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ORIGINAL ARTICLE

The impact and mode of action of phenolic compounds extracted from brown seaweed on mixed anaerobic microbial cultures

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antimicrobials, biodegradation, cell injury, mechanism of action, membrane.

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Abstract

Aims: This study discusses the effect of phenolic compounds extracted from brown seaweed (phlorotannins) on mixed microbial cultures found in anaerobic systems.
Methods and Results: Assays were conducted with phloroglucinol as the nonpolymerized form of phlorotannin and with phlorotannins extracted from the brown seaweed *Laminaria digitata*. Electron micrographs revealed that phlorotannins induce significant extra- and intracellular effects upon cells, with the disruption of cell membranes observed with most micro-organisms. Microscopy results were further confirmed by cell membrane leakage assays demonstrating that phloroglucinol strongly affects cell membrane permeability. However, cell membrane leakage could not be observed with phlorotannins as the cell suspension immediately started to coagulate and impaired spectrophotometric measurements.
Conclusions: Results suggest that the bactericidal activity of phlorotannins is a function of the level of polymerization of the compounds. By monitoring intermediary compounds during the anaerobic digestion of phlorotannins, it was also found that higher energy consumption is required by micro-organisms for survival under stress induced by phlorotannins.
Significance and Impact of the Study: The successful anaerobic degradation of brown seaweed is thus likely to be dependant on the concentration of phenolic compounds present and their bactericidal effect on micro-organisms. This is the first article to posit a probable mode of action for the antimicrobial effect of phlorotannins.

Introduction

Polyphenolic compounds are known inhibitors of anaerobic digestion systems (Chen *et al.* 2008). Their detrimental effects on anaerobic micro-organisms have been observed in anaerobic systems treating coal gasification wastewater (Wang and Han 2012), olive mill wastes (Fezzani and Ben Cheikh 2007) and wine distillery wastewater (Borja *et al.* 1993). Higher molecular weight polyphenols of natural occurrence have also been found to be toxic towards methanogens (Field and Lettinga 1987). Phloroglucinol-derived chemicals are polyphenolic compounds used in medicine, cosmetics, paints, pesticides and dyeing. The group includes about 700 naturally occurring variations (Singh *et al.* 2010). The phloroglucinol parent molecule (1,3,5-trihydroxybenzene) has been isolated from numerous natural sources (Singh and Bharate 2006) with the most complex polymerized forms, phlorotannins, exclusively found in brown seaweed species (Jormalainen and Honkanen 2008). Phlorotannins are categorized into different groups based on the chemical structure of the polymer (Fig. 1) and characterized by

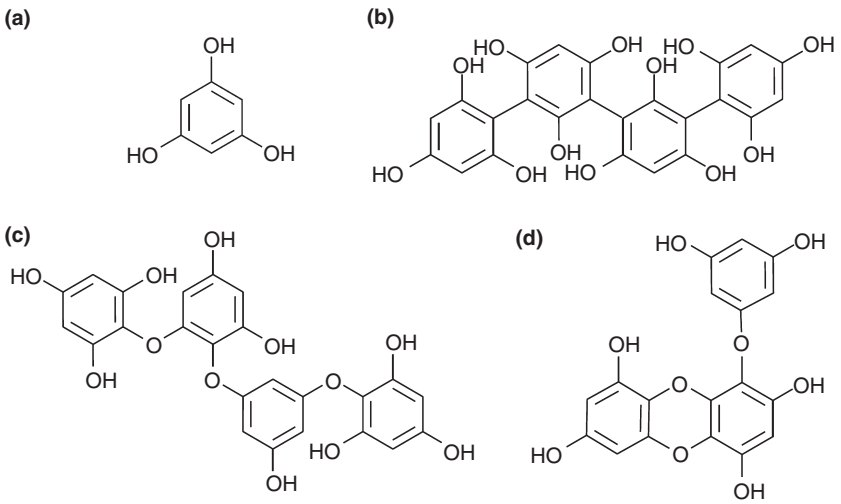

Figure 1 Structures of phloroglucinol (a) and polymeric derivatives examples: tetrafucol A (b), tetraphloretol B (c) and eckol (d).

molecular weights varying from 126 Daltons (Da) for the nonpolymerized form to 650 kDa, but it is usually found between 10 and 100 kDa (Boettcher and Targett 1993). Their occurrence in brown algae has been linked with defence mechanisms against predation (Haavisto *et al.* 2010) and they can account for up to 20% of the seaweed dry weight (Amsler and Fairhead 2006).

Nagayama *et al.* (2002) reported that crude phlorotannins extracted from the brown algae *Ecklonia kurome* showed an increase in bactericidal activity against pathogenic bacteria proportional to the degree of polymerization of phloroglucinol *viz.* increasing phlorotannins molecular weight. Dubber and Harder (2008) also found antibacterial effects of algal metabolites at and even below, algal tissue level concentrations from extracts of *L. digitata*. Recent studies have considered phlorotannin as a possible new source of natural antimicrobial agents for the pharmaceutical and food industries (Eom *et al.* 2012). In anaerobic systems, decreased methanogenic performance has been observed by Moen *et al.* (1997) during the digestion of *Ascophyllum nodosum* at phlorotannins levels ranging from 0·2 to 1·3 g l^{-1}.

By analogy with tannins from terrestrial sources, possible mechanisms to explain the antimicrobial activity of phlorotannins include (i) inhibition of extracellular microbial enzymes, (ii) inhibition of microbial metabolism or (iii) deprivation of the substrates necessary for microbial development (Scalbert 1991). Phlorotannins, as most phenolic compounds, are likely to affect cell membrane activity inducing leakage of intracellular constituents (McDonnell and Russell 1999; McDonnell 2007). The cytoplasmic membrane is highly active metabolically and acts as a selective permeability barrier with the cell's external environment that once damaged, results in the loss of intracellular potassium, 260-nm absorbing materials (mainly nucleic acids) and phosphates (Rye and Wiseman 1964; Elferink and Booij 1974; Al-Adham *et al.* 1998). Wang *et al.* (2009) observed damaged cell walls of *Escherichia coli* cells exposed to phlorotannins extracted from the brown seaweed *A. nodosum*.

However, the mode of action of phlorotannin on anaerobic micro-organisms remains obscure and there is little information available regarding their influence on mixed microbial cultures found in anaerobic digesters. Successful anaerobic degradation of brown seaweed is likely to be dependant on phlorotannin concentrations and their subsequent effect on the degradative micro-organisms employed. The aim of this study was to investigate the inhibitory effects and possible mode of action of phlorotannins extracted from brown seaweed (*L. digitata*) on mixed anaerobic microbial cultures. The relationship between antibacterial activity and the degree of polymerization of phlorotannin was also studied. Factors investigated include the production of methane at various polymerization levels and concentrations of phlorotannin and cell membrane damage through the monitoring of 260-nm absorbing materials and transmission electron microscopy observations.

Materials and methods

Phlorotannin extraction and quantification

The brown algae *L. digitata* was collected from the Westhaven beach (56°30′N, 2°42′W) near Dundee, Scotland, UK in October 2010. The seaweed (approximately 5 kg, but accurately weighed) was washed immediately after collection to remove marine salts and sediments, oven-dried at about 75°C for 24 h and pulverized (using a laboratory hammer mill) to reduce particle size to a maximum of 1 mm. The resulting algal powder was extracted three times with a 7:3 acetone/water solution as

recommended by Koivikko (2008) and left to evaporate in a fume hood. The resulting aqueous phase was frozen, freeze-dried and dissolved into water to obtain a final phlorotannin concentration of 11 mg g^{-1} seaweed dry weight measured by the DMBA (2,4-dimethoxy benzaldehyde) assay (Stern *et al.* 1996). The crude phlorotannin fraction was then diluted in deionized water to obtain a stock solution with a known phlorotannin concentration.

Batch assays of the biodegradation and impact of phlorotannins on acetoclastic methanogens

The experimental batch tests were designed to monitor the biodegradability and possible impact of phlorotannins on acetoclastic methanogens through the measurement of cumulative methane production. To investigate the effect of the degree of polymerization on antibacterial activity, tests were also conducted with anhydrous phloroglucinol (Sigma, Poole, UK) as a benchmark for the nonpolymerized form of phlorotannin. The assay was conducted according to the method described by Sklyar *et al.* (1999) with some modifications. Briefly, bottles with a capacity of 500 ml were used and 125 ml of inoculum was transferred to each bottle. The bottles were closed with rubber caps, flushed with N_2 and incubated (37°C ± 1°C). The inoculum, which consisted of anaerobically digested sludge obtained from a domestic wastewater treatment plant (Hatton, UK) operating at 37°C ± 1°C, was left to starve for 24 h in basal medium. Thereafter, different concentrations of phloroglucinol (50–1000 mg l^{-1}) or phlorotannin (20–200 mg l^{-1}) were added to the bottles together with 25 ml of sodium acetate solution [final concentration 2 g COD l^{-1}, where COD = Chemical Oxygen Demand]. Concentrations of phloroglucinol were selected in accordance with previous observations in which phloroglucinol was rapidly acidified and later converted to methane (CH_4) at concentrations as high as 1500 mg l^{-1} (Field and Lettinga 1989). The addition of a model substrate, such as acetate, was used to assess the activity of the trophic group of interest, that is, acetoclastic methanogens. The specific biogas potential of phloroglucinol and phlorotannin was not assessed. A nongrowth synthetic medium adapted from Akunna *et al.* (1993) was used to provide essential nutrients. The basal medium contained the following compounds (mg l^{-1}): KH_2PO_4 (2700), K_2HPO_4 (3500), $MgSO_4.7H_2O$ (5), $CaCl_2$ (0·5), $FeCl_2$ (0·5), KCl_2 (0·5), $CoCl_2.6H_2O$ (0·1), $NiCl_2$ (0·1). All the batch cultures were established and performed in duplicates. Controls containing no phloroglucinol or phlorotannin were also incubated for 30 days at 37°C ± 1°C. To avoid overpressure in the bottles, the gas produced was released by displacement of a syringe piston after methane measurement and added to cumulative biogas production.

Analytical methods

Methane production was measured by gas chromatography using a Hewlett-Packard 5890 Series II gas chromatograph with dual thermal conductivity detector and an Alltech Heliflex® AT-Alumina stainless steel capillary column. Injector, oven and detector temperatures were 120°C, 50°C and 150°C, respectively, and helium was used as a carrier gas. Gas samples were collected in a gas tight syringe, and 100 μl of sample was transferred to the gas chromatograph. Methane yields were converted to standard temperature and pressure (STP: 273·15°K; 1013·25 hPa). Total volatile fatty acids (VFA) were quantified by esterification (Montgomery *et al.* 1962) and colorimetric determination using a DR5000 spectrophotometer (Hach-Lange, Salford, UK). COD was measured using Hach-Lange cuvette tests (LCK 014), and samples were centrifuged for the determination of soluble COD (COD_S). Cell membrane leakage was assessed by measuring 260-nm absorbing material concentrations over time in a solution consisting of mixed anaerobic microorganisms and phloroglucinol/phlorotannins at different concentrations. To determine the leakage of 260-nm absorbing material, 4 ml of anaerobic digested sludge was placed in a beaker and magnetically stirred. At the beginning of the assay, 1 ml of phloroglucinol or phlorotannin solution was added to give a known final reaction concentration in 5 ml. The absorbance was measured at time intervals of 5 min by transferring 1 ml of sample in Eppendorf tubes. The tubes were centrifuged at 500 ***g*** for 2 min, and 1 ml of supernatant was transferred in a clean quartz UV cuvette for measurement. The absorbance of the cuvette was read at 260 nm in a calibrated spectrophotometer.

Transmission electron microscopy

Mixed anaerobic microbial cultures were obtained from the same source of inoculum used for the batch assays. Cultures were grown in a nutrient media under anaerobic conditions [using Oxoid anaerobic jars (HP0011) and AnaeroGen™ AN0035 kits; Oxoid, Basingstoke, UK] and for 24 h at 37°C and 125 ml was transferred to vials and fed with 25 ml of a sodium acetate solution (25 g l^{-1}) before being incubated under anaerobic conditions for a further 7 days. Cultures were then exposed to phloroglucinol (final concentration 1000 mg l^{-1}), phlorotannin (final concentration 18 mg l^{-1}) or water (control) for 5 h before being centrifuged (3000 ***g*** for 20 min). Pellets were washed twice with a solution of 0·9% w/v NaCl in sterile distilled water to remove test materials. The final pellets were resuspended in glutaraldehyde (2·5% v/v solution in normal saline) and incubated at room

temperature for fixation. The fixed pellets were resuspended in osmium tetroxide solution (0·2% OsO_4 in water) and left to fix/stain overnight. The suspensions were centrifuged (4000 ***g*** for 15 min) and rinsed twice with distilled water. Uranyl acetate solution (2% aq.) was then added to the pellets and left overnight. The pellets were then washed twice with distilled water. Cells were dehydrated through a graded ethanol series with two changes in absolute ethanol. The pellets were then placed in propylene oxide (100%) and infiltrated with a 1:1 (v/v) mixture of propylene oxide and Durcapan resin (Sigma) on a rotary wheel (4 rev min^{-1}) for 24 h at room temperature. The pellets were then infiltrated with 100% Durcapan resin, as described earlier, overnight. Pellets were transferred to flat embedding moulds with fresh resin and placed in a 60°C oven for 24 h to polymerize the resin. Sections were cut from the resin-fixed pellets using a Reichert OMU-3 ultramicrotome fitted with a diamond knife and mounted/collected on 50 mesh pioloform coated copper grids. After staining with uranyl acetate and lead citrate, sections were examined using a JEOL-1200 EX transmission electron microscope.

Statistical methods

Statistical analyses were performed using the SPSS 18.0 package (SPSS International, Chicago, IL, USA), the unpaired *t*-test was used to test the significance of differences between two samples means. The one-sample *t*-test was used to assess the significance of cell leakage results. All statistical analyses were preceded by the determination of model assumptions and tested at the 5% level of significance.

Results

Methane production and VFA levels

The cumulative methane production at different levels of phloroglucinol and phlorotannin is shown in Fig. 2.

Results are expressed as litres of methane measured per gram of COD added at STP (l CH_4 g^{-1} COD_{added}). The test was stopped after 30 days when a significant production of methane could no longer be observed. There was no statistically significant difference in observed methane production in the presence of phloroglucinol or phlorotannin, between the test and controls systems. However, ultimate methane volumes observed at the highest concentration of phlorotannin were 20% lower than the control, whilst no difference was observed at varying phloroglucinol concentrations. At a concentration of 1000 mg l^{-1} phloroglucinol, it can be noticed that the methane production rate was lower for the first few days

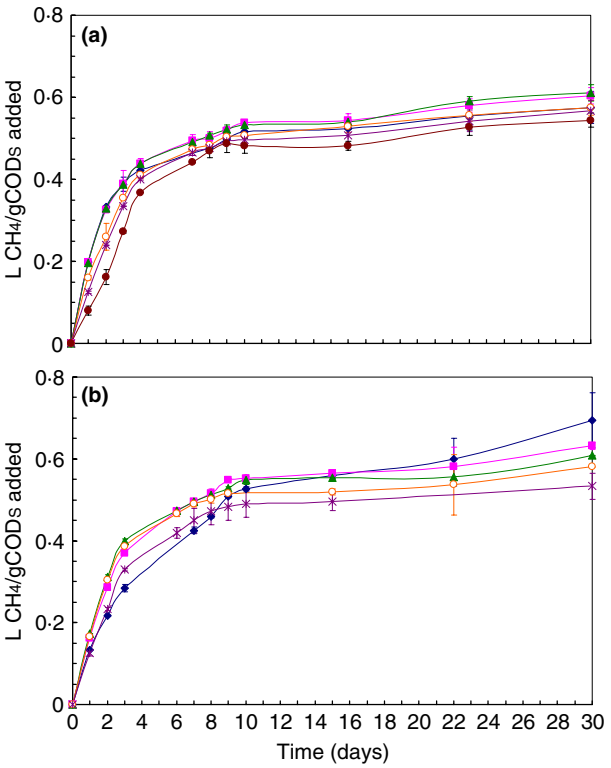

Figure 2 Cumulative methane production with different phloroglucinol concentrations (a): ◆ control; ■ 50 mg l^{-1}; ▲ 100 mg l^{-1}; ○ 250 mg l^{-1}; × 500 mg l^{-1}; ● 1000 mg l^{-1} and phlorotannins concentrations (b): ◆ control; ■ 20 mg l^{-1}; ▲ 50 mg l^{-1}; ○ 100 mg l^{-1}; × 200 mg l^{-1}. Error bars represent the standard deviation between duplicate experiments.

of the assay. This observation is confirmed by the value of the first-order degradation constant *k* found equal to 0·27 day^{-1} for the highest phloroglucinol concentration and 0·39 day^{-1} for the control. For phlorotannins, *k* was in the range of 0·32–0·41 day^{-1}, thus indicating the rapid biodegradation of phlorotannin.

Changes in VFA levels can be used to monitor the progress of anaerobic degradation, and Fig. 3 shows the variations of total VFA during the assay. It can be seen that VFA levels at the beginning of the experiment are similar for all groups and about 1000 mg l^{-1} as HAc. For cultures exposed to phlorotannins, VFA profiles show no accumulation and levels rapidly decreased for all groups presumably due to the rapid conversion of acids to methane. At the highest concentration of phloroglucinol, the decrease in VFA levels was slower than observed for the control, but concentrations were similar for all groups from day ten. This observation supports the delay observed for methane production at 1000 mg l^{-1} phloroglucinol during the first days of the assay.

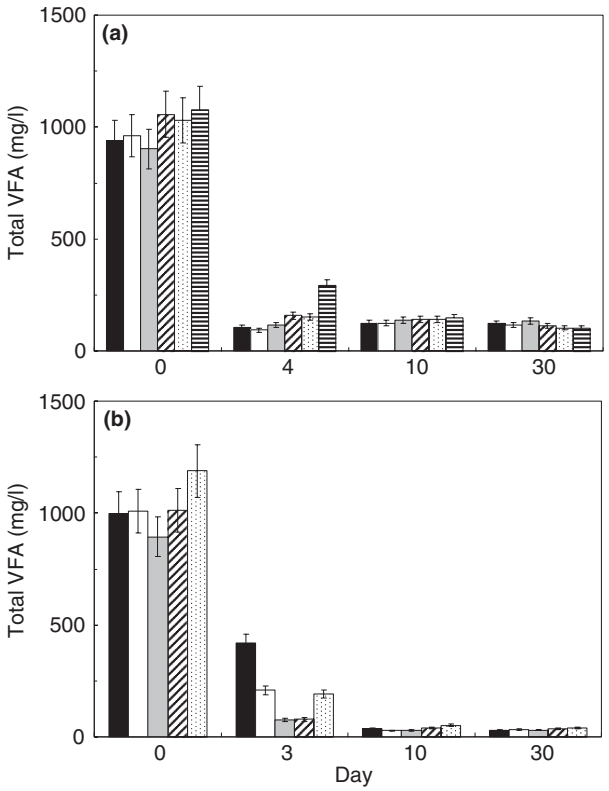

Figure 3 Volatile fatty acids profiles at different concentrations of phloroglucinol (a): the black, unfilled, grey, upward diagonal, dotted and dark horizontal bars represent concentrations of 0, 50, 100, 250, 500 and 1000 mg l^{-1}, respectively, and phlorotannins (b): the black, unfilled, grey, upward diagonal and dotted horizontal bars represent concentrations of 0, 20, 50, 100 and 200 mg l^{-1}, respectively. Error bars represent the standard error associated with the method of measurement.

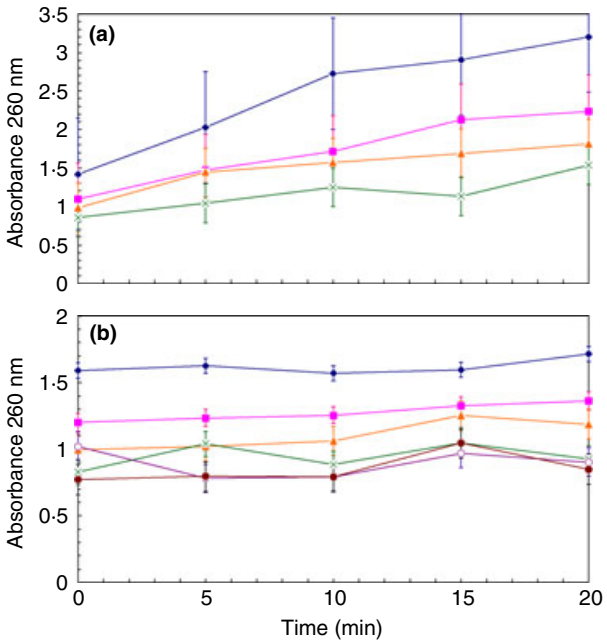

Figure 4 Leakage of 260-nm absorbing material from solutions of mixed anaerobic micro-organisms exposed to different levels of phloroglucinol (a): ◆ 700 mg l^{-1}; ■ 360 mg l^{-1}; ▲ 280 mg l^{-1}; × 140 mg l^{-1} and phlorotannins (b): ◆ 560 mg l^{-1}; ■ 280 mg l^{-1}; ▲ 140 mg l^{-1}; × 70 mg l^{-1}; ○ 36 mg l^{-1}; ● 28 mg l^{-1}. Error bars represent the standard error of the data set.

Cell membrane leakage

Figure 4 represents the absorbance at 260 nm for solutions containing mixed anaerobic cultures with different concentrations of phlorotannin and phloroglucinol. The absorbance at the beginning of the assay is different for all concentrations tested, as phlorotannin and phloroglucinol have been found to have absorbance peaks at 261 and 266 nm, respectively, thus impacting on the original absorbance monitored during leakage assays. The variations of absorbance were tested statistically against their initial value at the beginning of the assay. In solutions with different concentrations of phlorotannin, a significant variation in absorbance could only be observed from the solution containing 70 mg l^{-1} phlorotannin, where a peak in absorbance was found after 5 min of exposure. One explanation for the observed subsequent absorbance decreases has been proposed by Hugo and Longworth (1965), who suggested that this could be the result of the coagulation of protoplasmic contents resulting in smaller leakage rates. No significant increase in 260 nm absorbance could be found at other phlorotannin concentrations. However, it has been observed that after the addition of phlorotannin, the cell suspension immediately started to coagulate. Due to the experimental procedure used, the pellet formed was removed by centrifugation before measurement of the supernatant, which could have resulted in the removal of leaking material by sedimentation. Significant increases in absorbance were found at concentrations equal to or higher than 280 mg l^{-1} phloroglucinol, which suggests that phloroglucinol affected cell membrane permeability.

Transmission electron microscopy

Comparison of control cells in Fig. 5 and cells exposed to phloroglucinol in Fig. 6 suggests that micro-organisms were affected by the presence of phloroglucinol and supports the observations above regarding membrane leakage results. Field shots of bacterial cells from Fig. 5(a) (control) show the diversity of micro-organisms found in the mixed anaerobic culture with normal cellular morphology observed at higher magnification (Fig. 5c) and a smooth continuous cell envelope structure (Fig. 5d). Figure 6(a)

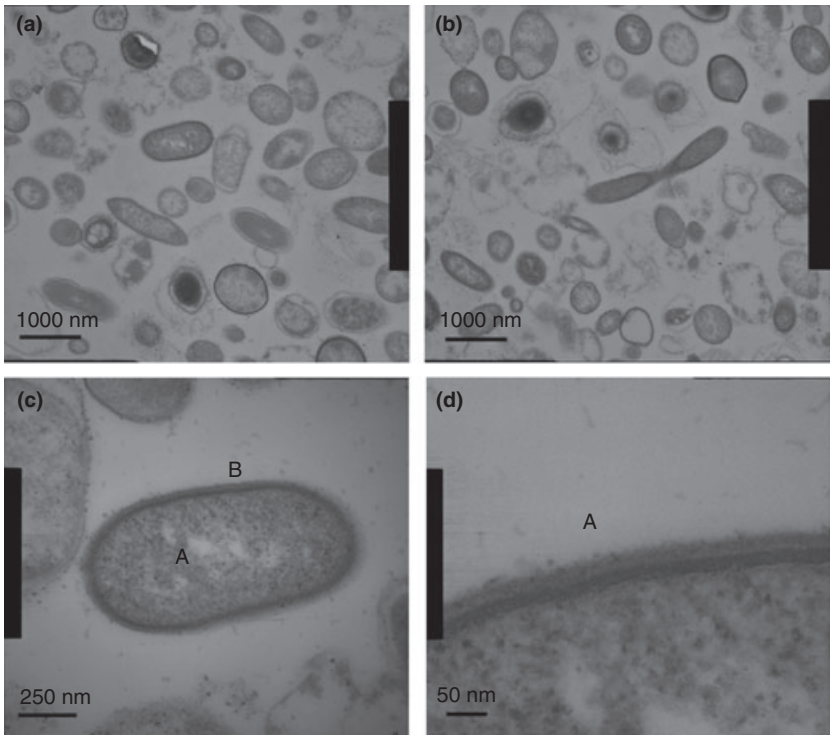

Figure 5 Transmission electron micrographs of mixed anaerobic microbial cultures exposed to water at 15 000× magnification (a & b), 60 000× magnification (c) and 200 000× magnification (d). Figure 5(c) shows normal cytosolic components (A) and an intact cell envelope (B). Figure 5(d) shows fine details of the intact envelope structure (A).

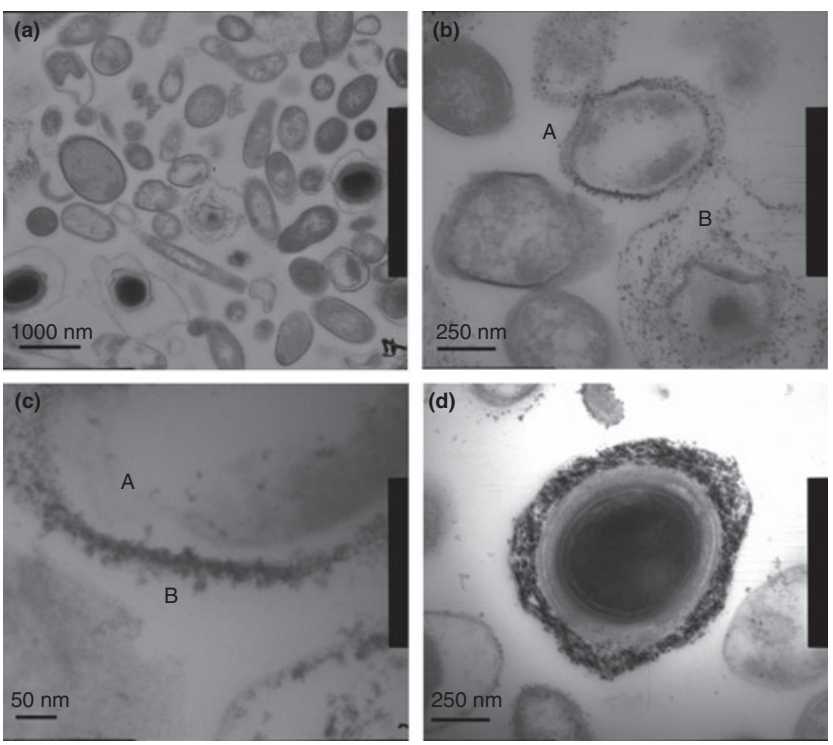

Figure 6 Transmission electron micrographs of mixed anaerobic microbial cultures exposed to phloroglucinol at 15 000× magnification (a), 60 000× magnification (b & d) and 200 000× magnification (c). Figure 6(a) shows densely packed micro-organisms with many spore-like structures. Figure 6(b) shows a disrupted outer membrane structure (A) and the coagulation of exopolysaccharides (B). Figure 6(c) shows fine details of crenation or separation of the cytoplasmic membrane from the cell envelope (A) and 'blebbing' (B). Figure 6(d) shows details of an endospore.

(phloroglucinol test) shows many spore-like structures and cells with disrupted outer membrane structures. These observations are supported at higher magnification in Fig. 6(b) with evident signs of membrane structure disruption (A) and the coagulation of exopolysaccharides (B). Cells exposed to phloroglucinol exhibit signs of

membrane dysfunction as seen in Fig. 6(c), with separation of the cytoplasmic membrane from the cell envelope (A) and 'blebbing' (B). Figure 6(d) shows fine details of an endospore created by micro-organisms under unfavourable conditions.

Morphological changes shown in Fig. 7 and 8 suggest strong extra- and intracellular effects induced by cell exposition to phlorotannins. Figure 7(a) is a field view of bacterial cells showing extensively disturbed cell morphology (A), but also some apparently intact cells (B). From Fig. 7(b), clear evidence of cell disturbance can be observed with what appears to be cells fused together as a result of membrane disturbance. Figure 7(c) shows details of laminar structures (A) with apparent cell membrane fusion.

Debris of what seems to be cell components coagulated by phlorotannin observed visually and mentioned earlier during leakage assays can be observed in most of the images with fine details shown in Fig. 7(d). Figure 8(a) shows evidence of crenation (A) and coagulation of cytosolic components (B). Similarly to the observation made after the exposition of cells to phloroglucinol, Fig. 8(b) shows spore-like structures (A) and a cell arrested at the early stage of the sporulation process (B). Figure 8(c) shows a higher magnification of a sporulating cell with bacterial DNA supercoiling (A) and an irregular cell envelope (B) as confirmed in Fig. 8(d) where electron dense precipitated deposits can be seen on the surface of the cell membrane.

Discussion

The pathway of phloroglucinol degradation under anaerobic conditions is believed to consist of its conversion to a phenol intermediate by removal of ring substituents, followed by ring fission and formation of cyclohexanol and cyclohexanone. Hence, yielding organic acid metabolites, which are later converted to methane through methanogenesis (Young and Rivera 1985). It is reasonable to assume that any inhibitory effect induced by phloroglucinol will tend to decrease once the benzene ring has been damaged and the original compound is metabolized to methane precursors by phloroglucinol-tolerant micro-organisms. This would explain the kinetic inhibition observed for methane generation and VFA removal during the first 8 days of our experiment and the similar levels of cumulative methane at the end of the experiment. The degradation of phlorotannins presumably follows a similar pathway, but the metabolism of high-weight phenolic compounds is likely to depend on polymerization levels with ring fission being more complex for highly polymerized compounds. However, similar initial biogas production and VFA removal show that no inhibitory effect could be observed during the fist few days of the test, whilst methane produced at the end of the experiment decreased almost proportionally to phlorotannin levels. During anaerobic digestion, biomass synthesis typically consumes 5% of the organic

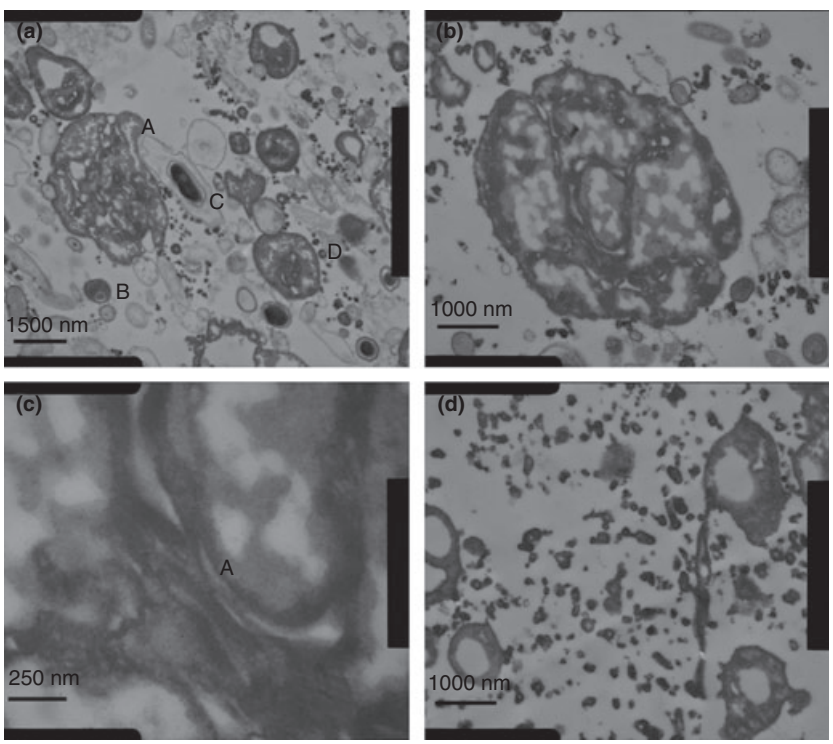

Figure 7 Transmission electron micrographs of mixed anaerobic microbial cultures exposed to phlorotannin at 7500× magnification (a), 15 000× magnification (b & d) and 60 000× magnification (c). Figure 7(a) shows affected cells (A), apparently intact cells (B), spore-like structures (C) and crenated cells (D). Figure 7(b) shows cells fused together. Figure 7(c) shows fine details of laminar structures resulting from cell membrane fusion (A). Figure 7(d) shows debris of cell components.

matter (Symons and Buswell 1933) and most of the remaining degradable fraction is expected to be transformed into methane if favourable conditions for methanogenesis are provided. An indication of organic matter is obtained through the monitoring of COD and a stoichiometric conversion to methane is usually assumed. In this experiment, final levels of COD_S were found to be similar (350 mg l^{-1}) in all phlorotannin-treated cultures, indicating that most of the organic matter was used for methane generation or biomass growth with only a small nonbiodegradable fraction remaining. This suggests that whilst most of acetic acid was removed and COD_S consumed, a smaller fraction of organic matter could be converted to methane in cultures containing phlorotannin. When assessing the impact of antimicrobials on acetoclastic methanogens, Cetecioglu *et al.* (2012) observed the removal of acetic acid, even when almost no methane was produced. The authors interpreted the phenomenon to be an uncompetitive inhibition caused by the antimicrobials tested. It is known that under unfavourable conditions, cells can synthesize organic solutes for survival, but energy requirements are consequently increased and metabolic activity is likely to be reduced (Muller *et al.* 2005). In the light of the COD levels found during this experiment, this latter theory is the most probable, as the methane production and VFA removal results indicate that higher energy consumption was required by micro-organisms for survival under stress induced by phlorotannins. Field *et al.* (1989) found that small oligomers seemed more toxic at early stage than larger sized compounds close to high molecular weight tannins. A similar observation can be made in this study with a kinetic inhibition triggered by phloroglucinol at the beginning of the assay opposed to the residual impact of phlorotannin.

Microscopy observations of cells exposed to phloroglucinol suggest that phloroglucinol interacts with the bacterial envelope and triggers survival mechanisms, such as endosporulation. However, cell membrane interactions are influenced by the exact nature of the bacterial cell envelope and cells seems to be affected to a different extent depending on their exact morphology. In the light of the cumulative methane production obtained during batch tests (Fig. 2a), this would confirm that the toxicity of phloroglucinol decreases through its degradation by tolerant micro-organisms inducing benzene ring fission and later conversion to methane.

Microscopy results also suggest that disturbance of the cell envelope is a key step associated with the bactericidal action of phlorotannins and similar effects have been reported by Wang *et al.* (2009) when studying the sensitivity of *E. coli* to phlorotannins. However, cell membrane disturbance could not be observed through 260–nm absorbing material leakage presumably due to the coagulation and sedimentation of phlorotannins with cell components that prevented their measurement *via* spectrophotometry. Final methane production (Fig. 2b) and VFA levels (Fig. 3b) suggest

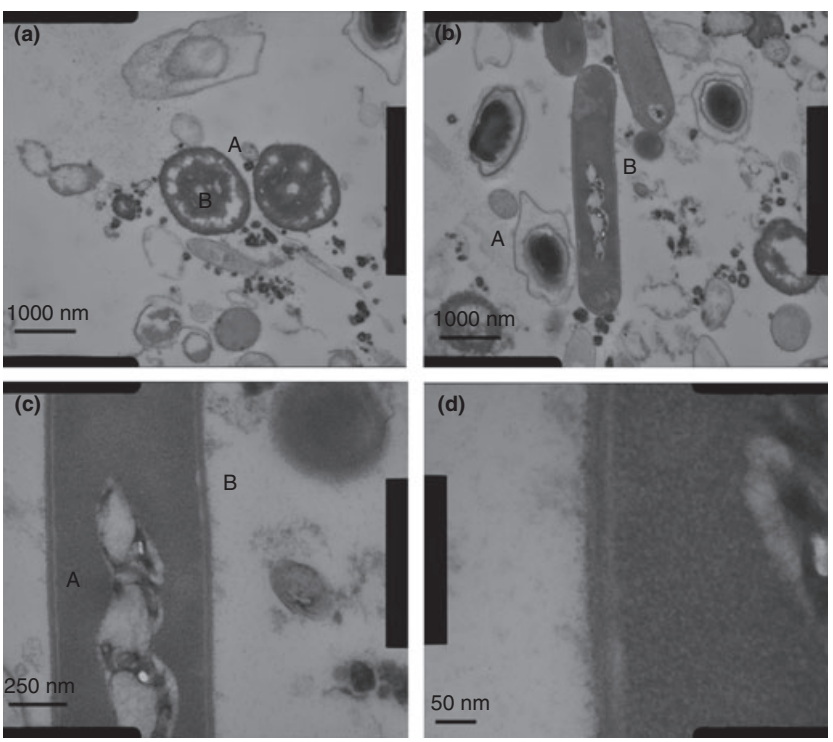

Figure 8 Transmission electron micrographs of mixed anaerobic microbial cultures exposed to phlorotannin at 15 000× magnification (a & b), 60 000× magnification (c) and 200 000× magnification (d). Figure 8 (a) shows two crenated cells (A) and the arrested early stage development of endospores (coagulated nucleic acids) (B). Figure 8(b) shows spore-like structures (A) with a cell arrested at the early stage of sporulation (B). Figure 8(c) shows DNA supercoiling (A) and irregular cell membrane structure. Figure 8(d) shows fine details of a cell membrane with electron dense precipitated deposits.

that, whilst strong cellular effects have been induced by phlorotannins on most of the cell, some micro-organisms proved less susceptible, as no intermediate components accumulated during the experiment and methanogenesis occurred at all levels tested. The decrease in methane production associated with the increase in phlorotannin concentration tends to show that a higher fraction of organic matter was consumed for biomass synthesis under unfavourable conditions and cell morphological changes observed by microscopy. From batch test results and microscopic observations, it appeared that phlorotannins induce a stronger inhibition on micro-organisms than phloroglucinol (nonpolymerized monomer of phlorotannin), which effect was overcome after a few days. Thus, the bactericidal activity of phlorotannins seems to be function of the level of polymerization of the compounds, which is in accordance with findings from Nagayama *et al.* (2002) who observed bactericidal activity of both crude and purified phlorotannins against pathogenic bacteria with the exception of phloroglucinol. This finding bears comparison with the known mode of action of some other phenolic compounds of a biocidal nature, such as polymeric biguanides and particularly polyhexamethylene biguanides (PHMB). It has been shown that their bactericidal properties are such that their activity increases in proportion to the increasing level of polymerization and its subsequent effect upon the bacterial inner membrane (Gilbert *et al.* 1990).

Results obtained in this study exhibit the effect of phlorotannins on mixed microbial cultures that can be found in anaerobic digesters. Through electron microscopic observations, it has been shown that phlorotannins induce significant extra- and intracellular effects upon cells, with the disruption of cell membranes observed with most micro-organisms. Results also suggest that the bactericidal activity of phlorotannin is function of the level of polymerization of the compounds. Phloroglucinol was found to cause a kinetic inhibition at the beginning of the biodegradation process, whilst phlorotannin had a residual impact on methane production. The use of brown seaweed as a potential sole and cosubstrate for anaerobic digestion is thus likely to be dependant on phlorotannin concentrations and their bactericidal effect on micro-organisms. However, inhibitory concentrations will be a function of polymerization levels and dominant microbial species found in anaerobic systems.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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