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Characterisation of thiocyanate degradation in a mixed culture activated sludge process treating coke wastewater

Eleanor Raper [a], Tom Stephenson [a], Raymond Fisher [b], David, R. Anderson [b], Ana Soares [a*].

[a] *Cranfield Water Sciences Institute, Cranfield University, Cranfield, MK43 0AL, UK.*

[b] *Tata Steel, Group Health Safety and Environment, Swinden Technology Centre, Rotherham, S60 3AR, UK.*

ABSTRACT

Microbial degradation of thiocyanate (SCN^-) has been reported to suffer from instability highlighting the need for improved understanding of underlying mechanisms and boundaries. Respirometry, batch tests and DNA sequencing analysis were used to improve understanding of a mixed culture treating coke wastewater rich in SCN^-. An uncultured species of *Thiobacillus* was the most abundant species (26%) and displayed similar metabolic capabilities to *Thiobacillus denitrificans* and *Thiobacillus thioparus*. Thiocyanate was hydrolysed /oxidised to NH_4^+-N, HCO_3^- and SO_4^{2-}. Nevertheless, at 360-2100 mg SCN^-/L a breakdown in the degradation pathway was observed. Respirometry tests demonstrated that NH_4^+-N was inhibitory to SCN^- degradation (IC_{50}: 316 mg/L). Likewise, phenol (180 mg/L) and hydroxylamine (0.25 - 16 mg/L) reduced SCN^- degradation by 41% and ca. 7%, respectively. The understanding of the SCN^- degradation pathways can enable stable treatment efficiencies and compliance with effluent of <4 mg SCN/L, required by the Industrial Emissions Directive.

*Corresponding author at Cranfield Water Science Institute, Cranfield University, Vincent Building, Cranfield, Bedfordshire, MK43 0AL, UK. Tel.: +44 (0) 1234 758121. E-mail address: a.soares@cranfield.ac.uk (A. Soares).

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1. Introduction

Production of coke for steel making, generates wastewater which contains significant quantities of thiocyanate (SCN^-) ranging from 50 to 400 mg/L (Pan et al., 2018a; 2018b; Raper et al., 2019; Staib and Lant, 2007; Vázquez et al., 2006). Thiocyanate is generated when cyanide and sulphur react under the high temperatures associated with the coke making process (Kim and Katayama, 2000). Emissions of SCN^- are regulated in coke making wastewater by the Industrial Emissions Directive (IED) and must be reduced to less than 4 mg/L (European Commission, 2013). Removal of SCN^- from the coke wastewater can be achieved through treatment in an activated sludge process (ASP), where biological flocs are mixed with the raw wastewater enabling degradation of target compounds in the wastewater. The treatment of SCN^- in an ASP however, is known to be sensitive and unstable, which can lead to treatment losses (Staib and Lant, 2007; Vázquez et al., 2006). Staib and Lant (2007) reported SCN^- degradation to be the most sensitive process after nitrification.

Due to its production in many industrial processes there has been an appreciable interest in SCN^- degradation (Combarros et al., 2015; Grigor et al., 2009, 2006; Hung and Pavlostathis, 1999; Watts and Moreau, 2016). Thiocyanate degrading bacteria have been isolated and identified from a variety of sources including the genera *Arthrobacter*, *Bacillus*, *Escherichia*, *Pseudomonas*, *Thiobacillus*, *Acinetobacter*, *Burkholderia*, *Chryseobacterium*, *Klebsiella*, *Ralstonia* and *Methylobacterium* (Boucabeille et al., 1994; Chaudhari and Kodam, 2010; Huang et al., 2013; Hung and Pavlostathis, 1997;

Kelly and Wood, 2000a; Kim and Katayama, 2000; Lee et al., 2008, 2003; Pan et al., 2018a; Sorokin et al., 2001). Numerous pathways have been identified for the biodegradation of SCN^-, including through the action of autotrophic and heterotrophic bacteria (Table 1). Autotrophic bacteria utilise inorganic carbon from SCN^- as a carbon source whilst heterotrophic SCN^- degraders utilise SCN^- as a source of nitrogen and use organic carbon as an energy source (Watts and Moreau, 2016). Autotrophic pathways are the most commonly reported, whereas heterotrophic pathways are less commonly reported and have mainly been linked with tests in synthetic wastewaters (Table 1) (Watts and Moreau, 2016). Several end products have been reported including ammonia (NH_4^+-N), sulphate (SO_4^{2-}), carbonyl sulphide (COS) and trithionate. Ammonia and SO_4^{2-} have both been reported to be produced under both aerobic and anoxic conditions. Carbonyl sulphide has only been reported to occur under aerobic conditions whilst trithionate has only been reported to arise under anoxic conditions whilst COS has only been observed to arise under aerobic conditions.

Additionally, a number of intermediate compounds have been reported including thiosulphate ($S_2O_3^{2-}$), tetrathionate ($S_4O_6^{2-}$) and cyanate (OCN^-). As there are several possible degradation pathways, a greater understanding of the degradation pathway and bacterial requirements will provide a better understanding of the requirements which need to be met to maintain stable operation in wastewater treatment plants.

Treatment of SCN^- in coke wastewater is further complicated by the presence of numerous species that are associated with toxicity. Coke wastewater contains NH_4^+-N, phenol, polycyclic aromatic hydrocarbons (PAHs) and trace metals (Bai et al., 2010; Marañón et al., 2008; Raper et al., 2017; Vázquez et al., 2006). Concentrations of such pollutants can vary significantly between and within different treatment works in

response to the changing coal blends and operational conditions (Marañón et al., 2008). Ammonia concentrations have been reported to vary between 50 and 500 mg/L with similar fluctuations observed for phenol concentrations (60 – 400 mg/L) (Bai et al., 2010; Marañón et al., 2008; Vázquez et al., 2006). The sum of 6 PAHs (sum of fluoranthene, benzo[b]fluoranthene, benzo[k]fluoranthene], benzo[a]pyrene, indeno[1,2,3-cd]pyrene and benzo[g,h,i]perylene) was reported at 179±35 $\mu g/L^{-1}$ whilst total trace metals (sum of Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Cd and Pb) were reported at 4216 µg/L (Raper et al., 2017).

Paruchuri, Shivaraman and Kumaran (1990) reported that a mixed culture containing *Pseudomonas* and *Bacillus* species was capable of degrading up to 1,400 mg/L of SCN^- in batch culture over 6 days. Furthermore, they investigated the impact of phenol and NH_4^+-N, demonstrating that the latter had no inhibitory effect up to 2000 mg/L, after which prolonged oxidation was required to maintain SCN^- degradation. Thiocyanate degradation was more sensitive to phenol, with 50 mg/L of phenol prolonging the oxidation time and 500 mg/L resulting in complete inhibition. In contrast, Staib and Lant (2007) suggested that under continuous treatment conditions phenol would exert no inhibitory influence as its degradation rate would exceed that of SCN^-. Jeong and Chung (2006) investigated the degradation of SCN^- in a laboratory-scale continuous process. Coke oven wastewater was diluted to create a wastewater characterised by SCN^- levels of 3000-7000 mg/L. The diluted wastewater was then passed through a fluidized biofilm reactor with a 40% filling ratio. When the volumetric loading rate of thiocyanate exceeded ca. 4 kg/m^3.d the biodegradation rate slowly declined demonstrating a substrate inhibition effect. Outlet SCN^- concentrations >50 mg/L were correlated with declining degradation rates.

Observations into SCN^- degradation to date have been controversial with further investigation being required. The objective of this study was to characterise the mixed culture responsible for SCN^- degradation in the ASP treating coke wastewater to enable a greater understanding of the conditions required to maintain stable treatment efficiencies and enable compliance with the IED emission limit of <4 mg/L. An uncultured species of Thiobacillus was identified which had similar metabolic characteristics to T. *thioparus* and T. *denitrificans*. Degradation of SCN- produced ammonia NH_4^+-N, HCO_3^- and SO_4^{2-}. Ammonia, phenol and hydroxylamine demonstrated inhibitory effects. The stable degradation of SCN^- required mesophilic temperatures.

2. Materials and Methods

2.1 Wastewater and activated sludge biomass

Coke wastewater and activated sludge biomass were collected from a full-scale ASP at a UK integrated steelworks. The ASP consisted of aeration tanks (combined volume of 2,280 m^3) receiving coke wastewater at an average flow rate of 680 m^3/d giving an average hydraulic retention time (HRT) of 21 hours. Activated sludge biomass was taken from the return activated sludge (RAS) line and was characterised by an average sludge retention time (SRT) of ca. 38 days. The MLSS of the RAS varied in response to sludge wasting and therefore varied between 16,000 and 33,000 mg/L.

The coke wastewater was characterised by a sCOD of 644±130 mg/L. Thiocyanate and phenol concentrations were 95±18 mg/L and 20±2 mg/L respectively. Ammonia contributed the highest concentration of nitrogen to the wastewater at an average of 91±24 mg/L as NH_4^+-N with small contributions from NO_3^--N (3±2.5 mg/L) and NO_2^--

N (3±2.9). The wastewater exhibited a pH of 7.8±0.3. Concentrations of pollutants therefore fell within the typical range for coke wastewater previously reported for this site as well as those reported in the wider literature (Marañón et al., 2008; Raper et al., 2019; Staib and Lant, 2007; Vázquez et al., 2006).

2.2 Impact of temperature on SCN^- degradation

Batch tests were conducted to investigate the impact of temperature and concomitant nitrification and SCN^- degradation. Batch tests with a 0.95 L working volume were completed using coke wastewater and activated sludge biomass to produce a mixed liquor suspended solids concentration (MLSS) of 4500 mg/L. Air pumps enabled dissolved oxygen to be maintained at ca. 3 mg/L. Samples were taken systematically to demonstrate the influence of temperature and concomitant nitrification. Temperature was maintained through a water bath at 25^{o}C to reflect the target conditions on the full-scale ASP, however, to investigate the impact of temperature some batch tests were left without heat provision. These batch tests were therefore subject to diurnal temperature variations ranging from 8 to 21^{o}C.

2.3 Respirometry

Activated sludge biomass was collected in advance of each run of the respirometer and was stored at 2 - 5^{o}C for a maximum of 48 hours. The respirometer (Environmental Services, UK) consisted of ten respirometric cells (450 ml working volume) positioned in a water bath maintained at a temperature of 25 ±1^{o}C to reflect the target operational conditions of the full-scale ASP. Temperature was controlled by a Grant thermostatic circulator (GD120), UK. Oxygen supply to the respirometric cells was enabled via the provision of a copper sulphate pentahydrate solution (25% w/v). Carbon dioxide was

removed by a 2M sodium hydroxide solution. Oxygen consumption data were recorded by a data logger at 20 minute intervals. Oxygen concentration within each respirometric cell was maintained via agitation using a magnetic stirrer. Activated sludge biomass and coke making wastewaters were combined to replicate the mixed liquor concentrations in the full-scale treatment process of 8300 mg/L.

In order to investigate the inhibition of SCN^- the mixed liquor from the coke making process was spiked with solutions of hydroxylamine hydrochloride (NH_2OH), potassium nitrite and potassium nitrate at concentrations varying from 0.25 – 16 mg/L. The impact of SCN^- was investigated through spiking potassium thiocyanate at concentrations from 250 - 2000 mg/L. The impact of ammonia and sulphate were investigated by spiking ammonium chloride and potassium sulphate at concentrations of 250 - 1500 mg/L NH_4^+-N and 1000 - 2000 mg/L SO_4^{2-} respectively. Inhibition tests were completed over 4-5 days and repeated in triplicate with decreased oxygen consumption in the test cells in comparison to the control cells representing inhibition of the biomass.

For each compound and concentration, the percentage inhibition was calculated takin in consideration the variation in oxygen consumption. The logarithm of the concentration of each compound was calculated and plotted against the observed inhibition. The half maximal inhibitory concentration (IC50) was then plotted and the inverse logarithm of the compound concentration calculated to provide the IC50."

2.4 Chemical analysis

Samples were filtered (0.45 μm syringe filters -VWR) and pH recorded (Jenway 3540, UK). Mixed liquor suspended solids were analysed according to standard methods

(Eaton, 2005). Merck cell test kits were used to determine the concentrations of NO_2^--N, NO_3^--N, NH_4^+-N, SO_4^{2-} and soluble chemical oxygen demand (sCOD) following the manufacturer's instructions. Thiocyanate and phenol were determined colorimetrically at a wavelength of 465 and 510 nm, respectively, using a Jenway 6300 spectrophotometer (Staffordshire, UK). Thiocyanate was determined by a method based upon the reaction of thiocyanate with iron (III) to produce an orange-red colour based on a red complex (The Institution of Gas Engineers, 1971) while phenol was determined using 4-aminoantipyrene based upon ISO 6439:1990 (ISO, 1990).

2.5 Molecular microbial ecology

Activated sludge biomass was taken from four respirometric cells which were operated under controlled conditions. Activated sludge biomass was analysed through polymerase chain reaction (PCR) in order to quantify the microbial diversity in the ASP mixed liquor. The biomass was placed into a lysing matrix tube and the deoxyribonucleic acid (DNA) extracted (MPBIO FastDNA Spin Kit for soil, Santa Ana, USA). The V4 and V5 regions of the 16S ribosomal RNA (rRNA) gene were targeted with the universal primers 515F and 926R (Quince et al., 2011). Error correcting golay barcodes enabled sample multiplexing (Hamady et al., 2012). Polymerase chain reaction products were purified using HighPrep magnetic beads (Magbio, Gaithersburg USA) and QuantiFluor ONE (Promega, Madison USA). An equimolar pool of amplicons was sequenced using Illumina MiSeq with 2x300 v2 chemistry (Illumina, San Diego USA). Quantitative Insights Into Microbial Ecology (QIIME) 1.9 (Caporaso et al., 2010) and the SILVA 16S rRNA gene database v123.1 (Quast et al., 2013) were used for sequence analysis. The 16S rRNA gene sequences were grouped at 97% similarity to create operational taxonomic units (OTUs). Representative sequences from each

OTU were then taxonomically assigned using the SILVA database. If the 16S sequences were not found in the database, these were described as “uncultured”, “ambiguous” or “other”. An uncultured sequence was one in which the sequence matched a database sequence but taxonomy was unavailable. An ambiguous species referred to a sequence which had more than a 97% similarity to more than one sequence of the genus. A sequence was referred to as “other” when the sequence could be identified no further than the genus level.

3. Results and Discussion

3.1 Thiocyanate degradation in the mixed culture

Respirometry tests showed that the mixed culture was capable of SCN^- removal at a range of initial SCN^- concentrations (Table 2). After 120 hours, removal of 110 mg/L SCN^- was complete. For initial SCN^- concentrations of 360 to 610 mg/L, the average SCN^- removal was 19 and 13%, respectively. Hence, as the initial SCN^- concentrations increased, removal efficiencies declined. Whether the observed decline in removal efficiency was as a result of toxicity or the requirement for longer degradation times deserves further investigation. Despite this, at an almost 20 times increase in the initial SCN^- concentration to 2109 mg/L, 58% of the initial SCN^- was degraded after 5 days demonstrating the ability of the mixed culture to cope with high SCN^- concentrations. The mixed culture therefore had a high SCN^- removal capacity similar to the mixed consortium investigated by Paruchuri, Shivaraman and Kumaran (1990). Similarly, a co-culture of SCN^- degrading bacteria *Klebsiella pneumoniae* and *Ralstonia* showed decreased removal efficiencies at increased initial concentrations in batch tests conducted by Chaudhari and Kodam (2010). Degradation efficiencies declined from

100% at 500 mg/L SCN^- to 76%, 57%, 42%, and 34% at 1000, 1,500, 2,000, and 2,500 mg/L SCN^- respectively.

On the other hand, different SCN^- initial concentrations resulted in different end products (Table 2). When the initial SCN^- concentration was 110 mg/L (control conditions), the ammonia was observed to increase from 70 mg/L to 110 mg/L. Ammonia is produced during the degradation of SCN^- through all reported degradation pathways (Table 1). For each mole of SCN^- degraded Kim et al. (2008) reported the production of 0.24 moles NH_4^+-N. The mixed culture in the present investigation produced 0.26 moles of NH_4^+-N from each mole of SCN^- degraded. Under control conditions, the production of NH_4^+-N was in line with the theoretical NH_4^+-N production expected (28 mg/L). As the initial SCN^- concentration increased, however, there was a decline in NH_4^+-N production suggesting a breakdown in the degradation process. Hung and Pavlostathis (1997) reported that SCN^- degradation proceeds in a series of steps (Table 1). Firstly SCN^- is hydrolysed forming OCN^- which is subsequently hydrolysed to form NH_4^+-N and bicarbonate (HCO_3^-) whilst sulphur is oxidised to produce SO_4^{2-}. Lower than expected concentrations of NH_4^+-N suggests that SCN^- hydrolysis occurred but OCN^- hydrolysis did not.

Cyanate is hydrolysed by the enzyme cyanase producing CO_2 and NH_4^+-N (Douglas Gould et al., 2012; Kozliak et al., 1995). The *E.coli* enzyme is the only cyanase which has been studied in detail (Walsh et al., 2000). Bicarbonate is believed to be involved in a nucleophilic attack on OCN^- which produces CO_2 and carbamate (Walsh et al., 2000). Decarboxylation then takes place producing CO_2 and NH_4^+-N (Walsh et al., 2000). Although cyanase is induced by OCN^-, high cyanate concentrations can equally have a toxic effect (Hung and Pavlostathis, 1997). Both HCO_3^- and OCN^- are capable of

binding at the other substrate binding site resulting in a dead-end complex (Anderson and Little, 1986). It is therefore suspected that at higher concentrations of SCN^- the hydrolysis of SCN^- proceeded more rapidly producing high concentrations of OCN^- which in turn led to inhibition of cyanase. As a result of the lower cyanase activity OCN^- would accumulate further and no NH_4^+-N would be produced (Hung and Pavlostathis, 1997).

The degradation of SCN^- during the treatment of coke wastewater has typically been reported to occur in aerobic conditions. Kim et al. (2011, 2008) reported that SCN^- degradation took place in the aerobic tank of the laboratory-scale anoxic-aerobic ASP. In contrast, however, previous work on the current activated sludge biomass revealed that the biomass was capable of completely removing SCN^- in both aerobic conditions and anoxic conditions (Raper et al., 2019, 2017). This SCN^- degradation in the anoxic cell of a pilot-scale anoxic-aerobic ASP was possible with biomass taken from an aerobic process without any acclimatisation period.

Table 2: Impact of initial SCN^- concentration on SCN^- removal and end product formation in the mixed culture after 5 days in the respirometer.

3.2 Impact of nitrogen compounds on SCN^- degradation

Nitrification is an essential process in the removal of nitrogen from coke wastewater. The degradation of SCN^- and nitrification both occur in the ASP under aerobic conditions. Therefore, compounds associated with the nitrogen cycle were assessed for their impact on SCN^- degradation. Nitrate, nitrite and hydroxylamine (NH_2OH) were

spiked into the mixed culture and the impact on SCN- degradation monitored through respirometry. After 4 days neither nitrite nor nitrate had any impact on SCN^- degradation efficiency at concentrations ranging from 0.25 mg/L to 16 mg/L. Hydroxylamine, an intermediate compound produced during nitrification(Gerardi, 2002)(Gerardi, 2002)(Gerardi, 2002)(Gerardi, 2002)(Gerardi, 2002)(Gerardi, 2002)(Gerardi, 2002)(Gerardi, 2002)(Gerardi, 2002)(Gerardi, 2002), can be found at low concentrations in nitrifying activated sludge processes (Gerardi, 2002) and as such was also investigated. Hydroxylamine resulted in a small inhibitory response leading to a 4 to 7% reduction in SCN^- degradation at concentrations of 0.25 mg/L to 16 mg/L. Inhibition did not increase with increased NH_2OH concentrations. Whilst NH_2OH may result in a low level of inhibition, SCN^- degradation proceeds faster than nitrification. This can be observed in Figure 1 which demonstrates an accumulation of ammonia (negative nitrification efficiency) after 24 hours due to degradation of SCN^- which produces additional ammonia to that already in the coke wastewater. SCN^- removal was complete at 72 hours whilst nitrification was <40%, taking 5 days to reach 90%. Any inhibitory impact of NH_2OH would therefore be minimal.

3.3 Impact of ammonia, sulphate and phenol on SCN^- degradation

The impact of phenol, NH_4^+-N and SO_4^{2-} on SCN^- degradation was also investigated as each of the compounds may be found at elevated concentrations in the mixed liquor due to their presence in the raw liquor or due to their production during SCN^- degradation. The average NH_4^+-N concentration at 0 hours in the respirometer was 82 mg/L (106

mg/L NH_4^+-N). An additional 250 - 1500 mg/L NH_4^+-N was subsequently spiked to the coke wastewater to assess the impact of NH_4^+-N on SCN^- degradation.

Ammonia was observed to have an inhibitory influence on SCN^- degradation. Thiocyanate removal efficiencies declined by 24, 19 and 22% upon the addition of 250, 500 and 1000 mg/L NH_4^+-N. At 1500 mg/L NH_4^+-N the degradation of SCN^- declined by 43%. Oxygen consumption in the respirometer tests was observed to decline (Figure 2) which was likely to be associated with NH_4^+-N toxicity. The calculated half maximal inhibitory concentration (IC_{50}) for NH_4^+-N was 316 mg/L NH_4^+-N. The mixed culture was therefore more sensitive to NH_4^+-N concentrations than the mixed culture described by Paruchuri, Shivaraman and Kumaran (1990). Ammonia loading to the ASP is therefore a critical parameter that should be controlled in the operation of ASPs treating coke wastewater.

Sulphate at concentrations between 80 - 2000 mg/L, had little impact on SCN^- degradation suggesting that no inhibition occurred as a result of its formation during the degradation process. The impact of varying phenol concentration on the SCN^- removal was investigated due to concerns around its toxicity. The results obtained indicated that phenol had an inhibitory role on SCN^- degradation with the addition of 80, 130 and 180 mg/L phenol resulting in a 29, 38 and 41% decrease in SCN^- removal respectively. Increased phenol results in higher organic carbon availability which can increase the growth of heterotrophic bacteria in turn increasing competition for dissolved oxygen with the slower-growing autotrophic bacteria (Kim et al. 2013a).

3.4 Temperature

The mixed culture was sensitive to process temperature (Figure 3). When the temperature was maintained at mesophilic conditions (25 ± 1^{o}C) SCN^- degradation was complete within 24 hours. When temperature was not controlled, it fluctuated between 8 - 21^{o}C (psychrophilic range of temperatures) and the SCN^- degradation was strongly affected decreasing to 2 - 26%. The optimal temperature for the mixed culture was therefore within the mesophilic range of temperatures, fitting into the typical reported optimal temperature range of 25 - 35^{o}C. (Robertson and Gijs Kuenen, 2006). Previous modelling of autotrophic thiocyanate degradation also suggested high temperature sensitivity (Kim et al. 2013b). It is therefore crucial that temperature is well controlled in treatment processes, such as activated sludge, to ensure effective degradation. This is particularly important in temperate climates, such as the UK, where the natural wastewater temperature is between 8 - 21^{o}C.

3.5 Molecular microbial ecology

Deoxyribonucleic acid sequencing analysis showed that the mixed culture was dominated by an uncultured species of *Thiobacillus* (26%) (Figure 4). The 16S sequence linked to SCN^- degradation in the mixed culture was previously identified by Bai et al. (2011), however, the sequence was not assigned to a species. The *Thiobacillus* genus was similarly the most abundant genus in a continuous flow bioreactor degrading SCN^- reported by Kantor et al. (Kantor et al., 2015). Furthermore, the activated sludge biomass contained a notable abundance of the genera *Mizugakiibacter* (13%), *Comamonas* (12%) and *Rhodanobacter* (11%) (Figure 4). *Mizugakiibacter* and *Rhodanobacter* are known for their iron-oxidising and nitrate reducing abilities (Wang et al., 2017) whilst *Comamonas* has been associated with a wide range of abilities including the degradation of phenol (Zámocký et al., 2001). Members of the

Rhodanobacter and *Comamonas* genera were also observed in the bioreactors reported by Kantor et al. (Kantor et al., 2015).

The mixed culture was shown to effectively degrade SCN^- over a range of initial concentrations (Table 2). The *Thiobacillus* genus has been recognised for SCN^- degradation for many years. Despite this, species within the *Thiobacillus* genus have been subjected to significant reclassification as from an original group of 17 species, only 3 species remain in the genera of *Thiobacillus* (*T*. *aquaesulis*, *T*. *thioparus* and *T*. *denitrificans*) (Kelly and Wood, 2000a). Of the *Thiobacillus* species, only *T*. *thioparus* and *T*. *denitrificans* have been documented to be capable of utilising thiocyanate as the sole source of energy (Kelly and Wood, 2000b) suggesting that the species present within the activated sludge were related to either *T*. *thioparus* or *T*. *denitrificans*. Whilst *T*. *thioparus* and *T*. *denitrificans* are genetically very similar (98% similarity) (Kelly and Wood, 2000b), *T*. *denitrificans* is distinguished from all other *Thiobacillus* species due to its ability to grow as a facultative anaerobe (Kelly and Wood, 2000b). *Thiobacillus thioparus* on the other hand is capable of reducing nitrate but not nitrite. Nitrite accumulation has been observed in the pre-denitrification ASP (Raper et al., 2019) which could suggest a metabolic similarity to *T*. *thioparus*. Despite this, an ambiguous species of the genus *Rhodanobacter* was also identified in the mixed culture (Figure 4). As some species of this genus are capable of nitrate reduction but not nitrite reduction (Lee et al., 2007) nitrite accumulation may also be attributed to other species in the mixed culture. As the SCN^- degradation investigation was focused on the properties of the mixed culture, it is therefore not possible to ascertain the full metabolic capability of SCN^- degraders. Further work is required to characterise the *Thiobacillus* species using pure cultures.

4. Conclusions

A mixed culture, taken from an aerobic ASP treating coke wastewater, was dominated by an uncultured species of *Thiobacillus* (26%) and was capable of degrading SCN^- at a range of concentrations (109 -2109 mg/L). Thiocyanate was hypothesised to be hydrolysed/oxidised to NH_4^+-N and sulphate, but at SCN^- concentrations >110 mg/L a rapid accumulation of OCN^- was believed to reduce cyanase activity preventing the formation of NH_4^+. The uncultured Thiobacillus displayed similar metabolic capabilities to *Thiobacillus denitrificans* and *Thiobacillus thioparus* with optimal degradation occurring at mesophilic temperatures. Phenol, NH_4^+-N and NH_2OH reduced SCN^- degradation efficiencies and should be controlled in the ASP influent to enable compliance with the IED emission limit of >4 mg/L.

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Figure 1

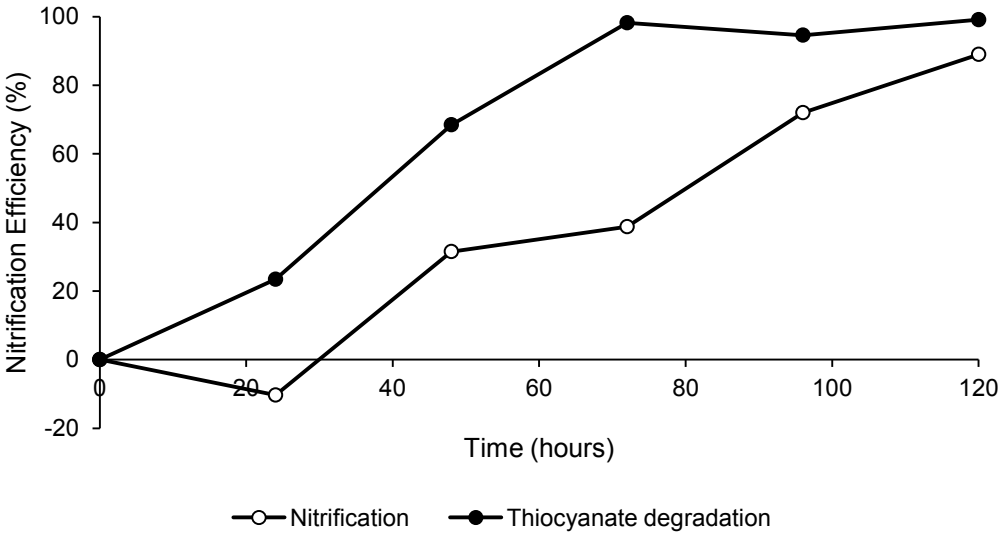


Fig. 1. Thiocyanate and nitrification treatment efficiency by the mixed culture during batch tests ●- SCN^- removal efficiency ○- Nitrification efficiency. SCN^- degradation proceeds faster than nitrification.

Figure 2:

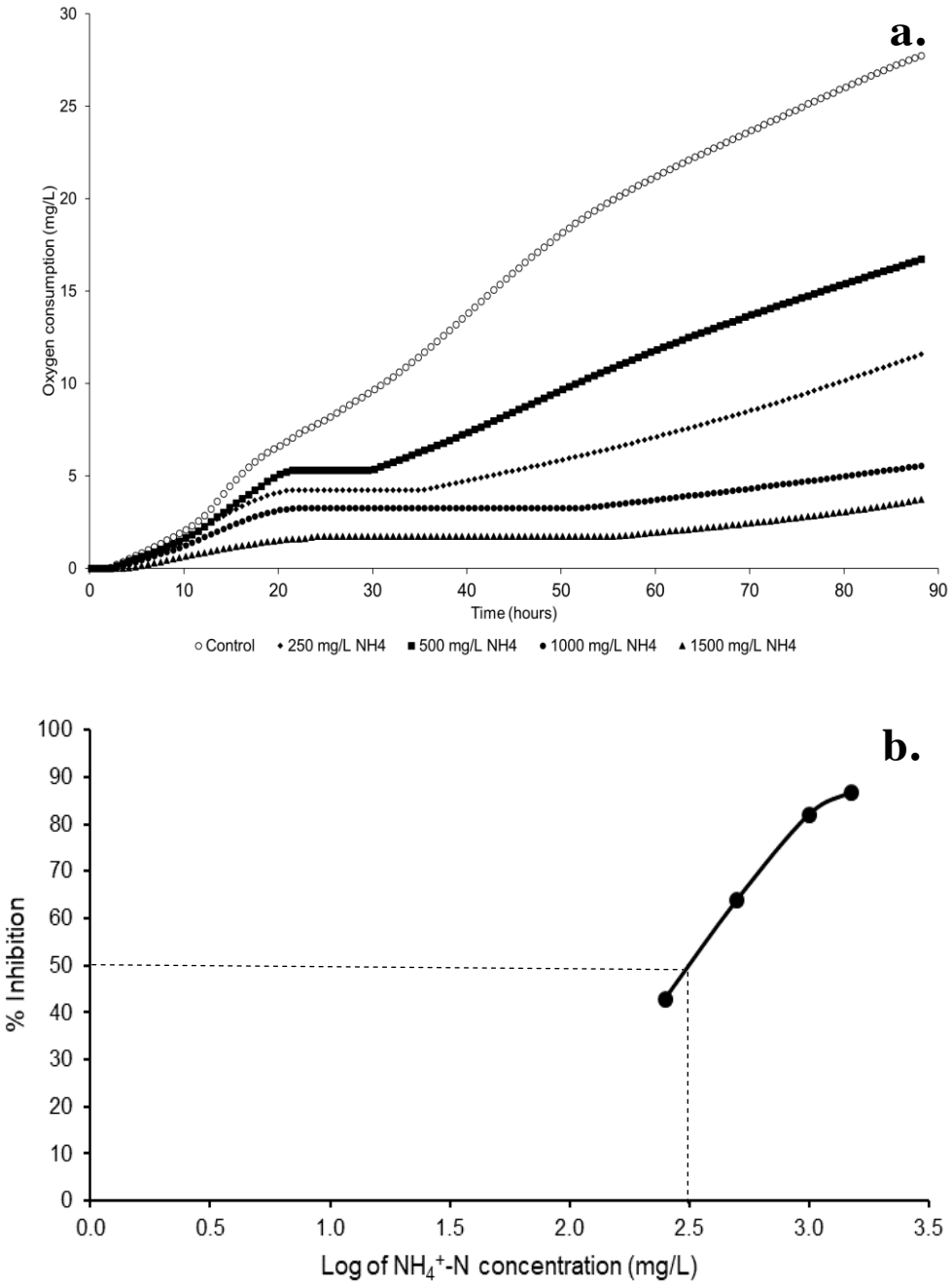


Fig. 2. Impact of NH_4^+-N addition on the mixed culture over a 4-day duration in the respirometer a. Reduced oxygen uptake as a result of increased NH_4^+-N concentrations b. A half maximal inhibitory concentration (IC50) of 312 mg/L of NH_4^+-N on SCN^- removal.

Figure 3:

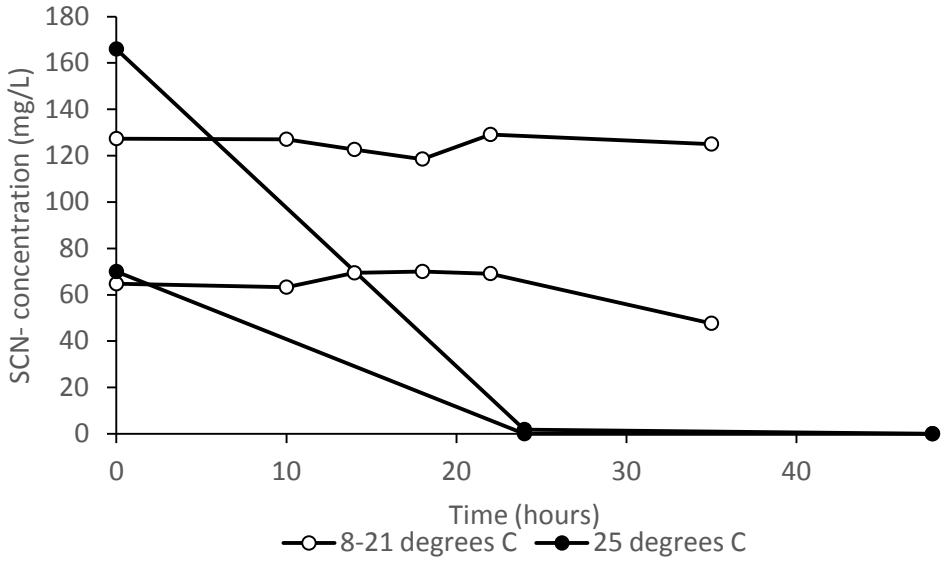


Fig. 3. Impact of temperature on SCN^- degradation in mixed culture batch tests. Thiocyanate degradation decreases when temperatures are sub-mesophilic.

Figure 4:

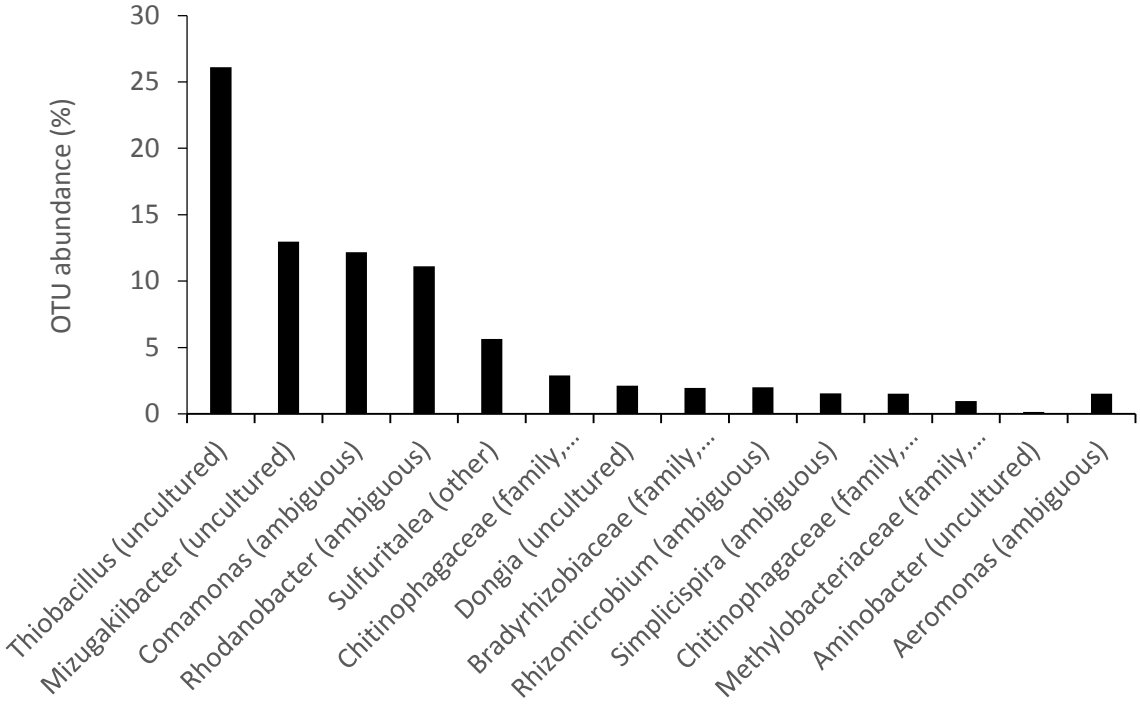


Fig. 4. Molecular microbial analysis displaying operational taxonomic units (OTUs) and relative abundance in the activated sludge mixed culture determined through PCR gene sequencing. An uncultured species of *Thiobacillus* was dominant in the mixed culture.

Table 1
Thiocyanate degradation pathway.

	Species	Oxygen availability	SCN^- degradation pathway	Type of wastewater and reference
Autotrophic	Mixed culture	Aerobic	$SCN^- + H_2O \rightarrow HCNO + HS^-$ $HCNO + H_2O \rightarrow NH_4^+ + HCO_3^-$ $HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$ Overall: $SCN^- + 2O_2 + 3H_2O \rightarrow NH_4^+ + HCO_3^- + SO_4^{2-} + H^+$	Photo-processing (Hung and Pavlostathis, 1997) Synthetic photo-processing (Hung and Pavlostathis, 1999) * Metallurgical (synthetic and reused water) (Grigor et al., 2009) Synthetic *Burkholderia* sp., *Chryseobacterium* sp., *Ralstonia* sp.(Huang et al., 2013)
	Mixed culture: dominated by *Pseudomonas* and *Bacillus*	Aerobic	$SCN^- + 2H_2O + 2O_2 \rightarrow CO_2 + SO_4^{2-} + NH_4^+$ $SCN^- + 2H_2O \rightarrow CO_2 + S^{2-} + NH_4^+$ $SCN^- + 3H_2O + 0.5O_2 \rightarrow CO_2 + S^o + NH_4^+ + 2OH^-$	Coke wastewater (Paruchuri et al., 1990)
	Mixed Culture	Aerobic	$SCN^- + 2H_2O + 2O_2 \rightarrow CO_2 + SO_4^{2-} + NH_4^+$	Coke wastewater (Staib and Lant, 2007)
	Thiobacillus thioparus	Aerobic	$SCN^- + 2H_2O \rightarrow COS + NH_3 + OH^-$	Synthetic (Katayama et al., 1992)
	Acinetobacter johnsonii and *Pseudomonas diminuta*	Aerobic	$SCN^- \rightarrow S_2O_3^{2-} \rightarrow SO_4^{2-}$	Synthetic (Boucabeille et al., 1994)
	Thialkalivibrio thiocyano denitrificans	Aerobic/ Anaerobic	$5SCN^- + NO_3^- + H_2O + 8H^+ + 5HCO_3^- \rightarrow 5SO_4^{2-} + 5NH_3 + 10CO_2 + 4N_2$	Soda lake sediment (Sorokin et al., 2004)
	Thiobacillus denitrificans	Anoxic	$5SCN^- + NO_3^- + H_2O + 8H^+ + 5HCO_3^- \rightarrow 5SO_4^{2-} + 5NH_3 + 10CO_2 + 4N_2$	(Robertson and Gijs Kuenen, 2006)*
Heterotrophic	*Pseudomonas putida* (strain 21) and Pseudomonas stutzeri (strain 18)	Aerobic	$SCN^- + H_2O \rightarrow NH_3 + CO_2 + S_2O_3^{2-}$ Further converted by P. putilda strain 21 to: $S_2O_3^{2-} \rightarrow S_4O_6^{2-} \rightarrow S_3O_6^{2-}$	Synthetic (Grigor et al., 2006)
	Soil isolate 26B	Aerobic	$SCN^- + H_2O \rightarrow NH_3 + CO_2 + S_2O_3^{2-}$	Synthetic (Stratford et al., 1994)*
	Klebsiella pneumoniae and Ralstonia sp.	Aerobic	$SCN^- + 2H_2O \rightarrow COS + NH_3 + OH^-$	Synthetic (Chaudhari and Kodam, 2010)

* Inferred from text

Table 2
Impact of initial SCN^- concentration on SCN^- removal and end product formation in the mixed culture after 5 days in the respirometer.

Start (mg/L)				End (mg/L)					
SCN^-	NH_4^+-N	NO_3^--N	NO_2^--N	SCN^-	NH_4^+-N	NO_3^--N	NO_2^--N	Theoretical NH_4^+-N concentration *	Difference between theoretical and empirical NH_4^+-N†
109	72	5	2	1	106	3.4	37	100	6
359	″	″	″	291	101	7.2	14	165	-64
609	″	″	″	527	98	10.6	17	230	-132
1109	″	″	″	839	15.4	18.2	21	360	-345
2109	″	″	″	890	4	25.4	17	620	-616

* Empirical data of SCN^- degradation by the studied mixed culture demonstrates that one mole of SCN^- forms 0.26 moles of NH_4^+-N. Theoretical NH_4^+-N concentration calculation: Start NH_4^+-N (mg/L) + (0.26 x Start SCN^- (mg/L).

† Difference between NH_4^+-N formation based on molar ratio of SCN^- to NH_4^+ and empirical NH_4^+-N concentration = End NH_4^+-N (mg/L) - (Start NH_4^+-N (mg/L) + (0.26 x Start SCN^- (mg/L)))



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Raper, Eleanor; Stephenson, Tom; Fisher, Raymond; Anderson, David R.; Soares, Ana

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