An Overview of the Effects of Heavy Metals Content in Wastewater on Anammox Bacteria

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Abstract
The application of Anammox process in treating nitrogen rich wastewater had been more preferable since the discovery of Anammox process and Anammox bacteria at 1999 due to the advantages of energy saving and cost reduction compared to the conventional nitrification/denitrification process. However, often nitrogen-laden wastewater such as metal refinery wastewater, swine, industrial wastewater, and landfill leachate contain various concentrations of heavy metal ions such as Cadmium, Copper, Lead, Mercury, Nickel, Zinc and Ferrous iron. Trace amount of Zn(II), Co(II), Mn(II), Cu(II) and Ni(II) are recommended to be added in substrate during cultivation of Anammox bacteria as essential micronutrients. These metals are crucial co-factors for certain enzymes and metalloproteinase. Nevertheless, regardless of stimulation effect of some metals on the growth of bacteria at low concentration, a high concentration of metals ions might cause a negative effect on long term. The inhibition effects of various heavy metals were compared in this study. It was found that nine heavy metal, Pb2+ has the lowest inhibition effect on Anammox process, Cu2+ might be having the most inhibition effect on Anammox with the IC50 inhibition concentration. The IC50 inhibition concentration of Fe (II) was found at 0.20 mM. It was found Pb2+ has the lowermost inhibition effect, AT > 75 mg/L of Pb2+, while, Cu2+ might be having the supreme inhibition effect with the IC50 inhibition concentration at 1.9 mg/L. The IC50 of Fe (II) was found at 55.6 mg/L. More effort should be dedicated to understand the profound knowledge of heavy metal on Anammox bacteria.

Keywords
Anammox, Wastewater, Heavy metal, Inhibition, IC50, Stimulation

Abbreviations
Anammox: Anaerobic Ammonium Oxidizing; AOB: Ammonia Oxidizing Bacteria; COD: Chemical Oxygen Demand; EDX: Energy Dispersive X-ray; Hao: Hydroxylamine Oxidoreductase; HDH: Hydrazine Dehydrogenase; hh: Hydrazine Hydrolase; HZO: Hydrazine Oxidizing Enzyme; HZS: Hydrazine Synthase; IC50: Half Inhibition Concentration; N: Element Nitrogen; NH4+: Ammonium; NO: Nitric Oxide; N2O: Nitrous Oxide; NOB: Nitrite-Oxidizing Bacteria; SAA: Specific Anammox Activity; TN: Total Nitrogen

Introduction
The concept where ammonium can be oxidized under anoxic condition initially came from calculations based on theoretical thermodynamic [1] and the Redfield ratio in marine ecosystems [2]. The concept was proven 20 decades later by Mulder, et al. [3] who discovers the anaerobic ammonium oxidizing (anammox) process (Eq. (1)) and Strous, et al. [4] who found the responsible microorganisms, anammox bacteria. Anammox bacteria are Planctomycete type bacterium with anaerobic (no need oxygen) and autotrophic (no need organic carbon) metabolism, which combines ammonium (as electron donor) and nitrite (as an electron acceptor) to generate dinitrogen gas in the absence of oxygen.

\[
\text{NH}_4^+ + 1.32\text{NO}_2^- + 0.066\text{HCO}_3^- + 0.13\text{H}^+ \rightarrow 1.02\text{N}_2 + 0.256\text{NO}_3^- + 0.066\text{CH}_2\text{O}_{0.5}\text{N}_0\text{H}_{0.15} + 2.03\text{H}_2\text{O} \quad (1)
\]

The process of Anammox has become essential at the global level as it contributes to oceanic nitrogen loss level
cess and anammox bacteria activities. and inhabitation effects of heavy metals on anammox process can provide a compressive review concerning the stimulation al activity and the performance of the reactor. This aims to knowledge about the effect of heavy metal on the microbiota in biochemical reactions dependent on the concentrations Heavy metals such as iron, zinc, copper, nickel, and cobalt in wastewater. However, these type of wastewa-
tables contain a high concentration of heavy metals [12-14]. Table 1 shows various concentration of nitrogen in different type of wastewater. However, these type of wastewaters contain a high concentration of heavy metals [12-14]. Heavy metals such as iron, zinc, copper, nickel, and cobalt can either cause stimulation, inhibition or even toxic effect in biochemical reactions dependent on the concentrations and species [13]. Therefore, it is crucial to obtain more knowledge about the effect of heavy metal on the microbi- al activity and the performance of the reactor. This aims to provide a comprehensive review concerning the stimulation and inhabitation effects of heavy metals on anammox process and anammox bacteria activities.

Removal of Nitrogen Compounds from Wastewater
The element nitrogen (N) in its various redox forms are essential macronutrients for all living organisms on earth. Our land’s atmosphere is made up of 78% of dinitrogen gas (N₂). However, living organisms cannot access the nutrients of nitrogen from the atmosphere directly. A specialized group of bacteria can fix dinitrogen gas from the air to ammonium (NH₄⁺), then, the fixed nitrogen, in the form of NH₄⁺, will either enter food chain directly by assimilation process for macromolecule biosynthesis or it will be used as a substrate for bacteria which acquired energy for growth throughout the oxidation. Figure 1 shows the nitrogen cycle in nature that is responsible for all the reaction of the bacteria that convert nitrogen to its various redox forms, from +5 (NO₃⁻) to -3 (NH₄⁺/NH₃). Nitrification (autotrophic conversion of ammonium to nitrite then further to nitrate), is a stepwise oxidation process where ammonia oxidizing bacteria (AOB) oxidize NH₄⁺ to NO₃⁻ and nitrite oxidizing bacteria (NOB) oxidize NO₂⁻ to NO₃⁻. Denitrification (heterotrophic conversion of nitrate to dinitrogen gas) is a stepwise reduction process where denitrifying bacteria reduced NO₃⁻ to N₂ with NO₂⁻, nitric oxide (NO) and nitrous oxide (N₂O) as intermediate. Furthermore, in the process of nitrogen fixation, some non-symbiotic and some symbiotic with leguminous plants will fixate the atmospheric N₂ to NH₄⁺. In Anammox process, autotrophic bacteria utilize NH₄⁺ as an electron donor and NO₃⁻ as an electron acceptor and convert into nitrogen gas and some nitrate under an aerobic conditions in the absence organic carbons.

Conventional nitrification/Denitrification process
Due to the elevation of industry or/and agriculture activities, and discharge of excess nitrogen from wastewater directly into water bodies such as river and lake without advanced treatment, nature suffered from eutrophication and hypoxia. Nowadays worldwide environmental authorities enforcing stringent nitrogen discharge criteria. The conventional nitrogen removal system implemented nitrification and denitrification process to helped in treating nitrogen-rich wastewater before discharging to nature. In autotrophic nitrification process, aeration is necessary as both AOB and NOB need either oxygen or sulfate and ferric as other oxidized compounds, as an electron acceptor to oxidize NH₄⁺ to NO₂⁻ and oxidize NO₂⁻ to NO₃⁻. While in heterotrophic denitrification process (from NO₃⁻ to N₂), addition external electron donor (e.g. organic carbon) such as methanol (in majority case) is required for the denitrifying bacteria. Intermediate such as NO₂⁻, NO and N₂O will be produced in denitrification process. When applying nitrification/denitrification in the process of nitrogen removal, a significant amount of oxygen is needed, large amount of sludge produced, and the considerable volume of N₂O generated a greenhouse gas with a potential roughly 300 times higher than CO₂.

Anaerobic ammonium-oxidizing (Anammox) process
Anammox bacteria is a coccoid-shaped Planctomyces type bacterium with anaerobic (absence of oxygen) and autotrophic (not require organic carbon) metabo-
Oxidation State

\[ \begin{align*}
+5 & \quad \text{NO}_3^- \\
+4 & \quad \text{NO}_2^- \\
+3 & \quad \text{NO} \\
+2 & \quad \text{N}_2\text{O} \\
+1 & \quad \text{N}_2 \\
0 & \quad \text{NH}_2\text{OH} \\
-1 & \quad \text{NH}_4^+ \\
-2 & \quad \text{N}_2\text{H}_4 \\
-3 & \quad \text{N}_2\text{H}_4^+ \\
\end{align*} \]

Figure 1: The Nitrogen Cycle. The proposed biochemical pathway of the anammox process is as follows: first, NO\textsubscript{2}\textsuperscript{−} is reduced to nitric oxide (NO) then, the produced NO is reduced to hydroxylamine (NH\textsubscript{2}OH) and coupled with NH\textsubscript{3} to form hydrazine (N\textsubscript{2}H\textsubscript{4}) by hydrazine synthase (HZS) and finally, the N\textsubscript{2}H\textsubscript{4} is oxidized to N\textsubscript{2} gas. However, in 2016, Oshiki proposed another pathway of Anammox bacteria with N-tracer experiments which demonstrated that “Candidatus Brocadia sinica” cells could reduce NO\textsubscript{2}\textsuperscript{−} to NH\textsubscript{2}OH, instead of NO, with as yet unidentified nitrite reductase(s).
there are several drawbacks in conventional nitrification/denitrification process in treating nitrogen rich wastewater include: i) Requirement of aeration for conversion of NH$_4^+$ to NO$_3^-$ in autotrophic nitrification process, ii) Heterotrophic denitrification process requires external carbon source (e.g., electron donor) such as methanol (in majority cases), that ultimately derived from fossil fuel and iii) Both process release N$_2$O, which is the primary cause of ozone depletion [16] and iv) Produce large amount of sludge. In addition, besides being a novel method, Anammox process are environmentally friendly and cost effective compared to conventional nitrification/denitrification. Anammox directly convert ammonium, NH$_4^+$ (electron donor) and nitrite, NO$_2^-$ (electron acceptor) to nitrate, NO$_3^-$ and dinitrogen gas, N$_2$ without both oxygen and organic carbon.

**Genera and Species of Anammox**

Ten species of Anammox bacteria had been identified: ‘Candidatus Brocadia’, ‘Candidatus Kuenenia’, ‘Candidatus Scalindua’, ‘Candidatus Anammoxoglobus’ and ‘Candidatus Jettenia’. Together, they form the monophyletic order Brocadiales that branches deeply in the phylum Planctomycetes [11]. Timeline of discovery of Anammox and Electro-dense Anammoxosome particles containing iron are illustrated in Figure 2.

**Conventional nitrification/denitrification vs. Anammox process**

Before the discovery of Anammox process and Anammox bacteria, conventional nitrification/denitrification process was one of the milestones in nitrogen removal technology that helped to prevent environmental disasters such as eutrophication and hypoxia. However, condensed to hydrazine (N$_2$H$_4$) through hydrazine synthase (HZS) enzyme (Eq (3)), followed by oxidation of hydrazine (N$_2$H$_4$) to nitrogen gas (N$_2$) catalyzed by hydrazine oxidizing enzyme (HZO) (Eq (4)).

\[
\begin{align*}
\text{NO}_2^- + 2\text{H}^+ + \text{e}^- & \rightarrow \text{NO} + \text{H}_2\text{O} \quad (E'_0 = +0.38 \text{ V}) \quad (2) \\
\text{NO} + \text{NH}_4^+ + 2\text{H}^+ + 3\text{e}^- & \rightarrow \text{N}_2\text{H}_4 + \text{H}_2\text{O} \quad (E'_0 = +0.06 \text{ V}) \quad (3) \\
\text{N}_2\text{H}_4 & \rightarrow \text{N}_2 + 4\text{H}^+ + 4\text{e}^- \quad (E'_0 = -0.75 \text{ V}) \quad (4) \\
\text{NO}_2^- & \rightarrow \text{NO}^- + 2\text{H}^+ + 2\text{e}^- \quad (E'_0 = +0.42 \text{ V}) \quad (5)
\end{align*}
\]

Until now five genomes have been identified: ‘Candidatus Brocadia’, ‘Candidatus Kuenenia’, ‘Candidatus Scalindua’, ‘Candidatus Anammoxoglobus’ and ‘Candidatus Jettenia’. Together, they form the monophyletic order Brocadiales that branches deeply in the phylum Planctomycetes [11]. Timeline of discovery of Anammox and Electro-dense Anammoxosome particles containing iron are illustrated in Figure 2.

**Figure 2:** Timeline of discovery of Anammox and Electro-dense Anammoxosome particles containing iron.

- 1880: Denitrification
- 1882: N-fixation
- 1888: Nitrification
- 1890: Anammox might exist [2]
- 1960: Theoretically Anammox possible and predicted [1]
- 1965: Discover of Anammox process in denitrifying fluidized bed reactor (FBR) [3]
- 1970: Discover of Anammox bacteria and indentified as Planctomycetales [4]
- 1995: Discover of Anammox process in denitrifying fluidized bed reactor (FBR) [3]
- 1999: Discover of Anammox bacteria and indentified as Planctomycetales [4]
Morphology of anammox and Anammoxosome

In Anammox cell, there are three compartments divided by bilayer membrane; they are from inside to the outside: The Anammoxosome, riboplasm and paryphoplasm [18]. While the function of paryphoplasm remains unknown [19]. Inside riboplasm, there isribosomes and nucleoid and the role of riboplasm is similar to the cytoplasm of other bacteria, in which the translation and transcription will take place. The mechanism that sorted and transports the protein synthesized in riboplasm to other compartment remain unknown [20]. Anammoxosome is a membrane-bounded intracytoplasmic compartment in Anammox bacteria, with the majority of its membrane in a curved configuration as shown in Figure 3. Anammoxosome occupied most of the volume of Anammox cell [21], and the catabolism metabolism of Anammox bacteria are assumed to take place inside this compartment [20]. The researcher had discovered the existence of the iron-containing electron-dense Anammoxosome particles in the Anammoxosome compartment [21]. The storage of iron inside Anammoxosome compartment was for the excess supply of iron for further Heme c synthesis [22].

Effect of Heavy Metals on Anammox Bacteria

Stimulation effect of heavy metals on Anammox

It was found that the existance of limited amount of several heavy metals during the cultivation of Anammox cell will enhance anammox activity [23]. The common trace element solutions added to substrate of Anam-
mmoxosome particles are for energy generation and as an iron storage facility for the heme-c enzyme involved in electron transport chain [21]. Therefore, Fe (II) can be the potential factor to affect the growth and activity of Anammox bacteria. Electron-dense An anammoxosome particles containing iron was found within the Anammoxosome compartment of Anammox [21]. Fe (II) is an essential nutrient for Anammox as it helps synthesize of heme c-containing enzyme of Anammox by forming the active region of heme c-containing enzyme. Hydrazine synthase (HZS), also named hydrazine hydrolase (hha) and hydrazine oxidizing enzyme (HZO) also called hydrazylamine oxidoreductase (hao), or hydrazine dehydrogenase (HDH) are two of the heme c-containing enzymes. In HZS enzyme catalyze formation of hydrazine (N₂H₄), the intermediates of Anammox from ammonia (NH₄⁺) and nitric oxide (NO), while hydrazine oxidizing enzyme (HZO) helps in the formation of dinitrogen gas (N₂) by oxidizing hydrazine, at the same time providing electrons needed for hydrazine synthase and nitrite reduction Figure 4. shows the morphology of Anammox cell and model for catabolic Anammox reaction.

In addition, Fe (II) is one of the essential nutrients for growth of Anammox. Since the discovery of Anammox process by Van de Graaff, et al. [26], the Fe (II) concentration was set as 0.03 mM or 0.04 mM in most of the feeding medium of enriched Anammox sludge system. Energy dispersive x-ray (EDX) analysis revealed that several electron-dense Anammoxosome particles contained iron. In which the possible function of these Anammoxosome particles are for energy generation and as an iron storage facility for the heme-c enzyme involved in electron transport chain [21]. Therefore, Fe (II) can be the potential factor to affect the growth and activity of Anammox bacteria. Electron-dense An anammoxosome particles containing iron was found within the Anammoxosome compartment of Anammox [21]. Fe (II) is an essential nutrient for Anammox as it helps synthesize of heme c-containing enzyme of Anammox by forming the active region of heme c-containing enzyme. Hydrazine synthase (HZS), also named hydrazine hydrolase (hha) and hydrazine oxidizing enzyme (HZO) also called hydrazylamine oxidoreductase (hao), or hydrazine dehydrogenase (HDH) are two of the heme c-containing enzymes. In HZS enzyme catalyze formation of hydrazine (N₂H₄), the intermediates of Anammox from ammonia (NH₄⁺) and nitric oxide (NO), while hydrazine oxidizing enzyme (HZO) helps in the formation of dinitrogen gas (N₂) by oxidizing hydrazine, at the same time providing electrons needed for hydrazine synthase and nitrite reduction Figure 4. shows the morphology of Anammox cell and model for catabolic Anammox reaction.

Since 2013, the researcher had evaluated the effect of different concentration of Fe (II) on Anammox. Liu, et al. [22] had study the relationship between the effect of various Fe (II) concentration and the growth rate of Anammox by batch test, and they found that growth rate (i.e. 0.172 d⁻¹) was maximum at 0.09 mM of Fe (II).
Similarly, Zhen, et al. [27] had investigated Anammox start-up period and they found that the shortest start-up (i.e. 50 d) was attained at 0.09 mM of Fe (II). Sen, et al. [28] investigated the total nitrogen (TN) removal percentage by batch test at different concentrations of Fe (II), and they found that the maximum total nitrogen removal percentage (63%) was observed at 0.09 mM of Fe (II) concentration. From the above studies, it can be presumed that 0.09 mM Fe (II) concentration has the most positive effect on Anammox.

The inhibition factors of heavy metal on Anammox bacteria

After the Anammox process and Anammox bacteria had been discovered, researcher from around the world have been tried to study Anammox due to the advantages of low cost and its capability of removing high ammonium nitrogen wastewater when comparing to conventional nitrification/denitrification process [29]. The Anammox process have been successfully applied from the lab-scale reactor to full-scale treatment plant to treat ammonium or nitrogen rich wastewater since the last two decades [30]. On the other hand, the application and operation of Anammox process in treating real wastewater can be restricted by few factor such as the slow growth rate of Anammox cell and the present of some inhibition factor in wastewater such as heavy metals, substrate, etc.

The inhibitory effects of heavy metals may show great variations in synthetic wastewater, contaminated wastewater and natural environment based on the type and concentration [31].

Toxicity occurs when microorganism’s uptake excess amount of heavy metals or metal partitioning through extracellular sorption, transmembrane transport, and intracellular accumulation [10,32,33]. Depending on the viability of the biomass and concentrations of the heavy metals, the transmembrane transport can active, passive or both. The toxicity of heavy metals towards Anammox cell was through their bioaccumulation in cells. When there is present of heavy metals, diffusion of metals will occur across the outer wall of Anammox bacteria through porins and subsequently enter transport across the cytoplasmic membrane in various ways. When the metal ions are inside the Anammox cell, they can interact with both nucleic acids, enzyme active sites and lead to a rapid decline in membrane integrity, which is usually demonstrated as leakage of mobile cellular solutes and cell death.

Zhen Bi, et al. [27] had investigated the inhibition effects of Cd, Ag, Hg and Pd on Anammox activity. Result of the study illustrated that deterioration of crude enzyme activity occurred due to the accumulation of heavy metals inside Anammox cell, which eventually lead to the decrease of nitrogen removal rate of the Anammox system. Furthermore, the heme c concentration also susceptible to short-term exposure to heavy metals. Therefore, investigations are necessary for the comprehend of the inhibitors so that the inhibition effect can be minimize at the same time improve Anammox process.

Specific Anammox Activity (SAA) Test

According to Daverey, et al. [34] the effect of heavy metals on Anammox bacteria is more prominent when the biomass concentration is low (< 2000 mg-MLVSS L⁻¹). Most of the specific anammox activity (SAA) tests perform according to Dapena-Mora, et al. [35] methods. The production of N₂ gas is normally tracked by measuring the overpressure in the headspace with a time-frequency depending on the biomass activity in each batch test.

Evaluation of SAA

The total amount of N₂ gas produced is normally calculated from the overpressure measured in the headspace of each serum bottle at the end of the assay by using the ideal gas law equation. The N₂ gas production rate, dN₂/dt can be calculated from the maximum slope of the curve describing the pressure increase in the vial along the time (a) (Eq. (6)) [35]

\[
\frac{dN_2}{dt} = \frac{\alpha \times V_g}{R \times T}, \text{molN}_2 \text{hr}^{-1}
\]

\( \alpha \) = slope of pressure increase in the bottle along the time (atm)

\( V_g \) = volume of gas phase (0.01 L)

\( R \) = ideal gas constant 0.0820575 (atm L mol⁻¹ K⁻¹)

\( T \) = temperature (K)

Then, SAA determined by dividing the N₂ gas production rate, dN₂/dt by the concentration of biomass in the serum bottle, X (g VSS L⁻¹) (Eq. (7)) [35]

\[
SAA = \frac{\frac{dN_2}{dt} \times 28}{X \times V_L} \times 24, \text{gN}_2\text{gVSS}^{-1} \text{d}^{-1}
\]

28 = molecular weight of N₂ (g N/mol)

24 = unit conversion factors from hour to days

\( X \) = biomass concentration in the bottle (g VSS L⁻¹)

\( V_L \) = volume of liquid phase in the bottle

Percentage of activity increase and IC₅₀

Comparison had been done between inhibition effect, IC₅₀ inhibition concentration of various heavy metals on Anammox bacteria based on specific anammox activity.
(SAA) as shown in Table 3. The inhibition effects of nine various heavy metals had been compared included ferrous ion (Fe^{2+}), Cadmium (Cd^{2+}), Copper (Cu^{2+}), Lead (Pb^{2+}), Mercury (Hg^{2+}), Molybdate (MoO_{4}^{2-}), Nickel (Ni^{2+}), Silver (Ag^{+}) and Zinc (Zn^{2+}) respectively. Among the nine heavy metal, Lead ion, Pb^{2+} has the lowest inhibition effect on Anammox process, in which even 75 mg/L of Lead ion do not cause any inhibition effects on Anammox bacteria, while copper ion, Cu^{2+} might be having the most inhibition effect on Anammox with the IC_{50} inhibition concentration ranged start from 1.9 mg/L. The IC_{50} inhibition concentration of Fe (II) was found to be approximately 0.20 mM, which, after conversion is equal to 55.6 mg/L [36]. Thus, from the comparison, the inhibition effects of Fe (II) on Anammox process is not as intense as most of the heavy metals tested and the inhibition effects of Fe (II) was found lower than Cadmium (Cd^{2+}), Copper (Cu^{2+}), Nickel (Ni^{2+}), Silver (Ag^{+}) and Zinc (Zn^{2+}) respectively. Among the nine heavy metal, Lead ion, Pb^{2+} has the lowest inhibition effect on Anammox process, in which even 75 mg/L of Lead ion do not cause any inhibition effects on Anammox bacteria, while copper ion, Cu^{2+} might be having the most inhibition effect on Anammox with the IC_{50} inhibition concentration ranged start from 1.9 mg/L. The IC_{50} inhibition concentration of Fe (II) was found to be approximately 0.20 mM, which, after conversion is equal to 55.6 mg/L [36]. Thus, from the comparison, the inhibition effects of Fe (II) on Anammox process is not as intense as most of the heavy metals tested and the inhibition effects of Fe (II) was found lower than Cadmium (Cd^{2+}), Copper (Cu^{2+}), Nickel (Ni^{2+}), Silver (Ag^{+}) and Zinc (Zn^{2+}).

### Table 3: Half inhibition concentration of heavy metals on Anammox bacteria.

| Heavy Metal ions | Half Inhibition Concentration, IC_{50} (mg/L) | References |
|------------------|-----------------------------------------------|------------|
| Cadmium, Cd^{2+} | 11.2                                          | Li, et al. [55] |
| Copper, Cu^{2+}  | 1.9-30                                         | Yang, et al.; Yang, et al. [9,39] |
| Lead, Pb^{2+}    | NT                                            | Li, et al. [55] |
| Mercury, Hg^{2+} | 60.35                                         | Li, et al. [55] |
| Molybdate, MoO_{4}^{2-} | NT | Li, et al. [55] |
| Nickel, Ni^{2+}  | 48.6                                          | Li, et al. [55] |
| Silver, Ag^{+}   | 11.52                                         | Li, et al. [55] |
| Zinc, Zn^{2+}    | 3.9-25                                        | Achlesh, et al.; Li, et al. [37,55] |
| Fe (II), Fe^{2+}  | 55.6 (0.20 mM)                                 | Mak, et al. [36] |

NT = was not toxic at the highest concentration tested (75 mg Pb/L and 23.8 mg Mo/L).

### Recovery

Achlesh, et al. [37] had studied the long term effect of zinc on SNAD system of Anammox bacteria, the system was able to recovered the nitrogen (total nitrogen and ammonium nitrogen) removal efficiency to around 90% after inhibited by 20 mg/L of zinc which had reduce the nitrogen (total nitrogen and ammonium nitrogen) removal efficiency to around 70%, due to the fact that the microbial communities in the reactor were well acclimated. In the study of Kimura and Isaka [38] a continuous Anammox bioreactor (lab-scale) with gel-carrier had been operated to investigate the effects of Ni, Cu, Co, Zn and Mo on Anammox activity. It was demonstrated that high concentrations of those heavy metal is inhibitory to Anammox cell, however the effects were reversible. Yet, the inhibition effect of Mo on Anammox cell was irreversible. Therefore, it is suggested that extra attention should be paid to Mo concentrations in the wastewater subject to Anammox bacteria. In addition, Anammox activity and performance subjected to copper inhibition for long time (almost 200 days) were restorable and the recovery process lasted for short time (nearly 50 days) due to the accumulation of Anammox cell [39].

### Conclusion

Anammox process has become essential in treating ammonium rich wastewater due to its multiple advantages compared to the conventional nitrification/denitrification process. Anammox process is a promising method for treating nitrogen rich and low COD content wastewater. The presence of heavy metals in wastewater can either affect stimulation, inhibition or even toxic in biochemical reactions. It was found that heavy metal ions can improve Anammox activity through stimulating the metabolism of Anammox cell as those metal ions can either be the component of many enzymes or the co-enzyme. Nevertheless, toxicity occurs when microorganism uptake extra quantity of heavy metal through extracellular sorption, transmembrane transport, and intracellular accumulation. In this review, IC_{50} inhibition concentration of various heavy metals on Anammox bacteria based on SAA. Based on literature review, it was found that Lead ion, Pb^{2+} has the lowest inhibition effect, in which even 75 mg/L of Pb^{2+} do not cause any inhibition effects on Anammox bacteria, while copper ion, Cu^{2+} might be having the most inhibition effect on Anammox with the IC_{50} inhibition concentration of 1.9 mg/L. The IC_{50} of Fe (II) was found at 55.6 mg/L. It can be concluded that that the high concentrations of those heavy metal has inhibitory effect on Anammox cell, on the other hand the effects are generally reversible.

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