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ENHANCEMENT OF AMETRYN BIODEGRADATION EFFICIENCY USING ANTHRAQUINONE-2,6-DISULPHONATE IN ANAEROBIC-AEROBIC TREATMENT

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Abstract

Ametryn (2-(ethylamino)-4-(isopropylamino)-6-(methylthio)-s-triazine), a herbicide present in different type of wastewater was treated using the anaerobic-aerobic batch reactor in the presence of anthraquinone-2,6-disulphonate (AQS) as a redox mediator. The anaerobic co-treatment process was conducted for an influent ametryn concentration of 8-10 mg/L during 280 days in previously acclimated biomass. Different intermediate compounds of ametryn were identified in the anaerobic effluent using the liquid chromatography-mass spectrophotometer (LC-MS). Low mixed liquor volatile suspended solids/mixed liquor suspended solids (MLVSS/MLSS) ratio between 0.71-0.81 indicated a stable anaerobic performance. Food to microorganism ratio (F/M), sludge age and solids retention time (SRT) for anaerobic reactors observed was 0.19 – 0.27, for 7 days and 64-190 days respectively. The aerobic reactor was coupled sequentially to remove trace organic matter and the intermediate compounds in the anaerobic effluent. In the aerobic reactor, MLVSS/MLSS ratio observed was 0.68-0.75, F/M ratio was 0.166, sludge age 15 days, and SRT 190-310 days. The overall removal efficiency of anaerobic-aerobic treatment was >99% for both ametryn and COD. Anaerobic-aerobic effluent was fed to the micro-algae *Chlorella vulgaris* and *Scenedesmus quadricauda*, and the effluent has contributed to algal growth.

Keywords: AQS, ametryn, biodegradation, redox mediator

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1. Introduction

The water released from agriculture and industrial activities contain different types of herbicides/pesticides compounds. Among the various type of herbicides being used, ametryn (2-ethylamino)-4-(isopropylamino)-6-(methylthio)-s-triazine) is consumed in large quantity worldwide to remove weeds in various crop fields (Peters et al., 2014). The presence of ametryn in the water ecosystem causes irreparable damage to the aquatic organisms including flora and fauna. The physico-chemical properties of ametryn (Table 1) allow it to remain present for a long time in the ecosystem, low solubility in water (209 mg/L at 25°C), and poor soil adsorption. Ametryn is a toxic compound that causes inhibitions not only to the weeds but also to non-targeted plants, animals, and humans (Hurley, 1988; USEPA, 2010). Ametryn is having a leaching potential of 6.9, which enables it to leach vertically and laterally (Jacomini et al., 2009). Ametryn is persistent, and it accumulates on the biotic and abiotic components, which can cause significant ecological damage. Ametryn has a maximum permissible limit of 1.4 µg/L for groundwater, and 14 µg/L for surface water (USEPA, 2010). The excessive usage of

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ametryn in crop fields such as sugarcane has led to rise in ametryn concentration up to 3.4 mg/L (Sangami and Manu, 2017). The consumption of herbicides is likely to be increased in the future to save the loss of crops. Presence of ametryn in the agriculture runoff often causes difficulty for nutrient removal processes (Malila et al., 2019) and also to avoid further water pollution and as a preventive measure, the runoff water from agriculture and manufacturing activities must be treated at the source.

Several researchers have reported the treatment of ametryn using physical, chemical, biological, and combination of all these methods. The treatment efficiency is found to be greater in physico-chemical method, but they are costlier due to chemical usage, production of a large quantity of sludge, intermediate compounds, which requires further treatment costs. Combinations of physico-chemical with biological methods were also being used to achieve maximum removal efficiencies but limited up to 61% removal (Navaratna et al., 2016). The biological treatment is considered as an economical, efficient, and sustainable method for the removal of ametryn from water and wastewater, if they are modified. A very few studies on biological treatment of ametryn have been reported in the literature, but most of the studies have highlighted the formation of intermediate compounds (Szewczyk et al., 2018) and which are more persistent than ametryn (Farré et al., 2002). Sandoval-Carrasco et al. (2013) have studied the compartmentalized biofilm reactor using isolated bacterial consortia to treat mixture of 2,4-dichlorphenoxyacetic acid and ametryn efficiently, but maintenance of such conditions in the real time application is difficult. Alternatively, use of activated sludge from the sewage treatment plants (STP) contains a stable community of active biomass, which can degrade the toxic compounds after a proper acclimation. The conventional treatment methods, such as combined anaerobic-aerobic treatment processes found to be effective in removing the ametryn concentrations up to 6 mg/L (Mahesh and Manu, 2019c).

With the co-treatment and combination of the anaerobic-aerobic treatment process, the biological treatment method often seems to be efficient in removing the herbicides of ecologically important. Co-treatment processes for the toxic compounds are considered to be efficient as they support biomass by providing a safe carbon source for adaptation under toxic condition (Xu et al., 2018). Application of anaerobic-aerobic treatment process for the removal of different halogenated herbicides such as ametryn, 2,4-d, and dicamba herbicides have been studied (Mahesh and Manu, 2019a; 2019b; 2019c). Complete removal of ametryn and COD was achieved from the sequential anaerobic-aerobic reactor, and the system helps to recover biogas and produce clean water than any other biological processes. The intermediates formed during the anaerobic biodegradation process were completely mineralized in the aerobic post-treatment. Though there are few studies reported in the literature for ametryn treatment under biological methods, the complete removal of ametryn has not been reported. In our previous study, 6 mg/L of ametryn was mineralized within 50 days of operation in a similar treatment process. Efficiency enhancement, reduction of toxicity on biomass, and removal of high concentrations of ametryn require the existing method to develop further. It has been reported that the reductive reactions in anaerobic reactors allow the biotransformation of halogenated pollutants including herbicides (Field and Brady, 2003). Anaerobic biotransformation of dicamba was carried out previously by Taraban et al. (1993); Weinberg and Teodosiu (2012), and reported that under anaerobic conditions the dehalogenation and dechlorination reactions take place which led to biotransformation of dicamba.

The use of redox mediators in biological (mainly anaerobic) reactors enhances the treatment performance of halogenated and persistent organic chemicals. Quinones such as anthraquinone-2,6-disulphonate (AQS) are may cause recalcitrance under aerobic conditions. But simultaneous reduction of quinones in the presence of nitrates and suphates under anaerobic digestion processes has also been reported (Cervantes et al., 2008). Under anaerobic condition, quinones act as redox mediators by shuttling reducing equivalents from anaerobic biomass in the presence of primary substrates like starch. At high concentration, AQS may be toxic to anaerobic biomass due to high redox potential but the bacteria can also degrade the AQS once it gets acquainted/enriched to the compound (Cervantes et al., 2003). Redox mediator (AQS) is widely used in the treatment of pollutants like azo dyes having complex organic structures that lead to difficulty for anaerobic degradation (Rau et al., 2002). Conversion of ametryn into carbon/nitrogen sources has been reported and conversion of quinones to hydroquinones and their further conversion to ammonium produce energy for bacteria under bicarbonate buffer condition (Field et al., 2000). The addition of AQS during the treatment of herbicide dicamba (belongs to the chloro-benzoate group) has contributed to enhanced removal and fast reactor recovery. Significance of AQS and its mode of action during the anaerobic treatment of dicamba have been discussed in Mahesh and Manu (2019b). The influence of AQS addition in removing herbicides is highly significant, but its use is limited and hence, more studies need to be conducted to explore its importance.

The microalgae such as *Chlorella vulgaris* and *Scenedesmus quadricauda* are considered as ecologically sensitive and can be affected by the toxicity of pesticides (Ma et al., 2012). These algal species are often used to treat different types of pesticides in water (Baglieri et al., 2016). The integrated system involves the sequence of the anaerobic-aerobic-algal treatment process, which may provide enhanced treatment efficiency of ametryn and its different transformation products.

This article elaborates on the enhanced ametryn treatment efficiency in a previously

acclimated laboratory-scale anaerobic-aerobic batch reactor. The impact of a high concentration of ametryn on the reactor performance, reactor biomass, and addition of AQS on reactor performance was studied. The paper also presents the outcome of batch experiments involving ametryn adsorption, and toxicity bioassay of treated effluent on micro-algal species *Chlorella vulgaris* and *Scenedesmus quadricauda*.

2. Material and methodology

2.1. Material

The reactor materials used are the glass containers and plastic beakers with an operating volume of 2 L. Starch and sodium bicarbonate were purchased from HI media, analytical grade ametryn, AQS, and tert butyl ether were purchased from Sigma Aldrich. Chromatography solvents like methanol and HPLC grade water were procured from Merck manufactured in India, and all other chemicals used to prepare Trace metal solution were of analytical grade.

The stock solution was prepared by diluting 250 mg of ametryn in 2 L freshwater, which was stored below -18 ºC, and the suitable stock was diluted with influent to yield 8 and 10 mg/L. The physico-chemical properties of ametryn are tabulated in the Table 1. The influent to R1 was prepared using 2 and 4 g/L of starch and sodium bicarbonate solution, while the influent to R2 was mixed with a suitable volume of ametryn stock solution.

A trace metal solution was prepared using the procedure mentioned in (Manu and Chaudhari, 2002). The influent to R1 reactor was prepared using 2 g/L of starch with COD of 2000 mg/L, 4 g/L sodium bicarbonate and the influent to R2 reactor was prepared using 2 g/L of starch and ametryn dose of 8 – 10 mg/L, which contained COD 2150 – 2200 mg/L. High ametryn dosages selected in the present study is to check the treatability potential of the sequential anaerobic-aerobic method and also to make it suitable for treating the effluents from manufacturing units containing high ametryn concentrations. An industrial effluent contained 500 mg/L of pesticides (Chiron et al. 2000). The influent water has pH: 7.7 – 8.5, alkalinity: 250 – 280 mg-$CaCO_3$/L.

2.2. Bioreactor set up and biomass acclimation

Fig. 1 shows the flow diagram of the treatment process carried out and the laboratory scale reactor set up. The acclimated biomass to 6 mg/L of ametryn during 150 days was used in the present research. MLSS and MLVSS concentration between 12.7 – 13.8, and 9.1 to 9.3 g/L for anaerobic reactors and that for the aerobic reactor was 4.3 – 6 and 2.5 – 4.5 g/L respectively on 150th day (steady-state condition). At steady-state condition of the anaerobic reactor (R2) the F/M was found to be 0.215 and sludge age was 7 days, similarly, aerobic reactor had F/M ratio 0.174 and sludge age of 12 days, which are within the required range for an efficient bioreactor (Shammas and Wang, 2009). The reactor set up and treatment process followed similarly as described in Supplementary Data. The biogas and methane gas produced was measured by passing the gas produced through a liquid containing 5% w/v of KOH (Isa et al., 1993). A set of batch reactors (M1 and M2) were linked to the existing anaerobic-aerobic reactor to access the possible impact of treated effluent on the micro-algae (M1 - *Chlorella vulgaris* and M2 - *Scenedesmus quadricauda*). About 1.8 – 2 g/L of each algal biomass was inoculated to M1 and M2 reactors and stabilized initially using fresh water for about 30 days to allow the growth of microalgae up to 2 – 3 g/L. After observing significant algal growth (formation of thick green biomass as visualized from the naked eyes), effluent from the anaerobic-aerobic reactor (R4) was fed to M1 and M2 reactors.

Table 1. Physico-chemical properties of ametryn

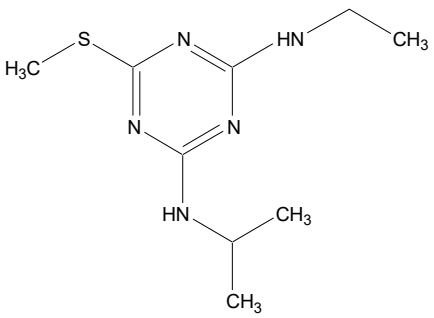
Property	***(2-ethylamino)-4-(isopropylamino)-6-(methylthio)-s-triazine (Ametryn)***
Structure	
Chemical formula	$C_9H_{17}N_5S$
Molecular Weight (g/mol)	227.35
Water solubility (mg/L)	209 at 25°C
Melting Point (ºC)	84
Boiling Point (ºC)	337

Fig. 1. Laboratory set of anaerobic and aerobic reactor

The adsorption study was carried out in four separate batch reactors with a capacity of 1L. These reactors were inoculated with fresh anaerobic biomass having MLSS 12.7 -13.1 and MLVSS 9 - 9.3 g/L, each reactor was initially fed with a known concentration of ametryn (S1 – 2, S2 – 4, S3 – 6 and S4 – 8 mg/L). The liquid and sludge samples were collected daily for about 10 days and analyzed in HPLC to find out the change of ametryn concentration.

2.3. Analytical methods

The determination of ametryn in the liquid samples was conducted using high-performance liquid chromatography (HPLC, Agilent). The formation of transformation products during the treatment process was determined using Liquid Chromatography-Mass Spectrophotometer (LC-MS, Shimadzu). The characterization of wastewater and sludge samples were conducted as per the standard methods adopted.

3. Results and discussion

3.1. Treatment of ametryn

Biodegradation occurs after the biomass gets acclimated to toxic conditions during any biological treatment process. The detailed discussion on reactor acclimation time required for different treatment conditions. (Nasseri et al., 2014) have reported more than 90 days of anaerobic reactor acclimation period for a low concentration of atrazine (1 mg/L). Up to 6 mg/L of ametryn is being acclimated sequentially in three stages, and an overall period of 150 days was required to achieve complete mineralization at 24 h hydraulic retention time (HRT) (Mahesh and Manu, 2019c). The same reactors were used continuing from 150 to 430 days, and the reactor was at the stable condition with a constant COD removal 85%, biogas production 790 mL/d, pH 6.5, ORP - 280 mV, temperature 30.4^{o}C, and alkalinity 1650 mg$CaCO_3$/L.

The performance of both anaerobic control (R1) and ametryn treating reactor (R2) is depicted in Fig. 2. Ametryn from 8 to 10 mg/L was treated over 280 days, starting from 151 to 431 days, with the organic loading rate 0.2 – 0.22 kg-COD/m^3/d. Since the complete removal achieved in 150 days for 6 mg/L of ametryn, it can be considered that reactor acclimation is achieved. Therefore, the further treatment process was carried out from the 150th day onwards for the influent concentration of 8 – 10 mg/L of ametryn. On the 151st day, the reactor performance dropped with ametryn and COD removal efficiency to 68 and 64%, respectively. Influent ametryn dosage 8 mg/L was fed from 151st day, and the removal efficiency dropped to 40 from 99%. Further, about 90% of ametryn was removed till day 245, and the COD removal efficiency observed was in the range 77 – 80%, biogas production in the range of 690 – 720 mL/d, and the ORP observed was in the range -290 to -300 mV.

After the introduction of 8 mg/L ametryn, the R2 reactor performance reduced significantly. Previously, 4 – 6 mg/L of ametryn was removed within 50 days, whereas the treatment of 8 mg/L required more than 90 days of operation to achieve 90% of the removal efficiency. The reasons for slow reactor recovery may be due to high influent ametryn concentration leading to biomass inhibition. Biomass can withstand the toxic load up to a certain saturation limit, and after that, it can cause toxicity, as there will be high concentrations of intermediate compound formation. It may be noted that the adapted biomass to a certain toxic condition may be able to remove high concentrations of such toxic compounds.

The formation of different intermediate compounds often accumulates over time and thus causes toxicity (Velisek et al., 2017); this can only be overcome after a certain time till the biomass gets acquainted with the existing reactor condition. Volatile fatty acids (VFA) and alkalinity of the anaerobic reactor effluent were in the range 950 – 1350 mg/L and 2100 – 2500 mg-$CaCO_3$/L indicating the accumulation of non-degraded organic compounds. Higher VFA and alkalinity concentration during the biological treatment processes may be an

indication of toxic conditions of the reactor (Chin et al., 2005; Shin et al., 2003).

The ametryn removal during this stage was limited to 90%, and to improve the reactor performance, about 5 mg/L of AQS was added from the 249th day onwards. The addition of AQS was able to enhance the reactor performance indicated by reduced ORP in the range -300 to -320 mV. This observation is supported by the previous studies reported by Da Silva et al. (2012); Field and Brady (2003). Ametryn removal was observed >99%; biogas production was >780 mL/d, COD removal >80% indicates the biodegradation of 8 mg/L of ametryn. At this stage, the effluents contained the VFA between 450 – 780 mg/L and alkalinity 1250 – 1400 mg-$CaCO_3$/L.

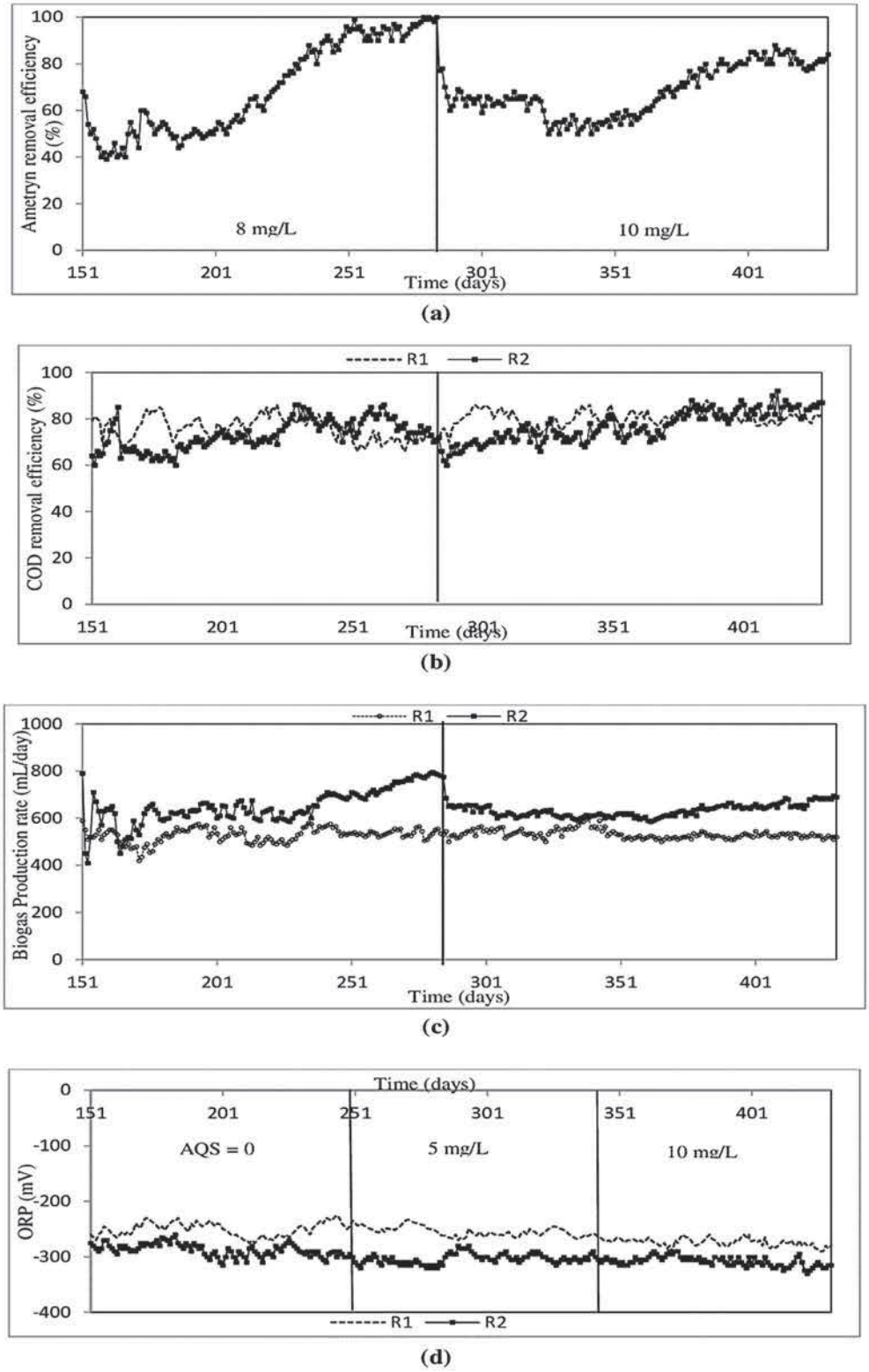


Fig. 2. Anaerobic reactors performance: comparison between ametryn treatment (R2) and control (R1) reactors

In another study, the improved removal efficiency of dicamba by 10 to 15% was achieved in the presence of 5 – 20 mg/L of AQS (Mahesh and Manu, 2019b). The granulation was observed continuously in the anaerobic reactor contributing high biogas production, indicating that ametryn being acted as a nutrient source to the anaerobic biomass. The disappearance of HPLC peak at 12.8 min for the R2 effluent indicated the degradation of ametryn, which was confirmed by subjecting the samples to MS analysis. These observations have also been reported previously during the treatment of triazine and ametryn (Derakhshan et al., 2018; Mahesh and Manu, 2019c).

Influent ametryn dose was increased to 10 mg/L from 284th day onwards. At this stage, the ametryn removal efficiency reduced to 77% and remains constant at around 65%. Further, reduced to 50% till 342nd day with COD removal 75 – 78%, and biogas production 600 – 630 mL/d. Influent AQS was raised to 10 mg/L from 342nd day and which has reduced the ORP to -300 to -320 mV. After an initial lag during bacterial adaptation, a maximum ametryn removal efficiency observed during the 126 days of operation was 85%, COD removal 85 – 90%, and biogas production of 670 mL/d. Consequently, high VFA (920 – 1400 mg/L) and alkalinity (2100 – 2650 mg-$CaCO_3$/L) concentrations in the effluent was observed. The granules formed previously started to deteriorate, indicating an unfavourable condition for the bacteria. Again after 375th days of operation, stable reactor performance was observed with a consistent ametryn removal 70 – 88%, COD 80%, and biogas yield of 625 mL/d. No further fluctuations in the removal efficiencies of ametryn and COD have been observed, mainly due to the bacterial adaptability and activation of inactive biomass for AQS addition. High concentrations of ametryn have appeared toxic and inhibited the biomass, which has led to slower reactor recovery and ametryn removal limited to 72% for the influent concentration of 25 mg/L (Mahesh and Manu, 2019a).

Under anaerobic conditions, ametryn was bio-transformed into its intermediate compounds such as a carbon/nutrient source and thus has been utilized by the anaerobes leading to 100% removal of ametryn within 50 days at 24 h HRT (Mahesh and Manu, 2019c). Some studies have reported HRT greater than 20 weeks for the removal of herbicide atrazine using anaerobic/mixed culture treatment (Ghosh and Philip, 2004; Keerthinarayana and Bandyopadhyay, 1997). High influent ametryn concentration creates greater accessibility to biodegradation, which may enhance the treatment efficiency (Baghapour et al., 2013). The same assumption seems to be impractical for anoxic membrane bioreactor, and it was able to treat only about 46% of ametryn (i.e., 1 – 2 mg/L) and appeared toxic to the biomass (Navaratna et al., 2016). Introduction of 8 mg/L to the biomass previously acclimated to 6 mg/L of ametryn for 150 days has shown a reduction in the reactor performance on initial days and required a long time for complete removal. This observation may be an indication of herbicide exposure and hence the biomass adaptability to high concentration levels. Upon an increase in the concentration to 10 mg/L similar treatment trend was observed, but during this stage, the maximum removal was limited up to 88% throughout 147 days of operation in the anaerobic reactor. The addition of AQS has significantly contributed to biotransformation of ametryn, COD removal efficiency and high biogas production rate.

3.2. Biotransformation and intermediate compounds of ametryn

According to the biodegradation pathway proposed, ametryn can easily be converted into carbon and nitrogen sources under anaerobic conditions (Mahesh and Manu, 2019c). The biotransformation followed by biodegradation was observed in the present study from 280 – 284 days, indicated by high biogas yield (>790 mL/day) and supported by the absence of HPLC peak.

It is evident that the formation of long-chain fatty acids, cyanuric acid, and biuret as intermediates of ametryn during the co-treatment under reducing conditions, which were further transformed into nitrogen and CO_2 (Sene et al., 2010). During the treatment of 10 mg/L of influent ametryn, the low biogas production up to 400 days suggested there may be the formation of intermediate compounds that are non-biodegradable. Therefore, the effluent samples of anaerobic reactors were analyzed in LC-MS to detect the intermediate compounds. The major intermediate compounds identified are indicated in the MS report (Fig. 3).

Some of the main compounds are 2-nitro-1-propanol, 2-chloro-n-ethylacetamide, ethyl 3-isothiocyanatopropionate, 4-nitrobenzoic acid, 3-chloroprop-2-enyl ester, methyl sester, dichloroacetamide, trichlamide, benzoylprop-ethyl, and pentadecyl ester. These compounds have accumulated in the anaerobic reactor led to reduced or lower reactor performance. The different types of intermediate compounds are expected to be produced in a biological reactor that may be much more toxic than the parent compound (Szewczyk et al., 2018). Over a certain period (after 20 – 30 days) of treatment, the appearance of compounds like cyanuric acid, allophanate, and biuret indicated the complete degradation of ametryn as observed from the biodegradation pathway (Fig. 4).

3.3. Impact of ametryn on anaerobic biomass

As the influent ametryn dose was increased to 8 mg/L, the reduction in MLVSS and reduction in the size of granules was observed, which leads to poor sludge quality. That was an unstable condition and is par agreement with the initial days of ametryn introduction in all the previous studies reported.

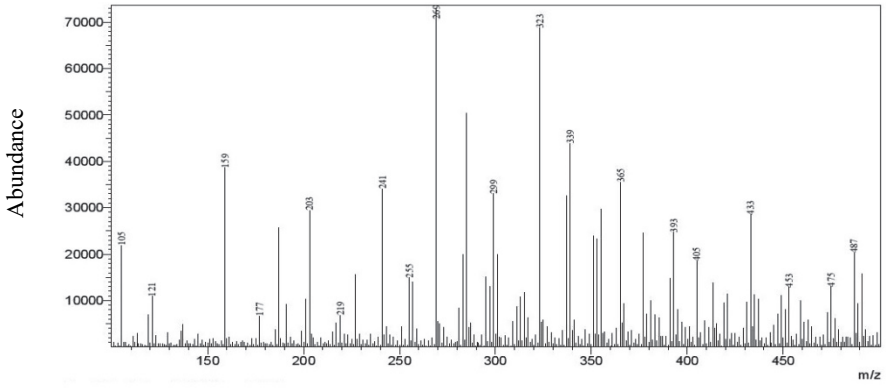


Fig. 3. The LC-MS report showing the major intermediate compounds of ametryn: 2-nitro-1-propanol (159), 2-chloro-n-ethylacetamide (203), ethyl 3-isothiocyanatopropionate (241), 4-nitrobenzoic acid (269), 3-chloroprop-2-enyl ester (299), methyl ester (323), dichloroacetamide (339), trichlamide (365), benzoylprop-ethyl (393), and pentadecyl ester (433)

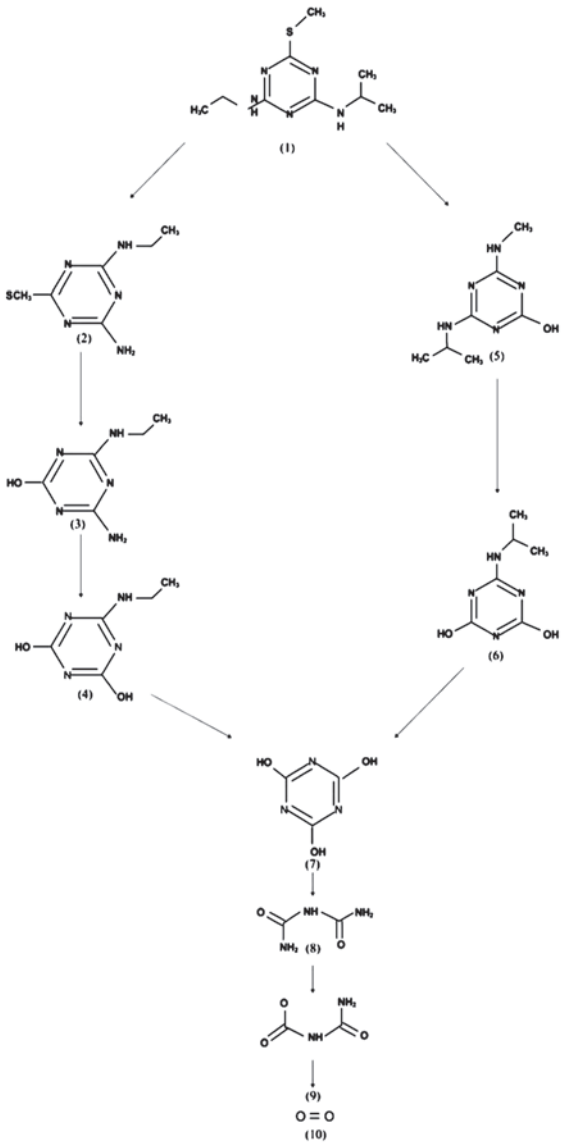


Fig. 4. Proposed biodegradation pathway of ametryn. (1) – ametryn, (2) - n-ethyl-6-(methylsulfanyl)-1,3,5-triazin-2,4-diamin, (3) – deisopropylhydroxyatrazine, (4) – 2,4-dihydroxy-6-(N'-ethyl)amino-1,3,5-triazine, (5) – hydroxyatrazine, (6) – n-isopropylammelide, (7) – cyanuric acid, (8) – biuret, (9) – allophanate, (10) – CO_2 (Adapted from Mahesh and Manu, 2019c)

Table 2. Ametryn adsorption, characterization of MLSS, MLVSS, and sludge stabilization ratio (MLVSS/MLSS) of anaerobic reactors

Run (Days)	*Ametryn adsorbed (mg/g.MLVSS)*	*MLVSS concentration (g/L)*		*MLSS concentration (g/L)*		*MLVSS/MLSS*	
Reactor	R2	R1	R2	R1	R2	R1	R2
151 – 210	2 – 4.5	9.1 – 9.3	7.6 – 8.1	12.7 – 13.8	9.7 – 10.2	0.67 – 0.71	0.78 – 8.1
211 – 250	1 – 2	9.1 – 9.4	7.8 – 8.4	12.5 – 12.8	10.1 – 10.6	0.72 – 0.73	0.77 – 0.79
251 – 280	0	9.2 – 9.5	9.6 – 10.8	13.9 – 14.2	13.2 – 14.2	0.66 – 0.69	0.72 – 0.76
281 – 320	2 – 4	9.2 – 9.3	7.2 – 9.4	12.7 – 13.1	9.0 – 12.9	0.7– 0.73	0.72 – 0.8
321 – 365	1 – 1.6	9.3 – 9.5	9.5 – 10.9	12.6 – 12.9	12.8 – 13.7	0.73 – 0.74	0.74 – 0.79
366 – 430	0	9.1 – 9.4	9.2 – 10.5	12.2 – 13	12.8 – 13.8	0.72 –0.74	0.71 – 0.76

Though the compound was used to treat ametryn during the past 150 days with stable bacterial adaptability, the reactor took more than 70 days to perform effectively. VFA concentration in the effluent was observed in the range 750 – 1200 mg/L, it has indicated the reactor inhibition (Shin et al., 2003). Addition of AQS and long operation period has contributed to the improved bacterial adaptability, growth of inactive bacteria and such observations have been reported previously by many researchers for treating different type of herbicides (Derakhshan et al., 2018; Mahesh and Manu, 2019b; Rau et al., 2002). The change in MLSS, MLVSS, MLVSS/MLSS ratio of R1, and R2 reactor throughout treatment are tabulated in Table 2. Sorption of ametryn on reactor sludge was studied at regular intervals. Adsorption on to the reactor sludge was found to be comparatively less; it was reported in the previous study.

A low concentration of ametryn 2 – 4.5 mg/g. MLVSS was sorbed till day 55 and almost reached to negligible later on due to desorption, biotransformation, and ametryn has high dissociation constant (pKa), which leads to weakly adsorbed (Frías et al., 2004). The variation in MLSS, MLVSS, and MLVSS/MLSS ratio during the study period was significant. High MLVSS/MLSS was observed every 30 – 40 days after the raise of influent ametryn concentration. The MLSS varied from 9 – 14.2, and MLVSS between 7.2 – 10.8 mg/L in the R2 reactor, while the R1 has 12.2 – 13.8 and 9.1 – 9.5 mg/L. Rise in influent ametryn concentration to 10 mg/L led to high MLVSS/MLSS ratio >0.80 and low SRT (<40 days). It was interesting to note the MLVSS/MLSS ranged between 0.71 – 0.81 and 0.66 – 0.74 for R2 and R1 respectively, low sludge stabilization ratio obtained after 90 days of raise in ametryn concentration indicating stable reactor condition (Derakhshan et al., 2018; Metcalf and Eddy, 1991). SRT observed for the R1 was in the range of 130 – 133 days and that of R2 was 64 – 190 days. Low SRT observed during initial days (up to 20 days) and SRT increased gradually attaining maximum between 260 – 280 and 410 – 430 days of operation. With continued operation the reactor biomass regenerated from the initial toxicity and supported the sludge granulation, which enhanced the sludge quality and settle ability.

An anaerobic batch reactor can provide enough SRT required for biomass to recover at a faster rate, which leads to reactor recovery from the toxic conditions (Koh et al., 2008; Wang et al., 2018). F/M and sludge age observed for the R1 reactor was 0.22 – 0.23 and 6 – 7 days and R2 reactor had significant variation in F/M and sludge age between 0.19 – 0.27 and 4 – 6 days respectively and all the values are within the required range for an efficient biological treatment (Shammas and Wang, 2009). Sludge age for biological treatment process should be between 3 – 15 days and it is 4 – 6 days for R2, which is found to be sufficient. Lower concentrations of MLSS and MLVSS indicated the toxic condition upon the increased ametryn concentration, while the high concentrations indicate stable reactor condition and bacterial adaptability. The size of granules differed initially, later stabilized, and retained the actual size 0.3 – 0.5 mm, no granulation observed in the R1 reactor throughout.

Outcome of the batch study experiment showed that ametryn initially adsorbed on biomass and successively desorbed from the sludge towards the end of 10 days. The liquid contained the actual concentration of ametryn and no sludge adsorption on the day of inoculation. Table 1S shows the adsorption pattern of ametryn on to reactor sludge. It may be observed that ametryn adsorption observed up to 5 days and reduced further gradually to zero from 9th day, this confirms that the compound is adsorbed initially and further biodegraded.

3.4. Effect of AQS addition on ametryn removal

AQS releases the free radicals and accelerates the oxidation-reduction reactions in anaerobic conditions in the presence of biodegradable and non-biodegradable compounds (Van der Zee and Cervante, 2009). The use of starch as a co-substrate also acts as an electron donor and transfers electrons to the ametryn (electron acceptor) (Da Silva et al., 2012). ORP observed in the absence of AQS in both the anaerobic reactors during the acclimation period (up to 150 days) was in the ranges of -220 to -290 mV.

The variation of ORP is directly proportional to redox reactions between the primary substrate (starch), and ametryn. Reactor outperformed during the low ORP range (-270 to -325 mV) during ametryn treatment as reported in the previous study, and therefore 5 mg/L of AQS was added from 251st day onwards. AQS addition significantly reduced the ORP, leading increased ametryn removal and complete removal observed after 290th day. The complete removal after 135 days may suggest that it was mainly due to bacterial adaptability supported by the enhanced redox reactions. This observation was slightly different during dicamba treatment, addition of AQS enhanced dicamba removal by >20% (Mahesh

and Manu, 2019b) Significance of AQS addition was also reported previously with enhanced color removal by Da Silva et al. (2012) and for the treatment of azo dyes by Rau et al. (2002).

After raise in the ametryn dose (10 mg/L) and AQS (5 mg/L), ORP increased from -310 to -280 mV, and the removal efficiency of ametryn reduced to 60%. Increased ORP may be an indication of a highly toxic condition leading to oxidation of biomass, which was also indicated by the poor sludge settling. Ametryn removal efficiency was almost constant up to 320 days around 65 – 68%, but further dropped to 50% from 326th day till 340th day, and the ORP varied during this period was between -290 to -315 mV. The influent AQS was raised to 10 mg/L from 342nd day, even then the ORP observed in the same range. The ametryn removal efficiency was observed to be 65% up to 365th day, and the COD removal efficiency is equal to 72%, which is lesser than the COD removal of the control reactor. The low ORP (<-30 mV than ORP of control) was observed for 10 mg/L of AQS, and corresponding high biogas production than the control may suggest that the acclimated biomass can resist the toxicity level in the present reactor condition. The high ORP observed on some days may be due to low temperature; this observation is in line with the studies reported (Field and Brady, 2003). AQS presence in the bioreactors may be viewed as a significant one, and a comparative observation can clearly show that in the absence of AQS the reactor efficiency dropped to 39% and took long time for 8 mg/L of influent ametryn. Faster recovery of the reactor and increased removal efficiency after a period of 30 – 35 days, the maximum reactor performance was observed on the 411th day. The high ametryn removal 88%, COD removal >90%, and high biogas production of 665 mL/d was observed at low ORP of -315mV.

3.5. Performance of sequential anaerobic-aerobic reactors

Aerobic reactors were operated simultaneously along with the anaerobic rectors using the anaerobic reactor effluent as feed. Some of the intermediate compounds detected in the anaerobic effluent of ametryn treatment are identified as the compounds similar to by-products of water disinfection process (Lin et al., 2016) and different type of VFA. VFA can be degraded under the aerobic treatment process by the aerobic and facultative bacteria, which was reported primarily by (Gaunt and Hester, 1989). The application of aerobic post-treatment to anaerobic effluent containing the metabolites of ametryn was proved to be efficient and it has been reported (Mahesh and Manu 2019c). The performance of aerobic control (R3) and reactor treating the effluent of ametryn (R4) are depicted (Fig. 5). Anaerobic effluent contained ametryn up to a detectable level for a longer time than the usual (about 40 to 60 days), and a maximum ametryn removal was achieved after the treatment in R4 reactor.

Under the aerobic condition, ametryn and its metabolic intermediates were removed more efficiently than the anaerobic treatment as the low ametryn concentration entering the aerobic reactor. During the first 5 days, higher ametryn removal (>80%) and low COD removal (70%) were observed, and it was mainly due to possible adsorption, as discussed earlier. Fluctuating treatment efficiency was observed up to the 201st day of operation. Further, the reactor performed at stable treatment outcome, indicating the bacterial adaptability digest the different intermediates fed. The HPLC and UV spectral analysis of influent and aerobic effluents indicates mineralization of ametryn. Higher COD removal efficiency >95% after 201 days of operation was attributed to the oxidation of VFA and other intermediates to end products like water and CO_2. (Navaratna et al., 2016) have conducted a study comprised of combination of membrane bioreactor/ultraviolet/granular activated carbon technique used to treat 5 mg/L ametryn exhibited 61% reduction.

During the previous study the reactor inhibitions were observed for 15 days of herbicide addition, but in the present study no such inhibitions have been detected. Lower COD removal efficiency with high VFA concentrations 400 – 700 mg/L observed till the 201st day may indicate non-degradable organic matter. After 201 days of operation the increased COD removal efficiency led to reduced VFA concentration. Aerobic treatment of refractory organic compounds such as azo dyes (Manu and Chaudhari, 2002), and herbicides like ametryn (Navaratna et al., 2012) have been proven to be inefficient. In the present study, aerobic treatment is found to be more efficient for treating the anaerobic effluent containing intermediates of ametryn and other organic pollutants. As a post-treatment, the aerobic reactor was able to remove ametryn and COD >99% after 239th day, indicated by the absence of intermediate compounds in the effluent.

A lag phase observed during the treatment of 10 mg/L of ametryn, mainly due to the increased load of intermediates and VFA compounds.

A low ametryn removal (70%) corresponding to low COD removal (75%) may indicate the toxic inhibitions. The conversion of long-chain fatty acids through β-oxidation pathway produces acetyl-CoA and then oxidized to CO_2 via the tricarboxylic acid cycle (Ratledge, 1992), leading to greater COD removal. On continuous operation, the removal efficiency of both ametryn and COD increased above 95% after 410th day. The effluent contained no identifiable compounds of ametryn when analyzed in the HPLC and LC-MS.

The sludge concentration in the R4 reactor was observed within the required range with MLSS and MLVSS 3.8 – 6.2 and 2.4 – 5.3 g/L and SRT varied from 190 – 310 days throughout the study period. Low SRT (190 days) observed during 10 – 20 days of ametryn load and gradually improved to high SRT values (310 days).

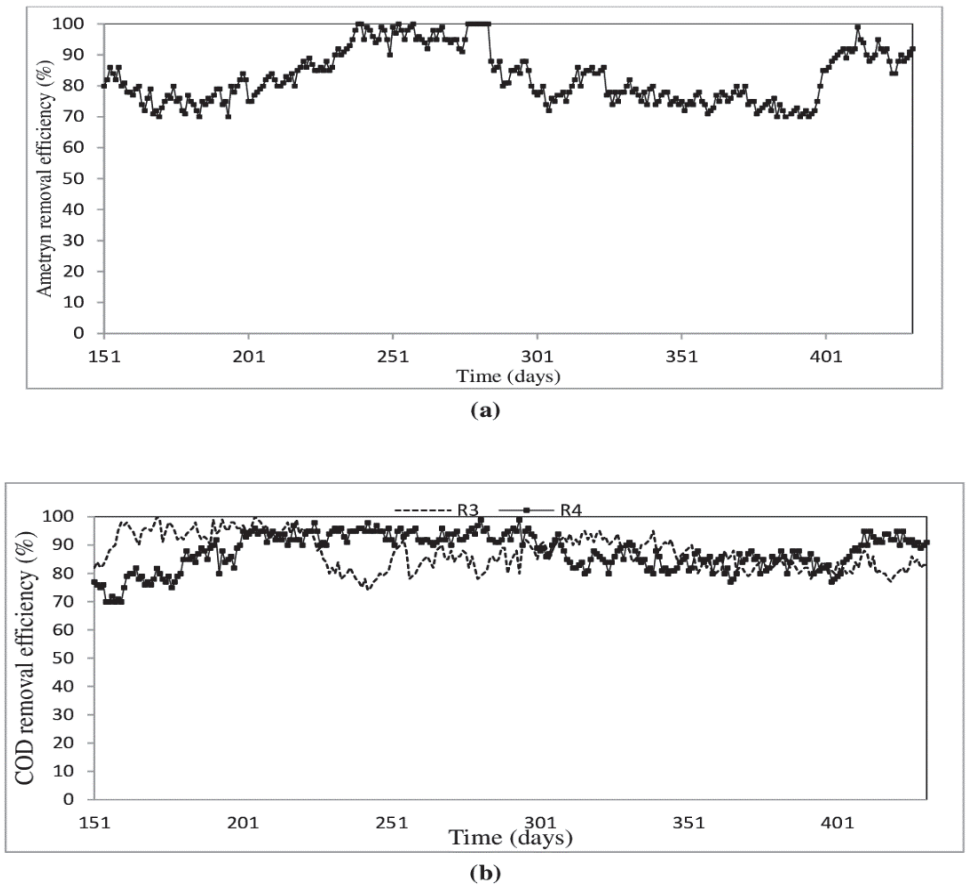


Fig. 5. (a) Aerobic reactors performance compared between ametryn treatment reactor (R4) and (b) control reactor (R3)

Long SRT observed with an increase in the MLSS and MLVSS concentration after the first 20 days (after ametryn load, 8 to 10 mg/L). At this reactor condition, F/M and sludge age were observed to be 0.07 - 0.166 and 9.5 – 15.5 days respectively. In the control reactor (R3) variation of MLSS and MLVSS 4 – 6.5 and 2.5 – 6.3 g/L with SRT being 40 – 65 days. F/M and sludge age observed as 0.06 - 0.1 and 12.5 – 15.7 days. A low F/M ratio indicated insufficient organic loading to the reactors (Shammas and Wang, 2009). Long SRTs and sludge in the R3 reactor than the R4 reactor may indicate that the reactor is performing better for the influent toxic feed.

3.6. Toxicity bioassay using micro-algae

The batch study conducted using the microalgae *Chlorella vulgaris and Scenedesmus quadricauda* in two separate reactors (M1 and M2). The thick green (*Chlorella vulgaris*) and pale yellowish (*Scenedesmus quadricauda*) algal biomass were spread across the reactor and in the suspension form on continuous aeration. The effluent of the anaerobic-aerobic reactor (R4 effluent) was fed from 225th day onwards; it was conducted to evaluate the consequences of treated effluent on micro-algae. After the introduction of R4 effluent to both M1 and M2 reactors, the algae started to form biomass balls and settled partially within the reactor. The algal biomass regenerated by itself after 60 days of operation with well-developed biomass concentration than before. This observation is attributed to the algal adaptability and reduced toxicity of the intermediates and which was also supported by increased algal biomass of M1 and M2 to 5.5 – 7 and 5 – 7.2 g after 60 days of operation. These algal species are often used to treat different types of pesticides in water (Baglieri et al., 2016). This batch study signifies the effectiveness of combined anaerobic-aerobic reactor in detoxifying the ametryn and its intermediate compounds. Thus the effluent water produced from this integrated treated system can be reused for various applications.

4. Conclusions

The results of this laboratory scale co-treatment study conferred that biodegradation of 8 – 10 mg/L ametryn occurred in the presence of AQS (5 – 10 mg/L) in anaerobic-aerobic treatment method. Overall removal occurred through biodegradation with no adsorption on to the reactor sludge. Low sludge stabilization ratio (MLVSS/MLSS) between 66 – 0.74 obtained after 90 days of operation for ametryn treatment reactor. VFA and intermediate compounds formed in the anaerobic reactor are degraded in the aerobic treatment. The bioassay results suggest that the effluent from anaerobic-aerobic reactor was non-toxic to the micro-algae *Chlorella vulgaris* and

Scenedesmus quadricauda with increased biomass concentration 5.5 – 7 and 5 – 7.2 g respectively. Thus, effluent containing high concentrations of ametryn can be treated using the novel and self-sustainable anaerobic-aerobic method for making water reusable. Use of AQS enhanced the reactor performance by 5 – 10% and reduced the biomass inhibition considerably. The bioreactors with acclimated biomass to high influent insecticide/herbicide dosages may become an efficient alternative to treat wastewater containing high concentrations of such compounds.

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