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NITRIFYING AND PATHOGENIC BACTERIAL FLUCTUATION DURING NITROGEN REMOVAL FROM WASTEWATER IN ZAKHO CITY/IRAQ

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ABSTRACT:

The high concentration of nitrogen (N) compounds in wastewater was found to be creating a reason of eutrophication, effect the biodiversity of aquatic ecosystems and human health. The aim of this study is to reduce harmful N forms from wastewater by biological nitrogen transforming (BNT) bacteria and to investigate the fluctuation of ammonium oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), and pathogenic bacteria during this process, so compost representative. A sample of municipal wastewater was taken, and then subjected to the treatment process of BNT, which was subdivided into three stages of ammonification, nitrification, and denitrification. The result revealed that nitrifying bacteria were flocculated during biological nitrogen transformation, like AOB and NOB isolated until obtaining colonies during the nitrification process, by liquid mineral solution and Agar-Agar then calculated. The AOB count was 468 CFU/ml and NOB was 2130 CFU/ml in the sample. The existence of coliform bacteria, especially E. coli raises the possibility of water contamination by pathogens bacteria that cause many serious illnesses in human. Therefore, Mac-Conkey agar was used for the isolation of total coliform bacteria through the BNT process because it is selective and also differentiating agar that specialized in the growth of gram-negative bacteria. Total coliform bacteria decreased gradually, before ammonification (556 CFU/ml), after ammonification (226 CFU/ml), after nitrification (154 CFU/ml) and after denitrification (45 CFU/ml) that have been determined and indicated that further chemical disinfection like chlorination or ozonation were required for complete sterilization of pathogenic bacteria. The study concluded that all harmful forms of N and total coliform bacteria that cause human and ecosystem deleterious were reduced by BNT.

Keywords: Biological nitrogen transformation BNT, AOB, NOB and Coliform Bacteria.

1. INTRODUCTION

Wastewater refers to water that has undergone physical, chemical, and biological changes as a result of the incorporation of certain substances, making it unfit for drinking or even other uses (Amoatey & Bani, 2011). Daily activities depends primarily on water to survive and therefore discharge waste into water (Crini & Lichtfouse, 2019). All liquid waste or sewage that originates from homes, hospitals, factories, or any other building using water through its facilities is referred to as wastewater. It is an unwanted byproduct of using water. The general composition of wastewater can include toxic chemicals, salts, oil/grease, heavy metals, solids, nutrients, acid/base and organic plus inorganic materials (Templeton & Butler, 2011).

The breakdown of organic wastes by bacteria, nematodes, or even other microscopic organisms using regular cellular processes is the foundation of biological treatments (Wang et al., 2022). Garbage, waste, and even partially digested food are just a few of the organic materials that are frequently found in wastewater. In addition, it might have poisons, heavy metals, and infectious organisms (Mittal, 2011).

The nitrogen N cycle includes the process of ammonification, which gives organisms the vital N they require to exist. Strock (2008) showed that Ammonification is indeed the process through which microscopic organisms, such as bacteria or even other sorts of decaying creatures, convert compounds containing nitrogen through dead organic matter into simple molecules such as ammonia. So, these tiny creatures support the whole ecosystem. Ammonification, to put it more simply, is just the process of turning naturally occurring nitrogen compounds into ammonia. Ward, Arp, & Klotz (2011) stated that reduced nitrogen molecules, primarily ammonia, are successively oxidized to nitrite and nitrate through the microbial process known as nitrification. Drinking water may contain ammonia due to naturally occurring activities or due to such addition of ammonia throughout secondary disinfection that creates chloramines. Two types of autotrophic nitrifying bacteria, which can create organic molecules utilizing energy via inorganic sources like ammonia as well as nitrite, are principally responsible for such a nitrification process Xiaohong, Juan, Lingli, Zhang, & Youbin (2021)

Hayatsu, Tago, Saito, & Nutrition (2008) mentioned that during the first step of nitrification, ammonia-oxidizing bacteria AOB oxidize ammonia into nitrite based on equation (1).

$$NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^- \quad (1)$$

Other genus, such as *Nitrosococcus* and *Nitrosospira*, have also been involved in this stage, *Nitrosomonas* is the one that has been detected as one of the most frequent bacteria in this stage. Some

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subgenera, including *Nitrosolobus* and *Nitrosovibrio*, can indeed oxidise ammonia autotrophically.

Through the second step of the process, nitrite-oxidizing bacteria NOB oxidize nitrite into nitrate based on equation (2).

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad (2)$$

The main method for removing ammonia mostly from wastewater includes biological nitrifying bacteria, which convert ammonia into nitrate through nitrite (Rani, Chowdhury, Tao, & Nedbalova, 2021)

Although slower than autotrophic species, some heterotrophic bacterial and fungal groupings are also capable of nitrification. According to nitrifying bacterial species found within drinking water systems, the proportion of heterotrophic nitrifiers to autotrophic nitrifiers may be very small. Contrarily, wastewater applications experience heterotrophic nitrification. By transforming nitrate (NO_3) to gaseous nitrogen, the nitrogen component is released back into the air atmosphere even during the denitrification process (N) (Zaidi & Sudthanom, 2011).

The soil-based *Thiobacillus* species *Clostridium & Pseudomonas* bacteria carry out the denitrification process when there is no oxygen present (Pan et al.2020). The above process results in the generation of nitrous oxide (N_2O) plus nitrogen gas, which also are ultimately released into the atmosphere, from nitrate compounds found in both soil and aquatic ecosystems. This process, often known as the microbial process, involves a wide variety of microorganisms (Einsle & Kroneck, 2004). Nitrite → Nitric Oxide → Nitrous oxide → Nitrogen gas.

The main objectives of this research are to reduce harmful nitrogen forms from municipal wastewater and to investigate the fluctuation of AOB, NOB, and Coliform bacterial communities during various steps of biological nitrogen transformation (BNT) as ammonification, nitrification, and denitrification processes from Zakho municipal wastewater in Iraq/Kurdistan Region.

2. MATERIALS AND METHODS

2.1 Description of study area

This study was approved in Zakho City (ZC), which is located in the Kurdistan Region of Iraq among both the latitudes of 37°22' and 37°14' N and 43°11' and 42°74' E and has an altitude of approximately 440m (Jindy et al. 2020).

One site that has a direct release of domestic, industrial and also agricultural wastewater from the point of water before reaching into the Khabor river in Zakho (Figure 1), located at 37°08'47.0"N 42°41'46.0"E, was registered by the Global Positioning System (GPS) program shown in Figure (2).

Figure 1. Wastewater sample location area.

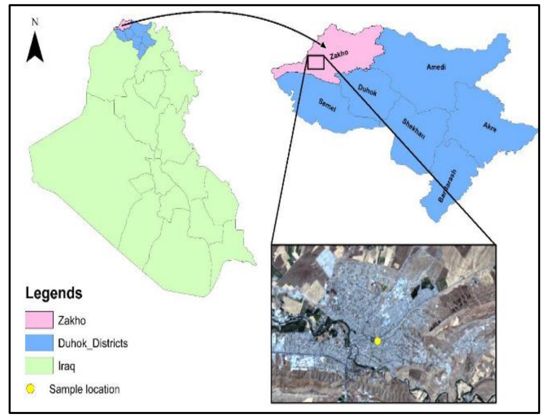

Figure 2. Sample location in Zakho city/Iraq.

2.2 Sample collection for biological nitrogen transforming and coliform during nitrogen removal experiment.

In this study, a wastewater sample from the Zakho municipal sewage system was collected, and eight clean plastic bottles of 20 L capacity were taken from one site location (see Figure 1). The sample was transported into the lab and put the wastewater sample in a container. Then subjected to the treatment process of biological nitrogen transformation which was subdivided into three stages ammonification, nitrification, and denitrification in which three replicates of each stage were conducted in container of 50L as shown in Figure (3). Primary treatment was carried out individually by allowing the grit but also sludge to settle mostly in the bottom of plastic containers. The upper supernatant was therefore transferred to plastic pans of 50 L, and indeed the secondary aerobic biological treatment was carried out by dissolved air flotation (DAF) and putting an electrically powered water pump throughout the pans to aerate the water and activate aerobic microbes to breakdown the organic waste like proteins by biological nitrogen transformation (BNT) to ammonium by ammonification, then this ammonium was converted to nitrite and nitrate through two steps of nitrification process, two weeks period for ammonification, then four weeks for nitrification . Then the water pump is released from the pans to create an anaerobic condition in the pans to allow denitrifying bacteria for another two weeks to convert this nitrate to the gaseous form of nitrous oxide and dinitrogen gas to be liberated to the air. The AOB, NOB, and coliform bacteria were isolated and enumerated to compare each steps of the process and for confirmation, these steps were repeated three times by five replicates of each specific culturing media, also the colour of and the smell of the water changed during this process as shown in Figure (3).

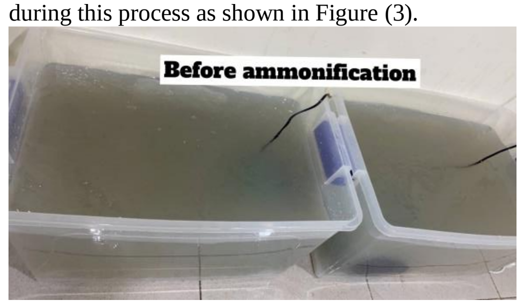

Figure 3. Compare between two steps before ammonification (as control) and after denitrification (final)

2.3 Isolation of Nitrifying bacteria (ammonium and nitrite oxidizing bacterial AOB and NOB) strains from water sample

Throughout the process of both isolating/quantifying selective bacteria, preceding cultures have been created within an enriched and selective AOB and NOB medium through ammonium or through nitrite and with bicarbonate as a source of carbon C, which might last for hours or days, subsequently this type of bacteria grows very slowly because of low energy produced in the process.

The composition of the liquid mineral medium was consisted of; 0.1 g $MgSO_4$ x $7H_2O$, 13.5 g Na_2HPO_4, 0.7 g KH_2PO_4, 0.01 g anhydrous $FeCl_3$, 0.18 g $CaCl_2$ x $2H_2O$, 0.5 g $(NH_4)_2SO_4$ and 0.5 g $NaHCO_3$ for the ammonium oxidizing bacteria AOB. For the nitrite-oxidizing bacteria NOB ammonium sulfate was replaced with 0.5 g of $NaNO_2$. The whole components were dissolved in 1L of distilled water. The final pH was measured by Model HI9024, HANNA Instrument, which have been calibrated by pH 10.0,7.0, plus 4.0 standard buffer solutions and adjusted as required to 7.5. Then the solution was filtered and followed by adjusting with filter paper into two sterile containers (RodríguezRodríguez, MauInchaustegui, PiedraCastro, JiménezMontealegre, & HerreraVargas, 2017).

Later on, a solid medium was prepared by adding Agar-Agar (15g 1L) into distilled water and autoclaved at 121^0C for 20 minutes and stored at 4 ^{0}C for further analysis. Both solutions-AOB and NOB-were diluted 2:1 with distilled water. To emphasize the sterility of the solid medium, Agar-Agar plate was incubated over night at 28^0C. 0.5 ml of the diluted AOB, then it was transferred and separated onto the incubated Agar-Agar plate and 0.2 ml of NOB and analysed in the same way as shown above. Both plates which were incubated at 28^oC for a week grown colonies were between 25-250 CFU. Identification and counting of natural nitrifying bacteria: the growth of the bacteria colonies was observed on the inoculated plates in this study where liquid mineral solutions were incubated at 28^0C. As this study shows, these techniques are generally time-consuming and also eliminate a portion of the actual nitrifying bacteria species through the process. However, during the process the technique of the counting-plate was used. Furthermore, the amount of 468 CFU/ml of water sample for AOB, and 2130 CFU/ml of water sample for NOB were counted Figure (4).

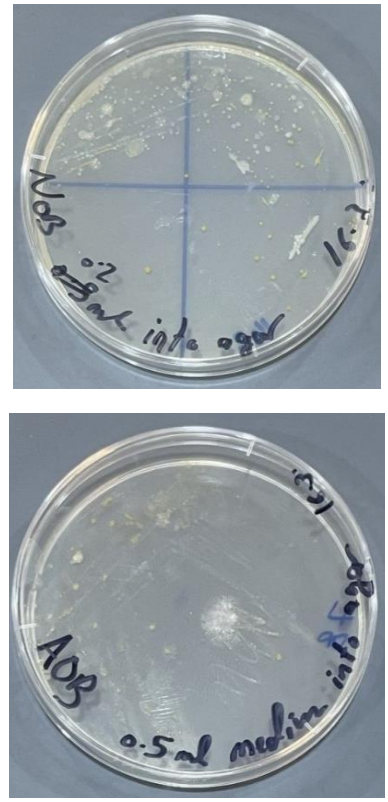

Figure 4. Plates of AOB and NOB.

3. RESULT AND DISCUSSION

3.1 Removal of harmful nitrogen forms.

The total Kjeldahl nitrogen is the most important to be eliminated from the rest of harmful nitrogen forms comes from this form. As indicated in Table (1), the total Kjeldahl nitrogen was reduced by ammonification significantly through a period of 2 weeks as a considerable amount of organic nitrogen converted to ammonia when it is activated by oxygen flotation in this stage. When aerobic condition is continued for another month, the nitrifying bacteria are able to convert the majority of total Kjeldahl nitrogen to nitrate. In the absence of oxygen in anaerobic conditions for another two weeks, the denitrifying bacteria are able to convert this nitrate to the gaseous form of nitrogen and to remove almost total Kjeldahl nitrogen from the wastewater (Yamashita & Yamamoto 2014). Table (1) shows that at the end of denitrifying process nearly half the of nitrite is converted to nitrate. Total nitrogen which includes all organic and inorganic forms of nitrogen in wastewater is significantly removed by BNT steps.

Table 1. Statistical description of nitrogen forms removal from wastewater.

Nitrogen Forms		Mean	± Std. Error	Sig.
Total Kjeldahl Nitrogen TKN mg/l	Control	120.00 a	.577	**
	Ammonification	94.00 b	.577	
	Nitrification	25.00 c	.577	
	Denitrification	15.00 d	.577	
Ammonium Nitrogen AN mg/l	Control	6.600 a	.0577	**
	Ammonification	7.200 a	.0577	
	Nitrification	5.500 b	.0577	
	Denitrification	4.000 c	.5774	
Total Nitrogen TN	Control	140.0 a	.577	
	Ammonification	117.0 b	.577	

mg/l	Nitrification	37.0 c	.577	**
	Denitrification	10.0 d	.577	
Nitrate Nitrogen NO_3-N	Control	16.00 c	.577	**
	Ammonification	18.00 b	.577	
	Nitrification	29.00 a	.577	
	Denitrification	4.00 d	.577	
Nitrite Nitrogen NO_2-N	Control	.140 b	.0057	**
	Ammonification	.0830 c	.0005	
	Nitrification	2.00 a	.5773	
	Denitrification	.006 d	.0005	
Organic Nitrogen ON	Control	112.00 a	.577	**
	Ammonification	85.00 b	.577	
	Nitrification	40.00 c	.577	
	Denitrification	21.00 d	.577	

Table (1) revealed that approximately total nitrogen is removed from waste water, (Rajpal et al., 2021) the total-N was removed around 90%.

As indicated in Table (1), the BNT is efficient in removing all forms of nitrogen from waste water, especially in the end of denitrification process, nitrite reduced about 96% and nitrate reduced to 75%. The organic form of nitrogen in wastewater is the source of continual water pollution by ammonia, nitrite, and nitrate, methane, hydrogen sulfide, and nitrous oxide because it undergoes continual degradation with the time by aerobic and anaerobic microbial respiration. A part of organic nitrogen frequently remains with discharged water even after different methods of treatment as some sorts of organic compounds are hard for degradation and need relative long time. Around 71–87%, 81–93% plus 65–79% of removal amounts for TN, NO3-N and NH4-N loads from sewage water treated reported by Asghari, Bochmann, & Tabari (2022)

As shown in Table (1), different BNT processes were able to reduce organic nitrogen by ammonification, nitrification, and denitrification to a large extend. Also Nourmohammadi (2013) found that the NH_4-N concentration decreased from 26.8 mg/L to 0.29 mg/L after biological treatment, and NO_3-N concentration increased from 8.8 mg/L to 27 mg/L after nitrification process.

3.2 Nitrifying and Pathogenic Bacteria

For *Nitrosomonas* sp. and *Nitrobacter* sp., the shortest times for creation or division discovered in a lab were between seven and ten hours, respectively as reported by Spieck & Bock (2005). However, in natural environments, the majority of known nitrifying bacteria take several days even weeks to complete a similar process, mainly depending just on substrate type, the quantity of oxygen present in the water or soil, temperature, and pH (Spieck & Bock, 2005; Werner & Newton, 2005). Similarly, these bacteria may be affected by the medium composition plus incubation conditions; therefore, it is possible that only some portion of the nitrifying population is considered when quantifying them. Almost every micro-organism has unique physiological, metabolic, as well as morphological traits that set it apart from species within the same genus, from many other genera, as well as from strains within the same species. They might also share traits with one another that serve an evolutionary purpose by giving them the variations and capacities they need to survive in their environment or perhaps to adapt to all of it (Madigan, Martinko, & Parker, 2003). The concentration range of DO in the medium is one of the important factors for the nitrifying process dynamics; the required concentration of oxygen of at least 2 mg/L must be present for the nitrifying method to be successful (Constantine, 2008).

Isolation and counting of pathogenic bacteria (total coliform bacteria) have done during the study by using Mac-Conkey agar, and the number of colonies decreased significantly which means that BNT process can also treat or decrease the water from pathogenic bacteria Figure (5). Therefore, coliform bacteria before ammonification have higher amount according to other steps it was 556 CFU/ml, after ammonification 226 CFU/ml, after nitrification 154 CFU/ml and after denitrification 45 CFU/ml.

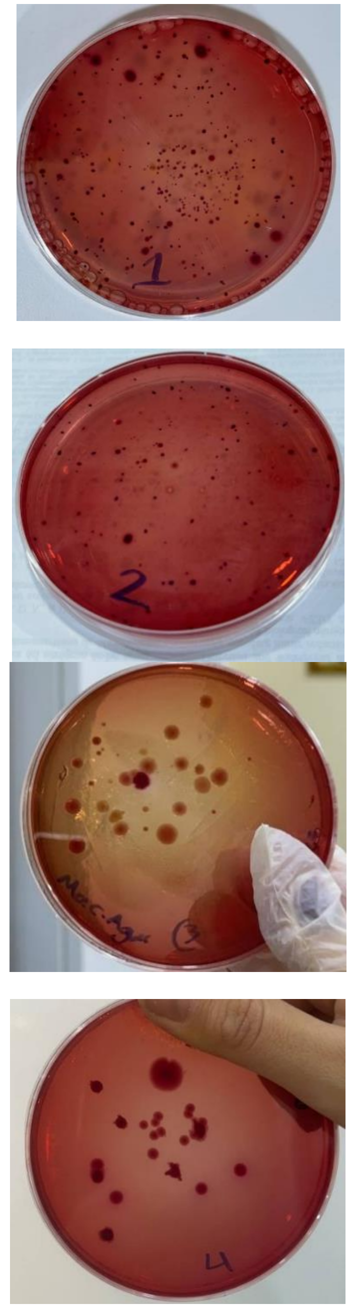

Figure 5. The growth of total coliform bacteria on Mac-Conkey. 1. Before ammonification, 2. After ammonification, 3. After nitrification and 4. After denitrification.

Then gram staining is applied to each of them, gram staining is one of the most used and important techniques used by microbiologists. After applying the stains, the pink color appeared under the microscope because of the gram-negative bacteria as shown in Figure (6).

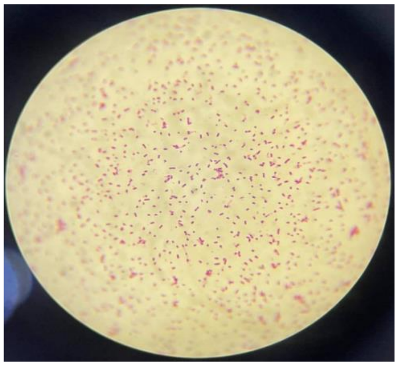

Figure 6. Gram-negative bacteria (total coliform) under microscope.

4. CONCLUSION

As the total amounts of harmful nitrogen forms are significantly removed from the wastewater. The nitrogen removal from wastewater by Biological Nitrogen Transformation (BNT) via ammonification, nitrification, and denitrification is an effective, economic, and eco-friendly method. The BNT was achieved firstly through the action of microorganisms (bacteria). Firstly, the ammonification process converts reduced organic nitrogen (R–NH_2) to reduced inorganic nitrogen (ammonium NH_4^+). Secondly, the nitrification process, which is aerobic, involves converting ammonium in wastewater into nitrates. Two types of bacteria are responsible for nitrification: *Nitrosomonas* and *Nitrobacter*. Finally, the denitrification process converts nitrate to nitrogen through an anaerobic process. So, further investigation on large scale about this process is needed for wastewater treatments. The study concluded that the number of pathogenic bacteria was also significantly reduced by BNT, but still need sterilization agent as chlorination or ozonation to completely lethal this harmful bacterium.

A further study should be conducted on municipal wastewater to analyze soil and sediments that were affected by pollutants. A storm water outlet remediation device should be designed with the purpose of decreasing discharges into Khabor River and efficiently removing dissolved toxic elements by using various filters and absorbents. Furthermore, the study recommends applying and using these procedures before discharging the wastewater into Khabor River.

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