Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunaliella tertiolecta* biomass

Aino-Maija Lakaniemi1*, Christopher J Hulatt2, David N Thomas2,3, Olli H Tuovinen1,4 and Jaakko A Puhakka1

**Abstract**

**Background:** Microalgae are a promising feedstock for biofuel and bioenergy production due to their high photosynthetic efficiencies, high growth rates and no need for external organic carbon supply. In this study, utilization of *Chlorella vulgaris* (a fresh water microalga) and *Dunaliella tertiolecta* (a marine microalga) biomass was tested as a feedstock for anaerobic H2 and CH4 production.

**Results:** Anaerobic serum bottle assays were conducted at 37°C with enrichment cultures derived from municipal anaerobic digester sludge. Low levels of H2 were produced by anaerobic enrichment cultures, but H2 was subsequently consumed even in the presence of 2-bromoethanesulfonic acid, an inhibitor of methanogens. Without inoculation, algal biomass still produced H2 due to the activities of satellite bacteria associated with algal cultures. CH4 was produced from both types of biomass with anaerobic enrichments. Polymerase chain reaction-denaturing gradient gel electrophoresis profiling indicated the presence of H2-producing and H2-consuming bacteria in the anaerobic enrichment cultures and the presence of H2-producing bacteria among the satellite bacteria in both sources of algal biomass.

**Conclusions:** H2 production by the satellite bacteria was comparable from *D. tertiolecta* (12.6 ml H2/g volatile solids (VS)) and from *C. vulgaris* (10.8 ml H2/g VS), whereas CH4 production was significantly higher from *C. vulgaris* (286 ml/g VS) than from *D. tertiolecta* (24 ml/g VS). The high salinity of the *D. tertiolecta* slurry, prohibitive to methanogens, was the probable reason for lower CH4 production.

**Background**

Photosynthetic biomass-based fuels are widely considered as viable contenders as sustainable alternatives to fossil fuels. Currently, the major share of biofuels and other forms of bioenergy are produced from terrestrial plants [1]. Microalgae may prove an alternative to terrestrial crops because they have higher photosynthetic efficiencies, higher yields and growth rates, and fewer requirements for cultivation land and they can be grown in saline waters and in arid and barren land areas [1,2]. Microalgal biomass is potent for anaerobic conversion as it can have a high content of lipids, carbohydrates and proteins, and does not contain recalcitrant lignin [1-3]. However, the robust cell walls of some microalgal species may limit digestibility [4,5].

Anaerobic digestion of microalgal biomass for CH4 production has been studied at various temperatures and with various pretreatments and cosubstrates [4,6-9]. For example, Chen and Oswald [4] reported that pretreatment of algal biomass at 100°C for 8 h increased digestibility by up to 33%, but the energy consumed in pretreatment was higher than the enhancement gained in CH4 production [8].

Some green microalgae, such as *Chlamydomonas reinhardtii* [10] and *Chlorella salina* [11] produce hydrogen under anaerobic conditions via direct photolysis [12]. However, despite extensive research this process has low yields and is rather feeble. It is filled with metabolic and technical obstacles [13] and remains an unlikely source of sustainable energy. Indirect photolysis of microalgal biomass by first hydrolyzing the biomass with lactic acid
bacteria followed by photosynthetic H₂ production resulted in H₂ yields up to 8 mol H₂/mol starch glucose from C. reinhardtii (66% starch conversion efficiency) [14]. Carver et al. [5] reported H₂ production from dark fermentation of Chlorella vulgaris and Dunaliella tertiolecta at 60°C. Further, Gfeller and Gibbs [15], Miura et al. [16] and Ueno et al. [17] reported hydrogen fermentation by microalgal cells under dark, anaerobic conditions.

The aim of this study was to examine the formation of H₂ and CH₄ from microalgal biomass. Two green microalgae, Chlorella vulgaris (a freshwater species) and Dunaliella tertiolecta (a marine species) were used as feedstocks. Experiments were carried out in batch bottles at 37°C without pretreatment of the algal biomass, and the microbial communities were characterized by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) profiling of 16S rRNA gene and sequencing.

Results

Algal biomass feedstocks

The chemical composition of the two microalgal biomass feedstocks was different. C. vulgaris contained 36%, 13% and 8% of proteins, lipids and sugars on a dry weight basis, respectively. The corresponding mass composition of D. tertiolecta was 15%, 11% and 4%, respectively. In general, these values are lower than previously reported in the literature (Additional file 1, Table S1). The compositional data for D. tertiolecta in particular may reflect loss of cellular constituents upon sample preparation and handling because the marine microalga does not have a rigid wall and is prone to lyse when the osmotic pressure changes. Growth conditions were not varied to determine the corresponding changes in cellular fractions.

Enrichment cultures

Four different cultures were enriched from the initial anaerobic digester sludge. Two H₂-fermenting cultures, one with C. vulgaris biomass, designated as B-C, and one with D. tertiolecta biomass as the substrate, B-D, and two CH₄-producing cultures, one utilizing C. vulgaris biomass, U-C, and one D. tertiolecta biomass, U-D. Methanogenesis was suppressed in the H₂-fermenting cultures by addition of 20 mM 2-bromoethanesulfonic acid (BESA). During enrichment phases 1-5 no H₂ was produced in any of the cultures, while in enrichment phases 6 to 9 low levels of H₂ were detected in B-C and B-D enrichments during the first few days, but usually by day 5 the H₂ level had decreased below detection limit (results not shown). No CH₄ was produced in the cultures with added BESA (Figure 1).

With U-C and U-D, the CH₄ production was higher from D. tertiolecta biomass than from C. vulgaris biomass in the first enrichment phase when tested with a combination of 25% algal biomass and 75% activated sludge (Figure 1A). From phase 2 onwards, when the proportion of algal biomass in the substrate was increased to 50% or higher, CH₄ production from C. vulgaris surpassed that from D. tertiolecta (Figure 1B-F). With 100% C. vulgaris and D. tertiolecta biomass the rates of CH₄ production ranged between 3.4-6.5 and 1.2-4.9 ml/day and the lag times between 2.6-5.1 and 5.3-10 days, respectively. The CH₄ yield and CH₄ production rate decreased and the lag time increased from D. tertiolecta as the enrichment proceeded. The CH₄ yields from C. vulgaris remained more or less constant after enrichment phase 4 (Figure 1).

H₂ and CH₄ production potential

Gas production potential from C. vulgaris and D. tertiolecta was studied using the enrichment cultures after nine passages. Some CO₂ was produced in all bottles indicating degradation in all cultures, including all controls with no anaerobic inoculum (Table 1, Figures 2B and 3B). CO₂ production was higher from C. vulgaris compared to D. tertiolecta.

H₂ was produced in all cultures including the controls on day 1. With glucose in particular, high levels of H₂ were produced during first few days. Over time H₂ decreased to undetectable levels in all cultures except those with algal biomass without inoculum and cultures with glucose and B-D. In the other cultures H₂ was consumed due to interspecies H₂ transfer, and cumulative H₂ production from algal biomass with the anaerobic inocula was negligible (Table 1). With no anaerobic inoculum, H₂ production was higher from D. tertiolecta biomass, 8.4 and 12.6 ml H₂/g volatile solids (VS), than from C. vulgaris biomass, 7.9 and 10.8 ml H₂/g VS, with and without BESA, respectively. Further enhancement of H₂ production was attempted by using these cultures as inoculum in batch incubations, but after four enrichment steps no increase in H₂ production was detected.

No CH₄ was produced in the cultures amended with BESA (Figure 2). A significant amount of CH₄ was produced only with C. vulgaris and U-C, glucose and U-C, and glucose and U-D (Table 1). Some CH₄ was also produced with D. tertiolecta and U-D as well as with chitosan and U-C (Table 1). CH₄ production from cellulose was negligible. CH₄ production from chitosan was significantly lower than that from microalgal biomass. Gas production in controls with no substrate but inoculum was very low, and was taken into account in calculation of the gas production yields (Table 2). Thus, CH₄ was produced from both C. vulgaris and D. tertiolecta biomass, while the yield was substantially lower with D.
tertiolecta than with C. vulgaris (Table 2). With C. vulgaris biomass 30.6% of organic carbon was released as CH$_4$ and 13.6% as CO$_2$, while with D. tertiolecta biomass the corresponding values were 5.2 and 2.6%, respectively. CH$_4$ production from C. vulgaris biomass was higher than in glucose controls, while CH$_4$ production from D. tertiolecta remained far below that of the glucose controls. With glucose, cellulose or chitosan, the H$_2$ production was generally higher with the B-D enrichment than with the B-C enrichment, but CH$_4$ production was generally higher with the U-C enrichment than with the U-D enrichment (Table 1).

The average chloride ion concentration in the anaerobic incubations was 0.7 and 4.8 g/l and sodium ion concentration was 2.3 and 2.1 g/l in bottles with C. vulgaris and D. tertiolecta as the substrate, respectively. The pH

![Figure 1](http://www.biotechnologyforbiofuels.com/content/4/1/34)
of the medium was not adjusted at the beginning of the anaerobic incubation. The initial pH was 8.0 in the cultures with algal biomass and 8.5 with the other substrates and the cultures with no substrate. With no substrate, cellulose and chitosan the pH changes were minimal, pH ranging from pH 8.0 to 8.5 during the incubation. With algal biomass, but no inoculum the pH varied between 7.5 and 8.0. With *C. vulgaris* and *U-C* the pH was 7.5-8.0, with *C. vulgaris* and *B-C* 7.0-8.0, with *D. tertiolecta* and *U-D* 8.0-8.5, and with *D. tertiolecta* and *B-D* 7.5-8.0. In cultures with glucose the pH varied between 6.0 and 8.5.

Organic acids accumulated in the cultures with the *B-C* and *B-D* enrichments as well as in the cultures with no substrate. With no substrate, cellulose and chitosan the pH changes were minimal, pH ranging from pH 8.0 to 8.5 during the incubation. With algal biomass, but no inoculum the pH varied between 7.5 and 8.0. With *C. vulgaris* and *U-C* the pH was 7.5-8.0, with *C. vulgaris* and *B-C* 7.0-8.0, with *D. tertiolecta* and *U-D* 8.0-8.5, and with *D. tertiolecta* and *B-D* 7.5-8.0. In cultures with glucose the pH varied between 6.0 and 8.5.

Organic acids accumulated in the cultures with the *B-C* and *B-D* enrichments as well as in the cultures with no substrate. With no substrate, cellulose and chitosan the pH changes were minimal, pH ranging from pH 8.0 to 8.5 during the incubation. With algal biomass, but no inoculum the pH varied between 7.5 and 8.0. With *C. vulgaris* and *U-C* the pH was 7.5-8.0, with *C. vulgaris* and *B-C* 7.0-8.0, with *D. tertiolecta* and *U-D* 8.0-8.5, and with *D. tertiolecta* and *B-D* 7.5-8.0. In cultures with glucose the pH varied between 6.0 and 8.5.

Table 1 Metabolite production in all cultures: cumulative gas production and accumulation of metabolites in the test cultures after 49 day of incubation

| Substrate | Inoculum | H₂ (ml) | CH₄ (ml) | CO₂ (ml) | Sum of VFA and alcohols (mM) |
|-----------|----------|---------|----------|----------|-------------------------------|
| None      | U-C      | 0.0 ± 0.0 | 0.2 ± 0.3 | 2.3 ± 0.4 | 0.6 ± 0.7                     |
| None      | B-C      | 0.0 ± 0.0 | 0.0 ± 0.0 | 4.7 ± 0.2 | 5.1 ± 0.5                     |
| None      | U-D      | 0.0 ± 0.0 | 0.0 ± 0.0 | 18 ± 0.0  | 0.5 ± 0.1                     |
| None      | B-D      | 0.0 ± 0.0 | 0.0 ± 0.0 | 45 ± 0.2  | 3.7 ± 0.4                     |
| *Chlorella vulgaris* | None | 2.1 ± 0.7 | 0.0 ± 0.0 | 108 ± 0.6 | 22.1 ± 1.8                    |
| *C. vulgaris* and *BESA* | None | 1.3 ± 0.2 | 0.0 ± 0.0 | 120 ± 0.7 | 19.1 ± 5.1                    |
| *Dunaliella tertiolecta* | None | 2.8 ± 0.1 | 0.0 ± 0.0 | 3.0 ± 0.4 | 5.1 ± 0.1                     |
| *D. tertiolecta* and *BESA* | None | 1.5 ± 0.3 | 0.0 ± 0.0 | 4.2 ± 0.2 | 4.0 ± 0.3                     |
| *C. vulgaris* | U-C      | 0.0 ± 0.0 | 74.9 ± 3.6 | 35.2 ± 0.3 | 3.8 ± 1.0                     |
| *C. vulgaris* | B-C      | 0.1 ± 0.0 | 0.0 ± 0.0 | 248 ± 0.0 | 31.2 ± 0.7                    |
| *D. tertiolecta* | U-D      | 0.0 ± 0.0 | 47 ± 0.2  | 49 ± 0.0  | 0.4 ± 0.1                     |
| *D. tertiolecta* | B-D      | 0.0 ± 0.0 | 0.0 ± 0.0 | 74 ± 0.8  | 8.9 ± 0.4                     |
| Glucose   | U-C      | 4.9 ± 0.4 | 56.4 ± 0.1 | 62.5 ± 0.4 | 29 ± 0.7                     |
| Glucose   | B-C      | 7.1 ± 0.4 | 0.0 ± 0.0 | 57.9 ± 0.9 | 46 ± 0.4                     |
| Glucose   | U-D      | 5.2 ± 0.9 | 38.5 ± 1.0 | 56.2 ± 7.0 | 13.3 ± 14.9                   |
| Glucose   | B-D      | 14.6 ± 2.3 | 0.0 ± 0.0 | 60.8 ± 0.5 | 44.4 ± 7.1                    |
| Cellulose | U-C      | 0.0 ± 0.0 | 0.3 ± 0.4  | 0.9 ± 0.1  | 0.7 ± 1.4                     |
| Cellulose | B-C      | 0.0 ± 0.0 | 0.0 ± 0.0 | 40 ± 0.0  | 7.6 ± 0.4                     |
| Cellulose | U-D      | 0.0 ± 0.0 | 0.1 ± 0.1  | 1.8 ± 0.1  | 0.0 ± 0.1                     |
| Cellulose | B-D      | 0.0 ± 0.0 | 0.0 ± 0.0 | 46 ± 0.3  | 5.4 ± 13                     |
| Chitosan  | U-C      | 0.0 ± 0.0 | 36 ± 4.1  | 2.4 ± 2.5  | 0.4 ± 1.5                     |
| Chitosan  | B-C      | 0.0 ± 0.0 | 0.0 ± 0.0 | 3.1 ± 0.3  | 6.9 ± 0.5                     |
| Chitosan  | U-D      | 0.0 ± 0.0 | 0.0 ± 0.0 | 1.0 ± 0.0  | 0.1 ± 0.1                     |
| Chitosan  | B-D      | 0.0 ± 0.0 | 0.0 ± 0.0 | 3.2 ± 0.1  | 5.6 ± 0.8                     |

A minus sign in front of sum of VFA and alcohols indicates that the sum of VFA and alcohols was higher on day 0 than on day 49. The values include standard errors.

H₂-fermenting cultures: *C. vulgaris* biomass as substrate = B-C; *D. tertiolecta* biomass as substrate = B-D; CH₄-producing cultures: *C. vulgaris* biomass as substrate = U-C; *D. tertiolecta* biomass as substrate = U-D.

BESA = 2-bromoethanesulfonic acid; VFA = volatile fatty acids.

The initial total chemical oxygen demand (CODₜₒᵗ) values were significantly higher in cultures with *C. vulgaris* than with *D. tertiolecta* in spite of identical initial concentrations of algal VS in all cultures. The addition of 20 mM BESA also increased the initial COD concentration. The CODₜₒᵗ concentrations decreased in all cultures between days 0 and 49, except in the case of no substrate and in cultures with *C. vulgaris*, BESA and no inoculum. The decrease in CODₜₒᵗ was greater in bottles with U-C and U-D than with B-C and B-D as inoculum, respectively (Figure 4A). CODₜₒᵗ reduction was 52% with *C. vulgaris* and U-C, and 57% *D. tertiolecta* and U-D, but only 21% with *C. vulgaris* and B-C, and 15% with *D. tertiolecta* and B-D, respectively. The ratio of soluble COD (CODₛ) to CODₜₒᵗ decreased with CH₄ production, but increased in the other cultures (Figure 4B).
The COD results were in line with the VFA and alcohol results.

**Microbial community composition**

Based on bacterial PCR-DGGE and sequencing, the initial anaerobic inoculum contained bacteria belonging to phyla Firmicutes, Bacteroidetes, Proteobacteria and Chloroflexi (Additional file 2, Table S2). No genus and species level information for these bacterial sequences were obtained from GenBank.

The bacterial community became enriched during the ten serial batch incubations. Bacterial DGGE profiles were different with the two algal biomass types. The addition of BESA also affected the bacterial community composition (Figure 5). For example, bands B13 and B29 were only clear with *C. vulgaris* and B-C, but not with *C. vulgaris* and U-C. Further, bands B18 and B21 were only clear in *C. vulgaris* and U-C, but not with *C. vulgaris* and B-C. In addition, bands B30-B33 were present in cultures with *D. tertiolecta*, but no corresponding bands were seen in cultures with *C. vulgaris* (Figure 5).

Most of the bacterial 16S rDNA sequences amplified from the anaerobic enrichments matched uncultured bacteria with no species-level information (Additional file 3, Table S3). The matches in the enrichments were *Petrimonas* spp. (band B14), *Bacteroides* spp. (B15), *Bilophila wadsworthia* (B26), *Wolinella succinogenes* (B34), *Oceanibulbus indolifex* (B35), and *Syntrophobacter* spp. (B39). *Petrimonas* spp. were present in all cultures with *C. vulgaris* and anaerobic inoculum, *B. wadsworthia* in *C. vulgaris* and B-C and *Bacteroides* spp. in *C. vulgaris* and U-D as well as in the duplicates of *D. tertiolecta* and U-D. *W. succinogenes*, *O. indolifex* and *Syntrophobacter* spp. were present in all cultures with *D. tertiolecta* and anaerobic inoculum (Figure 4).

A high diversity of bacteria was also present in cultures with no anaerobic inoculum (Figure 4 and Additional File 4, Table S4). These bacteria included *Acidobacterium* spp. (band B44), *Clostridium* spp. (B45,
B46, B47, B61), Clostridium celerecrescens (B48, B63), Brevundimonas spp. (B49), Hafnia alvei (B50, B54), Hafnia alvei or Obesumbacterium proteus (B51), Gordonia terrae (B56), Clostridium sulfidigenes (B57, B58, B59, B60), Oceanibulbus indolifex (B62), Roseobacter spp. (B65), Exiguobacterium spp. (B66), Bacillus thermoamylovorans (B67) and four unknown species (B52, B53, B55, B64).

**Figure 3** Metabolite production in the cultures without anaerobic enrichments. H$_2$ (A), CO$_2$ (B) and the main fermentation products acetate (C) and formate (D) where closed circles = Chlorella vulgaris and no inoculum, closed squares = C. vulgaris, 2-bromoethanesulfonic acid (BESA) and no inoculum, closed triangles = Dunaliella tertiolecta and no inoculum, and crosses = D. tertiolecta, BESA and no inoculum.

|                   | mmol x$^a$ per l | mmol x$^a$ per g volatile solids | mmol x$^a$ per g added COD$_{tot}$ | mmol x$^a$ per g removed COD$_{tot}$ |
|-------------------|------------------|-----------------------------------|-------------------------------------|--------------------------------------|
| CH$_4$            |                  |                                   |                                     |                                      |
| C. vulgaris and U-C | 5.96             | 11.9                              | 5.8                                 | 11.3                                 |
| C. vulgaris and B-C | 0                | 0                                 | 0                                   | 0                                    |
| D. tertiolecta and U-D | 5.1             | 1.0                               | 2.1                                 | 3.6                                  |
| D. tertiolecta and B-D | 0                | 0                                 | 0                                   | 0                                    |
| H$_2$             |                  |                                   |                                     |                                      |
| C. vulgaris and no inoculum | 2.3             | 0.45                              | 0.23                                | 1.7                                  |
| C. vulgaris, BESA and no inoculum | 1.6             | 0.33                              | 0.15                                | 4.0                                  |
| D. tertiolecta and no inoculum | 2.6             | 0.52                              | 1.6                                 | 21.1                                 |
| D. tertiolecta, BESA and no inoculum | 1.7             | 0.35                              | 0.42                                | 4.0                                  |

$^a$Where x is CH$_4$ or H$_2$, as indicated.$^b$H$_2$ yield per g removed COD$_{tot}$ could not be calculated as no COD$_{tot}$ reduction was detected.

BESA = 2-bromoethanesulfonic acid; COD$_{tot}$ = total chemical oxygen demand.
H₂ was produced from both *C. vulgaris* and *D. tertiolecta* biomass by the H₂ enrichment cultures (containing BESA), but it was subsequently consumed by non-methanogenic microorganisms. The pH was relatively high in these assays. In the cultures with added anaerobic inoculum, H₂ production was most sustained in the positive controls with glucose, where the pH was also the lowest. Karadag and Puhakka [18] showed with an anaerobic, moderately thermophilic (45°C) enrichment culture that the pH significantly affected H₂ production from glucose due to pH mediated shifts in fermentation pathways and the bacterial community composition. They reported pH 5.0 was optimal for H₂ production.

In the present work, several bacteria were identified from the anaerobic inoculum and algal biomass. These included *Petrimonas* spp. that have been previously shown to produce H₂ [19]. *Syntrophobacter* spp. have been shown to convert propionate to acetate, H₂ and CO₂, but only when cocultivated with H₂-consuming organisms [20,21]. *B. wadsworthia* and *W. succinogenes* utilize H₂ as their electron donor [22,23]. According to Chassard *et al.* [24] *Bacteroidetes* spp. can suppress H₂ production from cellulotic material in a mixed culture because they are non-H₂-producing bacteria with a relatively high cellulosic activity. *O. indolifex* is an obligately aerobic marine bacterium [25] with no activity under anaerobic conditions and thus it originated from the algal biomass slurry.

H₂ accumulated in the cultures supplemented only with algal biomass. These cultures formed CO₂ and accumulated organic acids and alcohols. Gfeller and Gibbs [15], Miura *et al.* [16] and Ueno *et al.* [17] reported hydrogen fermentation by microalgal cells under dark and anaerobic conditions, with H₂ yields up to 2 mmol H₂/g dry weight [16]. In this study, H₂ yields were approximately 25% of that in the cultures with no added anaerobic inoculum (Table 2). However, the DGGE profiles had matches with several H₂-producing bacteria such as *Clostridium* spp. [26,27] and *Hafnia alvei* [28], which are known H₂ producers. Some *Bacillus* spp., such as *B. cereus*, *B. thuringiensis* [29] and *B. megaterium* [30] also produce H₂. According to Combet-Blanc *et al.* [31], *B. thermoamylovorans* does not produce H₂. *O. proteus* is typical in breweries and is known to cause beer spoilage [32]. Some *Exiguobacterium* spp. such as *E. profundum* are facultatively anaerobic and produce lactate as the main fermentation product [33].

Carver *et al.* [5] used the same algal biomass stocks but a different source inoculum to monitor metabolite production under thermophilic (60°C) conditions. They reported H₂ production without anaerobic inoculum by heterotrophs associated with *C. vulgaris* biomass, but low H₂ production with heterotrophs associated with *D.
tertiolecta. In the present study, the D. tertiolecta-associated bacteria produced somewhat more H₂, but approximately 4.5 times less VFA and alcohols and approximately 3 times less CO₂ than the C. vulgaris-associated bacteria. The higher H₂ production from D. tertiolecta was likely due to the lack of proper cell wall in D. tertiolecta and differences in bacterial composition of the algal biomass slurry. However, the H₂ yields reported in this study were low. For comparison, Park et al. [34] reported the production of 28 ml H₂ per g dry weight of the macroalga Laminaria japonica pretreated by ball milling and heat treatment at 120°C for 30 min using anaerobic sewage sludge as an inoculum. Carver et al. [5] reported production of 82 and 114 ml H₂/g VS from C. vulgaris and 39 and 58 ml H₂/g VS from D. tertiolecta by only microalgal associated bacteria and by a thermophilic consortium at 60°C, respectively.

In the cultures with no added anaerobic inoculum, H₂ production was somewhat lower with BESA in the medium. This indicates that BESA was inhibitory to some bacteria involved in fermentation. Bacteria present in cultures with no added anaerobic inoculum were associated with the algal culture or were introduced during handling of the biomass.

CH₄ was produced from both C. vulgaris and D. tertiolecta biomass, but the yields were not comparable. CH₄ production was approximately 12 times higher from C. vulgaris than from D. tertiolecta per added VS but only approximately 3 times higher per added or removed CODₜot (Table 2). Based on the chemical composition (protein, lipid and sugar content) of the two algal biomass feedstocks, theoretical CH₄ yield according to Sialve et al. [35] would be 463 and 261 ml CH₄/g VS from C. vulgaris and D. tertiolecta, respectively. The CH₄ yields obtained (286 and 24 ml CH₄/g VS) were 62% and 9% of the theoretical for C. vulgaris and D. tertiolecta, respectively. However, the cellular composition and major cellular fractions are greatly influenced by storage and culture conditions and cell age. Storage enhances cellular leakage, which was more pronounced with D. tertiolecta than with C. vulgaris. Based on Becker [36], C. vulgaris composition varies on average
in the range of 51% to 58% protein, 14% to 22% lipids, and 2% to 17% carbohydrate on dry weight basis. Sydney et al. [37] reported 29% proteins, 11% lipids and 14% sugars for *D. tertiolecta* and the closely related *D. salina* contains 57% protein, 6% lipids, and 32% carbohydrate [36]. Additional file 1, Table S1 is a compilation of composition data pooled from specific studies; it is apparent that the bulk cellular composition is a variable parameter.

The large difference in CH₄ production between the two algal biomasses was likely due to inhibition of digester sludge enrichment by the salinity in the marine *D. tertiolecta* slurry flocculated with NaOH [6,35,38]. Salt toxicity towards methanogens is generally caused by the cation portion of the salt [38], which in this case is Na⁺. For example, McCarty [38] has reported 3.5 to 5.5 g/l Na⁺ to be moderately toxic and concentrations above 8 g/l highly toxic to methanogens. Similarly, high Cl⁻ levels can also cause inhibition of non-marine methanogens. The levels of dissolved Na⁺ in cultures with *D. tertiolecta* in this study were 2.1 g/l indicating non-toxic levels of Na⁺. However, Cl⁻ concentration was significantly higher in cultures with *D. tertiolecta* than with *C. vulgaris* as the feedstock. It was also clearly seen from freeze-dried *D. tertiolecta* that salts were bound on the surface of the biomass. Similar salt precipitation was not seen in *C. vulgaris* biomass. Another reason for low CH₄ production from *D. tertiolecta* biomass may be that *W. succinogenes* was identified from cultures with *D. tertiolecta* and U-D, but not from cultures with *C. vulgaris* and U-C. Coexistence of *W. succinogenes* has been reported to markedly reduce CH₄ production [39].

Chen and Oswald [4] reported 320 ml CH₄/g VS from biomass of a mixed microalgal culture from high-rate sewage stabilization ponds heat treated at 100°C for 8 h. Yen and Brune [8] reported 143 ml CH₄/g VS from an algal mixture including *Scenedesmus* spp. and *Chlorella* spp. without pretreatment. Thus, the CH₄ yield achieved from *C. vulgaris* was comparable with previous results, but the yield from *D. tertiolecta* was very low. *C. vulgaris* biomass also contained some chitosan, used in flocculation of the biomass. Co-digestion of algal biomass (N-rich material) with C-rich material such as cellulose or chitosan may enhance digestibility [8]. However, the anaerobic enrichments used in this study were not able to utilize chitosan very efficiently. Thus the co-digestion effect was negligible and CH₄ was mainly produced from the algal biomass.

The calorific yields calculated for the maximum H₂ and CH₄ yields were 0.14 kJ/g VS (for H₂ production from *D. tertiolecta* without added anaerobic inoculum) and 10 kJ/g VS (for CH₄ production from *C. vulgaris* with enriched digester sludge without BESA). Hydrolytic pretreatment of algal slurries could substantially improve H₂ production from complex biomass substrate. *C. vulgaris* biomass was shown to be amenable to methanogenic digestion without pretreatment, whilst the high salt content of *D. tertiolecta* biomass likely lowered the CH₄ yields. However, based on CODₜₐₜ, approximately 50% of *C. vulgaris* biomass was degraded during methanogenic fermentation. Therefore, pretreatment could also enhance CH₄ production from the biomass of thick cell walled algae, such as *C. vulgaris*, but the energy cost of the pretreatment need to be considered.

### Conclusions

CH₄ was produced from *C. vulgaris* and *D. tertiolecta* biomass by mesophilic municipal anaerobic digester sludge enrichments. H₂ was also produced with the anaerobic enrichments but was concurrently consumed by non-methanogenic microorganisms. H₂ was produced by satellite bacteria associated with algal biomass. PCR-DGGE profiling demonstrated the presence of H₂ producing (for example, *Petrimonas* spp., *Syntrophobacter* spp.) and H₂ consuming bacteria (for example, *Bilophila wadsworthia*, *Wolinella succinogenes*) in the anaerobic enrichments and H₂ producing bacteria (for example, *Clostridium* spp., *Hafnia alvei*) among the satellite bacteria of both microalgal biomasses. H₂ production by the satellite bacteria was comparable from *D. tertiolecta* and from *C. vulgaris*, but CH₄ production by the anaerobic enrichments was substantially higher from *C. vulgaris* than from *D. tertiolecta*. The CH₄ yield obtained from *D. tertiolecta* biomass with the inoculum originating from anaerobic digester was likely limited by the high salinity of the biomass, while the low protein, lipid and carbohydrate content of the *D. tertiolecta* further lowered the CH₄ yield.

### Methods

**Microalgal biomass production and harvest**

*Chlorella vulgaris* (Culture Collection of Algae and Protozoa, UK strain 211/11B) and *Dunalieilla tertiolecta* (Sammlung von Algenkulturen Göttingen, Germany, strain SAG 13.86) were grown photautotrophically in 20 l column (diameter 0.16 m) photobioreactors with 0.5vvm air sparging and photosynthetically active radiation at photon flux density averaging 225 μmol/m²s. *C. vulgaris* was grown in milliQ-water-based Jaworski’s medium (http://www.ccap.ac.uk/media/recipes/JM.htm) and *D. tertiolecta* in natural seawater from the Menai Strait, UK, treated by filtration (0.2 μm) and UV irradiation, with nutrients supplied according to Walne’s medium (http://www.ccap.ac.uk/media/documents/Walnes.pdf).

Algal biomass was harvested from 20 l cultures by flocculation followed by centrifugation. *C. vulgaris* was harvested by adding a chitosan stock solution (4 g
chitosan, 50 ml acetic acid, 950 ml water) to the culture at approximately 2% of the total volume and adjusting pH to 7 by adding 3 M NaOH to initiate the flocculation. *D. tertiolecta* was flocculated by adding 50-100 ml of 3 M NaOH to raise the pH to approximately pH 9.5 [40]. The biomass of both species was then collected and centrifuged at 1,000 g for 10 min to produce a thick paste. The pH of *C. vulgaris* and *D. tertiolecta* biomass was adjusted to 7.0 ± 0.2 with HCl and the biomass slurries were stored at -20°C until used in the gas production experiments. The algal biomass stocks were normalized by measurements of VS.

**Experimental conditions**

Anaerobic inocula were enriched from an anaerobic digester treating municipal wastewater sludge (City of Tampere, Finland). Serum bottle enrichments were prepared as series of batch incubations at 37°C with 5 g VS/l of substrate. In the first three phases the substrate consisted of 25% (VS/VS) algal biomass and 75% (VS/VS) of activated sludge, followed by 50% of algal biomass and 50% of activated sludge, and finally 75% of algal biomass and 25% of activated sludge. In the following enrichment phases, 100% of algal biomass was used. Four different cultures were enriched. Two H₂-fermenting cultures, one with *C. vulgaris* biomass, designated as B-C, and one with *D. tertiolecta* biomass as the substrate, B-D, and two CH₄-producing cultures, one utilizing *C. vulgaris* biomass, U-C, and one *D. tertiolecta* biomass, U-D. Methanogenesis was suppressed in the H₂-fermenting cultures by addition of 20 mM BESA. The medium was prepared according to Zehnder et al. [42] and Ejlertsson et al. [43].

Gas production potential from *C. vulgaris* and *D. tertiolecta* was studied after nine passages of the corresponding enrichment culture at 37°C in 120 ml anaerobic serum bottles with 50 ml of medium and 10% (v/v) inoculum. The incubations included two types of negative controls, with inoculum but no substrate and with 5 g VS/l algal biomass but without anaerobic enrichment inoculum. Three types of positive controls were prepared containing enriched anaerobic inoculum and either 5 g/l glucose, 5 g/l cellulose or 5 g/l chitosan.

**Chemical analyses**

The VS concentrations of the biomass samples were measured according to the Finnish Standard SFS 3008 [44]. Carbon and nitrogen were measured with Thermo-Electron Flash EA 1112 after drying the samples at 80°C for 72 hours. The elemental analyzer was calibrated using the standards sulfanilamide, 2,5-bis(5′-tert-butylbenzoxazolyl)thiophene and l-cystine. dl-methionine was used as a reference material. Mass composition of the two microalgal biomass feedstocks was determined with analytical methods generally used in microalgal studies and at least three replicate samples were included in all analyses. The total lipid content of biomass was measured by extracting the lipids from freeze-dried biomass with chloroform/methanol and determining the lipids gravimetrically [45]. The protein composition of the algal biomass was calculated by multiplying the total elemental nitrogen content by 4.44 [46]. Total carbohydrate concentration of the biomass feedstocks was determined by the phenol sulfuric acid method [47]. Prior to biomass analyzes *D. tertiolecta* biomass was washed with 0.5 M ammonium formate.

Gas production was measured according to Owen et al. [48]. The headspace gas composition (H₂, CH₄ and CO₂) was measured using Shimadzu gas chromatograph GC-2014 equipped with Porapak N column (80/100 mesh) and a thermal conductivity detector. The temperatures of the oven, injector and detector were 80, 110 and 110°C, respectively. N₂ was used as carrier gas at a flow rate of 20 ml/min. The formation of organic acids and alcohol (lactate, formate, acetate, propionate, butyrate and ethanol) was analyzed with a Shimadzu HPLC chromatograph with a Shodex Sugar SH1011 column (Showa Denko K.K., Tokyo, Japan) and a refractive index detector (Shimadzu, Kyoto, Japan). Mobile phase was 5 mM H₂SO₄ and flow rate 0.9 ml/min. COD was analyzed before (CODₜot) and after filtration (CODₜₐₜ) through 0.45 µm polyester syringe filter (Macherey-Nagel, Düren, Germany) with dichromate method according to standard SFS 5504 [49]. Concentration of dissolved chloride ions was analyzed with Dionex DX-120 ion chromatograph equipped with AS40 auto sampler and IonPac AS23 (4 × 250 mm) anion exchange column. The mobile phase was Na-carbonate/Na-bicarbonate solution containing 4.5 mM/l Na₂CO₃ and 3 mM/l NaHCO₃. Concentration of dissolved sodium ions was analyzed with inductively coupled plasma emission mass spectrometry according to industry standard DIN EN ISO 17294.

**Microbial community analyses**

Duplicate samples of 1.5 ml were taken from the original digester sludge and from batch bottles at the end of the 49-day incubation and stored at -20°C. Prior to DNA extraction samples were pelleted by centrifugation (10,000 g, 5 min) and the supernatant removed. DNA was extracted from the pellets with PowerSoil DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The extracted DNA sample was used as a template for the PCR. Partial bacterial 16S rRNA genes of the community DNA were amplified by using primer pair GC-BacV3f [50] and 907r [51] as described by Koskinen et al. [26]. DGGE was performed with
INGENyphorU2×2-system (Ingeny International BV, GP Goes, The Netherlands) using 8% polyacrylamide gels with denaturing gradient from 30% to 70% (100% denaturing solution contains 7 M of urea and 40% formamide). Gels were run at 60°C in 1 × TAE (40 mM Tris, 20 mM acetic acid, 1 mM ethylenediaminetetra-acetic acid (EDTA), pH 8.3) with 100 V for 22 h and stained with SYBR Gold (Molecular Probes Invitrogen, Eugene, OR, USA). The dominant bands were excised from the gels, eluted in 20 μl of sterile water at 4°C overnight, stored at -20°C and reamplified for sequencing. Sequencing was conducted at Macrogen Inc. (Seoul, Korea). Sequence data were analyzed with BioEdit software and compared with sequences in GenBank.

Calculations
Cumulative H₂ and CH₄ production were calculated according to Logan et al. [52]. The data were fitted to a modified Gompertz equation [53] by minimizing the square of the measurements and the estimates subtraction to give lag times and H₂/CH₄ production rates. The calorific yields from maximum H₂ and CH₄ yields were calculated from the lower heating values, 120 MJ/kg for H₂ and 50 MJ/kg for CH₄.

Additional material

Additional file 1: Mass composition of various microalgae. Mass composition (dry weight basis) data of microalgae pooled from literature sources.

Additional file 2: Bacterial band identities from the initial sludge.
Matches of selected band identities of PCR-denaturing gradient gel electrophoresis (PCR-DGGE) samples from the initial anaerobic digester sludge.

Additional file 3: Bacterial band identities from the cultures with algal biomass and anaerobic enrichment inocula.
Matches of selected band identities of PCR-denaturing gradient gel electrophoresis (PCR-DGGE) samples from cultures with algal biomass and enriched anaerobic inocula.

Additional file 4: Bacterial band identities from the cultures with algal biomass and no anaerobic enrichments.
Matches of selected band identities of PCR-denaturing gradient gel electrophoresis (PCR-DGGE) samples from the cultures with algal biomass and no anaerobic inoculum.

Acknowledgements
This research was funded by the Finnish Funding Agency for Technology and Innovation (Finland) and EPRSC (UK). Industrial Case Award with RWE npower. We would like to thank Dr Elena Efimova for assistance with the lipid analyses.

Author details
1Department of Chemistry and Bioengineering, Tampere University of Technology, PO Box 541; FI-33101 Tampere, Finland. 2School of Ocean Sciences, College of Natural Sciences, Bangor University, Menai Bridge, Anglesey LL59 5AB, UK. 3Finnish Environment Institute, Marine Centre, PO Box 140, FI-00251 Helsinki, Finland. 4Department of Microbiology, Ohio State University, Columbus, OH 43210, USA.

Authors’ contributions
AML carried out the anaerobic cultivations and all related analyses, microbial community analyses, data interpretation, and drafting and completion of the manuscript. CJH carried out microalgal biomass production and harvesting, and the elemental analysis of the harvested biomass and participated in the drafting of the manuscript. DNT participated in the design of microalgal biomass production and reviewed the manuscript. OHT participated in the design of the study and data interpretation, and thoroughly reviewed the manuscript. JAP conceived the study, participated in data interpretation and thoroughly reviewed the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 31 January 2011 Accepted: 26 September 2011 Published: 26 September 2011

References
1. Schenk PM, Thomas-Hall SR, Stephens E, Marx UC, Mussgnug JH, Posten C, Kruse O, Hankamer B: Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenergy Res 2008, 1:20-43.
2. Posten C, Schaub G: Microalgal and terrestrial biomass as source for fuels - a process view. J Biotechnol 2009, 142:64-69.
3. Fan LT, Gharpunary MM, Lee YH: Evaluation and pretreatments for enzymatic conversion of agricultural residues. Biotechnol Bioeng Symp 1981, 11:29-45.
4. Chen PH, Oswald WJ: Thermochemical treatment for algal fermentation. Environ Int 1998, 24:889-897.
5. Cavan SM, Hulst CJ, Thomas DN, Tuovinen OH: Thermophilic, anaerobic co-digestion of microalgal biomass and cellulose for H₂ production. Biodegradation 2011, 22:805-814.
6. Sandum R, LeDuy A: Influence of mechanical and thermochemical pretreatments on anaerobic digestion of Spirulina maxima algal biomass. Biotechnol Lett 1983, 5:671-676.
7. Sandum R, LeDuy A: Improved performance of anaerobic digestion of Spirulina maxima algal biomass by addition of carbon-rich wastes. Biotechnol Lett 1985, 7:677-682.
8. Yen HW, Brune DE: Anaerobic co-digestion of algal sludge and waste paper to produce methane. Bioresour Technol 2007, 98:130-134.
9. De Schamphelaere L, Verstraete W: Revival of the biological sunlight-to-biogas energy conversion system. Biotechnol Bioeng 2009, 103:296-304.
10. Kossov S, Tryankov A, Seiber M, Ghirardi ML: Sustained hydrogen production by Chloramydomonas reinhardtii; effects of culture parameters. Biotechnol Bioeng 2002, 78:731-740.
11. Chader S, Hacene H, Agathos SN: Study of hydrogen production by three strains of Chlorella isolated from the soil in the Algerian Sahara. Int J Hydrogen Energy 2009, 34:4941-4946.
12. Meli A: Green alga hydrogen production: progress, challenges and prospects. Int J Hydrogen Energy 2002, 27:1217-1228.
13. Benemann JR: Hydrogen production by microalgae. J Appl Phycol 2000, 12:291-300.
14. Ike A, Toda N, Tsuji N, Hirasaki K, Miyamoto K: Hydrogen production from CO₂-fixing microalgal biomass: application of halotolerant photosynthetic bacteria. J Ferment Bioeng 1997, 84:606-609.
15. Gótell RP, Gótzs M: Fementative metabolism of Chloamydomonas reinhardtii. Plant Physiol 1984, 75:212-218.
16. Miura Y, Ohta S, Mano M, Miyamoto K: Isolation and characterization of a unicellular marine green alga exhibiting high activity in dark hydrogen production. Agr Biol Chem 1986, 50:2837-2844.
17. Ueno Y, Kurano N, Miyachi S: Ethanol production by dark fermentation in the marine green alga, Chlorococcum littorale. J Ferment Bioeng 1998, 85:58-63.
18. Karadag D, Puhakka JA: Direction of glucose fermentation towards hydrogen or ethanol production through on-line pH control. Int J Hydrogen Energy 2010, 35:10245-10251.
19. Grabovski A, Tindall BJ, Bardin V, Blanchet D, Jeurthen C, Petrimonos sulphurgphil gen. nov., sp. nov., a mesophilic fermentative bacterium isolated from a biodegraded oil reservoir. Int J System Evolut Microb 2005, 55:1113-1121.
20. Boone DR, Bryant MP: Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov., from methanogenic ecosystems. Appl Environ Microbiol 1980, 40:626-632.

21. de Bok FAM, Plugee CM, Stams AJM: Interspecies electron transfer in methanogenic propionate degrading consortia. *Water Res* 2004, 38:1368-1375.

22. da Silva SM, Vencescas SS, Fernandes CLV, Valente FMA, Pereira IAC: Hydrogen as an energy source for the human pathogen *Bilophila wadsworthia*. Antone Van Leeuwenhoek 2008, 93:381-390.

23. Grass R, Simon J, Theis F, Kröger A: Two membrane anchors of *Wolinella succinogenes* hydrogenase and their function in fumarate and polysulfide respiration. *Arch Microbiol* 1998, 170:50-58.

24. Chassard C, Gaillard-Martine B, Bernaler-Donadille A: Interaction between *H*₂-producing and non-*H*₂-producing cellulytic bacteria from the human colon. *FEMS Microbiol Lett* 2005, 242:339-344.

25. Wagner-Döbler I, Rheims H, Felske A, El-Ghezal A, Flade-Schröder D, de Bok FAM, Plugge CM, Stams AJM: Interspecies electron transfer in a Propionate-degrading bacterium, *Syntrophobacter wadsworthii*. *Anaerobe* 2007, 13:87-93.

26. Porwal S, Kumar T, Lal S, Rani A, Kumar S, Cheema S, Purohit HJ, Sharma R, Patel SKS, Kalia VC: Determination of chemical oxygen demand (COD Cr) in water with the closed tube method. Oxidation with dichromate.[http://sales.sfs.de/index.jsp?lang=1].

27. Jeong T-Y, Cha G-C, Yeom SH, Choi SS, Kim Y, Sevilla JMF: Protein measurements of microalgal and cyanobacterial biomass. *Biotechnol Adv* 2010, 28:550-556.

28. Podesta JJ, Gutiérrez-Navarro AM, Estrella CN, Esteso MA: Interactions between *Clostridium* sp. and other facultative anaerobes in a self-formed granular sludge hydrogen-producing bioreactor. *Int J Hydrogen Energy* 2011, 36:804-811.

29. Waterman RP, Gannon CJ, Goodall GS: Determination of chemical oxygen demand (COD Cr) in water with the closed tube method. Oxidation with dichromate.[http://sales.sfs.de/index.jsp?lang=1].

30. Syntrophobacter wolinii: Bioassay for methanogenesis. *Appl Environ Microbiol* 2000, 59:1177-1184.

31. Int J System Bacteriol 1980, 124:91-94.

32. Int J System Evolut Microbiol 2004, 54:1177-1184.

33. Lobos M, Gilles KA, Hamilton JK, Rebers PA, Smith F: Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956, 28:550-556.

34. Porwal S, Kumar T, Lal S, Rani A, Kumar S, Cheema S, Purohit HJ, Sharma R, Patel SKS, Kalia VC: Two membrane anchors of *Wolinella succinogenes* hydrogenase and their function in fumarate and polysulfide respiration. *Arch Microbiol* 1998, 170:50-58.

35. Sialve B, Bernet N, Bernard O: *Bacillus thermoamylovorans* sp. nov., a moderately thermophilic, amyloidolytic bacterium. *Int J System Evolut Microbiol* 1995, 45:9-14.

36. Becker EW: Anaerobic digestion of microalgae as a source of protein. *Biotechnol Adv* 2007, 25:207-210.

37. Sydney EB, Sturm W, de Carvalho JC, Thomaz-Soccol V, Larroche C, Finnish Standards Association: *Determination of total residue and total fixed residue in water, sludge and sediment.* [http://sales.sfs.de/index.jsp?lang=1].

38. Waterman RP, Gannon CJ, Goodall GS: Determination of chemical oxygen demand (COD Cr) in water with the closed tube method. Oxidation with dichromate.[http://sales.sfs.de/index.jsp?lang=1].

39. Karlson A, Ejertsson J, Neizirievic D, Svensson BH: Degradation of phenol under meso- and thermophilic, anaerobic conditions. *Anaerobe* 1999, 5:25-33.

40. Karlsson A, Johansson E, Karlson A, Meyerson U, Svensson BH: Anaerobic degradation of xenobiotics by organisms from municipal solid waste under landfilling conditions. *Antone Van Leeuwenhoek* 1995, 69:67-74.

41. Finnish Standards Association: Determination of total residue and total fixed residue in water, sludge and sediment.[http://sales.sfs.de/index.jsp?lang=1].

42. Bigh EG, Dyer WJ: A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959, 37:911-917.

43. López OVG, García MCC, Fernández FGA, Bustos CS, Chisti Y, Sevilla JMF: Protein measurements of microalgal and cyanobacterial biomass. *Biotechnol Adv* 2010, 28:550-556.

44. Owen WF, Stuckey DC, Healy JB Jr, Young LY, McCarty PL: Bioassay for monitoring methane potential and anaerobic toxicity. *Water Res* 1979, 13:485-492.

45. Muyzer G, De Waal EC, Utterlinden AG: Profiling complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S *rRNA*. *Appl Environ Microbiol* 1993, 59:695-700.

46. Muyzer G, Hottentraeger S, Teske A, Waver C: Denaturing gradient gel electrophoresis of PCR-amplified 16S *rDNA* - A new molecular approach to analyse the genetic diversity of mixed microbial communities. *Molecular Microbial Ecology Manual*. Edited by: Akkermans ADL, van Elas JD, De Bruijn F. Dordrecht, The Netherlands: Kluwer Academic Publishers; 1996, 3:4/1-23.

47. Logan BE, Oh SE, Kim IS, van Ginkel S: Biological hydrogen production measured in batch anaerobic respirometers. *Environ Sci Technol* 2002, 36:2530-2533.

48. Chen C-C, Li C-Y, Lin M-C: Acid-base enrichment enhances anaerobic hydrogen production process. *Appl Microbiol Biotechnol* 2002, 58:224-228.

49. Karlson A, Ejertsson J, Neizirievic D, Svensson BH: Degradation of phenol under meso- and thermophilic, anaerobic conditions. *Anaerobe* 1999, 5:25-33.

50. Karlsson A, Johansson E, Karlson A, Meyerson U, Svensson BH: Anaerobic degradation of xenobiotics by organisms from municipal solid waste under landfilling conditions. *Antone Van Leeuwenhoek* 1995, 69:67-74.

51. Finnish Standards Association: Determination of total residue and total fixed residue in water, sludge and sediment.[http://sales.sfs.de/index.jsp?lang=1].

52. Bigh EG, Dyer WJ: A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959, 37:911-917.

53. López OVG, García MCC, Fernández FGA, Bustos CS, Chisti Y, Sevilla JMF: Protein measurements of microalgal and cyanobacterial biomass. *Biotechnol Adv* 2010, 28:550-556.

54. Owen WF, Stuckey DC, Healy JB Jr, Young LY, McCarty PL: Bioassay for monitoring methane potential and anaerobic toxicity. *Water Res* 1979, 13:485-492.

55. Muyzer G, Hottentraeger S, Teske A, Waver C: Denaturing gradient gel electrophoresis of PCR-amplified 16S *rDNA* - A new molecular approach to analyse the genetic diversity of mixed microbial communities. *Molecular Microbial Ecology Manual*. Edited by: Akkermans ADL, van Elas JD, De Bruijn F. Dordrecht, The Netherlands: Kluwer Academic Publishers; 1996, 3:4/1-23.

56. Logan BE, Oh SE, Kim IS, van Ginkel S: Biological hydrogen production measured in batch anaerobic respirometers. *Environ Sci Technol* 2002, 36:2530-2533.

57. Chen C-C, Li C-Y, Lin M-C: Acid-base enrichment enhances anaerobic hydrogen production process. *Appl Microbiol Biotechnol* 2002, 58:224-228.

58. Karlson A, Ejertsson J, Neizirievic D, Svensson BH: Degradation of phenol under meso- and thermophilic, anaerobic conditions. *Anaerobe* 1999, 5:25-33.

59. Karlsson A, Johansson E, Karlson A, Meyerson U, Svensson BH: Anaerobic degradation of xenobiotics by organisms from municipal solid waste under landfilling conditions. *Antone Van Leeuwenhoek* 1995, 69:67-74.

60. Finnish Standards Association: Determination of total residue and total fixed residue in water, sludge and sediment.[http://sales.sfs.de/index.jsp?lang=1].

Cite this article as: Lakaniemi et al.: Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunalieia tertiolecta* biomass. *Biotechnology for Biofuels* 2011, 4:34.