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Microbial electrolysis cell coupled with anaerobic granular sludge: A novel technology for real bilge water treatment

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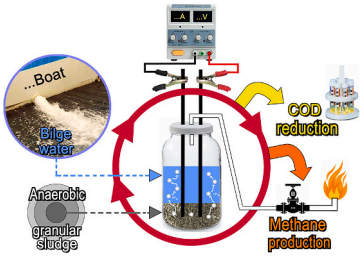
HIGHLIGHTS

- Undiluted real BW inhibited more Acetoclastics than Hydrogenotrophic methanogens.
- The dilution of BW resulted in lower inhibition to AD allowing BW biodegradation.
- MEC-AD at 1 V improved the decomposition of undiluted real BW vs. conventional AD.
- The recalcitrant nature of BW does not allow MEC-AD to balance the consumed energy.
- *Methanobacterium* and *Desulfovibrio* were mainly predominant in the cathodes of MEC.

GRAPHICAL ABSTRACT



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ABSTRACT

In the current study, treatment of undiluted real bilge water (BW) and the production of methane was examined for the first time using a membraneless single chamber Microbial Electrolysis Cell (MEC) with Anaerobic Granular Sludge (AGS) for its biodegradation. Initially, Anaerobic Toxicity Assays (ATAs) were used to evaluate the effect of undiluted real BW on the methanogenic activity of AGS. According to the results, BW shown higher impact to acetoclastics compared to hydrogenotrophic methanogens which proved to be more tolerant. However, dilution of BW caused lower inhibition allowing BW biodegradation. Maximum methane production (142.2 ± 4.8 mL) was observed at 50% of BW. Operation of MEC coupled with AGS, seemed to be very promising technology for BW treatment. During 80 days of operation in increasing levels of BW, R2 (1 V) reactor resulted in better performance than AGS alone. Exposure of AGS to gradual increase of BW content revealed that CH_4 production was possible and reached 51% in five days even after feeding with 90% of BW using simple commercial iron electrodes. Successful chemical oxygen demand (sCOD) removal (up to 70%) was observed after gradual biomass acclimatization. Among the different monitored volatile fatty acids (VFAs), acetic and valeric acids were the most frequently detected compounds with concentrations up to 2.79 and 1.81 g L^{-1}, respectively. The recalcitrant nature of BW did not allow the MEC-AD (anaerobic digester) to balance the consumed energy. Microbial profile analysis confirmed the existence of several methanogenic microorganisms of which *Desulfovibrio* and *Methanobacterium* presented significantly higher abundance in the cathodes compared to anodes and AGS.

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1. Introduction

BW (bilge water) is a mixture of seawater and numerous types of wastes such as diesel fuels, anionic surfactants, solid particles, lubricating and hydraulic oils from engine room, water leaks from internal pipes, PAHs, organic solvents, metals etc. (Jalkanen et al., 2021; Tiselius and Magnusson, 2017; Santisi et al., 2015). Its chemical composition varies greatly between vessels and from day to day within a vessel (Church et al., 2019; Tiselius and Magnusson, 2017). Usually, pH values range from 6.8 to 9.0 and oil content from 36 to 2953 mg L^{-1}. Salinity frequently fluctuates between 25 and 35 g L^{-1}, while its COD is also considered high (>3–15 gL-1) (Vyrides et al., 2018).

Up to now, several physicochemical methods have been used for the treatment of BW like membrane separation (Gryta, 2020; Tummons et al., 2016), electrocoagulation (Soeprijanto et al., 2019; Rincón and La Motta, 2014), centrifugation or addition of chemical agents (Church et al., 2019; McLaughlin et al., 2014). However, such methods often lead to secondary pollution and high operetional costs (Chen et al., 2017) making BW treatment unatisfactory. Biological technologies have also been applied. Biofilm membrane bioreactor (Sun et al., 2010), hybrid moving bed biofilm reactor (Mancini et al., 2012), up-flow anaerobic sludge fixed film reactor (Emadian et al., 2015a), hybrid up-flow anaerobic sludge blanket reactor (Emadian et al., 2015b) or more eco-friendly methods (Mazioti et al., 2020; Uma and Gandhimathi, 2019; Vyrides et al., 2018) are some examples. Despite the cost-effectiveness and the low-risk for secondary pollution, the performance of a biological system may be negatively affected in a dramatic extent due to salinity and toxic refractory organics of BW (Yan et al., 2017). Increase of suspended solids in effluent, reduction of organic load removal efficiency and inhibition of microbial community during treatment of saline wastewaters are often detected (Chen et al., 2017). Therefore, the investigation of alternative sustainable methods targeting to lower operational costs and greater treatment effectiveness are of high importance (Vyrides et al., 2018).

MEC (microbial electrolysis cell) is a promising technology gaining more and more researchers' interest during last years and so far the application of MEC-Ads (anaerobic digesters) for the treatment of high strength wastes or wastewaters originated from various sources have been reported (Yu et al., 2019; Park et al., 2018; Cerrillo et al., 2018). These systems in many cases overcome or significantly improve problems that conventional ADs faces like accumulation of short-chain VFAs, presence of toxic compounds or low performance with regards to recalcitrant substrates (Huang et al., 2020; Zakaria and Dhar, 2019). Furthermore, the use of AGS (anaerobic granular sludge) with layered functional microorganisms seems to exhibit advanced performance against degradation of recalcitrant organics and production of biogas (Zhu et al., 2020), while the existence of salinity in wastewaters decreases the internal resistance in a MEC reactor strengthening the effectivness of the system (Qu et al., 2021). Despite the obvious benefits, to the best of our knowledge MEC-AGS system has not been applied yet for the treatment of real undiluted BW and the possible methane generation. Moreover, available data regarding the effect of high saline BW on methanogens during long-term operation treatment are scarce.

Considering the above knowledge and gaps, the coupling of a single chamber MEC with AGS for the biodegradation of undiluted real BW and the production of methane was examined for the first time. The toxicity of saline BW on hydrogenotrophic and acetoclastic methanogens was also investigated for a long period using sodium formate and acetic acid as co-substrates. The performance of MEC-AD systems were monitored for a period of 80 days using varied strength of organic load. Two different external potentials were investigated as a strategy to increase CH_4 generation and COD (chemical oxygen demand) removal. Biogas production and composition, soluble COD (sCOD) and volatile fatty acids (VFAs) concentrations were measured at different time intervals, while the output voltage was also monitored. Finally, at the end of the experiments, the microbial profile was analyzed to gain insight into the process in samples taken from both the AGS and the electrodes.

2. Materials and methods

It should be mentioned that full details of all material and methods are given in the Supplementary Material (Section A. Analytical Methods). Below they are only described in breif.

2.1. Inoculum

AGS used as inoculum, was collected from a full-scale Internal Circulation bioreactor treating dairy wastewater at pH 7.0–7.5 (Charalambides Christis Ltd, Cyprus).

2.2. Bilge water (BW)

Real BW was provided from Ecofuel Ltd company (Zygi, Cyprus). Samples were taken only whenever there was a need for new supply. As a result, the physicochemical properties of the several collected batches differed from sampling to sampling (Table S1).

2.3. Anaerobic toxicity assay (ATA)

ATAs were performed following the protocol described by Owen et al. (1979). Assays were conducted using acetic acid (AA) or sodium formate (SF), as a substrate and real BW as the toxic agent. During Experiment 1 (phase I: 75 d, Phase II: 105 d, n = 3, Table S2) real BW was used at increasing percentages up to 100% and acetic acid as a co-substrate. At Experiment 2 (72 d, n = 3) the methanogenic activity was tested at three levels of BW (0–100%) in the presence of acetic and formic acid (examined separately).

2.4. Batch experiment

Batch lab-scale experiments were conducted in 1 L glass bottles (10 × 10 × 16cm) which served as membraneless MEC (working volume 0.7 L). Commercial iron rods (25 cm in length x 2.5 mm in diameter) were used as electrodes. As reported in the literature, zerovalent iron (ZVI), that was the only component of the used electrodes, acts as an electron donor and enhances the conversion of CO_2 into CH_4 (Zhang et al., 2020). The produced Fe^{2+} ions contribute significantly to microoganisms' proliferation and consequently to methanogens enrichment. As a result, iron electrodes are also used in bioelectrochemical processes to reinforce methane generation and improve salinity tolerance of microorganisms (Qu et al., 2021). In the present study, each reactor was fed with 7.1% AGS and BW at different rations (Table S3) for five consecutive batch cycles ~~(~~resulting in various strengths of organic load, ~~e~~external potential of 1 and 2 V was applied and gas and liquid samples were taken 4 times per week during the five cycles.

2.5. Analytical methods

Determination of total solids (TS), volatile solids (VS) and sCOD were performed according to Standard Methods (APHA, 2005). Biogas composition (H_2, O_2, N_2, CH_4, and CO_2 concentrations) analysis was performed using Gas Chromatographer - Thermal Conductivity Detector according to Vardanyan et al. (2018). VFAs (acetic, propionic, iso-butyric, butyric and valeric acid) were analyzed by High-Performance Liquid Chromatographer- UV detector based on Vyrides and Stuckey (2009).

2.6. Next-generation sequencing

At the end of all experiments, AGS samples (~250 mg) were collected from bioreactors and electrodes by scraping their surface with a sterile scalpel. Total genomic DNA was extracted using the DNeasy

PowerSoil Pro kit (Quiagen, Germany) according to manufacturers' instructions and the extracts were sent to DNASense Apps Company (Denmark) for analysis.

2.7. Calulations

Cumulative methane yield was calculated according to Charalambous and Vyrides (2021) as follows:

$$M_t = CH_{4,t-1} - CH_{4,t} \quad (1)$$

where Mt is the cumulative methane yield at time t (mL CH_4) and CH_4,t is the methane content in biogas at time t. Current (I) was calculated based on Xing et al. (2021), while the removal efficiency of sCOD was calculated according to Equation (2):

$$\%Removal_{sCOD} = \left(\frac{sCOD_o - sCOD_t}{sCOD_o}\right)x100 \quad (2)$$

C_0 and C_{eff} are the major pollutants' concentrations at the start and at the end of the experiment.

The software GraphPad Prism 5 for Windows was used for data evaluation.

3. Results and discussion

3.1. Anaerobic Toxicity Assays (ATAs)

3.3.1. ATAs at various BW levels and acetic acid

The effect of various levels of BW over time on acetoclastics is shown in Fig. 1a. Cumulative methane was higher than controls up to 75% of BW (100.4 ± 7.1 mL, Phase I) revealing that part of the BW was biodegraded by AGS. However, when the inoculum was exposed to 100% BW the cumulative methane was almost half compared to the control indicating severe inhibition of acetoclastics.

As expected, the higher the percentage of BW the longer the duration of lag phase, showing that acetoclastics need more time to be acclimatized as the BW content increased. Though, after their adaptation, the cumulative methane curve (Fig. 1a) exhibited a linear portion until steady-state was achieved. Even at 100% of BW, acetoclastics managed to cope with the refractory waste to some extent, producing methane which reached 51% at the end of phase I.

As for the effectiveness of feeding on 75 d and whether it could enhance methane production or not, results indicated that it was beneficial only for the assays of 25 and 50% BW. The cumulative methane in these assays found to be almost 71 and 59% higher,

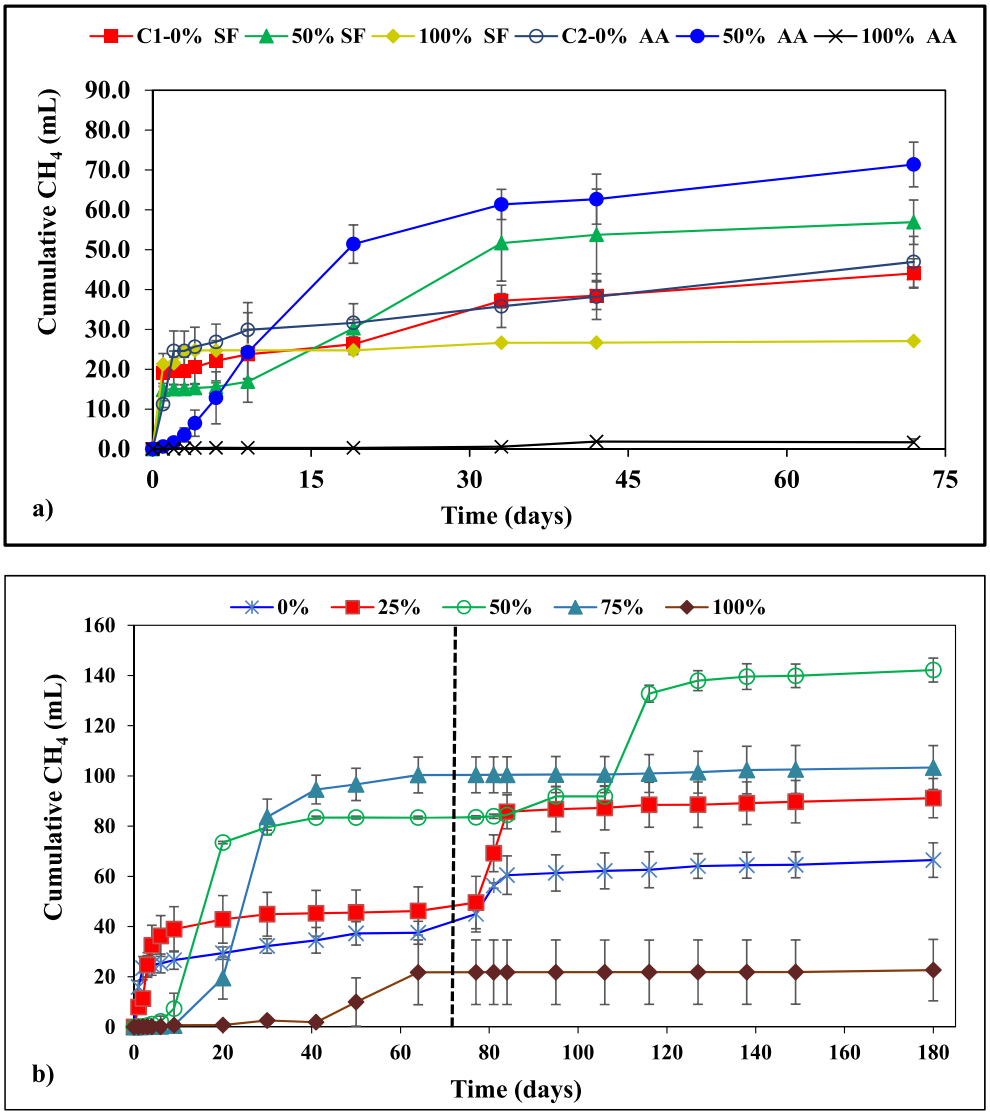

Fig. 1. Methane production (mL) accumulated in the methanogenic toxicity assays for different proportions of BW and a) CH_3COOH (Experiment I), b) both HCOONA and CH_3COOH (Experiment II) over time. Dot line represents the beginning of Phase II during Experiment I.

compared to the corresponding values of phase I probably due to a better adaption of biomass during phase I. Feeding of the assays containing 75 and 100% BW, proved unsubstantial since cumulative methane remained stable up to the end of the experiment (180 d) revealing inhibition of acetate conversion to methane. This might be attributed to the physicochemical properties of BW (Batch II) used (Table S1) and especially its salinity which was 2.75 (31.9 g L^{-1} NaCl) times higher compared to that of Batch I. Previous reports demonstrated that NaCl causes severe inhibition of methane production, while Zhao et al. (2017) claimed that as the concentration of NaCl increases methanogenesis decreases. Furhermore, Vyrides and Stuckey (2009) found that NaCl concentrations higher than 20 g L^{-1} inhibit CH_4 production and specially acetate conversion to methane by acetoclastics due to plasmolysis that is occurred at high salt levels and the consequent cell death because of the dramatic increase in osmotic pressure.

sCOD removal varied from 50.2 to 78.8 and from 26.4 to 80.9% for phase I and II, respectively. At 25 and 50% BW assays (Fig. S1) the % removal of sCOD was similar between the two phases (for the same level of BW), with no statistical difference. However, the use of more saline BW during phase II and the lack of biomass adaptation resulted in a significant decrease in sCOD removal (<30%) at assays containing 75 and 100% BW.

Acetic, propionic and valeric acid were the only VFAs detected (Table S4) with values from 43.6 mg L^{-1} (butyric acid, 75% BW) to 3.1 g L^{-1} (acetic acid, 100% BW). Propionic acid was detected in all assays at concentrations from 11.4 (25% BW) to 352.2 mg L^{-1} (100% BW). The other acids detected only at the assays containg 75 and 100% BW with accetic acid being one (100% BW) or two (75% BW) orders of magnitude higher than butyric acid. As the content of BW increased, their concentrations also increased indicating a distarbance of anaerobic procedure due to the raise of the toxic stress.

3.3.2. ATAs at various BW levels and acetic acid/sodium formate

Behavior of hydrogenotrophics vs. acetoclastics in the presence of BW, is presented in Fig. 1b. At 50% BW, methane production in SF assays found to be about 26% lower than that observed in AA assays at the end of the experiment. Calculated values of cumulative methane were 56.9 and 71.4 mL for SF and AA assays, respectively (72 d). Notably in SF assays, a steady state was achieved almost directly and lasted until 9 d. Afterwards, increase in methane production was observed up to the last day. On the contrary, in AA assays, a 2 days lag phase was observed but then methane production was continuously increased up to the end. Despite the difference in gas production, results indicated that both microorganisms were fast acclimatized in the presence of 50% BW. On the contrary, at 100% BW practically low (27.1 mL) or negligible (less than 1% CH_4) methane was produced throughout the experiment in assays containing SF and AA, respectively (Fig. 1b) and this fact indicated almost complete inhibition of acetoclastics.

sCOD removal (Fig. S2) found to be about 60% regardless the content of BW used in AA assays, whereas in SF assays the removal was increased with the increase of BW from 50 to 100%. Vyrides and Stuckey (2009) found that salinity inhibited acetoclastic methanogens more than hydrogenotrophic methanogens. Since bilge contains high salinity, the difference in CH4 production between hydrogenotrophic acetoclastic methanogens could be due to salinity.

Regarding VFAs, acetic, propionic and butyric acid were the most dominant. Propionic acid was detected in all samples from 24.3 to 202 mg L^{-1} and the values were proportional to BW levels (Table S4). Accumulation of propionic acid is a negative sign in the performance of anaerobic process and its further biodegradation is considered to be thermodynamically unfavorable unless low H_2 partial pressure is applied (Charalambous and Vyrides, 2021). Acetic acid was detected only at 100% BW assays at concentrations as high as 2.9 gL-1 indicating lack of AGS acclimation, possible weakness of acetoclastics to utilize acetic acid and biodegradation of BW to mainly propionic and acetic acid by anaerobic bacteria. However, the propionic acid utilizers (mostly syntrophic propionate biodegradation) and acetoclastic methanogens severely inhibited leading to accumulation of propionic and acetic acid. Butyric acid also detected in these assays but its concentration was orders of magnitude lower (up to 70.7 mg L^{-1}) than acetic.

3.2. Biogas production during microbial electrolysis experiment

Methane (%) production in the controls and MEC-AGS systems during 5 feeding cycles (totally 80 days) is given in Fig. 2. At 25% BW, methane production in R2 and R3 did not exceed 30% and was significantly lower compared to the controls. After reaching its peak values in both MECs, immediately started to probably due to weak acclimation of biomass as a consequence of the coexistence of toxic BW and electric current. High external current often generates environmental stress to the bacterial species, which affects their activity and growth and may even destroy bacterial cells (Luo et al., 2005). Nevertheless, since methane production in the controls reached a plateau, a second feeding of the reactors was followed with 50% BW. Besides successive feedings is a known strategy to check among other things, the possible adaptation of biomass. The interest for the next feeding depends on the results of the previous one (Arias et al., 2020).

At cycle B, methane production reached high values in all reactors just two days after feeding. Almost 62 and 57% methane were observed in R2 and R3, respectively (Fig. 12)indicating well acclimatization of biomass in the MECs and their benefit compared to conventional AD. However, no steady-state was achieved in any system during this cycle but a decline in methane production was observed after peaks possibly due to the lack of organic load. Thus, a new feeding was performed with a slightly higher quantity of BW (60%) to renew the carbon source. According to the results, methane varied from 24.3 (R0, 35 d) to 56.5% (R2, 37 d) throughout this cycle. Additionally, stabilization of the gas generation was observed almost in all reactors except R3, for which the CH_4 increase was continued until the end of this cycle. The highest value (56.5%) was observed in R2, four days after feeding and then it remained stable with minor fluctuations up to 44 d. In R3, methane production was unstable following an upwards trend until the end of this cycle.

Based on the above encouraging results regarding the tolerance of biomass in the presence of BW, an extra feeding was performed by increasing BW content to 90%. The results demonstrated the preeminence of R2 where the application of 1 V proved to be beneficial. The well acclimatized inoculum with the help of external current, managed to cope the “hostile” environment and to biodegrade BW leading to satisfactory methane production nearly three days after feeding. Methane levels varied at 51.3 ± 1.0% (or 299.4 ± 7.2 mL) and were similar to those observed during cycle C although 30% more BW was used. On the contrary, at R3 despite the observed anodic trend probably due to the applied voltage, methane production was extremely slow and did not exceed 6% suggesting strong inhibition of methanogens. Under 2 V, the production of O_2 in the anode may inhibit the methanogens (Chen et al., 2016). However, methanogens are in the core of anaerobic granular sludge and O_2 is likely to be utilized by facultative microbes in the outer surface of anaerobic granular sludge. Compared to other studies where enriched with nutrients (Mazioti et al., 2020) or initially treated aerobically (Vyrides et al., 2018) BW was used as a substrate for AGS in combination with 25 mg L^{-1} zero valent iron (Mazioti et al., 2020) or 1 mM glycine betaine (Vyrides et al., 2018) the observed values in R2 were higher. These findings revealed on one hand that a MEC system can accelerate the decomposition of recalcitrant substrates such as BW, producing substantial quantities of methane. On the other hand, it becomes evident that there is an upper limit in the applied voltages which can enhance methanogenesis. Choi et al. (2017) who studied the methane production in anaerobic digestion at different supplemental voltages observed that as the external voltage increased from 1 V to 1.5 V the MEC performance was declined. Other authors also came to the

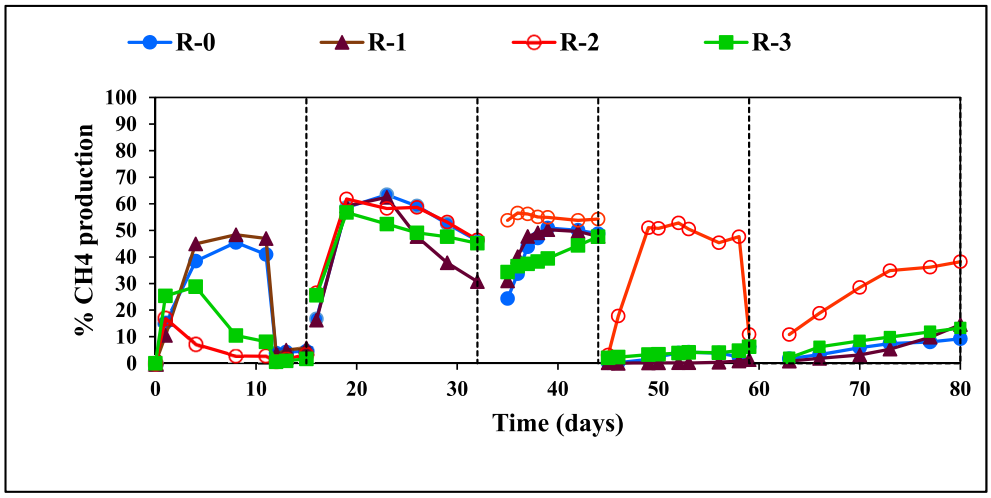

Fig. 2. Methane (%) produced in controls R0 (without electrodes inside) and R1 (with electrodes inside but without potential application) and MECs R2 (1 V) and R3 (2 V) over time. Dot lines represent the beginning of the next experimental cycle.

same conclusions (Zhao et al., 2016; Chen et al., 2016). Adversely effects on operation of a MEC-AD system at higher applied voltages (>1) could be attributed to several reasons such as the creation of alkaline conditions due to excessive consumption of H^+ for H_2 gas production via abiotic cathodic reaction which can increase pH to > 8 (Zakaria and Dhar, 2019; Zamalloa et al., 2013) or creation of micro-aerobic conditions as a result of the anodic water electrolysis that can have a negative impact and destroy the sensitive anaerobic microorganisms (Lee et al., 2017).

On 59 d, methane in R2 reduced suddenly to 10%, possibly due to depletion of the carbon source. Therefore, a further feeding was performed but with lower BW content (50%) to renew the carbon source but also to alleviate in a way the biomass from the pressure of the detrimental nature of BW. According to the results significant methane production was achieved only in R2, which reached its maximum performance practically on 73 d. From that day and until the end of cycle E (80 d) the gas percentage found to be 36.4 ± 1.7%. In R3, despite the observed anodic trend, methane generation was less than 15%. Lower values were probably due to the increase of osmotic pressure in the reactors as a consequence of the saline BW (Batch II) used in this cycle. As a result the anaerobic biomass might consume the substrate to produce compatible solutes and extracellular polysaccharides so as to survive in these high saline conditions rather than methanogenesis (Vyrides and Stuckey, 2017).

The overall findings confirmed the previous observations of AGS survival despite the fluctuations of the substrate's strength and salinity. Furthermore, it became clear that using MEC-AD at 1 V is more dignified and more efficient than only AGS in accordance with other studies which suggested electromethanogenesis as an effective treatment strategy to alleviate biomass from the inhibition of toxicants and recalcitrant compounds or to cope with failures in digesters' operation at mesophilic conditions (Yu et al., 2019; Luo et al., 2016).

3.3. Current generation and energy efficiceny

Regarding current production throughout the experiment in the two MECs (Fig. S3), the obtained results also confirmed the better performance of R2 (12.06 ± 1.8 mA) compared to R3 (0.01 ± 0.01 mA). Higher achievable current in R2 indicates quicker kinetic reaction (Lin et al., 2019), while the reduced current efficiency of R3 might be attributed to the growth of non-electroactive competitive bacterial communities in the anode as a result of the higher applied voltage which allowed their enrichment affecting adversely the electroactive bacteria (Zakaria and Dhar, 2019). However, the low energy efficiency (μE) of R2 (0.23) means that the incremental methane yield produced in the MEC can not cover the electrical energy consumed in MECs. Even so, MEC-AD performance was better than AD alone, but the recalcitrant nature of BW did not allow the system to balance the consumed energy. This is in line with the batch ATA results pointing out its inhibitory effect on methanogens.

3.4. COD removal in MECs

Comparing the two MECs, sCOD removal ranged from 49.6 to 70.9 and from 11.7 to 65% for R2 and R3 reactor, respectively (Fig. 3). At cycle A the sCOD removals in both MECs found to be similar to those observed in the controls with no statistical difference. During the next two cycles and despite the much higher BW content, the removal in R2 was further increased about 20% (cycle B: 70.9% and cycle C: 70.4%) indicating good performance of the reactor and excellant adaptation of biomass. Increase of BW to 90% resulted in reduced but still efficient sCOD removal (55%) in R2 and maintained hight (above 50%) until the end of the experiment, probably due to the better establishment of microbial communities at 1 V. In anaerobic digestion process, the removal of COD in conjunction with methane production is indicative of the effective microbial activity by methanogenic bacteria. Indeed, in the present study gradual increase of biogas production in R2 reactor, was supported by a simultaneous decrease of COD.

Compared with other studies the above findings were very promising regarding application of MEC-AD for real undiluted BW. Vyrides at al. (2018) reported that treatment of real BW with AGS plus glycine betaine resulted in low sCOD removal (around 35%) during 13 days of batch experiments and further increase was only possible thanks to extra aerobic treatment reaching a final sCOD removal of 55%. Mazioti et al. (2020) achieved 60% sCOD removal after treatment of BW with AGS during 80 d. However, this removal was achieved using notably lower organic load (50% diluted BW, initial COD: 1100 mg L^{-1}). The authors also reported that when BW with higher COD (>5000 mg L^{-1}) was used negligible COD removal was observed probably due to higher toxicity of the substrate toward AGS.

At R3 reactor, maximum removal was observed at the end of cycle C (60%). More increase of the organic load significantly reduced the sCOD removal (11%, cycle D) but futher decrease of BW content seemed to allevieate the biomass which appeared to start recovering and the sCOD removal was doubled (23%). These observations revealed a significant destruction of anaerobic microorganisms at 2 V, which resulted in weakness of this MEC system to successful continue decomposing BW and demonstrated that the choice of the external voltage is very crucial

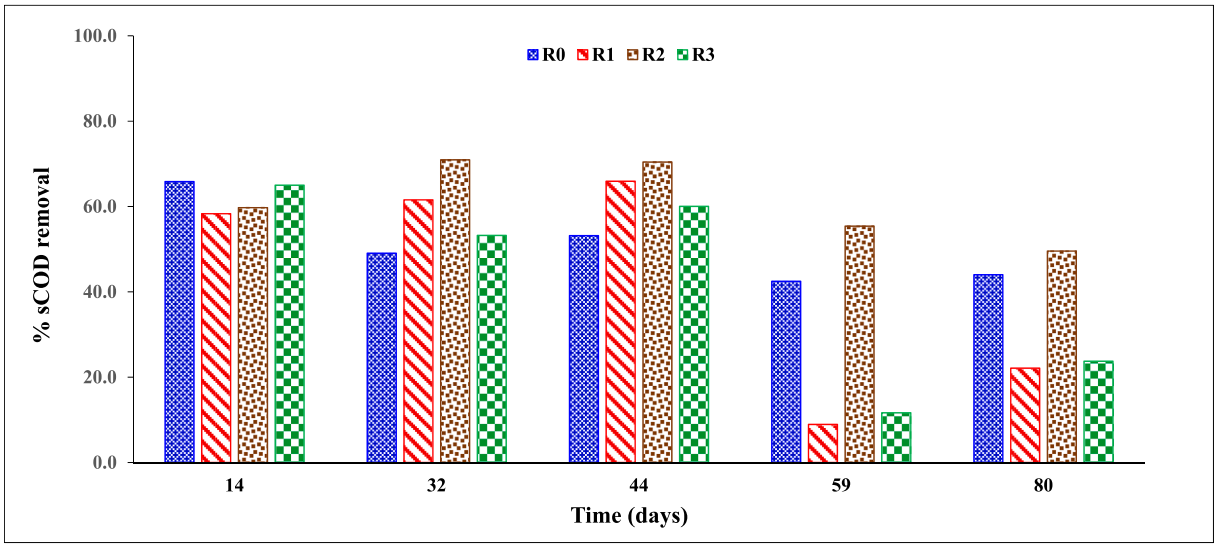

Fig. 3. SCOD removal (%) at the end of each experimental cycle A-E. R0: Control (without electrodes inside), R1: Control (with electrodes inside but without potential application), R2: MEC (1 V), R3: MEC (2 V).

for achieving high removal rates of pollutants or biogas generation (Yu et al., 2019).

3.5. VFAs

As shown in Fig. 4, acetic and valeric acids were the most frequently detected acids reaching 2.79 and 1.81 g L^{-1}, respectively. During the first three cycles a decreasing trend of VFAs was observed soon after feedings almost in all systems. This trend was continued in R2 reactor up to the end of cycle E. Acetic acid varied at lowest levels in R2 through 80 d, reflecting better acclimation of AGS and acetoclastics and more stable conditions dispite BW content fluctuations. This stability could be attributed to the production of carbon dioxide which was not removed as a gas but existed as carbonate/bicarbonate ions acting as buffer agent (Borja et al., 1998). Thus the methanogenic stage was balanced well enough making possible methane generation from these intermediates. It should be noted that the observed "peaks" in VFAs concentrations in R2 on 16, 35, 46, 49 and 63 d were mainly due to the increase of acetic acid at that time which resulted in inhibition of methane production (Fig. S4). The high concentration of acetic acid is in line with the results

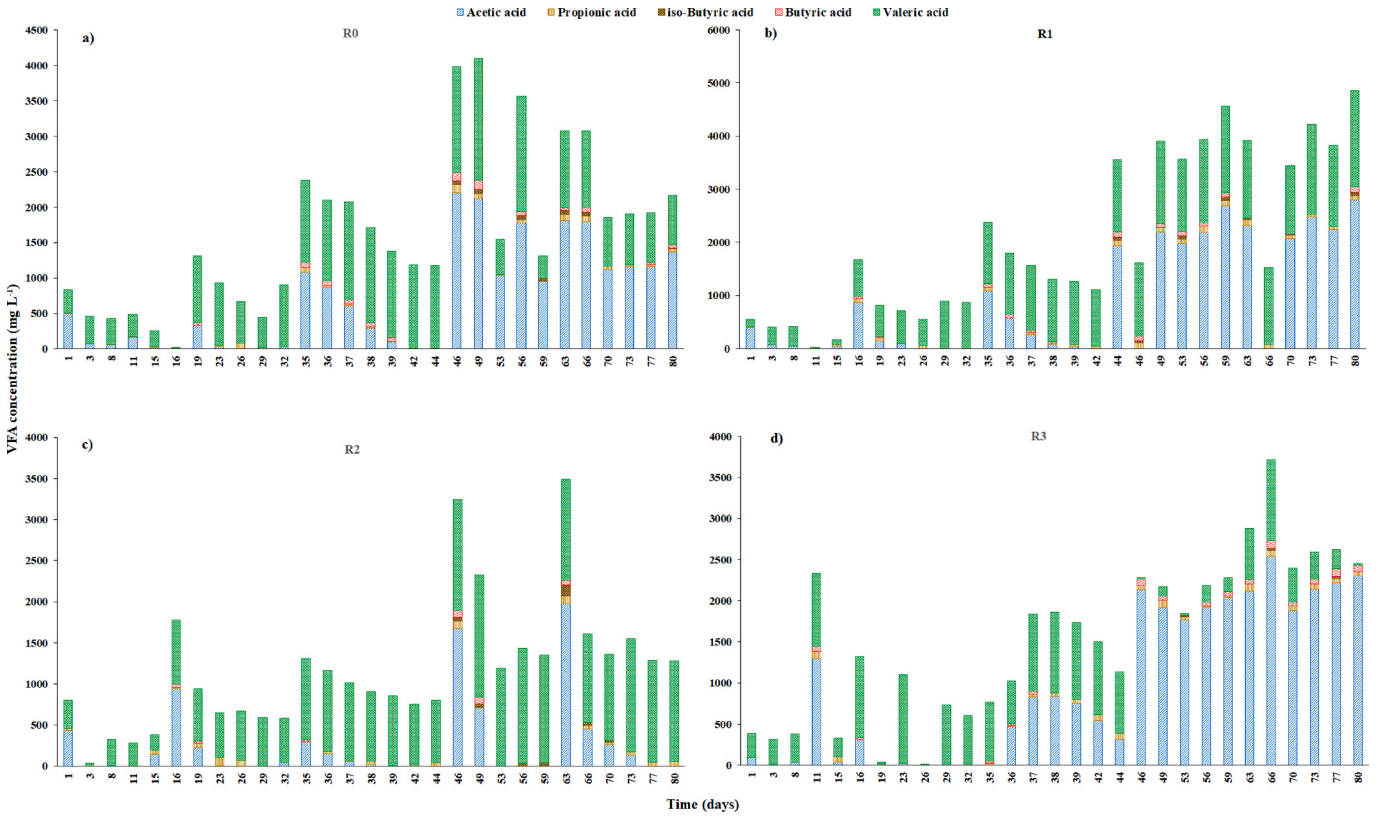

Fig. 4. Variation in VFAs composition (mg L^{-1}) over time in a) R0: Control (without electrodes inside), b) R1: Control (with electrodes inside but without potential application), c) R2: MEC (1 V) and d) R3: MEC (2 V).

from the ATA experiments (Section 3.1) so acetoclastic methanogens were severely inhibited by bilge, so the acetic acid is not utilized. In R3, a gradual accumulation of VFAs was observed especially in valeric acid after cycle C accompanied by negligible biogas production which reflected a kinetic uncoupling between acid producer and consumer microorganisms and is typically at stress and imbalance conditions (Ahring et al., 1995).

3.6. Microbial community analysis

Regarding the bacterial profile at the phylum level *Nitrospirae*, *Deltaproteobacteria* and *Thermotogae* were present in all samples with relative abundances up to 68.3% (Fig. 5). *Nitrospirae* are nitrite-oxidizing bacteria (Park et al., 2021) and were found mainly in AGS samples. Their relative abundance was remained unchanged in the controls (about 63%) and it was slightly increased in R1 (65.3%). However, in R3 they were significantly reduced (19.1%) confirming the previous observation that different applied voltages can boost different microbial communities.

Deltaproteobacteria were detected mainly in the electrodes. Among the different genera, *Desulfovibrio* a known metal reducing agent (Wang et al., 2017) was not identified in the controls but found to be predominant in cathodes and its relative abundance was enhanced as the applied voltage was increased. From 31.7% in R2-Cathode (1 V) reached 68.3% in R3-Cathode (2 V). The genus *Desulforomonas* was mainly detected in anodes and the obtained relative abundances were 31.6 and 49.1% for R2 and R3-Anode, respectively. According to Carmona--Martínez et al. (2015) several microorganisms within the deltaproteobacteria class have been proved to possess electroactivity. These microorganisms repeatedly appear in anodic biofilm communities and in well performing microbial electrochemical technologies (METs). Specifically, *Desulfovibrio* sp. are reported to be the dominant species in several MEC biocathode characterization studies. These species are recognized among detected genus in enriched mixed microbial communities capable of producing hydrogen when attached to an electrode surface. The hydrogenase surviving either on the cytoplasmic membrane or in the cytoplasm was proposed to reduce protons through an enzymatic reaction (Jafary et al. 2015, 2021).

Regarding *Thermotogae*, the predominant genera detected were *Mesotoga* and *Oceanotoga* (up to 14.4% relative abundance). Mazioti et al. (2020) also found high abundance of *Mesotoga* in AGS exposed to BW, while Nesbø et al. (2019) reported that *Mesotoga* are frequently identified in oil-polluted marine mesophilic anaerobic environments. *Mesotoga* although belong to the thermophilic phylum of *Thermotogae*, are the only strictly mesophilic genus (Pollo et al., 2015) and may take part in syntrophic acetate limitation (Nobu et al., 2015). They presented richer diversity at 1 V with relative abundance sligtly higher at R2-Anode (6.2%) compared to R2-AGS (5.8%) and R2-Cathode (4.9%). At 2 V the observed relative abundances were much lower (*R*-AGS: 3.1, R3-Anode:1.1, R3-Cathode: 1.7%). On the contrary, *Oceanotoga*, shown greater growth at 2 V and its relative abundance reached 14.4% (R3-Cathode), whereas at 1 V did not exceed 2.4%. Other phyla which were also identified at remarkable relative abundance (>10%) were *Marinimicrobia*, *Chloroflexi* and *Firmicutes*.

Regarding archaeal communities, apparently the diversity of *Euryarchaeota* was the most abundant phylum in all samples (Fig. 6). *Methanobacterium*, a typical hydrogen-utilizing methanogen that uses H_2 as an electron donor and CO_2 as an acceptor (Luo et al., 2018) was the most predominant one and its relative abundance was mainly higher in the cathodes (R2: 58.2%, R3: 49.9%) compared to the controls possibly due to the utilization of H_2 produced in the cathodes. The results are in accordance with Siegert et al. (2015) who reported cathodes's attachment mainly by the genus *Methanobacterium* while studing methane production in acetate-fed MECs systems. *Methanoculleus* and *Methanogranum* were also found at higher populations in R3-cathodes reaching 17.8 and 11.7% abundance, respectively. *Methanosaeta* (acetoclastic methanogen) was the next predominant genus identified at sufficient abundances (up to 34.8%) in AGS and anode samples (Fig. 6). Electrode samples analysis revealed that when 1 V was applied, *Methanosaeta* dominated the anode and the relative abundance found to be about 28% higher compared to AGS and cathode. On the contrary, at 2 V a decreasing trend was detected moving from AGS to anode and further

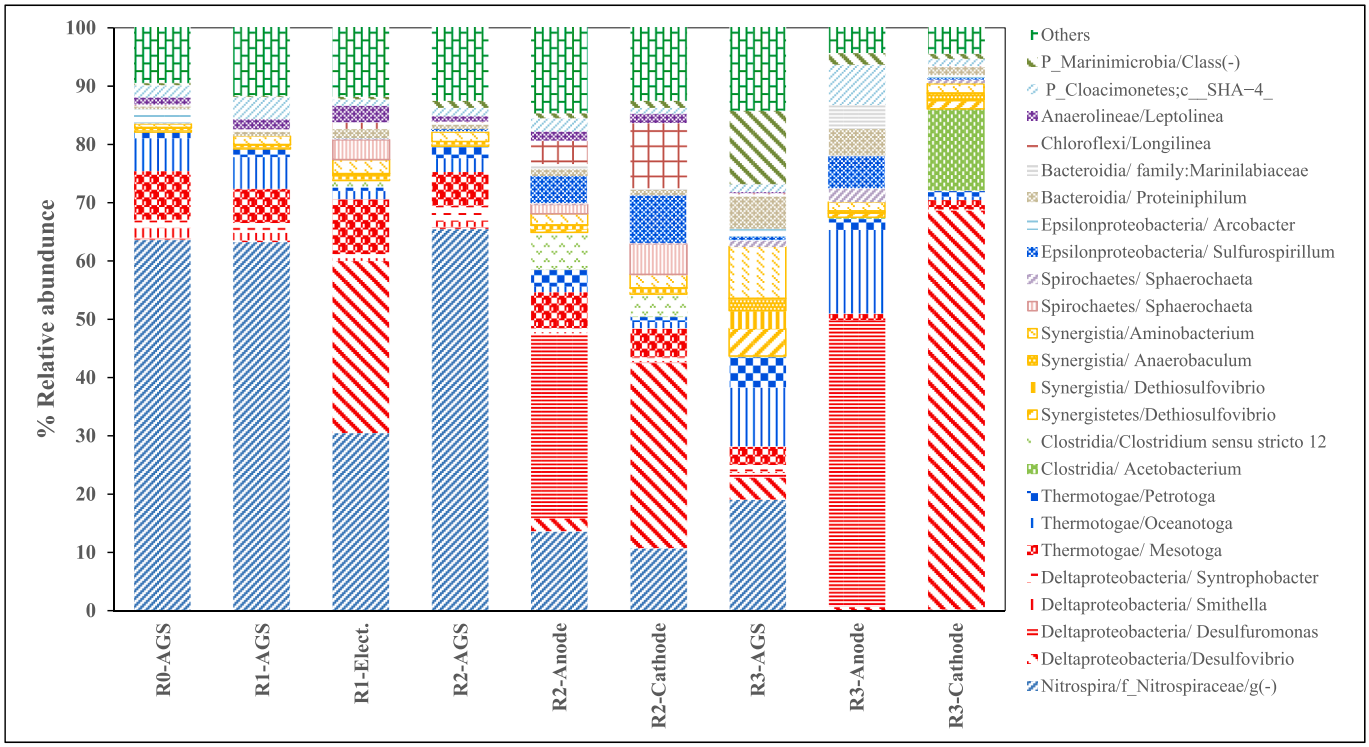

Fig. 5. Observed relative abundance of bacterial community at the class and genus level identified in the reactors.

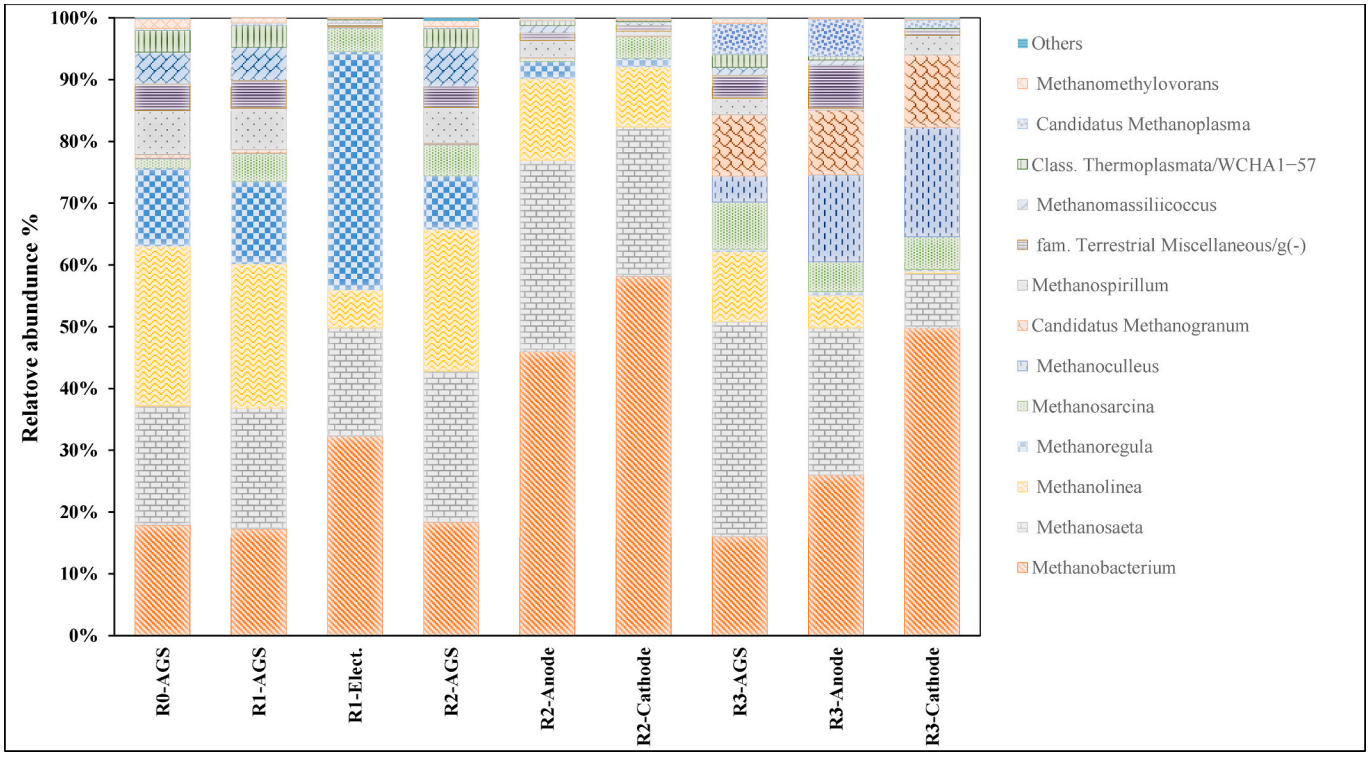

Fig. 6. The most abundant archaeal genera identified in the reactors.

to cathode. The highest abundance (34.8%) observed in R3-AGS is in consisent with the study of Mazioti et al. (2020) who also observed greater dominance of *Methanosaeta* in AGS samples, probably a result of their role in granule formation. Other genera which were identified at significant populations principally at AGS were *Methanolinea* (H_2-utilizing methanogen) and *Methanoregula*. *Methanosarcina* which is the only special anaerobic methanogen that can generate CH_4 by all metabolic pathways (using acetate, H_2/CO_2 and methylated carbon compounds) (Luo et al., 2018) also revealed higher abundance in AGS compared to electrodes and its growth seemed to be enhanced at 2 V. The overall microbial profile in MEC systems indicated that electrical stimulation or H_2 utilization in the cathodes can be very helpful in order to enrich some specific species selectively depending on what is desirable during BW treatmet.

4. Conclusions

ATA assays indicated that acetoclastics seemed to be more sensitive to BW exposure compared to hydrogenotrophic methanogens. However when diluted BW was used lower inhibition to anaerobic biomass was observed, allowing better BW biodegradation. Operation of membraneless MEC coupled with AGS using commercial iron rods as electrodes for real undiluted BW degradation, demonstrated significantly better performance than AGS system alone. After gradual acclimitization of biomass considerable COD removal and methane production was possible even when very high BW percentage was used. However, the recalcitrant nature of this waste did not allowed MEC-AD to balance the consumed energy. The microbiological analysis of the biofilm showed selection of specific species. *Desulfovibrio* and *Methanobacterium* were predominant in the cathodes, whereas these microbes were found in substantially lower abundances in the anodes and in the AGS. Overall the present study indicates that MEC-AGS sysems are very promising and can be efficiently applied for the treatment of high organic load saline wastewaters such as BW and production of biogas.

Author contributions statement

All authors contributed to this article. **Dr G. Gatidou** organized all the experiments. She run the ATA experiments, operated the MEC reactors, conducted the analyses of biogas, wastewater samples and microbial profile and wrote the major part of the article. **Ch. Samanides** contributed to DNA extraction of the samples. Assistant Professor **Dr M. Fountoulakis** contributed to the supervision of the operation of MECs systems. Assistant Professor **Dr I. Vyrides** organized and supervised the whole work of the present study and he substantially contributed to the article preparation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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