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Biotransformation studies of agricultural nitrogen pollutans in Keduang watershed

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ABSTRACT

The present study seeks to examine nitrogen biotransformation of agricultural wastewater carried out by nitrosomonas and nitrobacter into Ammonia (N-NH_3), Nitrite (N-NO_2), and Nitrate (N-NO_3) in Keduang watershed. Natural capability of the bacteria is necessary to find out to monitor assimilative capacity of the waterbody towards pollutants. Grab sampling technique was applied in agricultural land and Keduang watershed in reference to Indonesian National Standard (SNI) 6989.59:2008. Meanwhile, analysis of N-NO_2 was based on Indonesian National Standard (SNI) 06-6989.09-2004, N-NO_3 on SNI 6989.79-2011, and N-NH_3 on SNI 06-6989.30-2005. The nitrosomonas and nitrobacter were isolated and identified on NA medium considering methods of Capuccino and Sherman (2005). Afterwards, characterization of colony morphology variants was determined, and both gram stain and biochemical test were conducted. A number of 48.8 nitrosomonas colonies/100 mL were identified in samples of agricultural wastewater, which enable to transform Ammonia (N-NH_3) of 0.1390 mg/L into Nitrite (N-NO_2) of 0.0632 mg/L. Meanwhile, a number of 330 nitrobacter colonies/ 100 mL are capable of transforming Nitrite (N-NO_2) into Nitrate (N-NO_3) of 0.2168 mg/L. In conclusion, there is a positive relationship between nitrosomonas in transforming Ammonia into Nitrite and nitrobacter in converting Nitrite into Nitrate. Nitrogen pollutants of the agricultural wastewater in Keduang watershed are able to be reduced by both nitrosomonas and nitrobacter.

1. INTRODUCTION

Keduang watershed is located in Wonogiri regency, Central Java, Indonesia.The population is 339,074 people and the area covers 40,116.0735 Ha,36.7% of which is used for agricultural area. Agricultural activities that are not environmental-friendly are sources of wastewater, and these belong to non-point sources of pollution (NPS) (EPA-US, 2009). Dominant parameters of agricultural activity wastewater include sediment, nitrogen, phosphorus, pesticide, BOD and heavy metals. Non-environmental-friendly agricultural activities cause land erosion and sedimentation (Anshori, 2008), carbamatepesticideresidue (Manuba, 2009), and pollution source parameters of TSS, N-NO_3, P-PO_4 (KemenLH[1], 2010). According to Casali etal. (2010), agricultural activities produce sediment runoff, nitrate (N-NO_3) and phosphate (P-PO_4) running to river stream, and therefore cause water pollution. Keduang watershed outlet transports sediment run off and dissolved nutrients (N-NO_3, N-NH_3, H_2PO_4 and K) continuously about 32% from Keduang watershed, 18% from forests and 17% from agriculturalareas (Durn et.al., 2012). N-NH_3

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(ammonia) sources in river watery areas are resulted from agricultural activities using urea fertilizer intensively (Agustiningsih et.al, 2012).

Farmers make use of inorganic fertilizers, like urea and phonska (Pujiastuti, 2015), with high nutrient content. Urea fertilizers consist of 45-46% of N (Nitrogen), meaning that every 100 kg of urea contains 45-46 kg of Nitrogen. Phonska fertilizer is manure containing main elements of N, P and K with the proportion of 15% of N (Nitrogen), 15% of P (Phosphorus) and 15% of K_2O (Potassiumdioxide) (Maridi etal., 2012). The uses of fertilizer cause the absorption of nitrogen to environment and agricultural activities accelerate nitrogen transformation to water body (Xia etal., 2011). Only 30% of fertilizer is absorbed by roots of plan, and the remaining 70% of the fertilizer will run to rivers and dams as pollutant (Agustiningsih et.al., 2012).

Nitrogen contained in the body of water transforms into ammonia (N-NH_3), nitrite (N-NO_2), nitrate (N-NO_3) and $N_2$with the help of bacteria*Nitrosomonas sp* bacteria play important roles in transforming nitrite and nitrate (Saha etal., 2013). Ammonia and nitrite contents in water are toxics for fish (Titiresmi et.al, 2006), irritating gills and other tissues (Shen et.al., 2003), while nitrate providesnutrientenrichment of aquaticplants and this triggers eutrophication to happen. Eutrophication ofwater body will reduce dissolved oxygen, and therefore the self-purification ability of ecosystem becomes lower (Titiresmi et.al.,2006). This study aims at discussing mechanism of nitrogen biotransformation from agriculturalwastewater by *Nitrosomonas* and *Nitrobacter* bacteria intoammonia (N-NH_3), nitrite (N-NO_2) and nitrate (N-NO_3) in watershed in Keduang. The natural ability of bacteria needs to be observed to monitor assimilation capacity of water body towards pollutant.

2. METHODS

2.1. Sampling

Samples were taken representatively, based on Indonesian National Standard (SNI) of 06-6989.59:2008, from farming areas in watershed of Keduang, in Slogohimo (station 1), Jatiroto (station 2) and Sidoharjo (station 3) sub-districts, Wonogiri regency, Central Java, Indonesia. Water samples were taken in planting season and 7 days after fertilization. In every area, water samples were taken from 4 points, namely: A) farming outlet, B) farming outlet and river convergence, C) 50 m under farming outlet and river convergence, and D) before farming outlet and Keduang river convergence. Chemical and biological analyses were conducted on water samples in laboratory. The chemical analyses included: 1) pH, 2) Dissolved Oxygen (DO), 3) Ammonia (N-NH_3), 4) Nitrite (N-NO_2) and 5) Nitrate (N-NO_3). Meanwhile, biological analyses were carried out to the existence of *Nitrosomonas* and *Nitrobacter* bacteria.

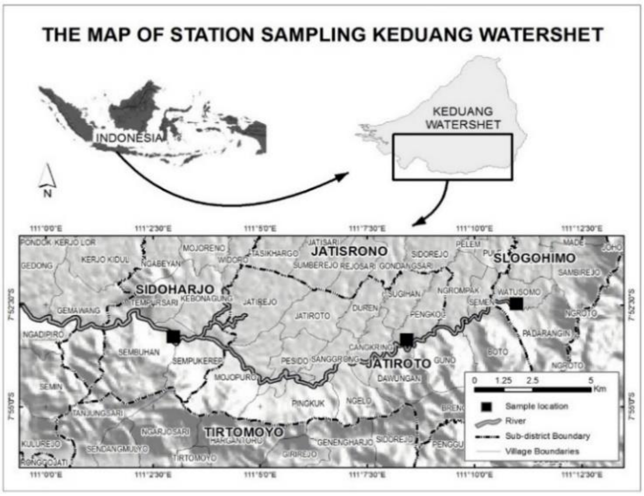

Figure 1. The map of station sampling Keduang watershed

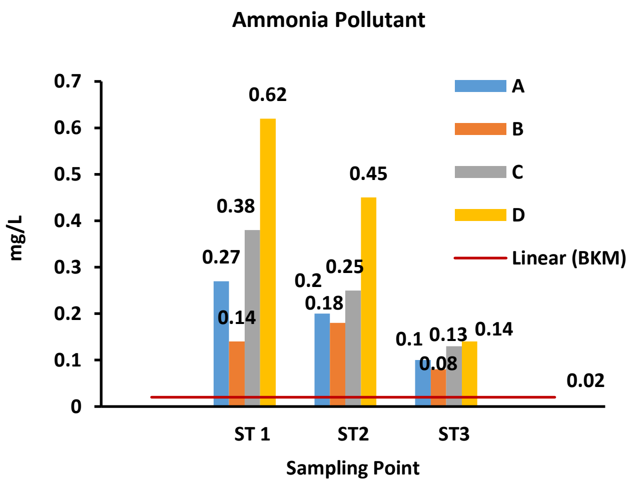

Figure 2. Ammonia pollutans in Keduang watershed from agricultural

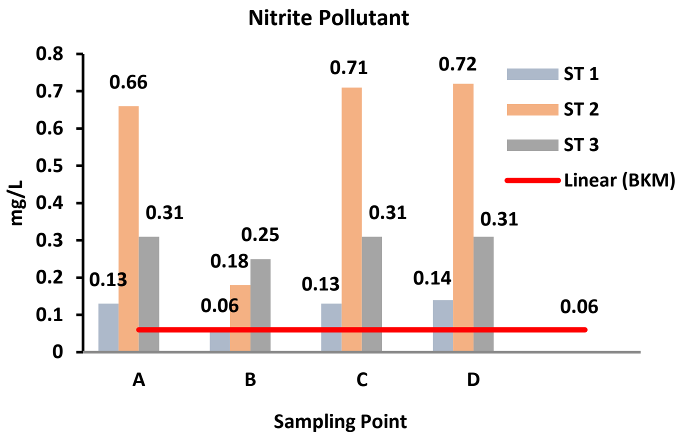

Figure 3. Nitrite pollutans in Keduang watershed

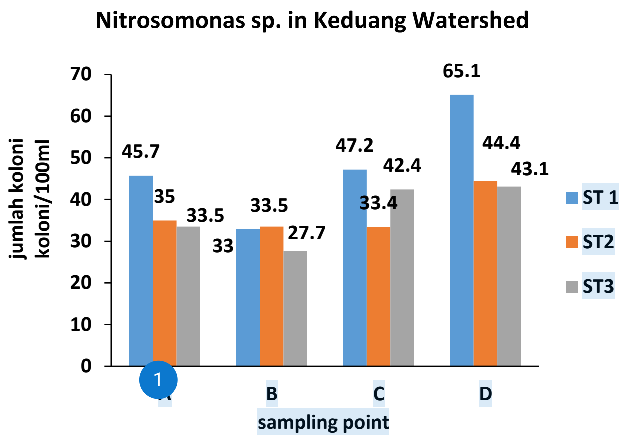

Figure 4. *Nitrosomonas sp.* in wastewater and river water

2.2. Chemical analysis

Analysis of ammonia (N-NH_3) based on Indonesian National Standard (SNI) of 06-6989.30-2005. Analysis of nitrite (N-NO_2) based on Indonesian National Standard (SNI) of 06-6989.09-2004. Analysis of nitrate (N-NO_3) based on Indonesian National Standard (SNI) of 6989.79:2011.

2.3. Biology analysis

The *Nitrosomonas*and *Nitrobacter*were isolated and identified on NA medium considering methods of Capuccino and Sherman (2005). The analysis included qualitative test with gram staining method and enrichment culture method to calculate the number of coloniesas well as qualitative analysis of *Nitrosomonas sp.* and *Nitrobacter sp.* bacteria with gram staining method.

2.4. Correlational analysis of bacteria in transformation process

Correlational analysis of *Nitrosomonas sp.* bacteria for transforming ammonia into nitrite with Chi-Square test using SPSS 17.0 version demonstrated the relationship among *Nitrosomonas sp.* bacteria in the transformation process of ammonia into nitrite. If the value of chi-square$_{count}$ was less than chi-square$_{table}$ (chi-square$_{count}$< chi-square$_{table}$), H_0 was accepted. If the value of chi-square$_{count}$ was greater than chi-square$_{table}$ (chi-square$_{count}$> chi-square$_{table}$), H_0 was rejected. In other words, when probability was greater than 0.05 (probability > 0.05), H_0 was accepted, but when probability was less than 0.05 (probability < 0.05), H_0 was rejected. In which, H_0 meant there was no positive relationship among *Nitrosomonas sp.* bacteria in agricultural water and water in Keduang river when transforming ammonia into nitrite. H_1 meant there was positive relationship among *Nitrosomonas sp.* bacteria in agricultural water and water in Keduang river when transforming ammonia into nitrite.

3. RESULT AND DISCUSSION

3.1. Ammonia pollutans in Keduang watershed from agricultural waste

The activities of non-environmentally friendly watershed, will cause the entry of pollutants into the receiving water body. Sources of ammonia pollutants in river come from agricultural activities that urea fertilizers and domestic activities that contain protein (Agustiningsih etal, 2012). Farmers make use of inorganic fertilizers, like urea and phonska (Pujiastuti, 2015). The ammonia pollutans in the agricultural wastewater and Keduang river at the sampling station are presented in **Figure 2.**

Keduang river water at the sampling point A, at a point before mixing with agricultural waste contains ammonia of 0.27 mg/L at station 1 Slogohimo, 0.20 mg/L at station 2 Jatiroto and 0.1 mg/L at station 3 Sidoharjo.These ammonia pollutants come from residual urea and phonska fertilizers used by farmers, which is not absorbed by the rice plant.Nitrogen fertilizer in rice field water will be degraded into ammonia. Only 30% of fertilizer is absorbed by roots of plan, and the remaining 70% of the fertilizer will run to rivers and dams as pollutant (Agustiningsih et.al, 2012).The use of fertilizer causes the absorption of nitrogen to environment and agricultural activities accelerate nitrogen transformation to the body of water (Xia et.al., 2011).

Nitrogen in body water transforms into ammonia (N-NH_3). The hydrolysis reaction of urea fertilizer in water is displayed below:

$$\underset{\text{Urea}}{CO(NH_2)_2} + H_2O \rightarrow \underset{\text{ammonia}}{2\,NH_3} + CO_2$$

Ammonia nitrogen in watery area is in the form of ammonium ion. The relationship between those two forms is in a balance system below (Titiresmi et.al., 2006):

$$NH_3 + H_2O \rightleftharpoons \underset{\text{ammonium ion}}{NH_4^+} + OH^-$$

Some of the ammonia in the water will undergo biotransformation into nitrite compounds, so the content of ammonia in water is reduced. Decreased ammonia content in outlet river and estuary, because the role of *Nitrosomonas sp.* bacteria in biotransformation process becomes nitrite compound (Saha et.al., 2013).

Ammonia at C sampling point, the meeting between agricultural wastewater with Keduang river water, has increased when compared with ammonia at the previous sampling point. Ammonia at station 1 was 0.38 mg/L, station 2 was 0.25 mg/L and station 2 was 0.13 mg/L. This is due to a meeting between agricultural wastewater with river water Keduang upstream, so that the ammonia content at that point has increased. However, this ammonia increase is not all ammonia to be an enumerating factor at the next sampling point, since some ammonia undergoes transformation by *Nitrosomonas sp.* bacteria into nitrites. Similarly, ammonia at the sampling point D, 5 m after point C, there is an increase in ammonia rather than the previous point. Ammonia point D at station 1 was 0.38 mg/L, station 2 was 0.45 mg/L and station 3 was 0.14 mg/L. The increasing pattern of ammonia at points C and D versus point A and B occurs in all sampling stations. Not all ammonia is transformed into nitrite compounds by *Nitrosomonas sp.* bacteria.

Table 1. The Characteristics of Cell Morphology Gram Staining

Station	Sampling Point	*Nitrosomonas*		*Nitrobacter*	
		Form	Gram	Form	Gram
1	A	Coccus	Negative	Short rod	Negative
	B	Coccus	Negative	Short rod	Negative
	C	Coccus	Negative	Short rod	Negative
	D	Coccus	Negative	Short rod	Negative
2	A	Coccus	Negative	Short rod	Negative
	B	Coccus	Negative	Short rod	Negative
	C	Coccus	Negative	Short rod	Negative
	D	Coccus	Negative	Short rod	Negative
3	A	Coccus	Negative	Short rod	Negative
	B	Coccus	Negative	Short rod	Negative
	C	Coccus	Negative	Short rod	Negative
	D	Coccus	Negative	Short rod	Negative

Table 2. Number of Colony of Nitrosomonas and Nitrobacter Bacteria

No	Analysis	Unit	Station 1				Station 2				Station 3			
			A	B	C	D	A	B	C	D	A	B	C	D
1	*Nitrosomonas sp*	Colony/100 mL	51.4	48.8	53.6	62.0	49.0	45.0	52.1	57.8	41.3	37.5	50.9	56.6
2	*Nitrobakter sp*	Colony/100 mL	45.7	33.0	47.2	65.1	35.0	33.5	35.4	44.4	33.5	27.7	42.4	43.1

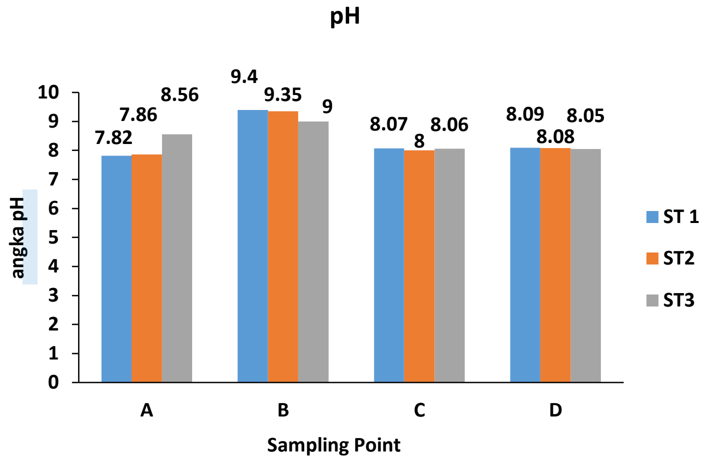

Figure 5. pH in Keduang watershed

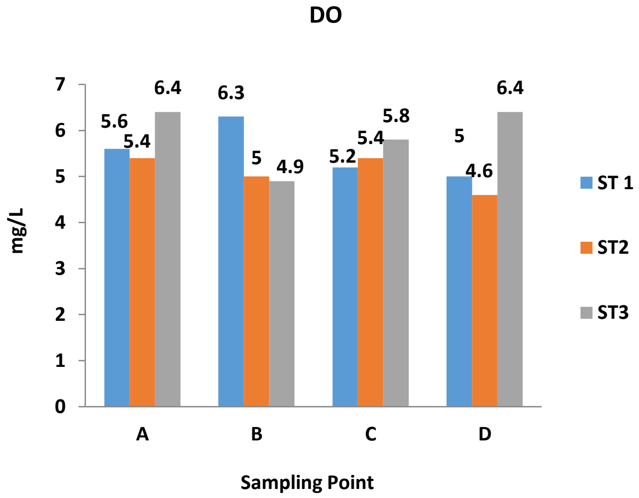

Figure 6. The spreading pattern of DO of theagricultural wastewater and the river water

Ammonia in water can negatively impact aquatic biota, if exceeded water quality standards have been set. Ammonia in water can negatively impact aquatic biota, if exceeded water quality standards have been set. Safe limit for fish to ammonia in water is 0.02 mg/L (Government Regulations No. 82/2001). The concentration of ammonia in the range of 0.2 to 2 mg/L cause toxicity in fish, damage to the gills so that the respiration of fish will be disrupted, and ranges from 0.2 to 9 mg/L of lead toxicity in higher organisms. Ammonia at sampling points C and D on station 1 and station 2 exceeds 0.2 mg/L, it can cause ammonia poisoning effect on the fish. Ammonia in the water of the Keduang river in Slogohimo and Jatiroto areas has the potential to cause ammonia poisoning in fish.

3.2. Biotransformation Amonia (N-NH$_3$) to Nitrite (N-NO$_2$) by Nitrosomonas sp

Ammonia is a nutrient for nitrogen bacteria (Aswadi, 2006). The aforementioned ammonia compound is naturally transformed by *Nitrosomonas sp.* bacteria, contained in the river water, into Nitrite (N-NO$_2$). The first stage of nitrogen biotransformation process is nitrification. It is an oxidation of ammonium ion (NH_4^+) into nitrite ion (NO_2^-) by *Nitrosomonas sp.* bacteria (Saha et.al., 2013). *Nitrosomonas sp.* bacteria need oxygen, the oxidation reaction converts ammonia into nitrite. The chemical reaction is as follows (Aswadi, 2006):

$$NH_4^+ + {}^1/_2O_2 + OH^- \rightarrow NO_2^- + 2H_2O + H^+ + 59.4\ Kcal.$$

The distribution pattern of Nitrite compound on the observed sample is shown in **Figure 3**.

In an aquatic environment with rich amount of ammonia, groups of nitrification bacteria (*Nitrosomonas sp.*) can be found in large number. Through the process of nitrification, the ammonia compound is transformed into nitrite that may be poisonous for the aquatic biota. Nitrite content in the wastewater in watershed of Keduang ranges from 0.06 to 0.25 mg/L. Based on the result of the laboratory analysis, it can be assumed that there is biotransformation process on the agricultural wastewater from ammonia into nitrite with the help of *Nitrosomonas sp.* On the basis of the observation of morphological characteristics of the cells through gram staining, it is found out that there are coccus gram-negative bacteria identifying the existence of *Nitrosomonas sp.*in all sampling points.

The sample of agricultural wastewater is positive with *Nitrosomonas sp.* bacteria, 37.5 - 48.8 colonies/100 ml. It means that in every 100 mL of the wastewater, there are approximately 37.5 – 48.8 colonies of *Nitrosomonas sp.* Once the agricultural wastewater assembles with river water, the content of nitrite ranges from 0.06 to 0.72 mg/L. Nitrite easily evaporates and is easily oxidized into nitrite compounds. It causes the content of nitrite in water to decrease. Nitrite is a toxic nitrogen compound, found in small concentration in the waters (Marganof, 2007). In waters with sufficient oxygen, nitrite is immediately oxidized to nitrate. Class 2 water quality stadard, based on Indonesia regulation No. 82 in 2001, maximum nitrite 0.06 mg/L. Keduang river waters have nitrite content above quality standards. This can endanger the water biota. Nitrite level of 0.5-5 mg/L can have a negative impact on fish life (Suriadarma, 2011). Nitrite is absorbed by fish, will react with hemoglobin, forming methemoglobin which cannot function as an oxygen transporter. Continuous absorption of nitrite by fish, will cause hypoxia and cyanosis. Blood of fish containing methemoglobin will be brown (Suriadarma, 2011).

3.3. Nitrosomonas sp. and Nitrobacter sp. in Keduang watersheed

The results of identification on the characteristics of cell morphology after gram staining were presented in **Table 1** and **Table 2**.

*Nitrosomonas sp.*are bacteriathat contribute to the oxidation process of ammonia into nitrite in nitrogen cycle. Morphologically, this bacterium has short steam, ellipse cell, motile and non-motile, consortium shape and is in pairs as short chain or individual. It is a gram-negative bacterium that has cyomembrane. The cells grow freely in the medium and form thin matrix (Fatmawati et.al., 2012; Masniawati et.al., 2017). This bacterium may optimally grow at 5 - 30°C and with an optimum pH of 5.8-8.5; and live in sea water, fresh water, and soil (Ramadhani, 2015). The spreading pattern of *Nitrosomonas sp.* in the agricultural wastewater and river water of Keduang is shown in **Figure 4**.

The spreading pattern of *Nitrosomonassp.*follows the spreading patterns of ammonia and nitrite. At station 1, every 100 mL of agricultural wastewater in Slogohimo sub-district contains 48.8 colonies of *Nitrosomonassp.*bacteria. In the Rhizosphere rice plants (*Oryza sativa* L.) have *Nitrosomonas*bacteria, around 14,8x10^5 CFU/mL - 19,74x10^5 CFU/mL (Masniawati et.al., 2017). These bacteria play a role in converting ammonia to nitrite. Every 1 liter of agricultural wastewater in Slogohimo sub-district contains 0.14 mg of ammonia, 0.06 mg of nitrite and 4.48 colonies of *Nitrosomonassp.* After the agricultural wastewater flows to Keduang river and assembles in the riverside, this water mixture increases the number of *Nitrosomonassp.*from 48.8 into 53.6 colonies/100 ml. *Nitrosomonassp.*also increases into 62.0 colonies when it arrives at the middle of the river. It also happens in station 2 and station 3 in which the contents of ammonia at the three stations also increase after converging with the river water. The number of *Nitrosomonassp.*influences the amount of nitrite in all sampling points, i.e. the amount of nitrite is lower than ammonia. It proves that *Nitrosomonassp.*can transform ammonia into nitrite. Indeed, it also proves that there is oxidation from nitrite into ammonia. The function of bacteria in transforming nitrogen compound is influenced by the content of dissolved oxygen in the water body. If the dissolved oxygen (DO) in the water is ≥ 4.0 mg/L, it can fulfill the oxygen needed bybacteria to breed and oxidize nitrite into nitrate. The agricultural wastewater and the river water samples contain 4.60 to 6.40 mg/L of DO.

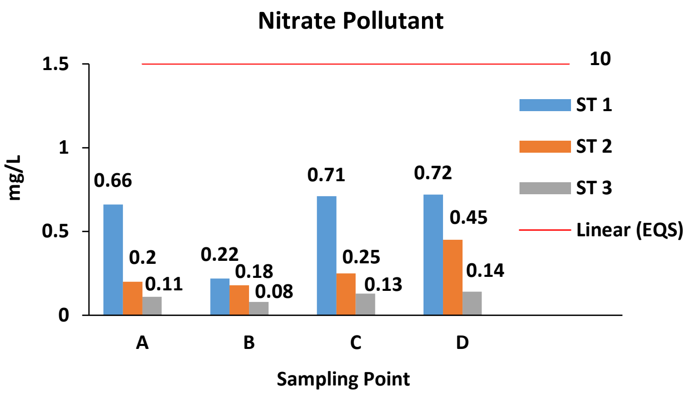

Figure 7. Nitrate in the agricultural wastewater and the river water

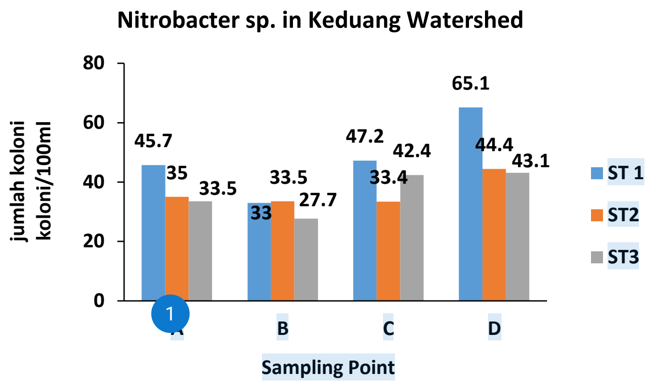

Figure 8. The spreading pattern of Nitrobacter sp. in wastewater and river water

The measurement of pH degree was conducted in the sampling withdrawal process. This step is conducted in order to validate the preciseness of pH values. The pH of the watershed of Keduang ranges from 9.0 to 9.40, higher than the pH of the river water before and after it converges with the agricultural wastewater. In this pH, the processes of nitrification and nitration can occur optimally, i.e. the efficiency rate of the biotransformation process is more than 90% (Shen et.al., 2003). The rate of the nitrification process can decrease at the pH of 6.3 – 6.7 and would be stopped at the pH of 5.0 – 5.5 (Titiresmi et.al., 2006).

The process of nitrogen compounds transformation in water is influenced by the dynamics of the DO (Mayo etal., 2014). DO concentration that is less than 2 mg/L (< 2 mg/L) in water can disturb nitrification process (Titiresmi et.al., 2006). DO samples of the agricultural wastewater and the river water observed range between 4.60 – 6.40 mg/L. It means that every 1 L of agricultural wastewater contains 4.60 – 6.40 mg of dissolved oxygen. The DO spreading pattern of the agricultural wastewater and the river water in three stations is shown in **Figure 6**. It is assumed that the DO values of all sampling points still meet the demand of the optimum DO range for nitrification process.

3.4. Biotransformation Nitrite (N-NO$_2$) to Nitrate (N-NO$_3$) by Nitrobacter sp.

Habitat of *Nitrobacter sp.* is spread in fresh water, sea water and soil (Kiding et.al., 2015). Nitrite compounds in water are transformed into nitrate through oxidation and accelerated by the existence of *Acetobacter sp.* bacteria. This biotransformation is called as nitration, the transform of nitrite compound into nitrate by nitrobacteria colonies, such as *Nitrobacter agilis* (Titiresmi et.al., 2006), *Nitrobacter winogradski* (Inamori et.al., 1997 in Titiresmi et.al., 2006), through the following reaction:

$$NO_2^- + 1/_2O_2 \longrightarrow NO_3^- + 18\ \text{Kcal}$$

Nitrate (N-NO$_3$) compounds resulted from nitrification process with *Nitrobacter sp.*are stable. Nitrite compounds in the agricultural wastewater of Keduang watershed range from 0.06 to 0.25 mg/L. They are transformed by *Nitrobacter sp.* into stableNitrate compounds with the contents of 0.08 – 0.22 mg/L. The meeting point area of the agricultural wastewater and the river water contains 0.13 – 0.71 mg/L of nitrate. The sample of the river water in station 3 contains nitrate compounds of 0.14 – 0.72 mg/L. The contents of nitrate in the agricultural wastewater and the river water are still under the second class of the water quality standard, i.e. of a maximum of 10 mg/L. The spreading pattern of *Nitrobacter sp.* in wastewater and river water, is shown in **Figure 8**.

3.5. The correlational analysisofNitrosomonas sp. and Nitrobacter sp. in the biotransformation of nitrogen compounds

Through chi-squaretest using SPSS, it is found out the value of chi-square$_{count}$of 16.661 for nitrification process and 109.904 for nitration process with the significance level of 0.000. As the significance level is under 0.0005, H_0 is declined which means that there is correlation between *Nitrosomonas sp.* And*Nitrobacter sp.* bacteria in the agricultural wastewater and the river water of Keduang within the process of ammonia biotransformation into nitrite and nitrate. The nitrification is done by *Nitrobacter sp.* bacteria. These bacteria use nitrite as the source of energy and oxygen is usedas the acceptorselectron (Sudarno, 2012). The transformation of nitrite into nitrate by *Nitrobacter sp.* can happen in an aerobic environment, i.e. an environment with the existence of oxygen to survive.

4. CONCLUSION

Nitrosomonas sp. and *Nitrobacter sp.* bacteria play an active role in the biotransformation process of nitrogen pollutans in agricultural wastewater in the Keduang watershed. The ammonia pollutans are transformed into nitrite pollutans by the role of *Nitrosomonas sp.* bacteria, then *Nitrobacter sp.* converted nitrite into nitrate. The samples of the agricultural wastewater and the river water of Keduang show that there are 48.8 – 62.0 colonies/100 mL of *Nitrosomonassp.* bacteria, *Nitrobactersp.* with the number of 33.0–65.1 colonies/100 mL, ammonia (N-NH_3) of 0.08–0.62 mg/L, nitrite (N-NO_2) of 0.06-0.72 mg/L, and Nitrate (N-NO_3) of 0.08 – 0.72 mg/L.Amonia and nitrite levels in the sample, above the class 2 Government Regulations No.82/2001 water quality standard, so that have can an impact on fish and others aquatic biota.

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