International Journal of Science and Research (IJSR)
ISSN: 2319-7064
ResearchGate Impact Factor (2018): 0.28 | SJIF (2018): 7.426

Anaerobic Digestion of Brown Algae with Trace Element Supplementation: Batch and Continuous Reactor Studies

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Abstract: ***This study investigates the effect of trace element supplementation (TES) on mesophilic anaerobic digestion treating Laminaria digitata (LD) in both batch and continuous (CSTR) reactors. Two set of Experiment 1 and 2 (batch and continuous) reactors were carried out with and without trace element addition, and their performance compared. In Experiment 1, five (batch 500 ml and 1L CSTR) reactors were operated with the addition of metals mixtures in Reactors (TES 1–4) and Reactor (TES 0) as control without addition. The results of first batch test (BT 1) show that TES 1-4 reactors achieved an increase in methane yield of between 17-26% compared to the control reactor without TES after 22 days of incubation. In CSTR reactors, the results show that daily trace elements addition with an HRT of 25 days allowed for a stable anaerobic digestion in three different combinations TES 2- 4 at an organic loading rate of 2 $gVS.L^{-1}.d^{-1}$ used throughout the experiment, but did not give any advantage over the reactor without TES 0. In Experiment 2, two (batch 500 ml and 1L CSTR) reactors were operated with and without TES 4 mix. Results of (BT 2), the TES 4 reactor achieved an increase in methane yield of 50% compared to the control reactor without TES 0 after 40 days of incubation. The second CSTR run, with an OLR from 2 - 5 $gVS.L^{-1}.d^{-1}$, and weekly addition of the TES 4 mix, showed stable performance compared to the reactor TES 0 without metal addition.***

Keywords: Anaerobic digestion; algae; batch; biogas; continuous reactors; trace element

1. Introduction

Nutrients are needed for all forms of life for their preservation and growth [1]. In anaerobic digestion (AD) processes, the nutrients required by various methane-forming bacteria are classified into macronutrients and micronutrients. Whereas the macronutrients such as carbon, nitrogen, phosphorus, and sulphur are required in large quantity, micronutrients also known as trace elements, for example, cobalt, molybdenum, nickel, iron tungsten, and selenium are required in relatively small quantities by most bacteria [2]. Trace elements are necessary nutrient for all microorganism and important for optimal cell metabolism [3-5]. They are regarded as "any chemical element that occurs in very small amounts in organisms but is essential for many physiological and biochemical processes" [1]. These essential trace elements are mostly metals and are often present in the enzyme system as part of a cofactor or they are of vital importance for the enzyme system [6]. On non-enzymatic forms, metals are involved in microbial respiration processes either with an electron transfer bound to the cell wall or extracellular electron acceptors [6]. The incorporation of micronutrients in enzyme systems is essential to ensure not only proper degradation of a substrate but also an efficient operation of the digester [2]. Anaerobic digestion and microbial growth depend on the availability and/or optimal supply of these nutrients [7]. The coenzymes are metal-laden organic acids that are incorporated into enzymes and allow the enzymes to work more efficiently. Coenzymes that are unique for methane-forming bacteria are coenzyme M and the nickel-containing coenzymes F_{420} and F_{430}. Coenzyme M is used to reduce carbon dioxide to methane. The nickel-containing coenzymes are only found in methanogenic bacteria [3], and are important hydrogen carriers [2]. Copper and cobalt are constituents of B_{12}-enzyme which catalyses the methanogens and molybdenum and selenium are subcomponents of formate dehydrogenase [3]. The coenzymes are components of energy-producing electron transfer systems that obtain energy for the bacterial cell and remove electrons from degraded substrate [2].

Macro and micronutrients are required for the stable growth of anaerobic microorganisms [2]. For the macronutrients, the approximate ratio of carbon to nitrogen and phosphate should be in the range of 75:5:1 to 125:5:1 [8]. Trace metals such as iron, nickel, cobalt, molybdenum, zinc, selenium, copper, boron, manganese and tungsten have been shown to be stimulatory to methanogens [4], and are necessary for stable AD level [9]. A literature survey about the stimulatory ranges of trace metals for anaerobic digestion of biomass for Co, Fe, Mo, Ni, and Se was reported to be 0.05 - 0.19,0 - 0.39, 0.16 - 0.3, 0.11 - 0.25, and 0.062 mg kg^{-1}, respectively [7]. It has been reported Fe, Co and Ni are required at the rates of 0.02, 0.04 and 0.003 mg/GM acetate respectively [4]. The unavailability of these elements in biogas digesters is probably the first reason of poor process efficiency without any other obvious reason [7]. Methane-forming bacteria are able to easily remove or "harvest" micronutrients from bulk of a solution through the production and excretion of extracellular "slime" that chelates and transports the nutrients into the cell. The use of extracellular slime permits "luxury" uptake of micronutrients, that is, the removal and storage of nutrients beyond the quantity that is needed [2]. Various researchers have studied the effect of trace metals on AD process. In their work on mesophilic digestion of *Napiergrass*, Wilkie, Goto [10] reported a 40% increase in methane production and a significant decreased in the VFA concentration by daily addition of micronutrients (nickel, cobalt, molybdenum, selenium, and sulphate). The addition of both

macro (N, K, P, and S) and micro-nutrients (Co, Cu, Fe, Mo, Ni, Se, W and Zn) during thermophilic pilot-scale digestion of the of organic fraction of municipal waste (OFMSW) helped to elevate the gas production rate by 30% and increase the stability of the digesters [11]. Zhang, et. al., [12] stated that selenium, molybdenum, and tungsten are essential trace elements for certain enzyme catalysing reactions, such as formate dehydrogenase (FDH) which is crucial for propionate oxidation, hence important for AD process. This study investigates the effect of these trace element on biogas production from macrolagae, a marine plant as a source of renewable energy.

2. Materials and methods

a) Substrates

Algal biomass *Laminaria digitata (LD)* used in the both the batch and continuous reactor experiments was collected from shallow water during low tide at Seaton sluice, 55.0836° N, 1.4744 ° W, Northumberland UK (NZ 3350) in December 2017. The seaweeds were transported in 30 L bags and were immediately washed to remove marine salts and sediments. The fronds were roughly chopped by hand to particle size of about 10 mm using knife and were oven dried at 70 °C for 24 - 48 h. This was then pulverized with a Kenwood 100 coffee blender to particle size generally < 1mm. All samples were stored at 4 °C in an airtight gas bag until required.

b) Inoculum

The inoculum used was collected from a full-scale running anaerobic digester operating on grass silage. It had following characteristics; pH 7.50, 21.2% TS, 60% VS (%TS), 0.019 Sulphur and C: N of 0.061. The initial trace element concentration of the inoculum is shown in Table 2.

c) Chemical analysis

The procedures as described in Standard Methods for the Examination of Water and Wastewater were used for physiochemical analysis of pH, Alkalinity, Solids, Chemical oxygen demand (COD), Ammonical nitrogen (NH3 -N), Total Kjeldahl Nitrogen (TKN), Total organic carbon (TOC) [34].

d) Volatile fatty acids (VFAs)

Volatile fatty acids (VFAs) was analysed on a Dionex ICS 1000 with an AS40 autosampler (Dionex, USA). Separation was carried out on an ionpac ICE-AS1 4 × 250 mm analytical column with a flow rate 16 ml min^{-1}; 1.0 mM heptafluorobutyric acid eluent; 5 mM tetrabutylammonium hydroxide suppressant regenerant; and a 10 ul injection loop. Supernatant of centrifuged samples liquors were filtered through a 0.20 µl syringe filter (VWR, UK), 0.4 mL of filtered samples were then diluted 1:1 with octanesulfonic acid, and sonicated (FS200B Sonic Bath, Decon Laboratories, Sussex, UK) for 40 mins to remove carbonate, which caused interference. The prepared samples were then transferred to 1 mL tubes with filter caps (Dionex, USA) before analysis.

e) Biogas and methane measurement

The percentage (%) methane from the biogas content was determined using a GC-FID analyser (Carlo-Erba 5160 GC) in split mode with the injector at 150°C and FID at 300°C.Using a 100 µl sample Lock syringe (Hamilton, USA), duplicate headspace samples (100 ul) were injected manually every 2 minutes into the GC with the split open 5 turns (100mls min-1). After the initial injection, the GC temperature programme and data acquisition commenced. Separation was performed on an HP-PLOT-Q capillary column (30m x 0.32mm id) packed with 20um Q phase. The GC was held isothermally at 35°C for 90min and heated to 250 °C at 10 °C min^{-1} and held at final temperature for 10 minutes with Helium as the carrier gas (flow 1ml min^{-1}, pressure of 50kPa, split at 100mls min^{-1}. The acquisition was stored on an Atlas laboratory data system. Methane standard were prepared prior to each analysis from 100% analytical grade CH4 (BOC Gases, UK) by injecting duplicate sample to make a five–point standard curve in the range 20 - 100% CH4. The volume of biogas produced was measured using a 100 mL BD Plastipak syringe from the gas bags. The % methane calculated was multiplied by the measured biogas volume giving the volume of methane produced [13].

3. Experimental Design

- ***Batch***

The batch tests (BT 1 and 2) were carried out according to Membere, E., et al., [13]. The inoculum to substrate ratio used was 3 gVS :1 gVS. Trace elements mix in four different combination TES 1 - 4 were added to the reactor bottles as shown in **Error! Reference source not found.**. The dose added were calculated based on trace metal content of the inoculum, algae substrate and stimulatory ranges reported in literature [7, 14], and also to avoid attaining toxic concentrations [15]. The tests were carried out by supplementing 370 mL of inoculum with the trace element matrix, before making the volume to 500 mL with distilled water. The reactors with TES 1 - 4 were compared to a control reactor without TES 0. For BT 1, 2 mL of each prepared mix were added in reactors TES 1 – 4, while in BT 2, only 2 mL of TES 4 mix which produced the highest biomethane in BT 1 were used and recessed for longer period of time. The inoculum used was acclimatised prior to the start of the experiment and allowed to degas for between 3 - 5 days. Biogas produced were collected using gas bags and all measured gas volume were normalized to standard temperature and pressure. In Batch 1 and 2, biomethane production potential was measured under controlled conditions (35 °C) for 22 and 40 days, respectively.

- ***Continuous stirred tank reactors (CSTR)***

The setup of the CSTR as described by Hinks et al. (2013) but modified; the continues study were performed in 1 L Quick fit® reactor vessels (800 ml working volume) with an impeller drive shaft passed into the reactor through a Quickfit glass stirrer gland and a water-seal to ensure the reactor remained gas-tight. Mixing was achieved with a 40 × 80 mm rectangular impeller rotating at 90 rpm.

The CSTRs were operated in semi-continuous batch mode, with daily feeding event being initiated by the removal of an

appropriate volume (Reactor Volume/hydraulic residence) of mixed liquors from the feeding/sampling point on the head plate of the reactor using a 100 ml plastic syringe. Stirring continued during sampling to prevent settling and fractionation of the reactor solids (Hinks et al., 2013), and the importance of mixing the reactors for efficient substrate conversion has been reported by many researchers (Nandi et al., 2017). An experimentally determined quantity of the algae substrate (expressed as dry weight (g VS / L) was made up to a specified volume of water (water volume dependent on hydraulic residence), to replace exactly the sample volume that had been removed from the reactor, and added manually through a head plate port. All samples were carried out in duplicate and standard deviation (SD) of the data shown in parenthesis.

Two set of CSTR (Experiment 1 and 2) were carried out.

In **Experiment 1**, five set of 1 L reactors (RTES 0 - 4) were operated with and without trace elements in four different combination (RTES 1- 4) as shown in **Error! Reference source not found.**. The reactors were inoculated with 1L of acclimatised inoculum and operated at a constant organic loading rate (OLR) of 2 $gVS.L^{-1}$ d^{-1} of the algae substrate. Feeding was carried out by daily removal of digestate through an outlet port followed by addition of the prepared substrate and 1 mL of the trace element mix in the reactor.

In **Experiment 2**, only two set of the 1 L reactors (R 1 and R 2) were used. In reactor 2, 5 ml of the TES 4 mix was added once a week. The OLR (g $VS.L^{-1}$ d^{-1}) was increased stepwise after acclimatization from 2 g $VS.L^{-1}$ d^{-1} on day 1 of the experiment to 3 g $VS.L^{-1}$ d^{-1} on day 26, thereafter, to 4 g $VS.L^{-1}$ d^{-1} on day 39 and, finally to 5 g $VS.L^{-1}$ d^{-1} on day 55, till the end of the experiment.

Table 1: Experimental design for both batch and CSTRs with trace element concentration.

Substrate and trace element additions	Batch	CSTR reactors
Algae (control)	TES 0	R TES 0
Algae + Se ,Mo	TES 1	R TES 1
Algae + Se , Mo, Co, W	TES 2	R TES 2
Algae + Se, Mo , Co, W , Fe , Ni	TES 3	R TES 3
Algae + Se, Mo, Co, W, Fe , Ni, Zn, Cu	TES 4	R TES 4

Trace element concentration added (mg/l)			
Selenium (Se)- $Na_2SeO_4.6H_2O$	0.1	Iron (Fe)- $FeCl_2.4H_2O$	0.5
Molybdenum (Mo)- $Na_2MO_4.2H_2O$	0.1	Nickel (Ni)- $NiCl_2.4H_2O$	0.5
Cobalt (Co)-$CoCl_2.6H_2O$	0.5	Zinc (Zn) $ZnCl_2$	0.1
Tungsten (W)-$Na_2WO_4.2H_2O$ -	0.1	Copper (Cu)- $CuCl_2.2H_2O$	0.05

4. Results and discussion

a) Biomethane potential

The daily, cumulative CH_4 and biogas production obtained for the TES 4 mix reactor for BT 1 and 2 is shown in Figure 1 A and B, respectively. Contribution from background CH_4 produced by the inoculum was deducted from the cumulative production in evaluating the data [13]. In BT 1, the control reactor (TES 0) without metals supplementation generated 237 ± 0.19 mL CH_4/g VS with methane content increasing up to 70%. The highest methane yield achieved was 299 ± 1.14 mL CH_4/g VS from the TES 4 reactor. This was followed closely by TES 1, 2 and 3 (293 ± 1.66, 284 ± 0.09, 278 ± 1.84 ml CH_4/g VS), respectively (data not shown). The results obtained compared to the control, shows supplementation with the various mix of metals improved methane yield by 17 – 26%. Results obtained from BT 2 carried out using only TES 4 mix which produced the highest methane yield from BT 1 is shown in Figure 2 B. The cumulative methane production after 40 days for TES 4 is 440 ± 0.49 ml CH_4/g VS compared to 293 ± 1.02 ml CH_4/g VS obtained for the reactor TES 0 without trace element addition. This represents an increase of about 50% in methane yield indicating trace element addition aided in more biogas production after longer period of digestion. Studies carried out by Facchin. V. et al., [16] using Co, Ni, M_o, Se, and W improved methane yield by 45 – 65% while the addition of Co and Ni mix has shown to increase methane yield by 13.5% [17]. Kayhanian and Rich [11] using Co, Fe, Cu, Ni, Mo, Se, W, and Zn nutrient addition also had elevated gas production by 30%, with increased digester stability.

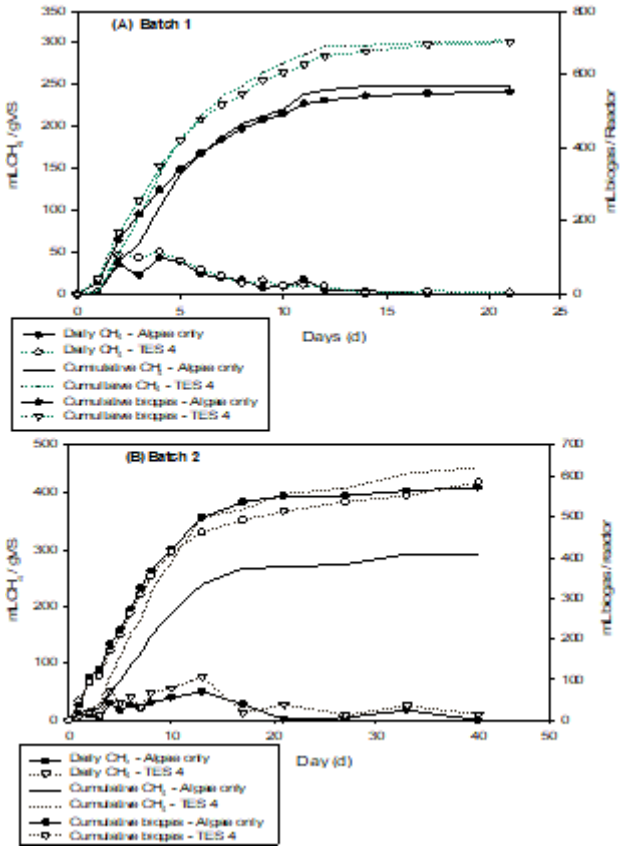


Figure 1: Cumulative biogas and methane yield for Batch test 1 and 2

The maximum rate of methane production was obtained in the TES reactors. For BT 1 and 2, 50 ml CH_4 /g VS.d was obtained on day 4 and 77 ml CH_4/g VS.d on day 13. The cumulative biogas production in BT 1 for the control and TES 4 are 551- and 686-mL biogas/reactor compared to BT 2, 573 and 586 ml biogas/reactor, respectively.

b) Continuous reactors (Experiment 1)

The continuous digesters (RTES 0 – 4) were fed once a day over a period of 90 days on the algae substrate. The

volumetric methane production rate of the reactors with and without supplementations is shown in Figure 2 and Figure 3, respectively. The cumulative methane production, % methane and H_2S content in the reactors are shown in Figure 4 and Figure 5.

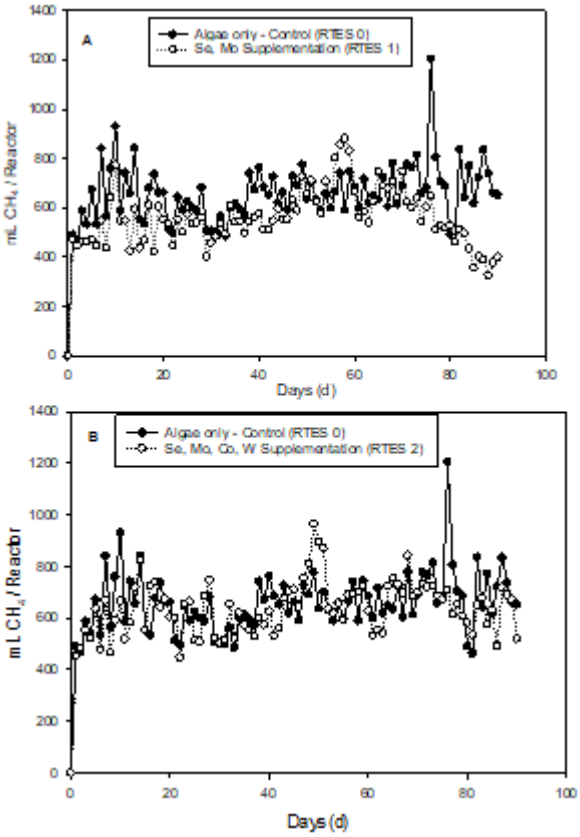


Figure 2: A), Daily volumetric methane production in continuous reactors (RTES 0 and RTES 1); B), Daily volumetric methane production in continuous reactors (RTES 0 and RTES 2).

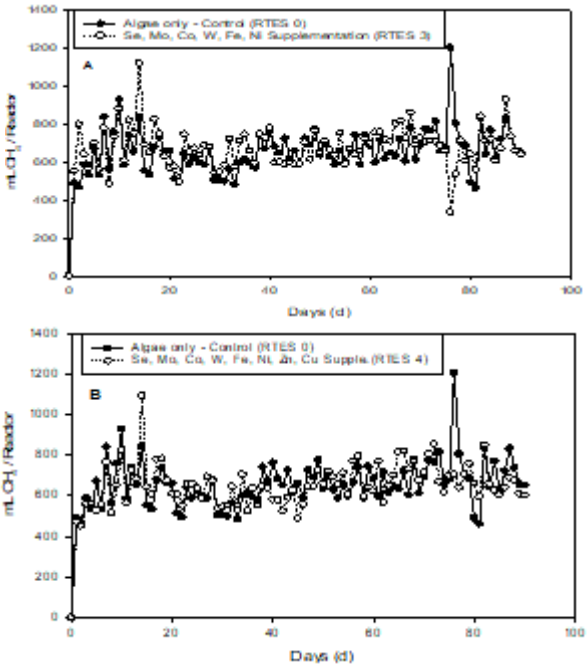


Figure 3: A), Daily volumetric methane production in continuous reactors (RTES 0 and RTES 3); B), Daily volumetric methane production in continuous reactors (RTES 0 and RTES 4).

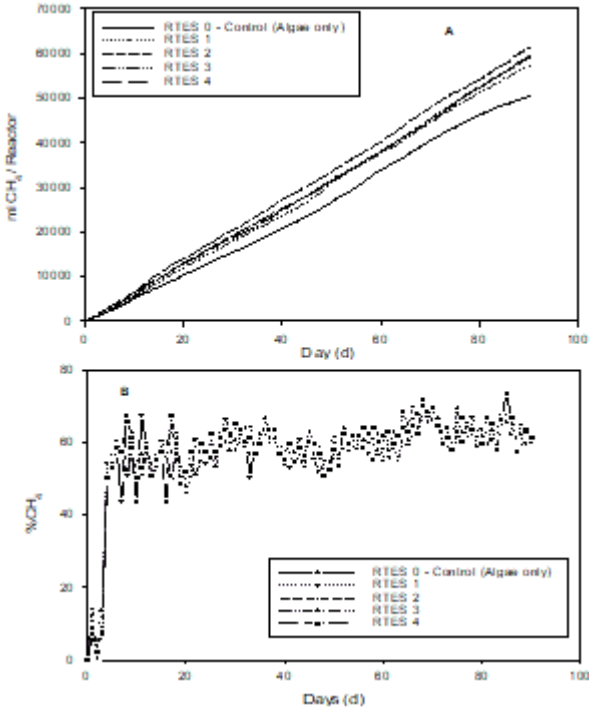


Figure 4: A), Cumulative methane production; and B), % Methane content in continuous reactors (RTES 0 – 4).

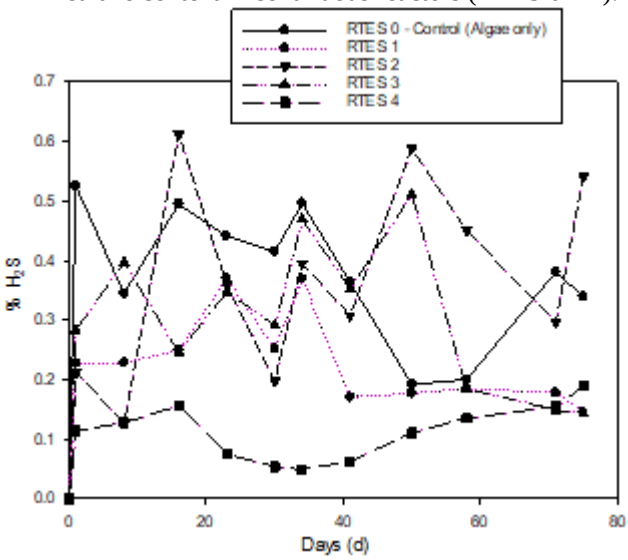


Figure 5: % Hydrogen sulphide production in continuous reactors (RTES 0 – 4).

Table 2 shows the trace element contribution from the algae and inoculum before the start of digestion and at the end of the test. The supplemented reactors performance was compared to the control. The methane yields for the reactors are obtained from average data of between 5 - 15 days of stable and pseudo-steady gas production, regarded as when the deviation is less than 5-10% for consecutive five days [20].

Table 2: Trace element concentration in algae substrate and inoculum at the start of the experiment, and the concentration in the reactors at the end of the experiment

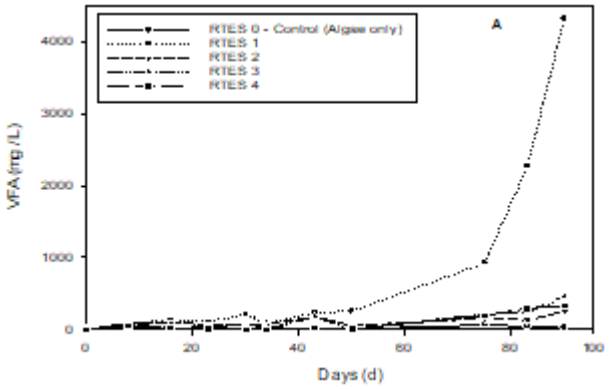
Start of Experiment			End of experiment				
Trace element	Algae	Innoculum / Control	Control RTES 0	Reactor 2 RTES 1	Reactor 3 RTES 2	Reactor 4 RTES 3	Reactor 5 RTES 4
	mg/l	mg/l	No metal additions	Se ,Mo (mg/l)	Se, Mo, Co, W (mg/l)	Se, Mo, Co, W, Fe, Ni (mg/l)	Se, Mo, Co, W, Fe, Ni, Zn, Cu (mg/l)
K	737	2597	963	1175	984	991	1083
Al	0.74	3.20	1.21	0.64	1.15	1.37	0.46
Ca	191	600	235	228	266	291	241
Cd	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Co	0.00	0.02	0.00	0.01	1.41	1.95	1.72
Cu	0.08	2.55	0.12	0.11	0.12	0.14	1.16
Fe	1.94	9.33	2.85	1.93	2.28	17	1.41
Mg	153	285	210	229	178	192	186
Mo	0.03	0.09	0.04	2.29	2.31	2.48	2.32
Na	143	464	206	252	208	207	225
Ni	0.13	0.31	0.14	0.12	0.11	10.15	8.95
P	32	186	45	35	39	44	30
Pb	0.00	0.06	0.00	0.18	0.01	0.01	0.00
S	48	63	47	51	39	47	42
Sc	0.00	0.01	0.01	1.37	1.43	1.89	1.59
W	0.00	0.19	0.01	0.02	0.38	0.50	0.44

c) Performance of the reactors (Experiment 1)

- **RTES 1 (Se and Mo mix)**

Both Se and Mo are component of an enzyme formate dehydrogenase (FDH) [11], which plays an essential role in energy supply to methylotrophic bacteria's [21]. They are part of metals needed for a balanced digestion process [16]. At the start of the experiment the initial inoculum concentration of Se and Mo are 0.01 and 0.09 mgl^{-1}, this was supplemented in the continuous reactor (RTES 1) by $Na_2SeO_4.6H_2O$ – Selenium (Se), and $Na_2MO_4.2H_2O$ - Molybdenum (Mo) at a dose of 0.1 mg L^{-1} daily. From Table 2, at the end of the experiment, concentration of Se and Mo had increased slightly to 1.4 and 2.3 mg L^{-1}, respectively. The stimulatory ranges reported for Se and Mo are 0.062 and 0.11 - 0.25 mg/kg^{-1}, respectively, and at Se concentration above 1.5 mg L^{-1}, Zhang et. al [12] has reported evidence of toxicity on digestion process. Figure 2 A shows the performance of the Se and Mo supplemented reactor compared to the control. The volumetric methane production evaluated for the supplemented and control reactors are 595 and 633 mL CH_4/reactor while the methane yield are 297 and 317 mL CH_4/g VS. The cumulative methane produced after 90 days of fermentation, shown in Figure 4 A was 5.04 and 5.94 L CH_4/reactor, respectively. The average methane content in the reactors fluctuated between 58 - 65%, and is similar for both reactors, Figure 4 B. The H_2S concentration in the gas phase with the RTES 1 reactor ranged between 0.14 to 0.36% (v/v) compared to the control reactor which peaked at 0.33% (v/v) within the duration of the experiment, Figure 5. The results show RTES 1 (Se and Mo) had a negative effect on methane production compared to the control reactor, a phenomenon which could be attributable to the negative or positive impact intracellular trace metal concentration can have on cell metabolism in AD [22]. Since, the trace metals Se and Mo are important in formate oxidation which is a breakdown product of propionic acid, the negative effect cannot be attributed to inhibition caused by propionic acid oxidation [23], because it is the lack of it that can trigger accumulation of formate [15]. Both Se and Mo are required in the synthesis of formate dehydrogenase, which is needed for formate oxidation and by extension the enzymes required for hydrogentrophic methane production [15].

The volumetric biogas and methane production for the RTES 1 reactor decreased from day 80 till the end of the experiment, Figure 2 A. This period was characterized by decline in pH below 6.7, Figure 7 A, when feeding was stopped, and an increase in volatile fatty acid (VFAs) production from < 0.5 up to 4.3 g L^{-1}, Figure 6 A. This is reflected in the FOS: TAC value > 0.5, Figure 6 B, showing the instability of the reactor supplemented with RTES 1 continued to increase from day 80, accumulating VFAs [24]. The VFA concentration is one of the most important parameters for the accurate control of anaerobic digestion The VFA/Alkalinity ratio is used to monitor the stability of the anaerobic process. It is a critical parameter and serves for fast evaluation of the digesters. It is also known as the FOS: TAC ratio and indicates the quantity of volatile organic acid (FOS) in relation to the buffer capacity of carbonate (i.e. total alkaline carbonate) [24]. Stable processes have a ratio between 0.1 - 0.25 without acidification risk, beyond 0.3-0.4 indicates the digester is upset, due to hyperacidity in the digester and a ratio of 0.8 and above, there is significant pH reductions and inhibition of methanogens, resulting in digester failures [24]. It has been shown that supplementation of Se and Mo in reactors help to prevent VFA accumulation [25] but VFA quickly build up when the organic loading rate (OLR) is high at which point increasing Se and Mo mix makes no difference, and other factors become limiting which are responsible for the VFAs accumulation [15]. In this study, using an OLR of 2 $gVS.L^{-1}.d^{-1}$, RTES 1 after day 50 began to show rapid buildup of VFAs. The performance of the continuous control reactor (RTES 0) could be attributed to the availability of rich nutrient compounds such as K, P, Mg and S needed by microorganism [11, 26, 27]. These compounds were present in high concentrations in the algae substrate and inoculum before and at the end of the experiment, Table 2, and could have played a stimulatory role in the stability of the control reactor. In their work Facchin, Cavinato [16] found out that supplementation of reactors with inoculum having high level of background trace element had a negative effect on biogas production. The pH of the control reactor declined slightly from 7.5 to 7.2, having a low VFAs concentration < 0.5 g L^{-1} and the FOS: TAC ratio remained below 0.5 over the duration of the experiment.



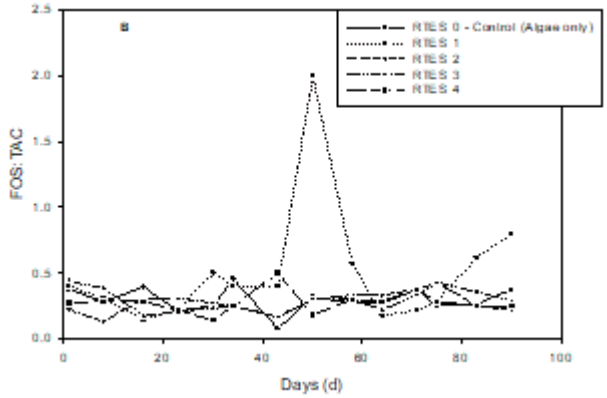


Figure 6: A), Volatile fatty acids profile; and B), FOS: TAC ratio in continuous reactors RTES 0 - 4.

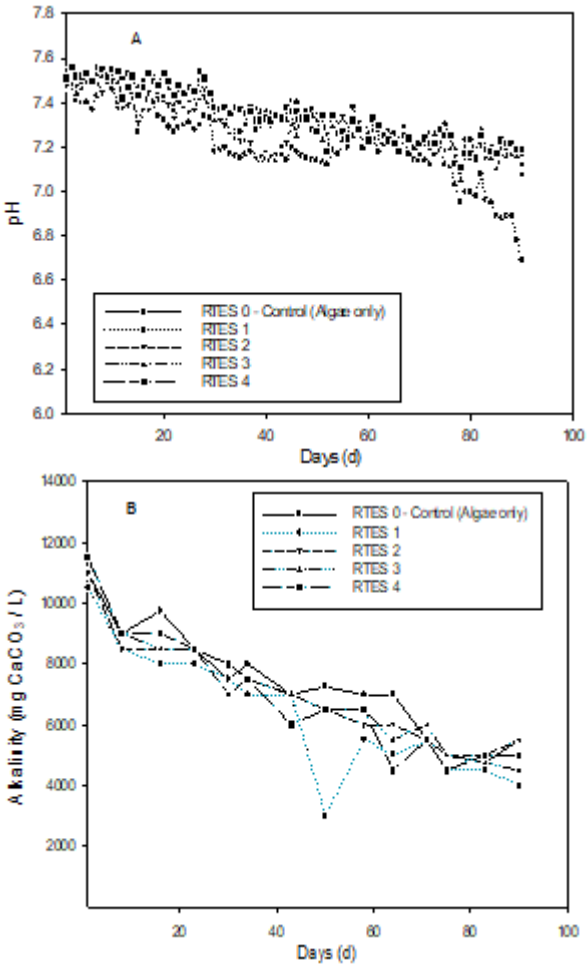


Figure 7: A), pH, and B), Alkalinity profile in continuous reactors RTES 0 – 4

- **RTES 2 (Se, Mo, Co, W mix)**

In addition to Se and Mo, both Co and W were added to the continuous reactor (RTES 2) in form of $CoCl_2$. $6H_2O$ – Cobalt (Co) and $Na_2WO_4.2H_2O$ – Tungsten (W) at a dose of 0.5 and 0.1 mg L^{-1} daily. Cobalt is present in methyl-H_4SPT, a coenzyme of M methyl-transferase complex of the methanogens [28]. It is also used by the enzyme, carbon monoxide dehydrogenase (CODH) which participates in acetate-formation, while Tungsten is a part of the FDH enzyme [11]. At the start of the experiment the initial inoculum concentration of Co and W are 0.02 and 0.19 mg L^{-1}, after addition of RTES 2 mix and at the end of the feeding period, their concentration increased to 1.41 and 0.38 mg L^{-1} compared to 0.01 and 0.02 mg L^{-1} for RTES 1, and 0.00 and 0.01 mg L^{-1} for the control reactor (RTES 0). Stimulatory concentration ranges reported are 0.05 - 0.19 mg kg^{-1} for W, and for Co it is 0.22 mg kg^{-1} [11, 15]. The results of methane production from the continuous reactors, RTES 2 compared to the control RTES 0 is shown in Figure 2 B. The volumetric methane production rate are 641 and 633 mL/reactor.d^{-1} with a methane yield of 321 and 317 mL CH_4/g VS, respectively. The cumulative methane produced, also shown in Figure 4 A are 5.7 and 5.9 L CH_4/reactor, respectively. The methane content is similar to that observed in RTES 1, averaging between 55 - 65% for both reactors, Figure 4 B. The H_2S concentration in the gas phase of the reactors fluctuated throughout the experiment between 0.19 - 0.58% (v/v) but peaked on day 20 at 0.61% (v/v) for the RTES 2 while in the control RTES 0 it ranged from 0.14 - 0.37% (v/v), Figure 5. The results obtained show the performance of the RTES 2 reactor and the control RTES 0 are similar.

- **RTES 3 (Se, Mo, Co, W, Fe, Ni mix)**

Additionally, Fe, Ni were added to the combination of elements used in the continuous reactor (RTES 2), and this mixture used to supplement continuous reactor (RTES 3). Fe was added in the form of $FeCl_2$. $4H_2O$ at a dose of 0.5 mg L^{-1}, and Ni as $NiCl_2$. $4H_2O$ at a of dose of 0.5 mg L^{-1}. While Fe is found in higher concentrations in methanogenic biomass and plays active roles in reduction processes, Ni is used by cells present in the compound F_{430}, a component of methyl-coenzyme M reductase complex used in catalyzing formation of methane [29]. Nickel is found in every methanogenic bacteria and in sulphate reducing bacteria through the enzyme carbon monoxide dehydrogenase (CODH) [11], which contains the factor F_{420} [29]. From Table 2, the initial concentration of Fe and Ni from the inoculum at the beginning of the fermentation process was 9.33 and 0.31 mg L^{-1}, respectively, which increased to about 16.45 and 10.15 mg L^{-1} for RTES 3 and 2.85 and 0.14 mg L^{-1} for RTES 0, respectively, by the end of the experiment. Kayhanian and Rich [11] has reported the stimulatory concentration range for Fe as 0 - 0.39 mg L^{-1} and Ni 0.11 - 0.25 mg L^{-1}. The daily volumetric methane production rate shown in Figure 3 A, for the RTES 3 and control RTES 0 are 668 and 633 mL CH_4/ reactor.d, respectively. Their methane yield is 334 and 317 mL CH_4/g VS while the cumulative methane produced after 90 days is 6.1 and 5.9 L CH_4/ reactor, respectively. The H_2S concentration in the gas phase for the reactors fluctuated throughout the duration of the experiment between 0.14 - 0.51% (v/v) but peaked on day 50 at 0.51% (v/v) for the RTES 3 while in the RTES 0 it ranged from 0.14 - 0.37% (v/v). The methane content (58 - 68%) and H_2S (0.14 - 0.51% (v/v)) obtained was similar to what was obtained in RTES 1 and 2. The results show the performance of the RTES 3 was similar to the control RTES 0.

- **RTES 4 (Se, Mo, Co, W, Fe, Ni, Zn, Cu mix)**

The continuous reactor (RTES 4), was supplemented further with Zn and Cu in addition to the combination mix used in RTES 3. Zn was added in the form of $ZnCl_2$ and Cu as $CuCl_2$. $2H_2O$ at a dose of 0.1 and 0.05 mg L^{-1}. Both Zn and Cu are found in large concentrations in methanogenic bacteria but reports of their stimulatory effects are scarce [11]. Figure 3 B shows the daily volumetric methane

production rate which averaged around 668 and 633 mL CH_4/reactor.d for the RTES 4 and RTES 0 continuous reactors, respectively. The methane yield obtained was 319 and 317 mL CH_4 /g VS, with a cumulative methane production of 5.90 and 5.94 L CH_4/reactor, respectively. The H_2S concentration in the gas phase was lowest for RTES 4 (0.04 - 0.18% (v/v)) compared to the other reactors. The methane content (53 - 67%) obtained was also similar to what was obtained in RTES 1, 2 and 3. The results shows performance of the RTES 4 compared to the control RTES 0 were also similar.

- **TES reactors process performance (Experiment 1)**

Results of other process performances for the five continuous reactors (RTES 0 – 4) are shown in Figure 6 - Figure 9. The total alkalinity values shown in Figure 7 B for the reactors including the control at the start of the experiment was around 10.0 g L^{-1}, and gradually reduced to 5.0 g L^{-1} except in the RTES 1 digester which dropped to around 2.8 g L^{-1} on day 50, reflecting the drop in pH and increase in VFAs, before recovering at day 58.

The total Kjeldahl nitrogen (TKN) and total ammonia nitrogen (TAN) concentration (Figure 8 A and B) in all the reactors were also similar, declining from a start value of ~ 2.2 and 1.7 g L^{-1} to 1.3 and < 0.2 g L^{-1}, respectively. Work by Banks, Zhang [15] showed continued reduction in TAN concentration in both supplemented and control reactors, with no direct reason being identified for the reduction in the supplemented reactor, while Lindorfer, Ramhold [30] tried to show a correlation between biological nitrogen fixation by TAN and an increase in microbial biomass in the effluent.

The VFAs profile (Figure 6 A) shows the starting inoculum in the reactors which had been acclimatized with algae substrate, contained a high concentration of VFAs which declined rapidly at the start of the experiment when OLR was increased in both the RTES 1 - 4 and control RTES 0 reactors. The VFA concentrations in all the RTES reactors, except the reactor with Se and Mo (RTES 1), were all below 500 mg L^{-1} at the end of the experiment. This agrees with results reported for digesters dosed with multiple trace elements where stable digestion was achieved, and VFA concentrations did not exceed 500 mg L^{-1} [15].

The soluble COD (sCOD) concentration profile for the digesters are shown in Figure 9. The average sCOD concentration at the start of the experiment was around 10.0 g L^{-1} which reduced to ~ 4.0 g L^{-1} except in RTES 1 (Se and Mo) where it was ~8.0 g L^{-1}. The RTES 4 digester performed better for sCOD reduction compared to all other reactors, with RTES 2 digester having the lowest performance.

d) Continuous reactors (Experiment 2)

The continuous digesters (R 1 and R 2) were fed with the algae feedstock once a day over a period of 85 days. In reactor 2, 5 ml of TES – 4 mix was added weekly. Table 3 shows the background trace element contribution from the algae and inoculum before the start of digestion and at the end of the experiment.

The variation in CH_4 production and Methane yield (MY) for R 1 and R 2 (TES – 4) with respect to increasing OLR from 2 - 5 $gVS.L^{-1}.d^{-1}$ over the length of the experiment is shown in Figure 10.

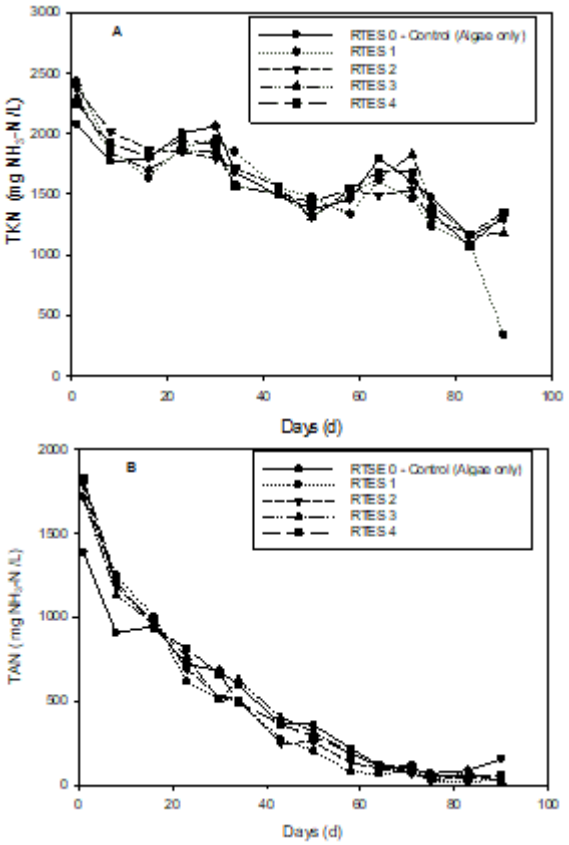


Figure 8: A), TKN; and B), TAN concentration profile in continuous reactors RTES 0 - 4.

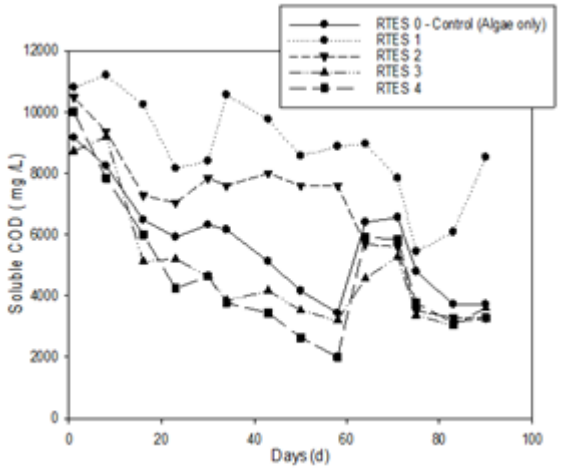


Figure 9: Soluble COD concentration profile in continuous reactors RTES 0 - 4

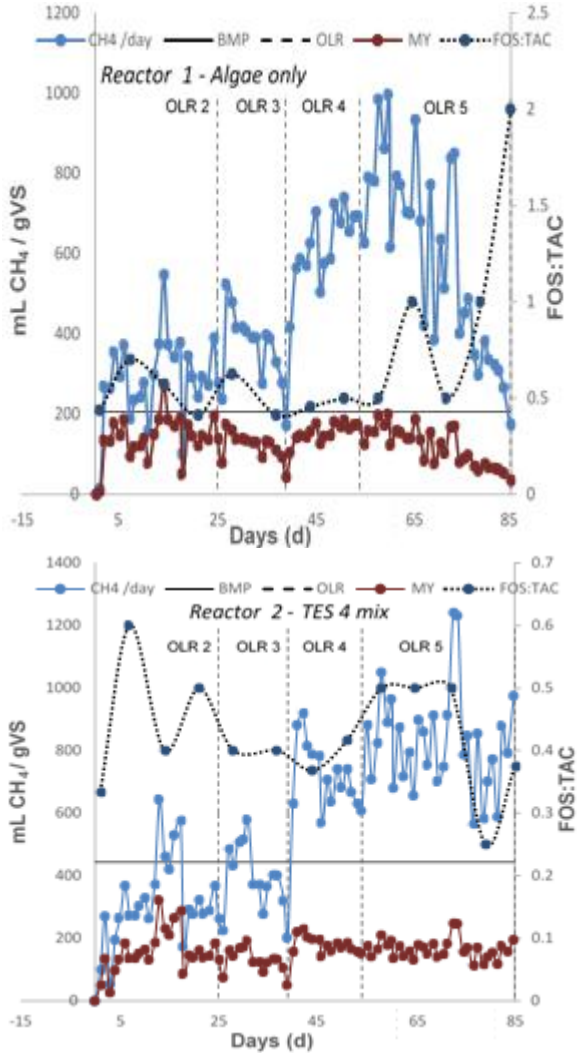


Figure 10: Assessment of continuous reactors, reactor 1 and reactor 2 (TES- 4) mix: Variations in CH_4 production, MY, BMP (mL CH_4/ gVS), and FOS: TAC ratio with increasing OLR (gVS.L^{-1}.d^{-1}). Vertical dashed line indicates organic loading rate (OLR).

A summary of the reactor performance is given in Table 4. It has been stated previously that for the continuous process, stable digestion is achieved with a FOS: TAC ratio is between 0.2 - 0.4 and when the MY value approaches the BMP value [31].

From Table 4, for R 1 and R 2 (TES 4 mix), the biomethane efficiency factor (BEF) averaged between 0.70 - 0.57 and 0.3 – 0.4, respectively, as the OLR was increased from 2 – 5 gVS.L^{-1}.d^{-1}. The BEF (ratio of the experimental to theoretical methane yield) shows that R 2 with TES 4 addition is not working at optimum yield conditions as higher value of BEF is an indication of better substrate degradation [32]. The methane yields obtained for R 2 at OLR 2, 3, 4 and 5 (159, 135, 172 and 166 mL CH_4/ gVS) were all higher than (144, 127, 148 and 119 mL CH_4/ gVS) obtained for R 1 (without TES mix) at the same OLR, indicating an increase of 11%, 6%, 16% and 39%, respectively. The % methane decreased slightly in both R 1 and R 2 (TES 4) from 60% to 48%, and from 61% to 56%, respectively.

The tVFA profiles, **Error! Reference source not found.** A show that as the OLR was increased, there was a gradual increase in the tVFA in both the TES 4 and control reactors, ranging from ~ 2.7 g L^{-1} and 4.7 g L^{-1}, respectively, on day 1, to 10.9 g L^{-1} and 5.2 g L^{-1} on day 51, then to 15.5 g L^{-1} and 3.7 g L^{-1} by day 85. The continued increase of tVFA from day 51 in the control reactor was characterized by a reduction in methane yield, increase in FOS: TAC ratio from 0.5 - ≥ 2 (Figure 10), indicating reactor instability. This caused an increase in COD from 17.8 – 28.8 g L^{-1}, Figure 12 B, and a drop in pH from 7.40 – 6.45, **Error! Reference source not found.** B, leading to reactor failure. However, the R 2 reactor with FOS: TAC ratio 0.5 - 0.25, COD 10.0 – 14.0 g L^{-1}, and pH within 7.52 – 7.58 from day 51, was relatively stable.

The total alkalinity values in the reactor R 2 and control at the start of the experiment were around 10.0 g L^{-1}, which gradually increased to around ~1.7 g L^{-1} before reducing to ~ 11.0 g L^{-1} in TES 4 and ~ 9.0 g L^{-1} in R 1, respectively, Figure 12 A. The TAN concentration in both reactors continued to decline as the OLR was increased from a start value of ~ 1.7 and 1.6 g L^{-1} to 0.86 and < 0.45 g L^{-1} for TES 4 and control reactors, respectively.

5. Conclusion

Trace elements are needed by microorganism for growth but if added in excess amounts to anaerobic digesters they can lead to inhibition [33]. The addition of both micro and macro nutrients for AD processes have been reported previously [27]. The results obtained from the current batch experiments show that the methane yield of the TES 1 - 4 reactors was between 17 - 50% higher than the control reactor (without TES). In the continuous reactors, Experiment 1, where the OLR of 2 gVS.L^{-1}.d^{-1} was maintained throughout the duration of the experiment, and where 1 mL of the TES 1 - 4 mix was added daily in reactors (RTES 1 – 4), results showed no significant difference in performance of these reactors compared to the control reactor (RTES 0) without metal addition. In the second continuous reactor (Experiment 2), where there was a step-wise increase in OLR from 2 - 5 gVS.L^{-1}.d^{-1}, and a weekly addition of 5 mL of TES 4 mix, the TES 4 reactor (R 2) showed better performance compared to the control reactor without TES (R 1), which was characterised by high tVFA, increased FOS: TAC ratio and a drop in pH,, leading to reactor failure.

Table 3: Summary of the results for the continuous reactors with and without trace element supplementation (Experiment 2)

Trace elements	Start of experiment Inoculum/ control mg/l	algae feedstock mg/l	End of experiment Control Reactor 1 No metal addition (mg/l)	Reactor 2 (TES 4 Mix) Se, Mo, Co, W, Fe, Ni, Zn, Cu (mg/l)
Al	11.61	0.00	2.52	2.86
As	0.00	0.00	1.02	0.0
B	0.74	2.10	4.39	4.69
Ba	0.92	0.18	0.96	0.49
Ca	355	206	479	371
Cd	0.00	0.02	0.04	0.0
Co	0.07	0.00	0.04	2.93
Cr	0.14	0.04	0.08	0.08
Cu	0.84	0.03	0.63	0.34
Fe	21.41	3.58	8.38	13.92
K	702	496	2229	3054
Mg	135	130	316	351
Mn	2.24	0.07	0.29	0.34
Na	78	93	270	338
Ni	0.12	0.10	0.20	3.33
Pb	0.28	0.00	0.17	0.0
Si	0.00	0.00	2.29	4.0
V	0.00	0.00	0.02	0.0
Zn	5.19	0.83	2.44	2.5
Ti	0.14	0.01	0.00	0.02
Se	0.77	0.00	0.00	0.63

Table 4: Summary of the results for the continuous reactors with and without trace element supplementation (Experiment 2)

OLR (kg VS / L / d)	BMP (L CH_4 / kg VS)	MY(L CH4 / kg VS)	CH_4 efficiency factor	CH_4 (%)	FOS:TAC	pH
R 1 Control- (Algae only)	207 ± 0.07					
OLR 2		144	0.70	60	0.40	7.38
OLR 3		127	0.61	57	0.50	7.36
OLR 4		148	0.72	53	0.47	7.34
OLR 5		119	0.57	48	1	7.11
Reactor 2 - TES 4 mix (Se, Mo, Co, W, Fe, Ni, Zn, Cu)						
OLR 2	440 ± 0.11	159	0.36	61	0.37	7.38
OLR 3		135	0.30	60	0.40	7.36
OLR 4		172	0.39	56	0.39	7.35
OLR 5		166	0.37	56	0.43	7.29

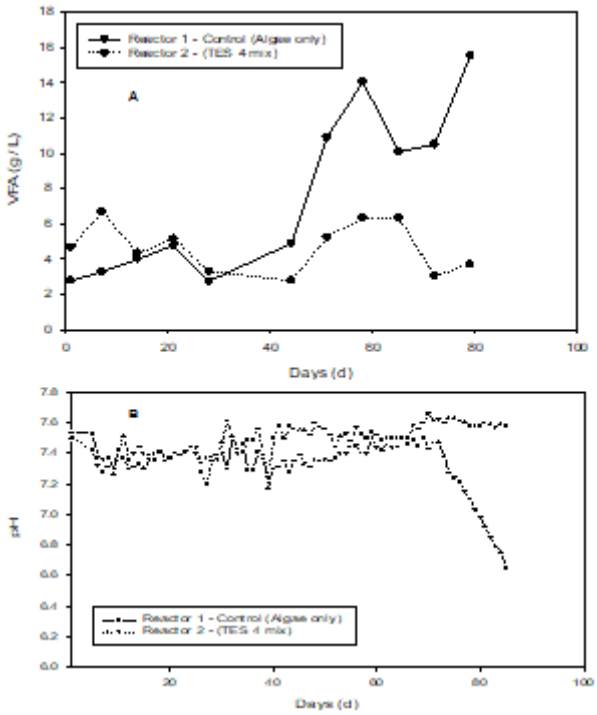


Figure 11: A), Volatile fatty acid; and B), pH concentration profile in continuous reactors R 1 and R 2 (TES 4 mix).

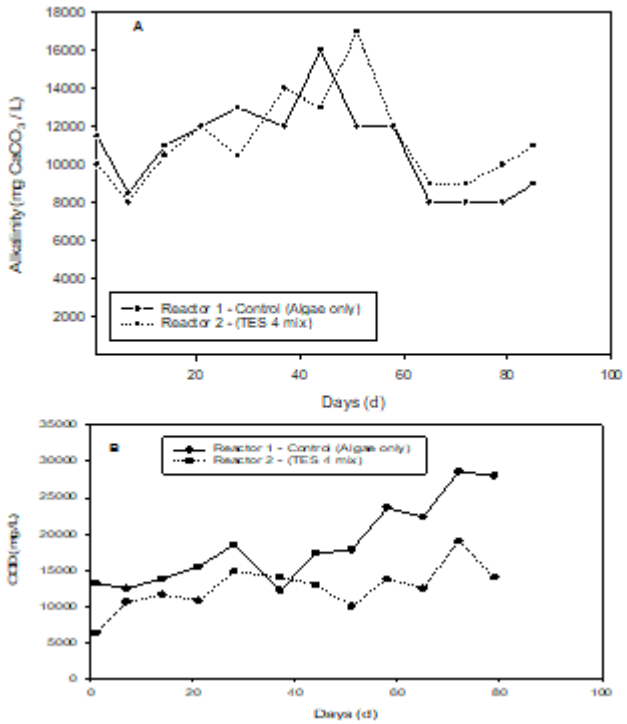


Figure 12: A), Alkalinity; and B), COD concentration profile in continuous reactors R 1 and R 2 (TES 4 mix).

6. Acknowledgement

We are grateful to Newcastle University, School of Civil and Geosciences. Also, the tertiary education trust fund (TETFUND) Nigeria for financial support.

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