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The Effect of Phenol on Heterotrophic Ammonia-Oxidizing Bacteria in Soils and Wastewaters

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ABSTRACT

Nitrification is an important biological process in soils which, ammonia convert to nitrite and subsequently to nitrate, oxidation of ammonia accomplish by Autotrophic and Heterotrophic oxidizer bacteria. In this study, Heterotrophic oxidizer bacteria enumerated in two soil and wastewater samples with different characteristic and displayed that heterotrophic nitrifers in soil samples were higher than number of those in wastewater samples. Different pollutants have effects on nitrification process, one of these are phenol that inhibitor for this process. Phenol is pertaining to poly aromatic hydrocarbons which are toxic chemical material for human and another organism. Add phenol with different concentrations to media of ammonia oxidizer bacteria and investigated decrease of ammonia removal percentage and percentage of nitrite product in soil and wastewater samples. This decline in ammonia removal and nitrite product was surveyed in heterotrophic nitrification. The percentage of elimination ammonia in soil samples were about 67.9 % in the absence of phenol and decreased to about 47.5% in 32 ppm phenol. The percentage of ammonia elimination in wastewater samples were about 91.3 % in the absence of phenol and decreased to about 71.6% in 32 ppm phenol. In soil samples was separated to two heterotrophic ammonia oxidizer bacteria, single and double rod with gram-positive test, this separation was because of vivid decreasing of ammonia removal percentage and in wastewater samples separated bacteria were gram-positive double rod and gram-positive cocci. Heterotrophic ammonia oxidizer bacteria in soil sample were had low resistance to phenol than heterotrophic ammonia oxidizer bacteria in wastewater samples and decreased the ammonia removal percentage in lesser phenol concentration.

***Keywords*:** Nitrification, Phenol, Heterotrophic ammonia-oxidizing bacteria, Ammonia

INTRODUCTION

In nature, the conversion of ammonia to nitrate is known as nitrification that consists of two stages. In the first stage ammonia is converted to nitrite by ammonia-oxidizing bacteria and in the second stage nitrite converts to nitrate by nitrite-oxidizing bacteria (Underhill, 1990). Nitrification process causes the accumulation of nitrate that is the main source of nitrogen for plant growth.

Nitrification occurs in the upper layers of an ecological environment that there is oxygen, while denitrification occurs in the lower layers that have aerobic conditions. Nitrate that was previously produced infiltrates from the upper to lower layers in order to do the denitrification (Knowles, 2000).

Ammonia-oxidizing bacteria are extensively found in soil, salt water and waste water. These bacteria can be found in aerobic environments that mineralizes the organic matter. In nature, these bacteria often found in an environment with unsuitable conditions, for example in conditions with low oxygen, acidic pH, in depth of the ocean where the temperature is less than 5 °C and in the desert with temperatures higher than 60°C, even though nitrifying bacteria that are obligate psychrophile or thermophile not separated (Watson,1989).

Nitrifying bacteria are often autotrophic but there are heterotrophic nitrifying too. Heterotrophic bacteria are also able to eliminate ammonia from soil and waste water, by nitrification and assimilation. In nature, autotrophic nitrifying bacteria are the most important group of nitrites and nitrate producing bacteria, although some of the heterotrophic bacteria and some of fungi are also able to do the nitrification process too (Watson, 1989). Numbers of heterotrophic bacteria can produce nitrite and nitrate from reduced nitrogen compound or from ammonia but their mechanisms are not yet known.

Distinguishing autotrophic and heterotrophic bacteria which accomplish nitrification process is not easy but in an investigative experiment low concentration of acetylene can be used as an inhibitor of ammoniamonooxygenase enzyme which is responsible for the conversion of ammonia to nitrite. Chlorite can also be used as a nitrite oxidation inhibitor; none of these materials have an effect on the nitrification process (Knowles, 2000).

The main objective of this study was the investigation of phenol effects on some industrial soils and wastewaters and activity of heterotrophic ammonia-oxidizer bacteria with regarding to ammonia removal.

MATERIALS AND METHODS

Soil and wastewater sampling

Soils and wastewaters used in this study were taken from chemical refinery Mobarakeh Steel Company in Isfahan.

Two different soils were used: first soil sample was collected from a green field in factory site where green trees were planted. Second sample was collected from the vicinity of the wastewater refinery pool. Both of the soils were taken from the depth of 0-20 cm. Also soil samples were retained in presterilized closed caps so that the moisture was kept until use for microbial analysis. Two wastewaters with different phenol concentration were used. Wastewater samples were collected from refinery pool and the time elapsing between collection and examination was not exceeding 24 hours.

Determination of chemical and physical characteristic of soils and wastewaters

Soil samples were air dried, crushed and passed a 2 mm sieve. Electrical Conductivity of the soil samples were determined by EC meter, model 644, in saturated soil extracts and in wastewater (Page, 1982). Soil acidity was measured in the soil extract samples with pH-meter model 262 (Page, 1982). Soil texture was determined by Hydrometer method. Cation Exchange Capacity was determined by Acetate Sodium method (Rhoades, 1986).

For measuring soil organic matter, wet oxidation was used. Kejeldal method, MgO and Devard alloy reagents are used to determine NH_4- NO_3 in soil samples. At first, soil samples are extracted with 2M KCl and then NO_3 and the NH_4 concentrations are measured **(**Henriksen and Selmer-Olsen, 1970). BOD_5 of the wastewater was determined by phosphate buffer solution (APHA, 1998). Closed inverse titration is used for measuring of COD in wastewater samples, (APHA, 1998).

Determination of phenol

Phenol was measured with Gibbs reagent (2,6-dichloroquinone-4-chloromide). 150 µl supernatant of solution was blended by 30 µl $NaHCO_3$ 1M and 20 µl Gibbs reagent was added. This solution was kept at room temperature for 15 to 45 minutes and then optical densities (OD) were read in 630 nm with spectrophotometer (Quintana, 1997).

Measuring of phenol in soil was performed with method by HPLC.

Determination of ammonia and nitrite

We determined ammonia with Nesler reagent method. This reagent indicates a yellow color in the presence of ammonia. Color absorbance is read at 410 nm with spectrophotometer (Greenbery, 1985).

For solution preparation in this method, 0.5 ml of Nesler reagent and 0.5 ml unknown blended solution were added to 9.5 ml distilled water then the OD was read by spectrophotometer (Greenbery, 1985). Nitrite is measured with α-naphtil amine and sulfanilic acid reagent, which indicates pink color in the presence of nitrite. 0.1 ml of the unknown sample is transferred to 9.9ml distilled water, and then 0.2 ml sulfanilic acid and 0.2 ml α-naphtil amine was added, after 15 to 45 minutes the result is read at 543 nm by spectrophotometer (Greenbery, 1985).

Ammonia and nitrite concentration in the unknown sample is determined with use of standard curve.

Enumeration of heterotrophic ammonia – oxidizing bacteria

Since heterotrophic nitrifiers are found in waste water and soil, the production of nitrite was used to enumerate the M.P.N[1] count of the nitrifying heterotrophic bacteria in media which included nutrient broth plus ammonium sulfate (1g/L) (Emtiazi, 1996). The dilution serial was supplied for waste water and 5-tubes for every dilution were prepared. For soil samples 10 g moisture soil was transferred to a blender and then, 95 ml of sterile 1mM phosphate buffer was added, then the serial dilution was prepared and 1ml aliquots to each of the five culture tubes. They were incubated at 25 to 30°C in the dark then, the pink color seen after instillation of sulfanilic acid and α-naphtil amine shows the positive reaction (Page, 1990).

Separation and isolation of heterotrophic nitrifying

1ml of media which produced nitrite was transferred to test tubes that had 4ml media .After 24 hours incubation samples were cultured by spread plate method (nutrient agar supplemented with ammonium sulfate (1g/l)) (Emtiazi,1996) and incubated at 30°C in the dark for two days.

The effect of phenol on nitrification

Isolated bacteria were selected for phenol consideration effect on nitrification process. For separating the

[1] Most Probable Number

nitrifying bacteria ammonia and nitrite of media was measured. Separated nitrifying bacteria were transferred to phenolic media for investigation of phenol effects on nitrification process. In this study, 1, 2, 4, 8, 16 and 32 ppm phenol were used.

Table 1. Soil properties

	pH	EC (dS/m)	NO_3^--N (mg/Kg)	NH_4^+ - N (mg/Kg)	CEC (Cmol/Kg)	OM (%)	Phenol (ppm)	Moisture content (%)	Soil texture
Soil 1	8.2	4.5	0.63	0.18	8.03	0.63	0	9.35	loam
Soil 2	8.0	0.1	0.27	0.72	12.86	0.1	0	10.8	Clay loam

Table 2. Wastewaters properties

	pH	EC	NH_4^+ - N	NO_3^- -N	phenol	COD	BOD_5
		dS/m	mg/l				
Wastewater 1	9.0	1.8	0.015	0.0085	0.78	28.8	0
Wastewater 2	7.8	1.5	0.028	0.0081	2.5	9.3	0

Table 3. Characteristics of heterotrophic ammonia –oxidizing bacteria in soil

	Soil bacteria	Shape	Gram test
Soil 1	Isolate 1	Short rod	Negative
	Isolate 2	rod	Positive
	Isolate 3	rod	positive
Soil 2	Isolate 4	cocci	Positive
	Isolate 5	rod	Positive

Table 4. Characteristics of heterotrophic ammonia –oxidizing bacteria in waste water

	Waste water bacteria	Shape	Gram test
Wastewater 1	Isolate a	Short rod	Negative
	Isolate b	rod	Negative
	Isolate c	rod	Positive
Wastewater 2	Isolate d	rod	Positive

RESULTS AND DISCUSSION

Characteristics of heterotrophic ammonia –oxidizing bacteria

Heterotrophic bacteria was isolated in soils were 5 types which three of them were from soil 1 (Isolate 1, 2, 3) and the others from soil 2 (Isolate 4, 5). Heterotrophic bacterial isolates in wastewaters were 4 types, isolate a, b and c, were from wastewater 1 and isolate d was from wastewater 2. Morphological characteristics of these bacteria are summarized in Table 3 and 4.

These bacteria are heterotroph, it means their sources of carbon and energy is organic matter which has nitrogen. These bacteria grow in a media that consists of organic matter and ammonia (nutrient agar and ammonium sulfate) and also removes ammonia and produces nitrite on nutrient broth supplemented with ammonium sulfate culture.

Results of enumeration of heterotrophic nitrifying bacteria

Heterotrophic nitrifying bacteria were determined by most probable number (M.P.N. method) and results are determined according to the numbers in each ml of media, Figure 1 shows enumeration of heterotrophic nitrifying bacteria in soils and wastewaters.

Heterotrophic nitrifying bacteria in soil 1 were more than soil 2, but the ammonia concentration in soil 2 was about 4 fold more than soil 1 that is reason for this significant difference in bacterial population (Figure 1).

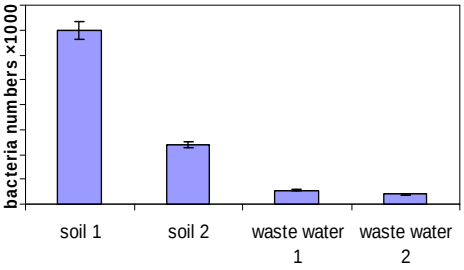

Figure 1. Enumeration of heterotrophic nitrifiers in soils and wastewaters

In soils the numbers of heterotrophic nitrifier were higher than autotrophic nitrifiers, in contrast to aquatic environments. Also the indicated number of heterotrophic nitrifier in garden soil, farm soil and compost soil are higher than their population in stream and river water (Emtiazi, 1996).

Removal of ammonia from soil and wastewater

Removal of ammonia did not occured at the initial stages but during the test, an increased percentage of ammonia elimination was seen (Figure 2). Due to having ammonia elimination percentages isolate 2 and 4 were selected from soil 1 and 2, respectively.

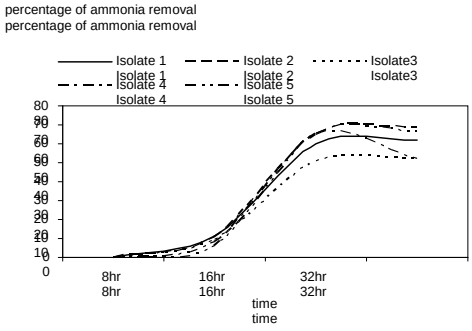

Figure 2. Removal ammonia *vs.* time

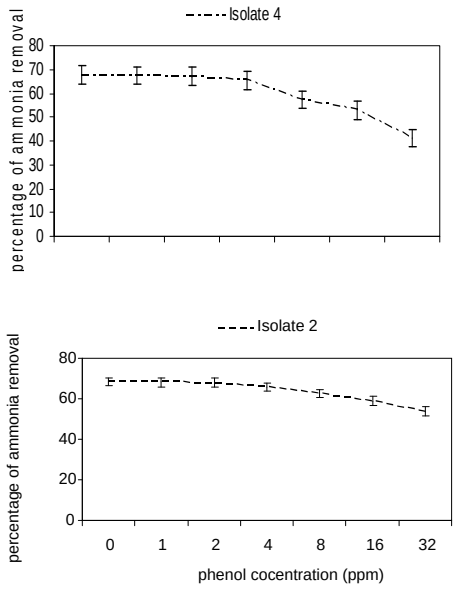

Figure 3. Removal ammonia *vs.* phenol concentration

The percentage of elimination ammonia in isolates 2 and 4 were 68.4 and 67.4 % in the absence of phenol and decreased to 53.8 and 41.2 % in 32 ppm phenol, respectively (Figure 3).

The nitrification inhibition test is used to determine the inhibitory effect of different phenolic compounds. The chlorinated phenols demonstrated an increased toxicity in the nitrification inhibition test, which was much more sensitive to these compounds than other bacterial toxicity tests (Strotmann, 1995).

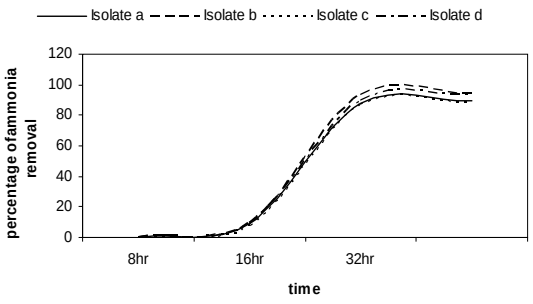

Figure 4. Removal of ammonia without phenol in wastewaters

Removal of ammonia did not occur at primary growth phase stage but it is started and continued at exponential growth phase (Figure 4). Percentages of ammonia elimination for a–d isolates at stationary growth phase were 89.1, 94.3, 89, and 93.6 respectively.

Isolates c and d were selected as wastewaters indicators. With increasing phenol concentration, the ammonia elimination percentage is decreased (Figure 5).

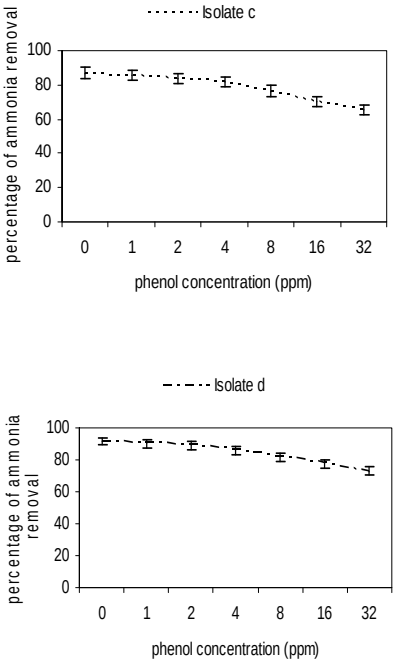

Figure 5. Removal of ammonia with phenol in media

The results showed that the percentage of elimination ammonia decreased from 89 and 93.6 to 67.2 and 76 in c and d isolates, respectively.

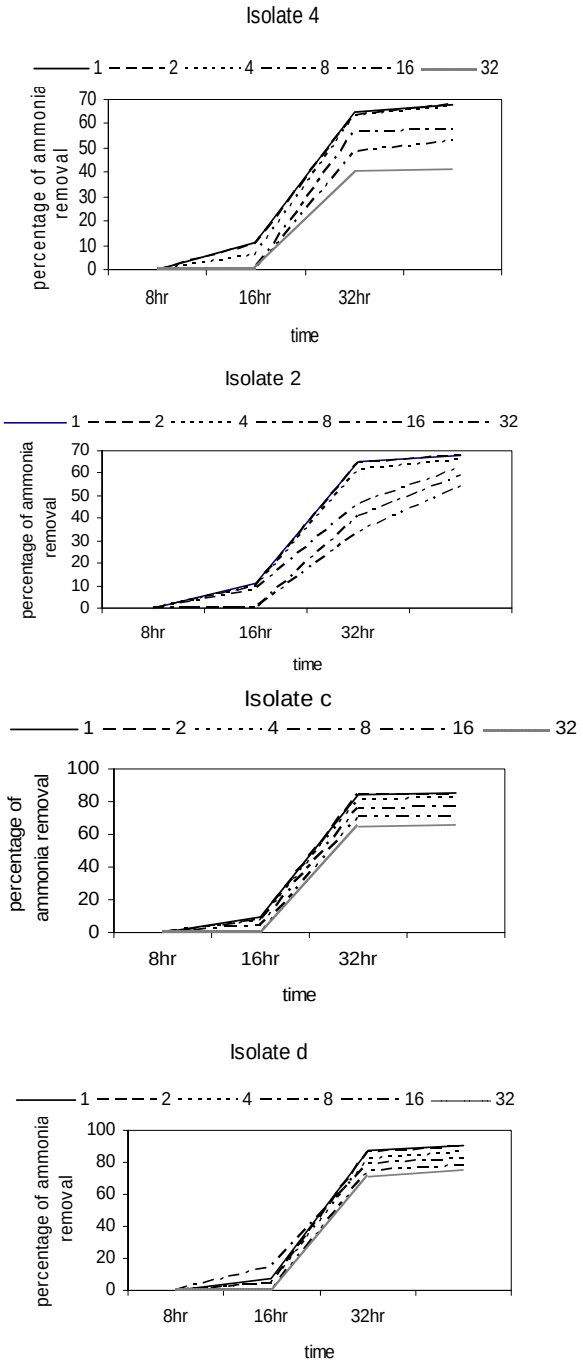

Figure 6. Removal ammonia in during of growth with phenol in media

The growth of ammonia –oxidizing bacteria and percentage of ammonia elimination decreased with increase of phenol in media .In both of isolates removal of ammonia began after 12 hours and the ammonia removal percentage decreases from 68.6 and 67.6 to 53.8 and 41.2 in isolate 2 and 4, respectively and for isolates c and d were 87 and 91.6 then decreased to 65.2 and 73, respectively.

Biological fluidized bed reactors are employed in the investigation of single-stage carbon oxidation and nitrification with the amines and phenol as the compounds targeted for removal (Nguyen, 1995). Also Ghanavati (2006) reported that ammonia removal is done by isolate nitrifying bacteria in two wastewaters with different phenol concentrations. But their results shown that nitrification is increased in low phenol concentration (Ghanavati, 2006).

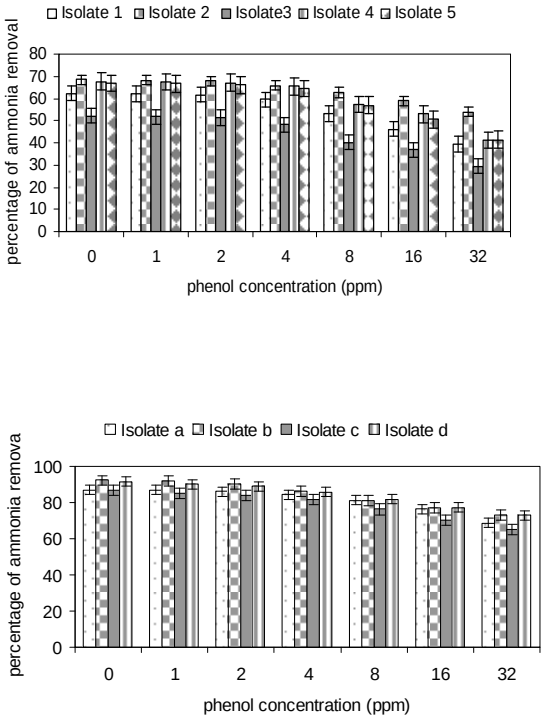

Figure 6.Comparison of averages for the influence of phenol on nitrification in soil and waste water's isolates.

Percentage of nitrite product is also measured. It indicated an obvious difference between percentage of ammonia removal and percentage of nitrite product. This difference maybe because of nitrate producing activity that immediately converts productive nitrite to nitrate.

Another explanation could be that the removal of ammonia occurred in another way (not through nitrification) which does not produce the nitrite with removal of ammonia.

Yamagishi (2001) showed that, it took 18 days for complete removal of ammonia by nitrification process in the presence of phenol (Yamagishi, 2001).

Nitrification process has been occasionally upset by serious inhibitory effects of toxic compounds, Phenol and p-cresol significantly inhibited nitrification above 200 mg/L and 100 mg/L, respectively (Young, 2008). In process of simultaneous removal of phenol, ammonium and thiocyanate from coke wastewater demonstrated a maximum nitrification efficiency (71%) is achieved when bicarbonate is added, the removal phenols being almost similar to those obtained in the absence of nitrification (Vázquez, 2006).

Different inhibitors are known for nitrification process, and one of these inhibitors is phenol. Inhibiting effect of phenol is because of its effect on ammoniamonooxygenase enzyme, alternating membrane and on proteins that bond to lipids in member, production of toxic metabolite and also variation the internal cell metabolite (Komarkova, 2003, Shiemke, 2004). In the highly concentrated phenol and copper medium, elimination of ammonia is decreased and hence the inhibition percentage on nitrification is increased (Kim, 2006).

CONCLUSION

Heterotrophic ammonia-oxidizing bacteria population decrease in the presence of high phenol concentration (wastewater 2). The rest bacteria were influenced. In presence of phenol, ammonia removal or nitrification process decreased. This decrease was identified in high phenol concentrations. In general the population of heterotrophic ammonia–oxidizing bacteria in soils was higher than those in wastewaters maybe due to the preparation of their sources of carbon and energy in soils relative to wastewaters.

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