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Ammonia inhibition and toxicity in anaerobic digestion: A critical review

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Abstract

As a waste management technology which offers environmental benefit and renewable energy production, anaerobic digestion (AD) has become the preferred technology for the treatment of organic waste. However, in such waste streams nitrogen contents are likely to be high. There is prevailing literature evidence suggests that high ammonia concentration especially its free molecular form (NH_3), derived from nitrogen content in substrates is the cause of inhibition and sudden failure of the AD process.

This paper comprehensively reviews previous knowledge from digestion studies using high nitrogen waste streams as feedstocks and critically analysed the considerable variations in the inhibition/toxicity levels reported for ammonia. Literature evidences suggest methanogens, particularly acetoclastic methanogens are most susceptible to ammonia toxicity, and therefore this review has a particular focus on the mechanism of the 'selective' inhibition to methanogens and the impact of ammonia toxicity to the overall methanogen population in an AD digester. This population change explains in many reported cases that sufficient acclimatisation can significantly alleviate the phenomenon of inhibition and specific requirement of certain trace nutrients. Currently available mitigation strategies for high nitrogen content feedstock digestion are reviewed and discussed in relation to the population change and trace nutrient requirements.

Keywords

Anaerobic Digestion; Ammonia; Inhibition; Toxicity; Methanogenesis

1. Introduction

The anaerobic digestion (AD) market is growing across Europe at around 10-12% (Gupta and Bais, 2017a) and is set to exceed $8BN by 2024 (Gupta and Bais, 2017b). Much of this growth has been driven through specific market segments, explicitly agriculture (farm slurries), food waste and industrial feedstocks (Holm-Nielsen et al., 2009; Zhang et al., 2016), in response to various legislative drivers, including incentivisation schemes for renewable source of energy in addition to restrictions imposed on disposal of food waste to landfill (Edwards et al., 2015). The commonality amongst these feedstocks is their high total ammoniacal nitrogen (TAN) concentration, which has created problematic operation in AD for specific organic wastes, including food waste, animal manure and slaughterhouse waste, as well as classical municipal AD which experience elevated ammonia concentrations often due to sludge imports. The inhibitory effect of ammonia is generally acknowledged to mainly inhibit the methanogenesis phase in anaerobic reactors (Calli et al. 2005; Koster and Lettinga, 1984; Angelidaki and Ahring, 1993; Schnurer et al. 1994; Hansen et al. 1998). Whilst ammonia toxicity is a known problem, the underpinning mechanism is not well defined (Rajagopal et al., 2013) and the general experience in identification of a 'critical' threshold concentration is seemingly difficult to ascertain with reported concentration in the range between 1500-7000 mg l^{-1} (Rajagopal et al., 2013). This review therefore focuses on providing a collective explanation for ammonia toxicity and further outlines prospective opportunities to limit the impact and implications associated with high TAN concentrations in AD feedstocks. In addition, through the critical thresholds identified within the AD literature, this review seeks to explain, together with the mechanisms underpinning both toxicity and the physical chemistry governing ammonia equilibria, why a consensus 'critical' threshold has yet to be approached.

2. Identification of ammonia equilibria and free ammonia concentration

In anaerobic digestion processes, ammonia is produced through the degradation of the nitrogenous matter in the feedstock, primarily in the form of proteins (Kayhanian et al., 1999; Kotsyurbenko et al., 2004). Consequently, operation of digesters treating high protein content substrates can be problematic as the ammonia released during the digestion of proteinaceous material can impose high ammonium concentrations. According to McCarty (1964), a low ammonium concentration

between 50 and 200 mg l^{-1} has beneficial effect on anaerobic processes as ammoniacal nitrogen is an essential element for synthesis of amino acids, proteins and nucleic acids, and therefore critical for bacterial growth. In addition, ammonia, as a base, neutralises the organic acids produced by fermentative bacteria, and thus helps maintain neutral pH conditions essential for cell growth. However, if present at high concentrations, ammonia is inhibitory to methanogenesis (McCarty, 1964; Gallert et al., 1998).

In aqueous conditions, ammonia exists mainly in two forms, as the ionised ammonium ion (NH_4^+) stable in the aqueous phase, and in gaseous form as free ammonia (FAN), as shown in Eq. [1].

$$NH_3 + H^+ \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} NH_4^+ \qquad [1]$$

The relative fraction of free ammonia nitrogen (FAN) relative to the total ammoniacal nitrogen concentration is related to the pH and temperature of the solution as shown in Eq. [2] (Koster, 1986).

$$FAN = TAN \times \left(1 + \frac{10^{-pH}}{10^{-(0.09018+\frac{2729.92}{T(K)})}}\right)^{-1} \qquad [2]$$

Where:

FAN = free ammonia nitrogen concentration (mg l^{-1})

TAN = total ammonia nitrogen concentration (mg l^{-1})

T (K) = temperature (Kelvin)

For example, for a solution at pH8, only 4% of TAN is available as FAN at 20°C, whereas at 40°C, 13% becomes available as FAN.

The proportion of free ammonia (FA) is of importance, as FA has been suggested as the main cause of inhibition (Kroeker et al., 1979, De Baere et al., 1984, Koster, 1986, Angelidaki and Ahring, 1993, Kayhanian, 1994, Kayhanian, 1999). Findings from several studies (Angenent et al., 2002, Koster, 1986) indicated that methane fermentation of high ammonia-containing wastes is more prone to inhibition at thermophilic temperatures than at mesophilic temperatures, supporting the view that it is the free ammonia which causes toxicity. Under normal operating conditions, digesters treating waste rich in proteins tend to have high pH values, often above pH 8 (Borja et al., 1996). An increase

in pH from 7 to 8 will lead to an eight-fold increase of the free ammonia concentration in mesophilic condition (Hansen et al., 1998), and even more at thermophilic condition.

With reference to the two parameters controlling the process, pH and temperature, the following considerations should be taken into account: 1) pH affects the growth of microorganisms as well as the partitioning of TAN (Hansen et al., 1999; Hashimoto, 1983; Kroeker et al., 1979). The equilibrium concentration between ammonium and FA follows the equation [1] and depends on pH. The FA form of ammonia has been suggested as the toxic agent, therefore an increase in pH would result in increased toxicity (Borja et al., 1996). At high pH values the unionised form, free ammonia, dominates and this form is more inhibitory than the ammonium ion (NH_4^+). 2) Temperature affects both microbial growth rates and FA concentration. A higher process temperature may increase the metabolic rate of the microorganisms but also results in a higher FA concentration. Some authors have found that anaerobic fermentation of wastes with a high concentration of ammonia was more easily inhibited and less stable at thermophilic temperatures than at mesophilic temperatures (Braun et al., 1981; Parkin and Miller, 1983). Other studies, however, indicated that thermophilic flora tolerated at least twice as much FA as compared to mesophilic flora (Gallert and Winter, 1997)

In addition, it is important to recognise that ionic strength, and explicitly total solids concentration, also influence the pKa, and introduce a transient solubility limit for these ammoniacal compounds ('salting out' of gases), such that estimations of FAN are difficult to obtain, with some studies suggesting an overestimation of FAN by up to 40% in different animal manures (Hafner and Bisogni, 2009; Lauterböck et al., 2012). In addition to this difficulty of accurate estimation of FAN, the dynamic shift in equilibrium coupled with transients induced by mixing and temporal feeding patterns in AD make it difficult to reconcile a generic critical ammonia concentration in such system.

Inhibitory thresholds for ammonia have been reported in a number of studies, but the concentrations found vary significantly. Koster and Lettinga (1984) indicated that under mesophilic conditions, the maximum methanogenic activity was unaffected at a TAN concentration of 680 mg l^{-1} (Free ammonia = 26.5 mg l^{-1}). However as the TAN concentration was increased to 1600 mg l^{-1} (Free ammonia = 60.3 mg l^{-1}), methanogenesis decreased to about 75% and decline further as

TAN continued to increase. Kayhanian (1994) revealed that under thermophilic conditions, methane production decreased at total ammonia nitrogen concentration of 1000 mg l^{-1} (Free ammonia = 60 mg l^{-1}) when the digester was operated at pH of 7.5 or higher. This comparison suggests a commensurate critical threshold for FAN, the distinction in TAN concentration being driven by the difference in physical chemistry. Other researchers have reported higher inhibitory thresholds at TAN concentrations of 1500-2500 mg l^{-1} (Kleiner, 1993, Wiegant and Zeeman, 1986). This discrepancy illustrates the difficulties associated with reporting ammonia inhibition based on TAN rather than free ammonia, as the total ammonia inhibitory concentrations reported from different studies are not comparable unless the pH and temperature conditions are also cited. In the above studies by Koster and Lettinga (1984) and Kayhanian (1994), although reported inhibitory concentrations of ammonia are different, if converted to free ammonia they are more consistent.

For anaerobic treatment of high nitrogen organic waste in typical continuous stirred tank reactors (CSTR), ammonia cannot be further degraded anaerobically. Some studies demonstrated of using anaerobic ammonium oxidation (ANAMMOX) bacteria with addition of nitrite or nitrate sources to removal ammonia (Kuenen, 2008). However, ANAMMOX process is unlikely to take place *in situ* during anaerobic digestion as the slow growth ANAMMOX bacteria cannot compete with the denitrifying organisms in anaerobic digester for limited nitrite or nitrate (Dong and Tollner, 2003). . Therefore once a high concentration of ammonia has been reached in digesters, the problem will normally persist unless ammonia can be removed via physical (e.g. air stripping) or physico-chemical means (e.g. acid scrubbing) (Lauterböck et al., 2012).

3. Mechanisms of ammonia inhibition to methanogenesis

In order to gain further understanding of the digester behaviour and successful operation of anaerobic treatment of organic waste with high nitrogen content such as food waste, a review of the ammonia toxicity mechanism towards anaerobic digestion, in particular its impact towards the methanogen population and potential for alteration of methanogenic pathway in anaerobic digesters is of great importance.

To understand the possible mechanisms of ammonia inhibition, it is important to consider the chemical interaction of ammonia and cells. For most of microorganisms, the energy equilibration in ATP is achieved by proton-translocating ATPases applying proton motive force ($\Delta_{\mu H}^{+}$) across the cell membrane. The energy in the $\Delta_{\mu H}^{+}$ is the sum of the energies in the trans-membrane pH gradient (ΔpH) and the trans-membrane electrical gradient (Δψ) (Wolin and Miller, 1982). For methanogens living in a slightly alkaline environment, studies indicated that the trans-membrane pH gradient (ΔpH) is small or even negative (Wolin and Miller, 1982); therefore those methanogens can grow with a near-neutral cytosol even when the external pH is above 7.

The mechanism of ammonia toxicity in methanogens is currently not clear; however a physical model (Figure 1) was proposed by Kayhanian et al. (1999). The model was based on the understanding of trans-membrane electrical and pH gradient theory and it convincingly described the entrance of the NH_3 molecule into cell and subsequently the internal accumulation of ammonia.

According the model, free ammonia molecules will diffuse readily through cell membranes into the cells of methanogens (De Baere et al., 1984, Kayhanian et al. 1999, Wolin and Miller, 1982), equilibrating the intracellular and extracellular concentrations of NH_3. On the other hand, ammonium (NH_4^+) does not readily diffuse through cell membranes. This in turn leads to a rapid increase of cytosolic concentration of un-ionized ammonia when a methanogen cell is exposed to an increased extracellular ammonia concentration (Kleiner, 1993). Under such a scenario, the intracellular and extracellular concentrations of NH_4^+ are dependent on NH_3 concentration and the local pH and temperature. Thus, cells with intracellular pH lower than the extracellular pH (i.e. negative ΔpH) would have an intracellular NH_4^+ concentration greater than that of their environment. In cells with a very negative ΔpH, cytosolic NH_4^+ may constitute a considerable fraction of the intracellular cations.

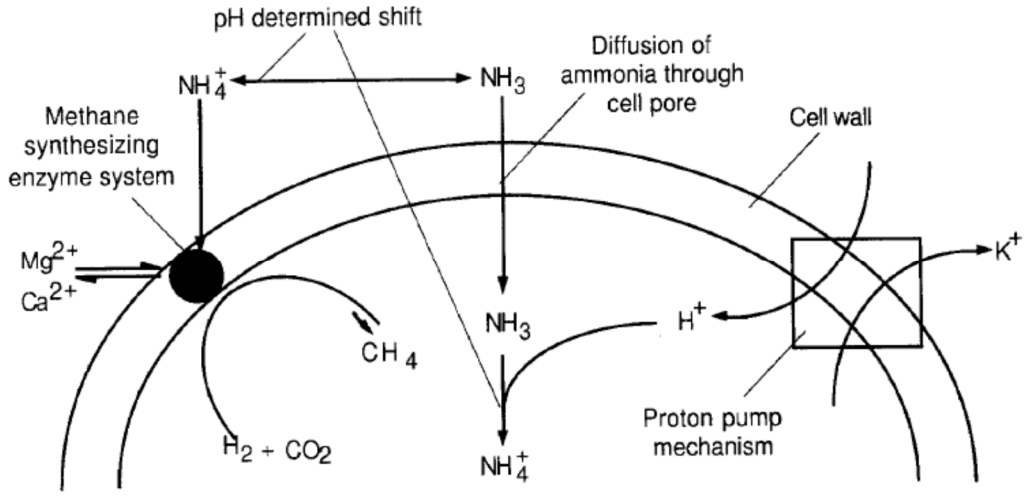

Figure 1. Proposed mechanism of ammonia inhibition in methanogenic bacteria (Kayhanian et al. 1999)

Due to the complicated procedures for measuring intracellular pH and cation concentrations, experimental work to support this hypothesis is very limited. A study carried out using the methanogen strain *Methanolobus taylorii* has shown that most of the energy in the $\Delta_{\mu H^+}$ of the methanogen cell is accounted for by its large $\Delta\psi$ (i.e., the outside of the cell is more positively charged); its ΔpH is therefore small or even negative (Jeris and McCarty, 1965). The study discovered that for the methanogen strain *Methanolobus taylorii,* the negative ΔpH allows it to grow with a near-neutral cytosol even when the external pH is above 7. The active extrusion of potassium by this methanogen was suggested as a mechanism by which it increases its $\Delta\psi$, thereby allowing it to grow with a low or negative ΔpH (Wolin and Miller, 1982, Koster, 1986).

Following the diffusion of ammonia into cell, at least two possible mechanisms of ammonia toxicity have been postulated (Kadam and Boone, 1996).

3.1 The direct inhibition of the activity of cytosolic enzymes by un-ionized ammonia

Kadam and Boone (1996) studied the active level of three ammonia-assimilating enzymes (glutamate dehydrogenase, glutamine synthetase, and alanine dehydrogenase) in three methanogen species of the family *Methanosarcinaceae*. The results showed diversified enzymatic responses in different species towards a high concentration of ammonia in the growth media, which implied variable tolerances to ammonia between the three species.

3.2 Intracellular accumulation of NH_4^+

When free NH_3 enters the cell, the lower intracellular pH causes the conversion of part of the free NH_3 into ammonium (NH_4^+), absorbing protons in the process. A number of previous studies proposed in order for the cell to maintain the intracellular pH, the cell has increased energy requirements for the potassium (K^+) pump to balance the increased number of protons. Therefore the elevated energy requirement, potentially causes inhibition of specific enzyme reactions (Gallert et al., 1998, Sprott et al., 1984, Whittmann et al., 1995). Furthermore, when the cell is exposed to high ammonia and the K^+ pump cannot keep up with the accumulated NH_4^+ inside cells, intracellular pH cannot be maintained, leading to cytotoxicity (Sprott and Patel, 1986). In a study conducted by Sprott et al. (1984) using pure culture *Methanospirillum hungatei* exposed to ammonia in a K^+ free buffer. It was observed that the methanogen lost up to 98% of the cytoplasmic K^+ through an ammonia/K^+ exchange reaction. The experiment also suggested that additions of NH_4OH or various NH_4^+ salts (or methylamine) were most effective in causing K^+ depletion in a medium of alkaline pH (i.e. containing a higher proportion of the unionised form free ammonia), suggesting that NH_3 was the active chemical species crossing the cell membrane and causing toxicity. Other essential cytosolic cations such as Mg^{2+} and Na^+ have also been reported to be affected in the same way by ammonia (Kadam and Boone, 1996). Based on the findings of these studies, it is reasonable to speculate that high ammonia could also affect the uptake of essential trace elements required for cell function and thereby cause micro-nutrients deficiency.

In either case, it is understandable that high pH and high total ammonia concentration could exert their toxicities synergistically. At higher pH values, a larger fraction of TAN is unprotonated (at 35°C, about 0.5% at pH 7 but almost 65% at pH 9, calculated using equation 2). Additionally, if methanogens growing at a higher pH establish a more negative ΔpH to maintain a near-neutral cytosol, then the potential toxicity due to NH_4^+ accumulation would also be greater.

4. Acclimation and microbial population adaptation to high ammonia condition

4.1 Operational consideration of acclimation to high ammonia concentration in AD reactors

Other factors such as microbial acclimatisation to the high ammonia concentration and cation antagonism effects (Kartal et al. 2010, Chen et al., 2008) could also contribute to the broad range of ammonia inhibitory thresholds reported in the literature. By acclimatising the anaerobic inocula to high ammonia concentration, higher tolerance can be achieved. Van Velsen (1986) conducted batch experiment inoculated with digested sewage sludge and digested piggery manure, acclimatised to low (815 mg N l^{-1}) and high (2420 mg l^{-1}) ammonia nitrogen respectively. The study found the reactor inoculated with digested sewage sludge showed a longer lag phase of methane production at increasing ammonia nitrogen concentrations in the range 730-4990 mg N l^{-1}, whereas with digested piggery manure inoculated reactor, methane formation started immediately without any lag phase.

A number of other continuous studies have also reported adaptation of methanogenesis to ammonia concentrations far above those believed to be inhibitory. Parkin and Miller (1987) reported that TAN concentrations as high as 8000–9000 mg N l^{-1} could be tolerated with no significant decrease in methane production after acclimation. The experiments clearly demonstrated the possibility of obtaining stable digestion of manure with ammonia concentrations exceeding 5000 mg N l^{-1}after an initial adaptation period. Hashimoto (1983) observed that ammonia inhibition began at about 2500 mg N l^{-1} and 4000 mg N l^{-1} for unacclimated and acclimated thermophilic methanogens, respectively. Hansen et al. (1998) conducted a batch experiment to determine ammonia toxicity using inoculums acclimated to high ammonia. The study demonstrated that after acclimatisation of the inoculum to high ammonia concentration, inhibition to the process started at a free ammonia concentration of 1100 mg N l^{-1} (TAN = 3400 mg l^{-1}). Below this value the specific apparent growth rate of methanogens was found to be constant.

4.2 Microbial population adaptation to high ammonia concentration

It is generally acknowledged that of all the anaerobic microorganisms involved in anaerobic digestion, the methanogens are the least tolerant to environmental inhibitors and the most likely to be affected by ammonia inhibition (Kayhanian, 1994, McMahon et al., 2001, Kotsyurbenko et al., 2004). Koster and Lettinga (1988) studied the microbial activity change of acidogenic and methanogenic population in granular sludge as ammonia nitrogen concentrations were increased within the range of 4051–

5734 mg N l^{-1}. The experiment showed the methanogenic population lost 56.5% of its activity; while the acidogenic populations were hardly affected. The particular sensitivity of methanogens to ammonia toxicity will lead to the cessation of the methanogenesis stage of anaerobic digestion, whilst acid-producing stage continues. The consequent build-up of organic acids resulting from inhibited methanogenesis will cause a rapid fall in pH and complete failure of the whole anaerobic process (McMahon et al., 2001).

Among the methanogenic strains, the tolerance towards ammonia toxicity varies significantly. In an early study (Wiegant and Zeeman, 1986), hydrogen-utilising methanogens were reported to be more susceptible to ammonia than acetoclastic methanogens under thermophilic conditions. In the same study, the author postulated that hydrogen accumulation due to the blockage of the hydrogenotrophic route caused a build-up of propionate which in turn, acts as an inhibitor of the acetoclastic methanogens. In a later study carried out by Angelidaki and Ahring (1993), however, specific methanogenic activity (SMA) was monitored for both acetoclastic and hydrogenotrophic methanogens under thermophilic conditions. It was observed in this study that the SMA of acetoclastic methanogens decreased more than hydrogenotrophic methanogens under ammonia stress. These experimental results therefore did not support the previous study by Wiegant and Zeeman (1986).

Increasing literature evidence indicating that acetoclastic methanogens are more sensitive to ammonia toxicity than hydrogenotrophic ones (Robbins et al., 1989, Schnürer and Nordberg, 2008, Sprott and Patel, 1986). Koster and Koomen (1988) studied ammonia inhibition specifically of hydrogenotrophic methanogens using sludge that had never experienced ammonia inhibition before. The hydrogenotrophic population was found to grow well at an ammonia concentration as high as 6300 mg N l^{-1}. Interestingly, in this study using only hydrogenotrophic methanogens strains, the methanogenesis started without a requirement for acclimation. Whereas in other studies using unacclimatised mixed populations of acetoclastic and hydrogenotrophic methanogens (Van Velsen, 1979, Koster and Lettinga, 1984, Koster, 1986), temporary cessation of methanogenesis was always encountered after exposure to ammonia and recovered only after an adaptation period. This again

supports other findings that the hydrogenotrophic methanogens are less susceptible to ammonia toxicity.

A small number of toxicity studies have been carried out studying the different inhibition thresholds of ammonia towards hydrogenotrophic and acetoclastic methanogens. Jarrell et al. (1987) used pure cultures to study the tolerance to ammonia in four methanogen strains commonly isolated from sludge digesters which can grow on H_2 and CO_2 (*Methanospirillum hungatei*, *Methanosarcina barkeri*, *Methanobacterium thermoautotrophicum*, and *Methanobacterium formicicum*). It has been observed that although being the most sensitive to ammonia of the four strains, *Methanospirillum hungatei* can tolerate ammonia concentrations up to 4200 mg N l^{-1}; whilst the other three strains tested were resistant to ammonia concentrations higher than 10000 mg N l^{-1}. In similar pure culture studies conducted on four strains of thermophilic hydrogenotrophic methanogens showed population growth could still be observed for some strains even at ammonia concentration of 9000 mg N l^{-1} (Hendriksen and Ahring, 1991). The concentrations of ammonia that could be tolerated by methanogens in those studies were significantly higher than in other studies using mixed cultures (Poggi-Varaldo et al., 1997), further indicating that hydrogenotrophic methanogens have higher tolerance towards ammonia toxicity. In a recent mix-culture continuous digestion study, Tian et al. (2019) added a hydrogenotrophic strain *Methanoculleus bourgensis* into an ammonia inhibited reactor (11000 g N l^{-1}) and observed a successful establishment of the *M. bourgensis.* In addition, methane production was restored after the bioaugmentation. This is attributed to the reduction of hydrogen partial pressure causing inhibition to syntrophic acetate oxidation bacteria (SAOB) as a result of bioaugmentation of *M. bourgensis*.

To the best of authors' knowledge, no literature to date has proposed a possible mechanism at a molecular microbiology level to account for the different sensitivities of the two groups of methanogens, or more specifically why ammonia should show greater toxicity to methanogens using the acetoclastic pathway. Some investigations have been conducted on the microbial response towards ammonia toxicity, as in Borja et al. (1996) who observed that inhibition of the acetoclastic populations showed a sigmoidal pattern. This finding coincided with results of the study conducted by Poggi-Varaldo et al. (1991) who found that the bacterial growth rate and the specific acetate-

uptake rate were affected by the free ammonia concentration in a three-stage pattern: initial inhibition, plateau and final inhibition. This inhibition pattern could indicate that two inhibition mechanisms are involved, acting at different concentration levels. The hydrogenotrophic populations, however, exhibited a more linear pattern of inhibition (Borja et al., 1996).

Some other studies have focused on microbial diversity and morphology at a genus and species levels. Sprott and Patel (1986) found that methane formation from obligate acetotroph *Methanosaeta concilii* was completely inhibited at a TAN concentration of 560 mg l^{-1}, while methane formation from *Methanosarcina barkeri* was not inhibited at a TAN concentration of 2800 mg l^{-1}.

Amongst the 83 species of methanogens discovered, the majority (61 species) are hydrogenotrophs that oxidise H_2 and reduce CO_2 to form methane, while only 9 species of acetoclastic methanogens are known that utilise acetate to produce methane (Garcia et al., 2000). Some researchers have concluded that cell morphology plays an important role in resisting ammonia toxicity. Demirel and Scherer (2008) attributed the particular susceptibility of acetate-utilising methanogens *Methanosaetaceae* to the cell morphology which is one of thin filaments. Due to the cell shape, *Methanosaetaceae* seemed to offer more surface area than the hydrogenotrophic methanogens which grow as rods, or *Methanosarcinaceae* which grow as thick clumps. The diffusion of free ammonia into the cells would be faster in the filamentous cells when expressed on the basis of kilograms of NH_3 entering per kilogram cell mass per hour. Similar speculation has also been by made by Wiegant and Zeeman (1986). A study by Hendriksen and Ahring (1991) even suggested that for some thermophilic hydrogenotrophic methanogens, high ammonia concentration in the growth medium can induce formation of large cell aggregates, which implied a possible defence mechanism for those strains against ammonia toxicity.

The selective inhibition of ammonia towards methanogens will have a profound impact on the diversity of the methanogenic population in an anaerobic digester treating an ammonia/ nitrogen rich substrate, i.e. under the influence of ammonia, hydrogenotrophic methanogens will gradually become the predominant species and as the result the major methanogenesis pathway will shift to hydrogenotrophic (Calli et al., 2005, Westerholm et al. 2011). Jiang et al. (2018) using C-14

radiolabelling technique on a selection of mesophilic digesters representing samples of low to high TAN concentrations (200–11100 mg N kg^{-1}). For high ammonia digesters (>4000 mg N kg^{-1}), $^{14}CO_2$ /$^{14}CH_4$ ratio found in the biogas was found in the range 2.1–3.0; indicating 68–75% of methane was produced via the hydrogenotrophic route; whereas in low ammonia samples (200-1600 mg N kg^{-1}) the ratio was 0.1–0.3, indicating 9–23% of methane was produced by the hydrogenotrophic route.

The application of advanced molecular microbiology techniques such as Fluorescent *in situ* Hybridisation (FISH) and Quantitative Polymerase Chain Reaction (q-PCR) have made it possible to monitor changes in the methanogenic population in anaerobic digesters exposed to a high concentration of ammonia. Results from these studies (Calli et al., 2005, Goberna, 2010, Schnürer and Nordberg, 2008, Westerholm et al. 2011) have clearly indicated the obvious change of dominate methanogen population to hydrogenotrophic after the exposure to ammonia.

Changes in the methanogenic population and major methanogenic pathway were examined during start-up of a full-scale anaerobic sequencing batch reactor (ASBR) treating swine waste (Angenent et al., 2002). It was observed that after the increase in total ammonia concentration during the start-up process, acetate degradation increased; however the acetoclastic methanogens population decreased based on 16S ribosomal RNA (rRNA) concentrations.

Karakashev et al. (2005) studied the influence of environmental parameters on the diversity of methanogenic communities in 15 full-scale biogas plants treating either manure or sludge as substrates under different conditions. The findings of this study indicated that in plants operating in the mesophilic range, where the free ammonia concentration was lower than in thermophilic plants, the diversity of the methanogenic population was broader. Dominance of the acetoclastic phylogenetic group *Methanosaetaceae* was observed in digesters fed with sludge (ammonia concentration at 0.03-0.3 g N l^{-1}). However, *Methanosaetaceae* was never found to be dominant in digesters treating manure (ammonia concentration at 2.1-6.1 g N l^{-1}). The authors reported that the inoculum type and loading rate did not affect the diversity of methanogens in biogas reactors, but the concentrations of ammonia and VFA were influential.

The consequences of the change in methanogenic pathway for the apparent behaviour and maintenance strategies of the anaerobic digestion process are significant. Under normal, uninhibited conditions, 70% of methanogenesis is attributed to the acetoclastic route and only 30% of methane is formed via the hydrogenotrophic route (Jeris and McCarty, 1965, Gujer and Zehnder., 1983). During anaerobic digestion, hydrogenotrophic methanogens play a crucial role to keep the hydrogen partial pressure low enough to make it thermodynamically possible for propionate and butyrate to be converted into the methanogenic substrates, i.e. acetate and hydrogen (Wolin and Miller, 1982). According to Gujer and Zehnder (1983), the hydrogen partial pressure should be maintained under 10^{-4} bar (0.1 kPa) in order to sustain a healthy digestion process; furthermore hydrogen partial pressure is the parameter that most promptly indicates a disturbance in the digestion process. Therefore if the digestion has been inhibited by ammonia and methanogenesis is reliant solely on the hydrogenotrophic pathway, any further disturbance to methanogenesis would cause accumulation of hydrogen which subsequently results in thermodynamic blockage of propionate degradation in a timescale of a second (Koch et al. 1983; Koster and Koomen 1988); whereas acetoclastic blockage affects anaerobic digestion by reducing pH, which normally takes about 1 day (Gujer and Zehnder, 1983). This could very likely be the reason why anaerobic digesters treating nitrogen-rich substrate such as food waste, are prone to rapid and irreversible operational failure (Neiva Correia et al., 2008; Banks and Zhang, 2010).

5. Impact of ammoniacal nitrogen on toxicity in various feedstocks

From the large number of previous literatures regarding the ammonia inhibition in AD, a broad range of high nitrogen substrates has been studied. These numerous substrates can be grouped into 2 types of waste streams, i.e. animal manures and municipal, commercial and industrial organic wastes.

5.1 Animal manure

A number of relevant studies on this topic were carried out on thermophilic digestion of manure. Zeeman et al. (1985) studied the influence of total ammonia concentration on thermophilic (50°C) digestion of cow manure, which in the Netherlands often contains total ammonia concentration up to 4000 mg l^{-1}. Results in a CSTR configuration showed inhibition at 1700 mg l^{-1} of TAN, but after

acclimation a constant methane production was achieved even at higher ammonia levels (up to 3300 mg l^{-1} as TAN). Zeeman et al. (1985) carried out batch digestion trials, and observed a fourfold increase in methane production if the pH was reduced from 7.5 to 7.0.

Angelidaki and Ahring (1993) investigated the effect of ammonia on cattle manure digestion and found that an ammonia concentration of 4000 mg l^{-1} inhibited the process, but stable digestion could be maintained at up to 6000 mg l^{-1} after 6 months of operation, with a consequent reduction in methane production.

The combined effect of temperature and ammonia were studied by Angelidaki and Ahring (1994) in CSTR treating cattle manure. They observed a decrease in biogas yields both for low (2500 mg l^{-1}) and high (6000 mg l^{-1}) ammonia load, when the temperature was increased from 55 to 64°C. A negative effect at high ammonia loadings was also evident at a temperature of 45°C. These conditions corresponded to a FA concentration of 600-800 mg l^{-1} which is higher than inhibition levels reported elsewhere for mesophilic digestion (80-150 mg l^{-1} of FA at pH 7.5) (Angelidaki and Ahring, 1994; Braun et al., 1981). Acclimation of the inoculum was suggested as the reason for this higher tolerance, as shown by Hashimoto (1986). Hashimoto (1986) found that if the inoculum was acclimatised at TAN between 1400 and 3300 mg l^{-1}, inhibition began at 4000 mg l^{-1} compared to 2500 mg l^{-1} without acclimation.

Borja et al. (1996) tested the influence of ammonia on a thermophilic UASB process treating cattle manure. An initial inhibition was observed at 5000 mg l^{-1} of TAN, but a stable digestion could be maintained at 7000 mg l^{-1} after 6 months of operation although with lower methane production and an increased VFA concentration. In addition, it was found that strong inhibition occurred with rapid temperature increases which could be avoided if the temperature was increased gradually.

Krylova et al. (1997) tested different ammonia concentrations (as NH_4Cl) in the AD of poultry manure (batch tests) and observed no inhibition up to 2600 mg l^{-1} of TAN but a reduction of 80-90% in biogas production for concentrations ranging from 2600 to 8000 mg l^{-1} of TAN. Hansen et al. (1999) observed a thermophilic AD inhibition treating swine manure in batch condition and found a limit value of 1100 mg N l^{-1} as free ammonia.

5.2 Municipal, commercial and industrial organic wastes

Liu and Sung (Liu and Sung, 2002; Sung and Liu, 2003) used acclimatized AD sludge at different ammonia concentrations with a substrate of non-fat dried milk, and observed in both batch and continuous tests that a strongly inhibitory TAN concentration for methanogens was between 8000 and 13000 mg l^{-1} depending on acclimation conditions and the pH of the system.

Gallert and Winter (1997) studied the AD of OFMSW in CSTR configuration at thermophilic and mesophilic temperatures. During the thermophilic process 1400 mg l^{-1} of ammonia was released, whereas in the mesophilic process only 1000 mg l^{-1} ammonia was generated, presumably from protein degradation. The results reported a 50% inhibition in methane production at 690 and 680 mg l^{-1} of FA at thermophilic temperature, and a 50% inhibition at 220 and 280 mg l^{-1} of FA at mesophilic temperature.

Kayhanian (1999) investigated ammonia inhibition in a pilot-scale high solids anaerobic reactor treating simulate OFMSW and showed an initial inhibition at a TAN concentration of about 1200 mg l^{-1} (FA 45 mg l^{-1} and pH of 7.2). Two strategies to mitigate ammonia inhibition were identified: the dilution of the digester contents with fresh water with the aim to correct ammonia overloads; and the adjustment of the feedstock C:N ratio from 27 to 32 (C:N ratio calculated using biodegradable carbon and total nitrogen values).

Climenhaga and Banks (2008) tested ammonia inhibition in bench-scale single-stage digesters treating food waste (a varied mix of fruits, vegetables, meats and fried foods). A constant organic loading rate (OLR) was maintained with different hydraulic retention times (25, 50, 100 and 180 days). The 100-day HRT reactors sustained total ammonia nitrogen (TAN) levels beyond 3000 mg l^{-1}, while in the 180-day HRT reactors anaerobic digestion continued at TAN concentrations exceeding 5700 mg l^{-1} at pH above 7.5. Free ammonia therefore exceeded 1000 mg l^{-1}, well beyond the inhibitory levels of 80-150 mg l^{-1} reported in early studies (Koster and Lettinga, 1984; McCarty and McKinney, 1961). In this investigation, TAN appears to be more beneficial than detrimental, as it provides buffering capacity in the long HRT reactors, as opposed to the 25-day HRT reactor in which TAN was washed out and declined through the trial.

Benabdallah El Hadi et al. (2009) performed some batch tests at both mesophilic and thermophilic temperatures using a synthetic substrate simulating OFMSW. It appeared that not only free ammonia affected the methanogenic fermentation, but the ammonium ion also had similar effects. A 50% inhibition of biomethane production was observed at level of 215 and 468 mg FA l^{-1} under mesophilic and thermophilic conditions. However, methane generation under mesophilic and thermophilic conditions was reduced by 50% when the ammonium ion reached concentrations of 3860 and 5600 mg l^{-1} under mesophilic and thermophilic temperature conditions, respectively.

5.3 Ammoniacal nitrogen inhibitory concentrations of various waste streams

From the data in Tables 1 and 2, it can be concluded that in a CSTR configuration the inhibiting range for anaerobic digestion of cattle manure is 600-900 mg/l as free ammonia, whilst for swine manure it is from 1600 to 2600 mg l^{-1} of FA. Inhibition levels reported for batch assays of manure ranged between 2600 and 8000 mg TAN l^{-1} or 1100 mg N l^{-1} as free ammonia. Anaerobic digestion of OFMSW in CSTR configuration appear to show the following inhibitory concentration range: 45-1000 mg FA l^{-1} and 1830 – 5700 mg TAN l^{-1}, while anaerobic digestion of the same substrate in batch assays showed inhibitory concentrations in the range: 215-468 mg FA l^{-1} and 4080-6070 mg TAN l^{-1}.

As expected, a wide range of inhibitory concentrations of ammonia were reported in literature as shown in Table 1 and 2. The significant difference in ammonia concentration can be attributed to the differences in substrates and inocula, environmental conditions (temperature, pH) and acclimation periods. The importance of acclimatisation is due to different level tolerance to ammonia toxicity within methanogen species (Raynal et al., 1998, Koster and Koomen, 1988, Borja et al., 1996). According to Hansen et al., (1999), at 1100 mg l^{-1} of FA ammonia affects organisms such as the acetate utilizing methanogens that are normally responsible for approximately 70% of methane production in the digestion of sewage sludge, while inhibition of the H_2-utilizing methanogens occurred at a higher FA concentration (>1200 mg l^{-1}). The acclimatisation of anaerobic process to high concentration of ammonia is likely to lead to the internal changes in the predominant species of methanogens and a shift in the methanogenic pathway (Raynal et al., 1998).

To summarise, the previous literature on anaerobic digestion shows considerable variations in the inhibition/toxicity levels reported for FA and TAN. The reason for these variations is the complexity of the anaerobic digestion process where mechanisms such as antagonism, synergism and acclimatisation can significantly affect the phenomenon of inhibition.

Table 1. Inhibition limit of FA and TAN in continuously fed reactors

Temp. °C	Substrate	Reactor	Inoculum	Inhibition limit FA mgN l^{-1}	Inhibition limit TAN g N l^{-1}	% reduction in CH_4 production	pH	References
55	Soluble non-fat dry milk + NH_4Cl	CSTR	Acclim		5.77	64	6.40	Sung and Liu, 2003
55	Cattle manure	CSTR	Acclim	600-800	NR		7.4-7.9	Angelidaki et al., 1994
55	Cattle manure	UASB	Acclim	500	7.00	72		Borja et al., 1996
50	Cattle manure	CSTR	NR	NR	1.70	Initial inhibition	NR	Zeeman et al., 1985
55	Cattle manure	Continuously fed reactor	NR	900	4.00	25	NR	Angelidaki and Ahring, 1993
55	Swine manure	CSTR	NR	1600	NR	70	7.97	Hansen et al., 1998
60	Swine manure	CSTR	NR	2600	NR	96	8.15	Hansen et al., 1998

55	OFMSW	Complete-mix reactor	NR	45	1.2		7.20	Kayhanian et al., 1999
37	Food waste	CSTR	NR	> 1000	5.7	NR	>7.5	Climenhaga and Banks, 2008
55	OFMSW	CSTR	Acclim	680-690	3.4-3.5	50	7.60	Gallert and Winter, 1997
37	OFMSW	CSTR	Acclim	220-280	3.0-3.7	50	7.60	Gallert and Winter, 1997
55	OFMSW	CSTR with waste recirculation	Acclim	251	1.83	NR	NR	Gallert et al., 1998

Note: Acclim = acclimatised; FA= Free Ammonia; NR= Not reported

Table 2. Inhibition limit of FA and TAN in batch reactors

Temp. °C	Substrate	Inoculum	Inhibition limit FA mgN l^{-1}	Inhibition limit TAN gN l^{-1}	% reduction in CH_4 production	pH	References
52	Glucose+Na_2SO_4+ ammonia	Acclim	620	NR	21	NR	Siles et al., 2010
55	Soluble non fat dry milk + NH_4Cl	Acclim	NR	10.00	100	NR	Liu and Sung, 2002
55	Cattle manure + NH_4Cl	Acclim	NR	4.00	NR	7.2	Hashimoto et al., 1986
55	Swine manure	NR	1100	NR	NR	NR	Hansen et al., 1998
55	Poultry anure+NH_4Cl	NR	NR	2.6-8	80-90	NR	Krylova et al., 1997
55	Synthetic OFMSW	NR	468	6.07	50	7.5	Benabdallah El Hadi T. et al., 2009
37	Synthetic OFMSW	NR	215	4.08	50	7.5	Benabdallah El Hadi T. et al., 2009

Note: Acclim = acclimatised; FA= Free Ammonia; NR= Not reported

6. Mitigation of Ammonia Inhibition to Anaerobic Digestion

Dilution of high nitrogen content waste streams with either water or low nitrogen materials (co-digestion) can reduce the concentration of ammoniacal nitrogen produced during the anaerobic digestion process. Both options are commonly employed to optimise solids loading into municipal AD, or to control organic loading in food waste AD, for example, where the feed mixture can be adjusted to some extent through regulation of sludge imports from different sources. It is worth noting that this practice seeks to maximise the financial return on capital infrastructure by seeking to optimise hydraulic or organic loading, without risking asset failure. When there is co-substrate available, then co-digestion of high nitrogen content substrate with nitrogen deficient substrate can be considered for adjustment of the C:N ratio, to sustain the ammoniacal nitrogen concentration below the inhibitory concentration. For example, Wang et al. (2012) investigated the C:N ratio for the co-digestion of dairy manure, chicken manure and wheat straw in a batch experiment under mesophilic anaerobic conditions. The authors reported stable digestion for C:N ratios of 25, 30 and 35, which corresponded to FAN concentrations of 9.1, 7.5 and 2.2 mg l^{-1}. However, when the C:N was reduced to 15, both the TAN and FAN increased to concentrations of 2614 and 223 mg l^{-1} respectively, introducing digestion failure.

However, if the sole purpose of diluting the feed is to manage free ammonia below a critical threshold, this practice will inevitably reduce the return on capital investment, and is not likely to be favoured.

Physico-chemical methods for the selective separation of either the free ammonia or ammonium have also been trialled, focussing in at various stages of integration, either as a pre-treatment, in-situ, side-stream or post-digestion process (Hansen et al., 1999; De La Rubia, et al. 2010; Zhang & Jahng, 2010; Walker et al., 2011; Lauterbock et al., 2012; Mcleod et al., 2016; Garcia-Gonzalez et al., 2016; Serna-Maza et al. 2017). The separation of free-ammonia is conventionally facilitated using a phase separation, in which a two-stage packed column comprising an air stripper stage is used to separate NH_3 whilst the second stage stabilises the

gas phase ammonia as ammonium in a concentrated acid (Kinidia et al., 2018). This two-stage system has been applied at full scale for TAN removal from a poultry litter AD with a capacity of 29,000 tonnes per annum (Burns, 2012). Teichgräber and Stein (1994) proposed stripping with steam, the advantage being the recovery of a highly concentrated waste ammonia product, which could represent a valuable by-product, complimented by the use of waste heat as the driving force which could also limit chemical consumption. Similarly, Walker et al. (2011) investigated the feasibility of biogas stripping to remove ammonia in the anaerobic digestion of source segregated food waste. The authors indicated technical suitability for TAN reduction, when applied at numerous integration points, however, inclusion of sufficient specific surface area and energy requirements for stripping, coupled with the subsequent management of the downstream gaseous phase demands consideration (Heile et al., 2017). Several authors have also proposed immersed solutions for direct contact with sludge using membrane technology, either for in-situ treatment (Lauterbock et al., 2012; Garcia-Gonzalez et al., 2015) or as a side-stream process (Mcleod et al., 2016). García-González et al. (2015) reported on application of gas selective membranes for digested swine manure. Whilst successful, the separation is necessarily limited by gas permeability of the polymer. Lauterbock et al. (2012) instead demonstrated immersed hydrophobic hollow-fibre membranes, where the sludge contacted the outside of the fibre and acid was recirculated on the insider of the fibre. This enables an analogous technology to two stage air stripping/acid scrubbing but within a single engineered stage, and evidenced a 70% reduction in free ammonia which led to considerably higher gas yields. This technology has been successfully demonstrated at scale for a broader range of industrial applications (Ulbricht et al., 2013), but as with two-stage contactors, the final ammonia-acid product is difficult to dispose of and will require tankering. Mcleod and McAdam (2016) therefore introduced an antisolvent into the acid phase which, when combined with the reduction in free energy barrier by the hydrophobic membrane, enabled the recovery of crystalline ammonium sulphate, which reduces waste volume and simplifies product disposal. There is also value in the removal of ammonia from

AD slurries for post-treatment disposal as the spreading of AD slurries to land is primarily constrained by the nitrogen content.

Chemical methods have also been applied for the separation of ammonium which rely on valence state (NH_4^+) rather than volatility as with the FAN fraction. Thornton et al. (2007) trialled a synthetic clay ion exchange media (MesoLite) for the separation of ammonium from digested sludge liquors, which expressed a cation exchange capacity of 51 g NH_4^+-N kg^{-1} and evidenced over 95% ammonium nitrogen removal at a flow rate 0.5 m^3 h^{-1}. Whilst the technology cannot be directly applied to a high solids fraction for risk of clogging and fouling of the IEX media, suitable pre-treatment technology could make this suitable as a sidestream solution for specific applications. Chemical precipitation of ammonium with Mg and P to form magnesium ammonium phosphate hexahydrate ($MgNH_4PO_4 \cdot 6H_2O$), or struvite, as a low water soluble crystal has also been trialled. By integrating controlled struvite precipitation into AD, Uludag-Demirer et al. (2008) enabled ammonia removal between 11 and 23%. Whilst many promising management solutions are available, each offering quite different value propositions (trade-off between cost, energy resilience, chemical consumption and final product quality), the synergy between the reduction in toxicity, simplification in sludge disposal and the realisation of a final product with a potential resale value (e.g. ammonia) may see the value proposition shift in favour of technologies for ammonia separation in AD.

In addition to physico-chemical method, studies also suggested that supplementing certain trace elements to anaerobic digesters with high ammonia concentration could significantly improve stability of the operation at increased organic loading rate (OLR). In a study carried out by Bayr et al. (2012), trace element addition on process stability of mesophilic anaerobic digestion pig slaughterhouse waste was investigated using laboratory-scale CSTRs. The ammonia concentrations in digesters were increased to 2500 mg l^{-1} and 3500 mg l^{-1}. The study found that the use of additive (Fe, Co, Ni, Se W and HCl) prevented the build-up of VFAs and enabled higher OLR with stable performance compared to the digester without additive. Banks et al. (2012) investigated effect of trace element to the performance of CSTR digesters treating

food waste where ammonia concentrations are in the range of 5000- 6100 mg l^{-1}. The study found that adding critical trace element Co and Se, the maximum OLR can be achieved was 5 g VS l^{-1} day^{-1}, whereas the trace element deficient control digester could not sustain at the OLR at 2 g VS l^{-1} day^{-1}. Using molecular microbiology technique, only hydrogenotrophic methanogen can be observed in these high ammonia concentration digesters. This suggested the acclimatised hydrogenotrophic methanogens in the high ammonia digesters were specifically dependent on certain types of trace element to maintain their biological function.

7. Conclusion

Anaerobic digestion of high nitrogen content waste streams such as animal manure and municipal or industrial organic waste will lead to high ammonia concentration during the digestion process. Ammonia, especially in the free molecular form (NH_3), is a potent inhibitor to methanogens responsible for the methanogenesis stage of the AD. Process inhibition is related to several operational parameters such as pH, temperature, acclimatisation of inoculums and concentration of ammonium ion and free ammonia. The mechanism of ammonia toxicity is not yet clear; however theories have been put forward which includes

Within the 2 major groups of methanogens, i.e. acetoclastic and hydrogenotrophic, acetoclastic methanogens are more sensitive to ammonia inhibition, therefore after adequate acclimatisation period dominant methanogen and methanogenic pathway in high ammonia anaerobic digester will shift to hydrogenotrophic. No definitive explanation for this selective inhibition has been proposed so far. Considerable further work on microbiological or molecular biology level is required to improve our understanding of the mechanism of ammonia toxicity. Several solutions have been reported to mitigate the adverse effect of high ammonia, such as C:N ratio adjusting, gas stripping of ammonia from digestate, chemical precipitation as struvite, and have proven to be technologically viable. However, the implementation of these technologies to larger scale operation has to be taken into consideration the overall energetic and economic benefits. It is noteworthy that supplementation of certain trace element can significantly improve operation stability and performance under high ammonia concentration.

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