after 24 h was investigated and the results are as shown in FIG. 1C. Without the catalyst Fe-TAML, the conversion of aniline was only 15.9%. The result suggests that the oxidation of aniline using H₂O₂ alone was too slow. In contrast, in the presence of 1 mg/L of Fe-TAML, the conversion was 64.6%. The result shows that 1 mg/L Fe-TAML was insufficient and more Fe-TAML was needed to complete the reaction in 1 h. FIG. 1C shows that the conversion increased with increasing Fe-TAML dosages. When 3 mg/L of Fe-TAML was used, the aniline conversion reached 98.0%. Hence, the Fe-TAML dosage was chosen for subsequent studies.

[0040] 1.2 Formation of solid precipitates

[0041] It has been shown that, in the presence of H₂O₂ and Fe-TAML, aniline could be oxidized, and the solution turned dark after 1 h. However, the reaction products became

suspended in the solution and could not be separated easily. To remove them from the solution, a membrane filtration or a centrifugation process is required. This additional step is costly and poses a practical limitation on the process for wastewater treatment applications.

[0042] Hence, in the present disclosure, a treatment method has been developed to form solid precipitates at the end of the reaction, which can be removed more easily. It was found that, when the sample was left standing for 1 day without stirring, a layer of solid precipitate was observed, after which the supernatant was decanted to recover the solid precipitates. Suspended solids in the supernatant was also recovered using a centrifugation process. Both the solid precipitates and the suspended solids were dried and weighted.

[0043] Herein, the total solid weight (wt.) is defined as:

[0044]
$$w_t = w_p + w_s \quad (2)$$

[0045] where w_p and w_s are dry weights of the solid precipitates and the suspended solids in the supernatant, respectively.

[0046] FIGs. **2A** to **2C** show that the amount of total solid weight increased with reaction time. At 20°C, the total solid weight was only 0.330 ± 0.099 g after 1 h of reaction, and it increased to 0.79 g after 24 h of reaction (FIG. **2A**). The same trend was observed at 40°C and 60°C (FIGs. **2B** and **2C**) as well. The result shows that the production of solids was much slower than the oxidation of aniline. There was probably a further oxidation reaction of the initial reaction products that led to the formation of the solid precipitates. Furthermore, as shown in FIG. **2C**, the total solid weight after 4 h of reaction reached 0.44 g, which was slightly higher than the total solid weights at 20°C and 40°C. It indicates that a higher temperature benefited the formation of solid in the reaction, but the effect was marginal.

[0047] To obtain more solids in the reaction, the H_2O_2 concentration was increased and the reactions at 20°C, 40°C and 60°C were repeated. FIG. **3** shows that with an increase H_2O_2 concentration, the amounts of solid precipitates also increased. The effect was apparent at 60°C

with 8 g/L of H₂O₂ added, at which a thick layer of solid precipitate was formed at the bottom. Meanwhile, the supernatant showed a purple color, suggesting that a higher amount of H₂O₂ caused further oxidation of the reaction products in the solution.

[0048] 1.3 Two-step treatment process for aniline wastewater

[0049] Because the oxidation of aniline was very fast and could be completed in 1 h, but the formation of solid precipitates occurred on a much longer timescale, a two-step treatment process was developed for the treatment of aniline wastewater.

[0050] In the first step, 3 mg/L of Fe-TAML and 1 g/L H₂O₂ were added to the solution to react with the aniline. The solution was kept under ambient temperature for 1 h. It was noted that after the addition of the Fe-TAML catalyst, the solution turned to a black color within 1 hour as shown in FIG. 4A. However, there was no solid precipitates at the bottom. In the second step, another dosage of H₂O₂ (1 g/L) was added to continue the oxidation reaction, and the temperature was raised to 80°C as shown in FIG. 4B.

[0051] After the reaction, a layer of solid precipitate was formed at the bottom, and the solution turned clear with some black particles suspended in the solution. Subsequently, the supernatant was decanted, and the solid precipitates at the bottom were recovered. After drying and weighting, the solid precipitate accounted for 62.5% of the aniline added. Based on mass spectroscopy results, it is hypothesized that the solid particles are aniline oligomers with 4-6 units. These molecules have a N-phenylphenazine or phenazine structure, whose larger conjugated macrocyclic structure makes precipitation more easily through π - π stacking. Meanwhile, the supernatant was filtered to remove suspended particles in the solution. The solid on the filter paper was dried and weighted. It accounted for 1.3±0.2% of aniline. Combining the solid precipitate at the bottom and suspended solid particles gives a total solid yield of 63.8±1.6%. This number indicates the amount of aniline that can be converted to solid and recovered through filtration. Overall, the total aniline conversion could reach 96.8±0.1%.

[0052] In the filtered supernatant, 17.62 mg-N/L of nitrate and 3.28 mg-N/L of ammonium were detected, suggesting that part of the aniline can be converted to inorganic nitrogen species through a series of oxidation reactions. The presence of nitrate (or other inorganic nitrogen species) can be treated using a biological process with a highly enriched culture as will be described in the following section.

[0053] In the second step, the effect of temperature was further studied. Table 1 shows that a high temperature in the second step is beneficial to both precipitation and total solid yield. When the temperature was 20°C (i.e. no heating in the second step), the precipitate yield was only 19.1±1.1%. However, the total solid yield was 56.5±1.8, which was only slightly lower than that at 80°C. The result suggests that reaction products at a lower temperature were suspended solid particles which did not precipitate by themselves. However, they still could be separated from the solution using filtration. Based on the result, an optimized condition (3 mg/L Fe-TAML, extra 1 g/L H₂O₂, 80°C) was selected for the treatment of real aniline wastewater.

[0054] **Table 1:** Effect of heating temperature on the precipitation yield, total solid yield and total aniline conversion in a 2-step chemical treatment process for both synthetic and real aniline wastewater. During the heating step, another dosage of 1 g/L H₂O₂ was added.

	Heating temperature (°C)	Precipitation yield (%)	Total solid yield (%)	Total aniline conversion (%)
Synthetic aniline solution	80	62.5±1.4	63.8±1.6	96.8±0.1
	60	32.0±0.5	55.4±0.6	91.6±0.2
	40	26.8±1.6	59.5±2.0	96.8±0.2
	20	19.1±1.1	56.5±1.8	95.6±0.2
Real wastewater	80	55.4±0.6	56.3±0.9	89.4±0.5

* The “±” presented the standard error (n=3).

[0055] 1.4 Treatment of Real Aniline Wastewater

[0056] The optimized procedure was then applied for the treatment of a real aniline wastewater obtained from a local wastewater treatment plant. The sample contained approximately 1 g/L of aniline (992.8 mg/L by HPLC analysis). As shown in Table 1, after the treatment, the aniline conversion and the precipitation yield were 89.4% and 55.4%, respectively, which are slightly lower than the results of the synthetic aniline solution. These results could be caused by interference from other pollutants in the wastewater sample. The result shows that the process can be applied for the treatment of real industrial wastewater.

Example 2: Biological process for the treatment of inorganic nitrogen species

2.1 Simultaneous removal of ammonium and nitrite using a highly enriched culture

[0057] To obtain a highly enriched culture for denitrification, activated sludge (5mL) was transferred from inside an anaerobic chamber to a sealed serum bottle containing 45 mL sterile medium and agitated to obtain a homogeneous suspension. Nitrogen was purged into the medium to maintain an anaerobic condition before bringing the medium into the anaerobic chamber. After several days of incubation, the concentrations of nitrite and ammonium were measured to determine the nitrogen removal activity. Cultures with the highest nitrogen removal rates and efficiencies were transferred to fresh medium (10% V/V). Transfers were repeated until sediment-free cultures were obtained. Dilution-to-extinction (from 10^{-1} to 10^{-9}) of sediment free cultures was conducted and the highest dilution exhibiting nitrogen removal activity was transferred to fresh medium (10% V/V). The dilution-to-extinction process was repeated three times until a highly enriched, anammox bacteria-containing culture with a high nitrogen removal rate and short doubling time was obtained. This highly enriched culture contains two species *Thauera* sp. N101 and *Brocadia* sp. G56 based on 16S rRNA gene sequences.

[0059] In the presence of 100 mg-N/L ammonium and nitrite, the highly enriched culture was able to remove nitrite and ammonium simultaneously in the absence of carbon source.

After 24 h, the ammonium and nitrite concentrations decreased to 18 mg-N/L and 0 mg-N/L, respectively. Meanwhile, the nitrate concentration increased to 62 mg-N/L. Since both ammonium and nitrite were consumed by the bacterial culture, another dosage of 100 mg-N/L ammonium and nitrite were added. Both of ammonium and nitrite were again depleted in 1 day. This result shows that the bacterial culture was able to remove ammonium and nitrite simultaneously and converted them to either nitrate or nitrogen gas. The experiment was continued for 6 days, with the addition of 100 mg-N/L ammonium and nitrite each day. At the end of 6th day, a total of 600 mg-N/L of ammonium and nitrite were consumed by the bacterial culture, and 143 mg-N/L of nitrate was produced.

[0060] 2.2 Effect of carbon-to-nitrogen (C/N) ratio on nitrite and nitrate removal

[0061] Highly enriched culture was used to study the effect of C/N ratio on nitrogen removal. Acetate was used as a sole carbon source in the study. Concentrations of ammonium and nitrite were maintained at 100 mg-N/L each. No nitrate was added to the solution.

[0062] When the C/N ratio was 0.2, the nitrite concentration decreased to 6.6 mg-N/L after 24 h, and the nitrate concentration increased to 42.8 mg-N/L. This result suggests that nitrite could be converted to nitrate during the process, and the microbial culture was unable to convert nitrate to nitrogen gas.

[0063] When the C/N ratio was increased to 0.5, the nitrite concentration decreased to 2.3 mg-N/L, and the nitrate concentration decreased to 21.0 mg-N/L, suggesting that a higher C/N ratio is beneficial to the removal of both nitrite and nitrate. The result also suggests that in the presence of carbon source, nitrate could be converted to nitrogen gas.

[0064] When the C/N ratio was 1.5, both nitrite and nitrate could not be detected after 24 h.

[0065] For the removal of ammonium, the rate was slower. At the C/N ratio of 0.2, the remaining ammonium concentration was 12.5 mg-N/L after 24 h. In contrast, at a C/N ratio of 1.5, 33.5 mg-N/L of ammonium remained in the solution after 24 h. This result suggests that a

high C/N ratio has an adverse effect on the ammonium removal, but it is needed for the removal of nitrate.

[0066] **Table 2:** Effect of C/N ratio on the removal of nitrite and ammonium by the highly enriched culture over 24 h. The initial nitrite, nitrate and ammonium concentrations were 100 mg-N/L, 0 mg-N/L, and 100 mg-N/L, respectively.

C/N ratio	Nitrite (mg-N/L)	Ammonium (mg-N/L)	Nitrate (mg-N/L)
0.2	6.6	12.5	42.8
0.5	2.3	20.2	21.0
1.0	0.8	29.3	10.3
1.5	0	33.5	0

[0067] 2.3 Removal of nitrate in wastewater after a chemical pretreatment process

[0068] It was shown that wastewater containing 1 g/L of aniline could be treated with H₂O₂ and Fe-TAML to produce a layer of solid precipitate. Meanwhile, 17.62 mg-N/L of nitrate and 3.28 mg-N/L ammonium were detected in the solution as a side product of the oxidation reaction. To remove nitrate and ammonium from the wastewater, 1 L of the supernatant was taken, and 20 mL of the enriched culture was added to the solution with 100 mg of sodium acetate. The solution was kept in a closed bottle, and samples were taken from time to time to analyse the nitrate, nitrite and ammonium concentrations. As shown in Table 3, both nitrate and ammonium decreased quickly over time. After 24 h, only 8.34 mg-N/L of nitrate and 1.02 mg-N/L of ammonium remained in the solution. Meanwhile, 13.27 mg-N/L of nitrite was detected. The result shows that nitrate can be converted to nitrite by the bacterial culture in the presence of carbon source. Finally, all nitrogen species were removed with 2 days by the highly enriched culture.

[0069] **Table 3:** Removal of nitrate and ammonium from aniline wastewater using an enriched bacteria culture with additional carbon source (1 g/L sodium acetate)

Time	Remaining nitrate (mg-N/L)	Nitrate removal (mg-N/L)	Nitrite (mg- N/L)	Ammonium (mg- N/L)
0	17.62	0	0	3.28
8	17.07	0.55	0	3.15
12	17.13	0.48	0.42	2.56
24	8.34	9.28	13.27	1.02
36	0	17.62	3.08	0.23
48	0	17.62	0	0

[0070] In summary, a chemical-biological pretreatment process for nitrogen pollutants was developed. In the process, only low-level of hydrogen peroxide and a Fe-TAML catalyst is needed. After the pretreatment, the nitrogen pollutants are oxidized to form a layer of solid precipitates which can be separated easily. The supernatant contains inorganic nitrogen species such as ammonium, nitrite and nitrate, which can be further treated using a highly enriched microbial culture containing two species belonging to the genera *Thauera* and *Brocadia*, in particular, *Thauera* sp. N101 and *Brocadia* sp. G56, wherein the microbial culture exhibits aerobic denitrification behaviour. All three inorganic nitrogen species (i.e. ammonium, nitrite and nitrate) can be converted into nitrogen gas with only a minimal amount of carbon source.

[0071] The method of the present disclosure is generally applicable for the removal of nitrogen pollutants from wastewater including industrial and domestic wastewater. In some embodiments, the biological process can be used independently to remove inorganic nitrogen pollutants from agriculture and aquaculture wastewaters.

[0072] Although embodiments of the invention have been shown and described, the invention is not limited to the described embodiments. Instead, it would be appreciated by those skilled in the art that various modifications and variations can be made to the embodiments of the invention without departing from the scope of the invention, the scope of which is set forth in the following claims.

CLAIMS

1. A chemical-biological treatment method for removing nitrogen pollutants from nitrogen-containing wastewater, the method comprising:
 - pre-treating the nitrogen-containing wastewater with an oxidizing agent in the presence of a catalyst to form an oligomer mixture;
 - precipitating the oligomer mixture by further addition of the oxidizing agent to obtain a solid precipitate and a supernatant, wherein the supernatant comprises one or more inorganic nitrogen compounds;
 - separating the supernatant from the solid precipitate; and
 - contacting the supernatant with a microbial culture to convert the one or more inorganic nitrogen compounds into nitrogen gas,
 - wherein the catalyst comprises iron-tetraamido macrocyclic ligand (Fe-TAML) or peroxidase; and
 - wherein the microbial culture comprises denitrifying bacteria and anammox bacteria.
2. The method of claim 1, wherein the denitrifying bacteria are of the genus *Thauera* and the anammox bacteria are of the genus *Brocadia*.
3. The method of claim 1, wherein the precipitating step is performed at a temperature ranging from 60°C to 80°C.
4. The method of claim 1, wherein the one or more inorganic nitrogen compounds comprise ammonium, nitrite and nitrate.
5. The method of claim 4, wherein the supernatant has a carbon-to-nitrogen ratio of less than 0.5 for converting the nitrate in the supernatant into nitrite.

6. The method of claim 4, further comprising:
adding a carbon source prior to or during the step of contacting the supernatant with the microbial culture to adjust carbon-to-nitrogen ratio of the supernatant to less than 0.5 for converting the nitrate in the supernatant into nitrite.
7. The method of claim 5 or 6, wherein the ammonium and the nitrite in the supernatant are converted simultaneously into the nitrogen gas under an anaerobic condition, in the absence of carbon source.
8. The method of claim 1, wherein the nitrogen-containing wastewater comprises aniline having a concentration of 1 g/L.
9. The method of claim 1, wherein the oxidizing agent comprises hydrogen peroxide.
10. The method of claim 1, wherein the pre-treating step is performed using the oxidizing agent having a concentration of 1 g/L.
11. The method of claim 1, wherein the oxidizing agent added during the precipitating step has a concentration ranging from 1 g/L to 10 g/L.
12. The method of claim 1, wherein the catalyst has a concentration of 3 mg/L.