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TECHNICAL ARTICLE

Acute and Chronic Toxicity of Acid Mine Drainage to the Activated Sludge Process

Theresa A. Hughes · N. F. Gray

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Abstract The combined treatment of acid mine drainage (AMD) and municipal wastewater using the activated sludge process is an innovative approach to AMD remediation. The toxicity of synthetic AMD to activated sludge was evaluated using oxygen uptake rate (OUR) inhibition tests, which showed that activated sludge can withstand high proportions of AMD (EC_{50} 19–52% AMD by volume). The EC_{50} values of municipal and industrial activated sludges were significantly different ($p < 0.05$), with municipal sludges exhibiting higher tolerance to AMD. Although the EC_{50} values for heterotrophic and nitrifying activated sludges were not statistically significantly different, the EC_{50} values for heterotrophic bacteria were generally higher. Laboratory-based sequencing batch reactors were used to examine the treatability of AMD. Increased concentrations of COD and suspended solids, associated with turbidity and poor floc morphology, were observed in the final effluent after extended AMD loading. Protozoan community structure changed during the AMD loading period, and overall abundance tended to decrease over time. OUR decreased in the AMD-loaded reactors, particularly in the reactor receiving the highest AMD load, indicating reduced biomass activity over the acclimatization period. Results from OUR inhibition tests on the acclimatized activated sludge indicated that over a relatively short timescale (21 days), the activated sludge microbial community can adapt to AMD sufficiently so that shock loads of metals and acidity do not significantly inhibit OUR. These preliminary studies indicate that it is possible to treat AMD successfully in admixture with municipal wastewater using the activated sludge process.

Keywords Acid rock drainage · Acclimatization · Inhibition · Ireland · Sewage · Toxicity · Treatment

Abbreviations

AMD	Acid mine drainage
ATU	Allyl thiourea
BOD	Biochemical oxygen demand
COD	Chemical oxygen demand
EC_{50}	Effective concentration causing 50% inhibition
EPS	Extracellular polymeric substances
MLSS	Mixed liquor suspended solids
MWW	Municipal wastewater
OUR	Oxygen uptake rate
OUR_{max}	Maximum oxygen uptake rate
SVI	Sludge volume index
TSS	Total suspended solids
WWTP	Wastewater treatment plant

Introduction

Co-treatment of acid mine drainage (AMD) and municipal wastewater (MWW) using the activated sludge process is an innovative approach to AMD remediation that utilizes the alkalinity of MWW and the adsorptive properties of the activated sludge flocs to remove acidity and metals from AMD-impacted waters. In theory, co-treatment of AMD and MWW should be highly effective, because compounds that are high in one effluent stream tend to be low in the other. For example, (1) sewage effluent with relatively high

T. A. Hughes (✉) · N. F. Gray
Water Technology Research Group,
Centre for the Environment, School of Natural Sciences,
Trinity College Dublin, Dublin 2, Ireland
e-mail: hughesta@tcd.ie

concentrations of suspended solids may enhance Fe oxyhydroxide precipitation by encouraging Fe, which is often present in high concentrations in AMD, to form flocs (Johnson and Younger 2006), and (2) phosphate, which is present in high concentrations in sewage effluent, can be sorbed onto the Fe oxyhydroxide precipitates (Sibrell et al. 2009; Wei et al. 2008) or react with Al to form hydroxyphosphates (Omoike and Vanloon 1999). Rao et al. (1992) evaluated AMD as a coagulant and reported that it was as effective as the commercial coagulant $FeCl_3$ for turbidity removal from MWW.

A key function of wastewater treatment is to remove organic matter, which is typically quantified in wastewaters in terms of chemical oxygen demand (COD) or biochemical oxygen demand (BOD), parameters that are related to the degradation of organic compounds (Ekama and Wentzel 2008). Passive co-treatment of MWW and AMD in a multi-stage system consistently achieved high BOD and nutrient removal efficiency (Strosnider et al. 2011b), removed significant concentrations of dissolved Al, As, Cd, Fe, Mn, Pb, and Zn, and produced a net-alkaline effluent (Strosnider et al. 2011a). Other recent reports of passive co-treatment indicated that AMD enhanced coagulation, sedimentation, and pathogen removal during wastewater treatment (Neto et al. 2010; Winfrey et al. 2010). Johnson and Younger (2006) reported removal of Fe and Mn from net-alkaline coal mine AMD, as well as phosphate, nitrate, and suspended solids removal, in a wetland co-treating AMD with secondary sewage effluent. Metal removal in the activated sludge process is believed to occur primarily by entrapment and settlement of particulate non-settleable metal-containing solids in the sludge floc matrix, as well as by binding of soluble metal to extracellular polymeric substances (EPS; Brown and Lester 1979; Santos and Judd 2010). Pamukoglu and Kargi (2009) demonstrated that Cu concentrations up to 30 mg L^{-1} could be tolerated by an activated sludge system with no detrimental effects on COD removal efficiency or sludge settling, by using a premixing stage to remove some Cu by biosorption onto dried waste sludge. Finally, MWW is alkaline, and therefore has a high acid-neutralizing capacity; furthermore, dilution of AMD H^{+} concentrations on mixing with MWW causes the pH to increase, thereby decreasing the pH-dependent solubility of many metals (Strosnider et al. 2011c).

Three key elements to consider in co-treatment of AMD and MWW are: (1) the treatability of AMD by activated sludge, (2) the metal removal and neutralization capacity of wastewaters, activated sludge, and digested sludge, and (3) the impacts of AMD loading on wastewater treatment performance. This study investigated the first aspect, i.e. the treatability of AMD by activated sludge. The activated sludge process is widely used to treat municipal and industrial wastewaters, many of which contain metals that can potentially inhibit microbial activity and growth (Alkan et al. 2008; Pai et al. 2009) and aerobic and anaerobic processes that are critical for plant performance (e.g. removal of organic matter, suspended solids, and nutrients; Chua et al. 1999; Ong et al. 2003, 2004; You et al. 2009). Successful co-treatment requires that the activated sludge microbial community is not adversely affected by the loadings of acidity, metals, and sulphate.

Microbial inhibition can cause decreased activity, changes in community structure, and loss of floc structure, leading to poor settling and loss of biomass from the system (Love and Bott 2000; Neufeld 1976). The sensitivity of activated sludge to different toxic substances can be influenced by previous exposure and acclimatization: the population of microorganisms can be modified and selected owing to the presence of industrial effluents (Christofi et al. 2003). Alternatively, the activated sludge bacteria can adapt to protect themselves; Guibaud et al. (2005) observed increased metal binding capacity by EPS produced by activated sludge microorganisms that had been exposed to Cd, Pb, and Ni, when compared to EPS on pure bacterial cultures that had not been previously exposed to metals. Acclimatization of activated sludge to high metal loads (Neufeld and Hermann 1975; Sorour and Sayed-Ahmed 2005) and sulphur (Burgess and Stuetz 2002) has been reported; furthermore, adding trace concentrations of metals may stimulate microbial growth and actually improve biological treatment of wastewater (Aragón et al. 2010; Cabrero et al. 1998; Gikas 2008; Jefferson et al. 2001; Nicolau et al. 2005; Wang et al. 2010; Yetis and Gokcay 1989).

This study investigated the acute and chronic toxic effects of AMD on activated sludge from different WWTPs, to determine what AMD concentrations cause significant inhibition of microbial activity and to assess recovery from toxic effects and the potential for acclimatization of activated sludge to AMD loading. To assess acute toxicity, high-strength synthetic AMD was used in batch experiments with activated sludge from WWTPs receiving municipal and industrial effluents. Oxygen uptake rate (OUR) is associated with microbial metabolic activity and organic substrate removal, and thus serves as a useful parameter in screening tests to evaluate toxic effects (Kilroy and Gray 1992; Spanjers et al. 1998; Madoni et al. 1999). To examine chronic toxicity, acclimatization of activated sludge to continuous AMD loading was examined using standard activated sludge biomass assessments [i.e. mixed liquor suspended solids (MLSS), sludge volume index (SVI), and floc morphology], effluent analyses (COD and suspended solids), microscopic analysis of protozoan communities, and OUR inhibition tests. Protozoa are important microorganisms in the wastewater treatment process because they graze on viruses and dispersed bacteria, thus improving final effluent quality (Nicolau et al. 2005), and protozoan community structure can be used as an indicator of functional conditions in a WWTP (Madoni et al. 1996). The goal of the present investigation was to observe the effects of AMD loading on activated sludge microorganisms; the fate of AMD metals and metal uptake by activated sludge biomass during co-treatment will be presented as a separate study.

Experiments were designed to test the following hypotheses:

- Whether AMD affects the OUR of activated sludge from municipal and industrial wastewater treatment plants differently.
- Whether nitrifying sludge (i.e. a mixed population of nitrifiers and carbonaceous bacteria) is significantly more inhibited by AMD than non-nitrifying sludge (i.e. a mixed population of heterotrophic bacteria with no nitrifiers).
- Whether activated sludge can acclimatize to AMD without chronic toxic effects, e.g. decreased removal of COD and suspended solids, undesirable changes in floc morphology and settleability, or significant impacts on the protozoan community.

Materials and Methods

Activated Sludge Sampling

Activated sludge samples were taken from the aeration tanks of four wastewater treatment plants (WWTPs) with different influent characteristics (Table 1).

Samples were aerated with porous ceramic air diffusers and stored at 20° ± 2°C until used. Mixed liquor suspended solids (MLSS) concentration was determined gravimetrically according to standard methods (Eaton et al. 2005; Method 2540D).

Table 1 Location, population equivalent (p.e.) and process type of the four WWTPs where activated sludge was sampled for the OUR inhibition tests and acclimatization studies

Plant name/location	Population equivalent	Process type	Proportion of domestic sewage (%)
Kilcoole	3,000	Extended aeration	100
Leixlip I	45,000	Completely mixed	80
Leixlip II	35,000	Plug flow	30
Swords	60,000	Extended aeration	95

Synthetic Acid Mine Drainage and Wastewater Solutions

For the OUR inhibition tests, a high-strength AMD formulation (Table 2) was used because AMD with sufficient toxicity to activated sludge to cause significant OUR inhibition was required. For the acclimatization studies, synthetic AMD simulating the AMD emanating from two major adits near the abandoned Cu sulphide mines in Avoca, SE Ireland, was used (Table 2) (Gray and O'Neill 1995). Synthetic AMD was prepared fresh daily from stock metal solutions of 1,000 mg L^{-1} made using $Fe(SO_4)\cdot 7H_2O$, $Al_2(SO_4)_3\cdot 18H_2O$, $Cu(SO_4)\cdot 5H_2O$, $Zn(SO_4)\cdot 7H_2O$, $Mn(SO_4)\cdot H_2O$, $PbCl_2$, and $CdCl_2\cdot H_2O$, and distilled, deionized water. All the chemicals used in the study were of analytical reagent grade. Where necessary, pH was adjusted by adding sulphuric acid (H_2SO_4) (1 M).

Synthetic wastewater (OECD 1984) was prepared as a 100-fold concentrate solution, by dissolving peptone (16 g), meat extract (11 g), urea (3 g), sodium chloride (NaCl) (0.7 g), calcium chloride ($CaCl_2\cdot 2H_2O$) (0.4 g), magnesium sulphate ($MgSO_4\cdot 7H_2O$) (0.2 g), and potassium monohydrogen phosphate (K_2HPO_4) (2.8 g) in 1 L distilled water.

Acute Toxicity Tests

The 'Activated Sludge, Respiration Inhibition Test' (OECD 1984) was used to measure the toxic effect of AMD on activated sludge from the different WWTPs. This test measures the effect of a test substance on microorganisms by comparing the OUR of activated sludge under defined conditions in the presence of five different concentrations of the test substance with that of a two control samples made up with synthetic wastewater and distilled water. After a 3 h incubation period, a 20 mL subsample was taken from each incubation flask using a pipette, and

Table 2 Physicochemical characteristics of the high-strength and simulated Avoca mine synthetic AMD used in OUR inhibition tests and acclimatization studies

Parameter (units)	High-strength AMD	Simulated Avoca AMD
pH	2.7	3.1
Fe (mg L^{-1})	620	130
Al (mg L^{-1})	200	150
Cu (mg L^{-1})	30	5
Zn (mg L^{-1})	70	90
Mn (mg L^{-1})	30	6
Pb (µg L^{-1})	60	1,500
Cd (mg L^{-1})	0	0.2
SO_4 (mg L^{-1})	5,120	1,670

the OUR was measured using a Strathtox® respirometer (Strathkelvin Instruments, Glasgow, UK). Dissolved oxygen was measured over a 10 min period, and the dissolved oxygen concentration was plotted against time. For each concentration of the test substance, the OUR was estimated as the slope of a regression line fitted to a series of data points, and this value was used to calculate $\%I$, the percentage inhibition (Eq. 1):

$$\%I = 100 * \left(1 - \frac{2 * R_S}{R_{c1} + R_{c2}}\right) \quad (1)$$

where R_S = OUR at test concentration, R_{c1} = OUR of Control 1, and R_{c2} = OUR of Control 2.

The percentage inhibition was calculated for each test concentration, and the EC_{50} was determined after plotting the percentage inhibition against toxicant concentration on a log-normal graph. Allyl thiourea (ATU) was used to inhibit nitrifying bacteria in tests comparing the OUR inhibition of heterotrophic and autotrophic bacteria in activated sludge from Swords and Leixlip WWTPs (OECD 2010). Statistical analyses were performed using Minitab 15® statistical software (Minitab Inc., State College, PA, USA).

Acclimatization Procedure

The acclimatization studies were performed using a bench-scale, sequencing batch reactor (SBR) system, comprising four 4 L HDPE containers operated at constant temperature (20 ± 2°C) on a fill and draw system, made up of four phases: fill (10 min), react (22.5 h with aeration), settle (1 h) and decant (20 min). Porous ceramic air diffusers were used to aerate and mix the contents of the reactors. There was no sludge recirculation. Activated sludge from Leixlip I WWTP was used to seed the reactors at start-up. The reactors were operated with a food-to-microorganism (f/m) ratio of 0.13 kg COD kg^{-1} day^{-1} using the synthetic wastewater described above, with sodium bicarbonate ($NaHCO_3$) added to final concentration of 0.3 g L^{-1} synthetic wastewater to prevent a drop in pH due to nitrification (Christofi et al. 2003). Distilled, deionized water was used to prepare the synthetic wastewater and for dilution to the desired COD. Sludge retention time (Θ = 8 days) was controlled by manual sludge wasting. Mixed liquor suspended solids (MLSS) was maintained at approximately 3,000–3,500 mg L^{-1}. Hydraulic retention time (HRT) was 24 h.

After a start-up period of 2Θ, to allow the sludge to adapt to synthetic wastewater, the acclimatization process began. Beginning on Day 16, synthetic AMD, simulating Avoca AMD (Table 2), was added to Reactors B, C, and D at loading rates of 5, 10, and 25%, respectively, as a percentage of the influent volume. Synthetic wastewater dilutions were adjusted accordingly to maintain equal BOD and nutrient supply in all reactors. Metal loading continued for 26 days. Reactor A was the control and received no AMD. The pH in all reactors was maintained between pH 6.5 and 8.0 by the addition of $NaHCO_3$ as needed.

Standard methods (Eaton et al. 2005) were used to analyze mixed liquor suspended solids (MLSS; Method 2540D), effluent total suspended solids (Method 2540D), and settled sludge volume (Method 2710C). Sludge volume index (SVI) was calculated as (Eq. 2) (Method 2710D):

$$\mathrm{SVI}\left(\mathrm{mL\,g^{-1}}\right) = \text{settled sludge volume}\left(\mathrm{mL\,L^{-1}}\right)/\mathrm{MLSS}\left(\mathrm{g\,L^{-1}}\right) \quad (2)$$

Chemical oxygen demand (COD) measurements were made colorimetrically using COD digestion vials (HACH LANGE GmbH; HACH Company 2007). Microscopic analysis was used to examine protozoa abundance, floc morphology, and filamentous growth. For microscopic analysis, 25 μL sub-samples were obtained using a gravimetrically calibrated automatic micropipette. Protozoan enumeration was performed on each sub-sample using phase contrast microscopy at 100× magnification (Dubber and Gray 2009). Protozoa were counted and categorized according to major habit groupings, i.e. sessile, crawling, or free-swimming, and the relative abundance of each grouping and total protozoa abundance were determined. Filament abundance was scored from 0 to 6, according to the subjective scale in Jenkins et al. (2004), with a score of 0 indicating that no filaments were observed, and scores of 1–6 indicating few, some, common, very common, abundant, and excessive filaments, respectively. Finally, OUR_{max} was tested to assess activated sludge activity, and periodic OUR inhibition tests were performed to examine changes in response of acclimatized activated sludge to spiked additions of synthetic AMD.

Results

Acute Toxicity Tests

Comparing OUR Inhibition of Activated Sludge from Municipal and Industrial WWTPs

Activated sludge from four different WWTPs was used to evaluate the toxicity of high-strength AMD (Table 3). Prior to each test, the MLSS concentration of the activated sludge sample was adjusted to 4 g L^{-1}, either by dilution with distilled water or by settling and discarding supernatant. Inhibition tests were performed using concentrations of AMD ranging from 10 to 100% (i.e. AMD concentration in the test mixture). In all cases, the pH of the activated sludge and AMD mixtures was greater than 6.0 after

Table 3 Mean, median, and range of EC_{50} (% AMD) results of OUR inhibition tests using high-strength synthetic AMD and activated sludge samples from municipal and industrial WWTPs

WWTP location	n	Mean EC_{50} (% AMD)	SD	Median	Range	
					Minimum	Maximum
Swords (sample 1, week 2)	4	51.6	9.9	53.7	39.1	59.7
Swords (sample 2, week 7)	6	41.4	1.8	40.7	40.3	44.8
Swords (sample 3, week 8)	6	34.8	11.3	32.3	22.2	49.9
Swords (sample 4, week 9)	6	44.5	6.5	44.5	36.4	51.9
Swords (sample 5, week 11)	6	20.3	6.4	18.6	13.3	32.2
Kilcoole (week 12)	4	39.5	7.9	37.6	32.1	50.6
Leixlip I (sample 1, week 1)	4	33.0	3.6	33.6	28.1	36.8
Leixlip I (sample 2, week 13)	3	35.3	3.9	33.8	32.4	39.7
Leixlip II (sample 1, week 5)	6	29.5	10.6	30.6	14.0	42.4
Leixlip II (sample 2, week 6)	6	19.1	7.1	19.8	8.2	26.5

Samples from Swords WWTP were obtained over a 10-week period, and samples were obtained from Leixlip I and II WWTPs over 13- and 2-week periods, respectively

n number of replicates, *SD* standard deviation

mixing. EC_{50} results for all sludges ranged from approximately 19–52% AMD (Fig. 1).

Activated sludge from Swords WWTP was sampled on five different dates (spanning 10 weeks) to investigate how the response of activated sludge from the same WWTP varied over time. Significant differences were observed among results, with the EC_{50} for sample 3 significantly less ($p < 0.05$) than sample 1, and the EC_{50} for sample 5 significantly less ($p < 0.05$) than samples 1, 2, and 4. One-way ANOVA on the entire dataset indicated statistically significant differences among means. Tukey multiple comparison tests indicated that the EC_{50} for activated sludge from Leixlip II (sample 1) was significantly less ($p < 0.05$) than the EC_{50} for Swords (samples 1 and 4). The EC_{50} for activated sludge from Leixlip II (sample 2) was significantly less ($p < 0.05$) than all sludges except Swords (sample 5) and Leixlip II (sample 1). The acute toxicity of AMD to activated sludge varied significantly, not only in time (as shown by results from Swords and Leixlip II WWTPs), but also between WWTPs, with activated sludge from Leixlip II showing less tolerance to AMD than other sludges.

Comparing OUR Inhibition of Heterotrophic and Autotrophic Bacteria

Experiments were performed using nitrifying activated sludge from Swords and Leixlip I WWTPs to test for significant differences in OUR inhibition between nitrifying and non-nitrifying (ATU-inhibited) sludges. In these experiments, although the EC_{50} concentrations were higher for ATU-inhibited sludge in both experiments, indicating that heterotrophic bacteria are less sensitive to AMD, there was no statistically significant difference ($\alpha = 0.05$) between the results (Table 4).

Acclimatization

Organic Matter Removal Efficiency

The COD value of the synthetic wastewater was 900 ± 19 mg L^{-1}. Until Day 36, COD removal efficiency remained high (approximately 82–93%) in all reactors, although over time COD removal generally became less efficient in reactors receiving AMD loading when compared

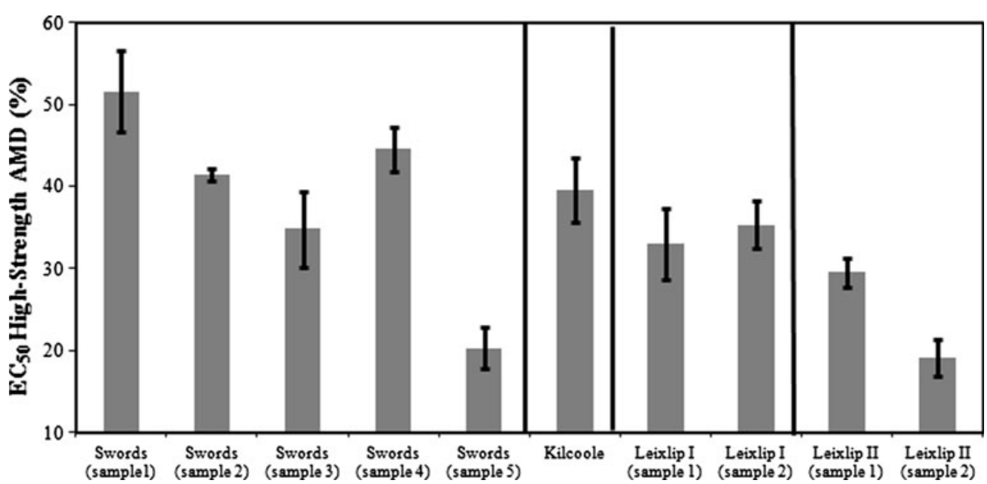

Fig. 1 Comparison of AMD concentrations (% volume in test solution) causing effects of 50% inhibition in OUR inhibition tests using high-strength synthetic AMD mixed with activated sludge samples from municipal and industrial WWTPs (Table 1). *Standard error* of results is shown by *error bars*

Table 4 Mean, median, and range of EC_{50} (% AMD) results of OUR inhibition tests using high-strength synthetic AMD and activated sludge samples from two different municipal WWTPs with nitrifying activated sludge, i.e. Swords and Leixlip WWTPs

WWTP location	*n*	Mean EC_{50} (% AMD)	SD	Median	Range	
					Minimum	Maximum
Swords (sample 5)	6	20.3	6.4	18.6	13.3	32.2
Swords (sample 5, ATU-inhibited)	4	38.6	12.5	34.0	29.5	57.0
Leixlip I (sample 2)	3	35.3	3.9	33.8	32.4	39.7
Leixlip I (sample 2, ATU-inhibited)	3	41.5	4.3	42.8	36.7	45.0

Allyl thiourea (ATU) was added to a subsample of activated sludge from each WWTP to inhibit the activity of nitrifying bacteria, and the test results for ATU-inhibited and non-ATU-inhibited activated sludge are presented

n number of replicates, *SD* standard deviation

Table 5 Percentage of organic matter removal performance, measured in terms of COD removal, in Reactor A (Control), and Reactors B, C, and D, which were loaded with 5, 10, and 25% AMD as a fraction of total influent volume during the acclimatization study

Day	Days of AMD loading	Reactor A, COD removal	Reactor B, COD removal	Reactor C, COD removal	Reactor D, COD removal
19	3	92.7	88.4	90.4	92.3
26	10	89.8	91.3	88.4	92.3
28	12	89.9	88.5	87.5	86.2
30	14	92.1	91.4	82.3	89.1
36	20	92.2	89.2	87.6	85.2
42	26	91.3	75.8	35.7	63.3

Final effluent samples were analyzed throughout the AMD loading period, which began on Day 16

to the control reactor (Table 5, Fig. 2). On Day 42, COD removal efficiency was dramatically lower in the reactors receiving AMD loading, with Reactor C achieving only 36% COD removal.

Reactors C and D, receiving the highest AMD loads (10 and 25%, respectively), had the poorest performance in terms of COD removal throughout most of the AMD loading period.

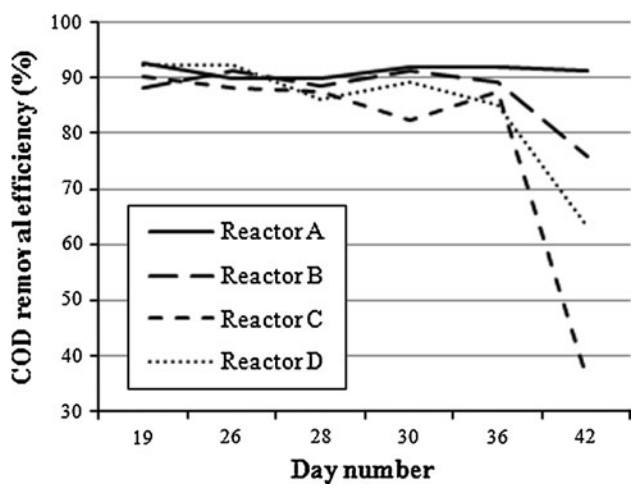

Fig. 2 Organic matter removal performance, measured in terms of COD removal, in Reactor A (Control), and Reactors B, C, and D, which were loaded with 5, 10, and 25% AMD as a fraction of total influent volume during the acclimatization study. Final effluent samples were analyzed throughout the AMD loading period, which began on Day 16

Effluent Suspended Solids

Effluent suspended solids concentrations were variable over the period of AMD loading, and did not exhibit a clear relationship with either AMD load or time (Fig. 3). Overall, effluent suspended solids concentrations from all reactors remained greater than typical discharge limits (e.g. 30 mg L^{-1}) throughout the AMD loading period. On Day 42, the TSS concentration was highest in Reactor C.

SVI and Floc Morphology

During the start-up period (Days 1–15), SVI values ranged from approximately 110–186 mL g^{-1} MLSS. The supernatant was consistently clear after settleability tests, with no particles visible in suspension. Floc morphology was generally irregular, with variations from compact to diffuse structure evident in all reactors, and filament abundance values were consistently equal to 4.

Changes were observed in floc morphology and SVI values in all reactors after AMD loading began on Day 16. For optimum settling and suspended solids removal, compact activated sludge floc structure is most desirable. Flocs

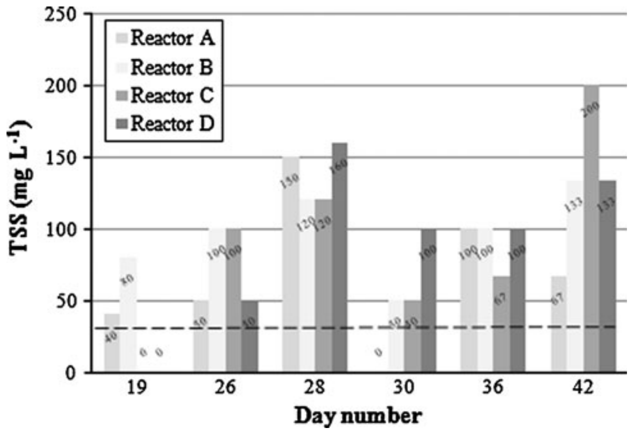

Fig. 3 Total effluent suspended solids (TSS) (mg L^{-1}) in Reactor A (Control), and Reactors B, C, and D, which were loaded with 5, 10, and 25% AMD as a fraction of total influent volume during the acclimatization study. The *dashed line* at 30 mg L^{-1} indicates the typical discharge limit for WWTPs [e.g. 40 CFR 133.102(c) (Code of Federal Regulations (CFR) 2006)]. Final effluent samples were analyzed throughout the AMD loading period, which began on Day 16

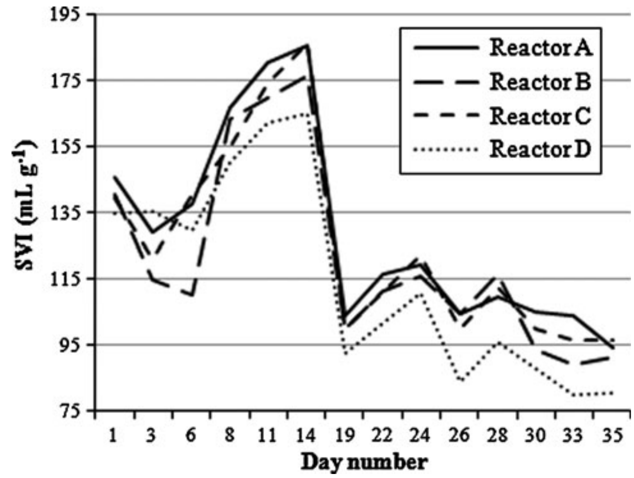

Fig. 4 Sludge volume index (SVI) values of activated sludge in Reactor A (Control), and Reactors B, C, and D, which were loaded with 5, 10, and 25% AMD as a fraction of total influent volume during the acclimatization study. Start-up period: days 1–15; continuous AMD loading began on Day 16

with diffuse structure and/or very small 'pin' flocs develop when flocculation is poor, leading to poor settling and loss of biomass in treated effluent. In Reactors A (control) and B, the floc morphology deteriorated throughout the acclimatization period, changing from compact to diffuse structure, with pin flocs eventually developing. In Reactors C and D, pin flocs were observed earlier, but subsequently disappeared; however, overall floc morphology changed from compact to diffuse in these reactors as well.

The SVI of activated sludge from all four reactors (including the control) followed identical trends (Fig. 4). Before AMD loading began, there was a brief period of high SVI values (Days 11–14). After Day 14, the SVI dropped, remaining between 80.0 and 122.2 mL g^{-1} for the remainder of the acclimatization period. Reactor D had the lowest SVI. Although the SVI was below the bulking threshold of 120 mL g^{-1}, the sludge supernatant in all reactors changed from clear to turbid on Day 24 and remained turbid for the duration of the study. Filament abundance also remained generally constant at 4, with only a slight decrease observed in Reactors A and B on Day 33.

Protozoan Community

The protozoan community in each reactor was monitored for qualitative changes using broad habit groupings. Sessile, crawling, and free-swimming protozoa were present in all reactors throughout the AMD loading period (Fig. 5). Shifts in protozoa species distribution, as indicated by changes in major habit grouping dominance, were evident in all reactors. In all reactors, total protozoa abundance dropped during the start-up period but then increased after AMD loading began, with peak abundance observed on Day 19. During start-up, however, loss of sessile species occurred in all reactors. After AMD loading began, crawling and free-swimming species gradually became more dominant, particularly in Reactor C, which experienced a large increase in protozoa abundance with free-swimming species being the most dominant. Overall, species distribution changed significantly in all reactors, including the control reactor. Protozoa abundance was variable but generally constant in Reactor A, but followed decreasing trends in Reactors B and D. An apparent decreasing trend in Reactor C until Day 24 changed as free-swimming species became increasingly abundant and eventually highly dominant.

Effect of AMD Acclimatization on OUR_{max} and Respiration Inhibition

The OUR_{max}, an indicator of activated sludge activity, did not change significantly during the AMD loading period in Reactors A (control), B, or C (Fig. 6). However, the OUR_{max} in Reactor D dropped from 61.7 to 42.2 mg O_2 L^{-1} h^{-1}. This change indicated decreased activity of the activated sludge microbial community in the reactor receiving the highest AMD load.

OUR inhibition tests were conducted using spiked additions of simulated Avoca AMD. Results for Reactors B, C, and D indicated that the EC_{50} value was approximately 70–100% for all reactors at Day 15 (immediately before the addition of AMD to the reactors; Table 6).

All reactors then experienced increased inhibition, as reflected by lower EC_{50} values, at Day 24 (8 days after AMD loading began). The drop in EC_{50} value was most

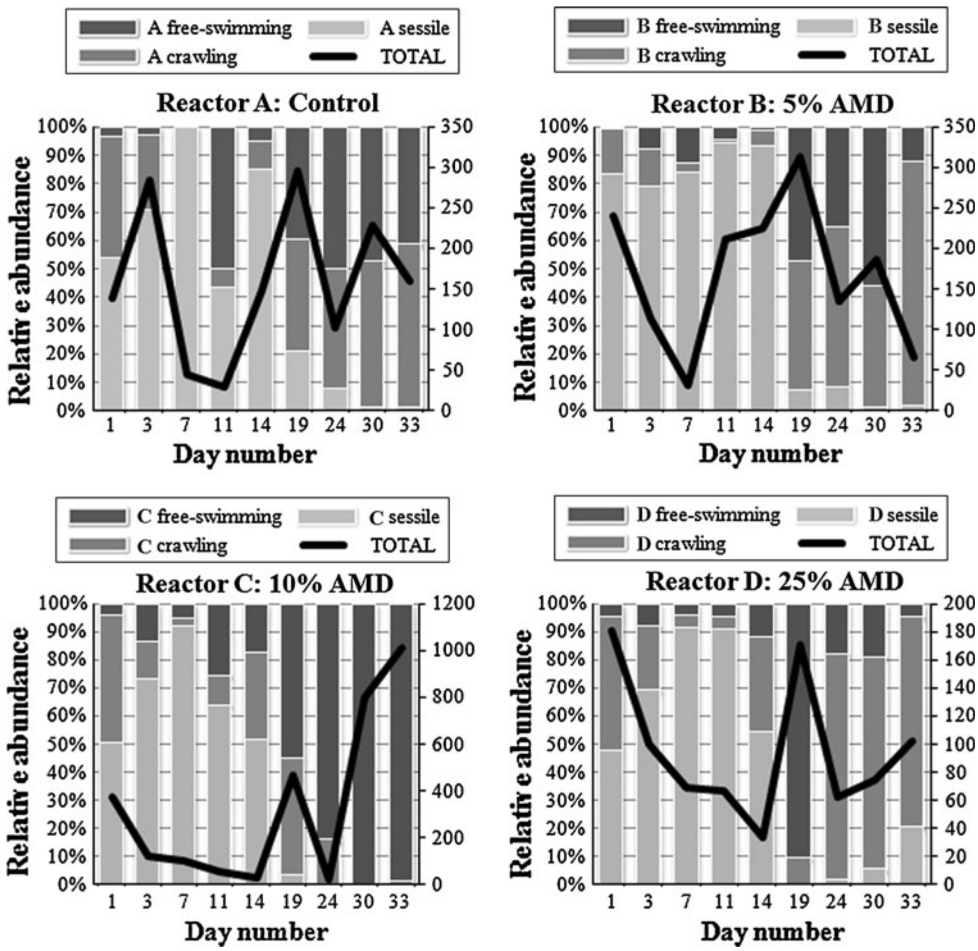

Fig. 5 Relative abundance of major habit groupings and protozoa counts for Reactor A (Control), and Reactors B, C, and D, which were loaded with 5, 10, and 25% AMD as a fraction of total influent volume during the acclimatization study. Start-up period: days 1–15; continuous AMD loading began on Day 16

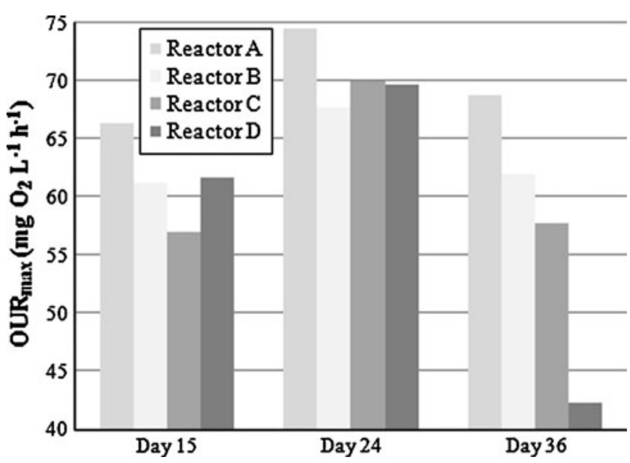

Fig. 6 Maximum OUR in activated sludge samples from Reactor A (Control), and Reactors B, C, and D, which were loaded with 5, 10, and 25% AMD as a fraction of total influent volume during the acclimatization study. Start-up period: days 1–15; continuous AMD loading began on Day 16. Tests were performed during the start-up period, and 8 and 20 days after AMD loading began

evident in Reactor D, the reactor receiving the highest influent AMD concentration. On Day 36 (20 days after AMD loading began), all reactors were less inhibited by AMD than on Day 15, as shown by higher EC_{50} values. Adding synthetic AMD to Reactor D on Day 36 made no discernible difference in OUR, so no EC_{50} value could be calculated. This indicated that Reactor D was the most acclimatized to synthetic AMD. Interestingly, the OUR of the control reactor was also slightly less inhibited by AMD on Day 36 than on Day 15. The reason for this is not known, although an increase in substrate-selective bacteria adapted to the OECD synthetic sewage may have slightly masked inhibition effects. OUR inhibition tests on the acclimatized activated sludge indicated that the activated sludge microbial community could adapt to AMD over a relatively short timescale (approximately 3 weeks), such that shock loads of metals and acidity did not significantly inhibit OUR.

Discussion

Acute Toxicity Tests

Acute toxicity test results demonstrated the high tolerance for unacclimatized activated sludge to shock loads of high-

Table 6 Results of OUR inhibition tests performed with simulated Avoca AMD and activated sludge samples from Reactor A (Control), and Reactors B, C, and D, which were loaded with 5, 10, and 25% AMD as a fraction of total influent volume during the acclimatization study

Day number	Number of days of AMD loading	Reactor A EC_{50} (% AMD)	Reactor B EC_{50} (% AMD)	Reactor C EC_{50} (% AMD)	Reactor D EC_{50} (% AMD)
15	0	69.5	87.6	100.7	86.7
24	8	104.5	74.7	88.9	66.0
36	20	93.0	104.3	108.1	No inhibition

strength synthetic AMD containing high concentrations of six metals, including Cu and Zn, which are known to exert toxic effects on activated sludge. For example, from previous studies, EC_{50} values for Cd, Cu, and Zn range from 15 to 60 mg L^{-1} (Alkan et al. 2008; Kelly et al. 2004; Gutiérrez et al. 2002; Sher et al. 2000). However, EC_{50} values in the current study ranged from 19 to 52% AMD. In terms of metal concentrations, the activated sludge from municipal WWTPs tolerated combined metal loads in excess of 300 mg L^{-1} Fe, 100 mg L^{-1} Al, 15 mg L^{-1} Cu, 35 mg L^{-1} Zn, 15 mg L^{-1} Mn, and 30 μg L^{-1} Pb. The acute toxicity of a multi-metal solution to activated sludge is difficult to predict owing to the complexity of interactions between metal ions and wastewater, interactions between metal ions and activated sludge biomass, and interactions among metal ions. Toxic effects of metals can be synergistic or antagonistic; empirical determination of EC_{50} concentrations for a specific AMD (or any metal-containing wastewater) is recommended.

Toxicity test results showed that the ability of activated sludge to withstand elevated metal concentrations and acidity, such as those found in AMD, can vary significantly from one WWTP to another. Although differences between toxic effects on sludge from different source WWTPs have been reported elsewhere (Gernaey et al. 1999), many previous studies have found that activated sludge from different sources can be used indistinctly in toxicity studies, giving similar results in respiration inhibition tests (Christofi et al. 2000; Dalzell and Christofi 2002; Gutiérrez et al. 2002). The EC_{50} results using Swords activated sludge and Leixlip II activated sludge sampled on different dates indicated that the toxic effect of synthetic AMD to activated sludge sampled from a single WWTP can also change significantly over time. Industrial sludges often show very little variation in their characteristics, especially where there is a continuous production line producing the same effluents. In contrast, MWWs have wide variability and contain a wide range of contaminants, allowing more rapid acclimatization and a greater ability to deal with shock loadings. In this study, the OUR inhibition caused by high-strength AMD was significantly different for municipal and industrial activated sludge. Rather than demonstrating a higher tolerance to shock loads of metal contaminants, the activated sludge from the industrial WWTP (Leixlip II) was more inhibited by AMD than activated sludge from municipal WWTPs. It is likely that the population of microorganisms in the industrial sludge has adapted to the presence of a specific industrial effluent (Christofi et al. 2003), and consequently has a decreased resilience to shock loads of other toxicants. Using activated sludge from WWTPs treating MWW can ensure that the population of microorganisms has not become adapted to a specific industrial wastewater and is therefore more robust to changes in metal concentrations or pH shocks caused by adding AMD.

Results of acute toxicity tests comparing OUR inhibition of heterotrophic and nitrifying bacteria were not significantly different ($p < 0.05$), but a trend of lower EC_{50} values for ATU-inhibited activated sludge suggested that AMD will cause a reduction in nitrification. Nitrifying bacteria have been reported to be more sensitive than heterotrophic bacteria to many toxic substances, including metals (Blum and Speece 1992; Juliastuti et al. 2003; Stasinakis et al. 2003; Principi et al. 2006), as well as being sensitive to low-pH environments. In contrast, Madoni et al. (1999) reported that nitrifiers were no more sensitive than heterotrophs to Cd, Cr, Cu, Pb, and Zn. Possible elimination of nitrification should be considered if AMD is added to full-scale plants and the long-term impacts of metal exposure on nitrifying bacteria should be monitored. The present study highlights the importance of testing AMD treatability to identify an acceptable loading regimen for individual WWTPs.

In the acute toxicity tests, the mixture of activated sludge and AMD reached pH > 6 in all cases. This increase in pH indicates that neutralization of AMD occurs when it is mixed with activated sludge. Metals are removed from solution in WWTPs via several mechanisms, including adsorption onto activated sludge biomass and EPS (Battistoni et al. 1993; Brown and Lester 1979; Cheng et al. 1975; Guibaud et al. 2003), precipitation and entrapment by activated sludge flocs (Chang et al. 2007; Pagnanelli et al. 2009; Sterritt et al. 1981), and chelation onto organic compounds in wastewater (Arican and Yetis 2003; Kunz and Jardim 2000; Nicolau et al. 1999; Sterritt and Lester 1984). The pH is an important factor in all of these metal removal mechanisms. It is likely that each of these processes occurred as AMD was added to the activated sludge during toxicity tests, thereby decreasing dissolved metal concentrations and limiting toxic effects (Dalzell and Christofi 2002; Kelly et al. 2004; Madoni et al. 1996; Pambrun et al. 2008; Pai et al. 2009). Çeçen et al. (2010) studied toxicity of Ag, Cd, Cr, Hg, and Pb to nitrifying sludge, considering theoretical metal speciation as well as respiration inhibition. They concluded that the speciation is in fact the primary factor for inhibition, rather than total metal concentration. In their study, Pb was almost entirely removed from solution via carbonate precipitation, leading them to conclude that where alkalinity is present in stoichiometric amounts, Pb is not likely to cause significant inhibition. Theoretical metal speciation and the removal of dissolved species after high-strength synthetic AMD is mixed with MWW and activated sludge biomass are the subjects of further investigation.

Acclimatization

During the acclimatization study, effluent concentrations of COD and suspended solids were monitored as indicators of performance. Over the AMD loading period, COD removal efficiency decreased in the reactors receiving AMD, and was generally lowest in the reactors receiving the highest AMD loads. Until Day 36, COD removal remained high, indicating that the activated sludge was still effectively removing the organic components of wastewater in the presence of AMD. Katsou et al. (2011) also reported high COD removal in membrane bioreactors receiving moderate concentrations (3–15 mg L^{-1}) of Cu, Pb, Ni, and Zn, and Arican and Yetis (2003) observed a stimulating effect on COD removal after sludge was acclimatized to a continuous feed of Ni over an extended period (>100 days). However, COD removal in Reactors B, C, and D dropped on Day 42. This coincides with the findings of Beyenal et al. (1997), who reported reduced substrate removal efficiency by activated sludge that had been acclimatized to Cu and Zn. Özbelge et al. (2007) also reported that inhibition effects of Cu and Zn on activated sludge became more pronounced with an increased period of acclimatization, leading to a drop in COD removal efficiency, possibly owing to changes in the microorganism population as well as changes in microbial enzymatic pathways caused by long-term metal exposure. Removal of organic substances may also be reduced if the metals compete with organic substances for binding sites (Chua et al. 1999; Xie and Nakamura 2002).

Effluent suspended solids concentrations from all reactors exceeded typical discharge limits (i.e. 30 mg L^{-1}) throughout the AMD loading period. The poor removal of suspended solids during AMD loading coincided with increasing turbidity, which became apparent in all reactors on Day 24. Despite these indications that performance was adversely affected by AMD loading, settleability (measured in terms of the SVI) improved after AMD loading began, and subsequently remained within the normal range, showing improvement with time. However, the increased turbidity and elevated effluent suspended solids may indicate decreased populations of floc-forming bacteria associated with increased concentrations of dispersed bacteria and pinpoint flocs in the mixed liquor. The presence of cations, and metals in particular, affects the settleability and compressibility of activated sludge. Calcium, Mg, Al, and Fe have been reported to significantly improve sludge settleability and compressibility (Jin et al. 2003). Those researchers suggested that bridge binding between negatively charged polymeric groups and cations and/or surface charge reduction as cations bind to negatively charged sites on the floc surface bring flocs closer together and aid settling. Alternatively, Ong et al. (2005) suggested that the likely cause for observed improvement in settleability after adding metal solutions to activated sludge reactors was a decrease in filamentous bacteria. In the present study, consistent filament abundance values do not indicate loss of filamentous bacteria; settleability most likely remained good because of cations in the AMD aiding coagulation (Jin et al. 2003; Rao et al. 1992) and the flocculation of Fe oxyhydroxide precipitates onto suspended solids (Johnson and Younger 2006).

The OUR_{max} of Reactor D (25% AMD) decreased during the acclimatization period, indicating a drop in sludge biomass activity; however, spike loads of AMD in toxicity tests on Day 36 caused no respiration inhibition in activated sludge from this reactor. These results, together with the decreased COD removal performance for Reactor D, suggest that over time, the AMD loading caused a change in the microbial population, as metal-tolerant species were selected. Sorour and Sayed-Ahmed (2005) also used OUR to assess the effect of shock doses of Cd and Zn on acclimatized sludge, and reported a similar trend of decreased toxicity with increasing influent metal concentrations. Heavy metals have previously been shown to cause changes in the community structure of activated sludge protozoa (Madoni et al. 1996; Nicolau et al. 2005; Ong et al. 2004) and microscopic observations during this study indicated a dramatic shift from predominantly sessile to predominantly crawling and free-swimming species. Loss of protozoa, leading to decreased removal of dispersed bacteria, has been identified as a cause of increased turbidity (Curds and Cockburn 1970). In the present study, abundance of sessile ciliates tended to decrease in reactors receiving AMD loading, turbidity increased, and effluent suspended solids increased. However, the continued presence of ciliated protozoa in all reactors during AMD loading indicates that although some species are more sensitive to AMD, and therefore become less dominant or disappear over time, a population of microorganisms can become acclimatized to loads of metal and acidity. The increased abundance of some species under the 10% AMD loading regime suggests some possible stimulatory effects, perhaps as trace metals are used for microbial growth by specific species (Nicolau et al. 2005; Yetis and Gokcay 1989). Previous work supports the occurrence of acclimatization: Abraham et al. (1997) observed that ciliates in a municipal WWTP could withstand variable influent concentrations of Fe, Zn, Cu and Cr, including sudden peaks, and Neufeld and Hermann (1975) proposed maintaining a culture of acclimatized activated sludge biomass to handle slug doses of toxic metal-laden wastewater, such as AMD.

Conclusions

The key findings from this study are:

- Acute toxicity of synthetic AMD to activated sludge was evaluated with oxygen uptake rate (OUR) inhibition tests; the results showed that activated sludge can withstand high proportions of AMD (EC_{50} values ranging from 19 to 52% AMD by volume).
- The EC_{50} values of municipal and industrial activated sludge were significantly different ($p < 0.05$), with municipal activated sludge EC_{50} values generally higher, showing increased tolerance of municipal activated sludge to large proportions of AMD flow.
- The EC_{50} values of heterotrophic and nitrifying activated sludge were not statistically significantly different, although the EC_{50} values for heterotrophic bacteria were generally higher.
- The processes of neutralization, metal precipitation, and adsorption, which occur after mixing AMD with activated sludge, are evidently very important factors in determining overall toxicity.
- Increased concentrations of COD and suspended solids were observed in the final effluent, associated with turbidity and poor floc morphology, after extended AMD loading.
- Protozoan community structure changed during the AMD loading period, and overall abundance tended to decrease over time. OUR_{max} values also decreased in the AMD-loaded reactors, particularly in the reactor receiving the highest AMD load, suggesting losses in activated sludge biomass activity over the acclimatization period.
- Results from OUR inhibition tests on the acclimatized activated sludge indicate that over a relatively short timescale (i.e. approximately 3 weeks), the activated sludge microbial community can adapt to AMD sufficiently that shock loads of metals and acidity do not significantly inhibit OUR.

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