energies

MDPI

Article

Hydrocarbon Toxicity towards Hydrogenotrophic Methanogens in Oily Waste Streams

Bruno P. Morais †, Valdo Martins, Gilberto Martins, Ana Rita Castro, Maria Madalena Alves, Maria Alcina Pereira and Ana J. Cavaleiro *

CEB—Centre of Biological Engineering, Campus de Gualtar, University of Minho, 4710-057 Braga, Portugal; brunodpmorais@ua.pt (B.P.M.); valdomartins1991@gmail.com (V.M.); gilberto.martins@deb.uminho.pt (G.M.); ritacastro@deb.uminho.pt (A.R.C.); madalena.alves@deb.uminho.pt (M.M.A.); alcina@deb.uminho.pt (M.A.P.)

* Correspondence: acavaleiro@deb.uminho.pt; Tel.: +351-253604423

† Current affiliation: Department of Chemistry, CICECO—Aveiro Institute of Materials, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal.

Abstract: Hydrocarbon-containing wastes and wastewaters are produced worldwide by the activities of the oil and gas industry. Anaerobic digestion has the potential to treat these waste streams, while recovering part of its energy potential as biogas. However, hydrocarbons are toxic compounds that may inhibit the microbial processes, and particularly the methanogens. In this work, the toxicity of hexadecane (0–30 mM) towards pure cultures of hydrogenotrophic methanogens (*Methanobacterium formicicum* and *Methanospirillum hungatei*) was assessed. Significantly lower ($p < 0.05$) methane production rates were only verified in the incubations with more than 15 mM hexadecane and represented up to 52% and 27% inhibition for *M. formicicum* and *M. hungatei*, respectively. The results obtained point out that 50% inhibition of the methanogenic activity would likely occur at hexadecane concentrations between 5–15 mM and >30 mM for *M. formicicum* and *M. hungatei*, respectively, suggesting that toxic effects from aliphatic hydrocarbons towards hydrogenotrophic methanogens may not occur during anaerobic treatment. Hydrocarbon toxicity towards hydrogenotrophic methanogens was further assessed by incubating an anaerobic sludge with H_2/CO_2 in the presence of a complex mixture of hydrocarbons (provided by the addition of an oily sludge from a groundwater treatment system). Specific methanogenic activity from H_2/CO_2 decreased 1.2 times in the presence of the hydrocarbons, but a relatively high methane production (~30 mM) was still obtained in the assays containing the inoculum and the oily sludge (without H_2/CO_2), reinforcing the potential of anaerobic treatment systems for methane production from oily waste/wastewater.

Keywords: methanogens; hydrocarbons; hexadecane; toxicity; anaerobic digestion

Citation: Morais, B.P.; Martins, V.; Martins, G.; Castro, A.R.; Alves, M.M.; Pereira, M.A.; Cavaleiro, A.J. Hydrocarbon Toxicity towards Hydrogenotrophic Methanogens in Oily Waste Streams. *Energies* **2021**, *14*, 4830. https://doi.org/10.3390/en14164830

Academic Editor: Byong-Hun Jeon

Received: 2 July 2021
Accepted: 5 August 2021
Published: 8 August 2021

1. Introduction

Petroleum-derived oils are the most important primary energy source in our society, and represent an important fraction of the economic markets, as shown by the fact that five of the ten largest companies by revenue in the world are oil companies [1]. The oil industry produces a multitude of wastes and wastewaters, most of which are hazardous to the environment and to human health [2–4]. The typical range of hydrocarbon concentrations in wastewater from the petroleum industry average 200–500 mg L^{-1}, and only in very specific situations it exceeds 1500 mg L^{-1} [5–7]. Anaerobic treatment of these oily-waste/wastewater can be an attractive alternative to the conventional aerobic systems, since it can potentially couple organic treatment with the recovery of bioenergy through methane production.

Hydrocarbons are common in many subsurface environments where their conversion to methane by methanogenic microbial consortia is known to occur [8–12]. Hydrocarbons' biodegradation to methane is performed by different groups of microorganisms, which interact through a series of metabolic steps that end up in the production of methane. At

least three different functional groups, including fermenting bacteria, syntrophic bacteria, and methanogens, are generally involved in the methanogenic degradation of hydrocarbons. First, hydrocarbons need to be activated. Different mechanisms have been proposed for hydrocarbon activation in anaerobic biodegradation pathways [13], from which fumarate addition is the most frequently reported. After hydrocarbons' activation, these compounds are converted into smaller molecules such as short-chain fatty acids, alcohols or hydrogen by fermentative bacteria. Further degradation involves reactions that are endergonic and become thermodynamically feasible only when the end products (particularly hydrogen or formate) are kept at low concentrations [14]. For example, the methanogenic transformation of alkanes is possible at hydrogen partial pressure lower than 4 Pa [15]. Under methanogenic conditions, this is generally accomplished by hydrogenotrophic methanogens. Therefore, close syntrophic relationships between bacteria and methanogenic archaea have been reported as essential for complete hydrocarbons conversion to methane [16–18]. As such, the transformation of hydrocarbons is driven by bacteria, but it is the activity of methanogens (particularly hydrogenotrophs) that allows the cascade of metabolic reactions to flow. This is also the case for the majority of other easily biodegradable organic compounds, for which the activity of methanogens and the occurrence of syntrophic relationships is essential for their complete conversion to methane.

Petroleum hydrocarbons degradation by anaerobic microorganisms can be influenced by several factors, including their toxic effects towards bacteria and archaea. Within anaerobic microbial communities, methanogens are generally more susceptible to toxic compounds than the other microorganisms [19]. In most cases, acetoclastic methanogens are more sensitive to toxicants than hydrogenotrophs, but syntrophic metabolism will be hindered mainly by inhibition of the latter. This justifies the relevance of studying the toxicity of hydrocarbons, or other compounds whose degradation proceeds via syntrophic interactions, towards the specific group of hydrogenotrophic methanogens. Few studies have addressed the potential toxicity of hydrocarbons towards pure cultures of hydrogenotrophic methanogens. Short-chain linear hydrocarbons [20], polyaromatics (PAH), and BTEX [19,21,22] have all been shown to inhibit methanogenesis in mixed cultures. Direct inhibition of methanogens may occur, but indirect inhibition is also possible, by disrupting the microbial relationships necessary for the complete degradation of hydrocarbons to methane. Other compounds such as halogenated aliphatic are also very toxic due to their highly reactive halogen group (mainly Cl and Br) [23–26]. Biodegradation of these compounds was reported to occur at slow rates and it is suggested that prolonged acclimation may help the communities to adapt [27–29].

The main goal of the present work was to investigate the potential inhibitory effects of hydrocarbons on the activity of hydrogenotrophic methanogens. A first experiment was performed with pure methanogenic cultures, and hexadecane was chosen as the model compound, considering that aliphatic hydrocarbons represent the largest fraction of crude oil and petroleum-derived products. Then, the feasibility of producing methane from H_2/CO_2 by a mixed anaerobic community (granular sludge) in the presence of a real complex mixture of hydrocarbons was further assessed in batch assays.

2. Materials and Methods

2.1. Growth Conditions

Methanobacterium formicicum (DSM 1535^T) and *Methanospirillum hungatei* JF-1 (DSM 864^T) were obtained from Leibniz-Institute DSMZ, Braunschweig, Germany. Methanogens were cultivated under strict anaerobic conditions in saline bicarbonate-buffered mineral medium, supplemented with a cocktail of vitamins as described elsewhere [30]. Serum bottles (120 mL total volume, medium volume of 55 mL) were sealed with butyl rubber stoppers and aluminum crimp caps and pressurized with a mixture of H_2/CO_2 (80:20% *v/v*, 1.7×10^5 Pa final pressure). Acetate was added at 2 mM for internal cell maintenance. The medium was reduced with 0.8 mM of sodium sulfide ($Na_2S{\cdot}7–9H_2O$) prior to inoculation. All solutions added were previously sterilized by filtering or autoclaving. The

incubations were performed at 37 °C, under agitation (120 rpm), and in the dark. For maintenance and preparation of the pre-inoculum used in the toxicity assays, cultures were transferred (10% *v/v*) to fresh media after reaching the exponential growth phase (5–7 days) and incubated again. To verify the growth stage of the cultures, a visual assessment was performed and confirmed with a headspace pressure measurement using a pressure transducer (Centerpoints Electronics; Galway, Ireland).

2.2. Toxicity Assays with Pure Methanogenic Cultures and Hexadecane

Toxicity assays were performed in triplicates, in closed bottles prepared as described in Section 2.1. A mixture of H_2/CO_2 (80/20% *v/v*, 1.7×10^5 Pa final pressure) was used as substrate. Increasing hexadecane concentrations of 1 mM, 5 mM, 15 mM, and 30 mM were added to the bottles with a glass syringe before autoclaving. Triplicate bottles without hexadecane were prepared as well (controls). Bottles were inoculated with 10% (*v/v*) of pre-grown cultures of *M. formicicum* or *M. hungatei*. Incubations were performed at 37 °C and 120 rpm, in the dark. CH_4 production and H_2 uptake were monitored during the experiment. At the end of the assays, oxidation-reduction potential (ORP) and pH were measured.

2.3. Toxicity Assessment with Granular Sludge and a Real Complex Mixture of Hydrocarbons

Anaerobic batch assays were prepared in 120 mL bottles containing 55 mL working volume. Anaerobic granular sludge from a brewery wastewater treatment plant (Porto, Portugal), with a volatile solids (VS) content of 0.08 g g^{-1} (wet weight), was used as inoculum. After collection, the inoculum was stored at 4 °C, and was mechanically disrupted immediately before use. The inoculum was added to the vials at a final VS concentration of 4 g L^{-1}. Oily sludge, collected from a full-scale treatment plant performing *ex situ* aerobic bioremediation of petroleum-contaminated groundwater located in France, was used as a real complex mixture of hydrocarbons. Total solids (TS) and VS were 76 ± 2 g L^{-1} and 21 ± 0 g L^{-1}, respectively. The presence of hexane-extractable hydrocarbons in the oily sludge was confirmed by GC analysis (Figure 1) and corresponded to a total petroleum hydrocarbons (TPH) concentration of 80 ± 30 g kg^{-1} (wet weight). The oily sludge was added to the vials at a final VS concentration of approximately 0.5 g L^{-1}. An anaerobic bicarbonate-buffered mineral salt medium was used [30], and a mixture of H_2/CO_2 (80/20% *v/v*, 1.7×10^5 Pa final pressure) was added as substrate. Assays containing only the inoculum or the inoculum plus oily sludge (i.e., without H_2/CO_2) were also prepared. An additional set of bottles was setup containing inoculum, oily sludge, and hexadecane (1 mM). The headspace of those bottles was flushed and pressurized with N_2/CO_2 (80:20% *v/v*, 1.7×10^5 Pa final pressure). Triplicate assays were prepared for all the conditions studied, and incubations were performed at 37 °C and 120 rpm, in the dark. The CH_4 produced was measured over time, and the specific hydrogenotrophic methanogenic activity (SHMA) was calculated from the highest slope of the linear initial region of the cumulative methane production curve (mL d^{-1}), divided by the mass unit of VS of inoculum sludge (g) [31]. The values obtained were corrected for standard temperature and pressure (STP) conditions. ORP and pH were determined at the start and end of the experiment. TPH were also analyzed at the end of the incubations.

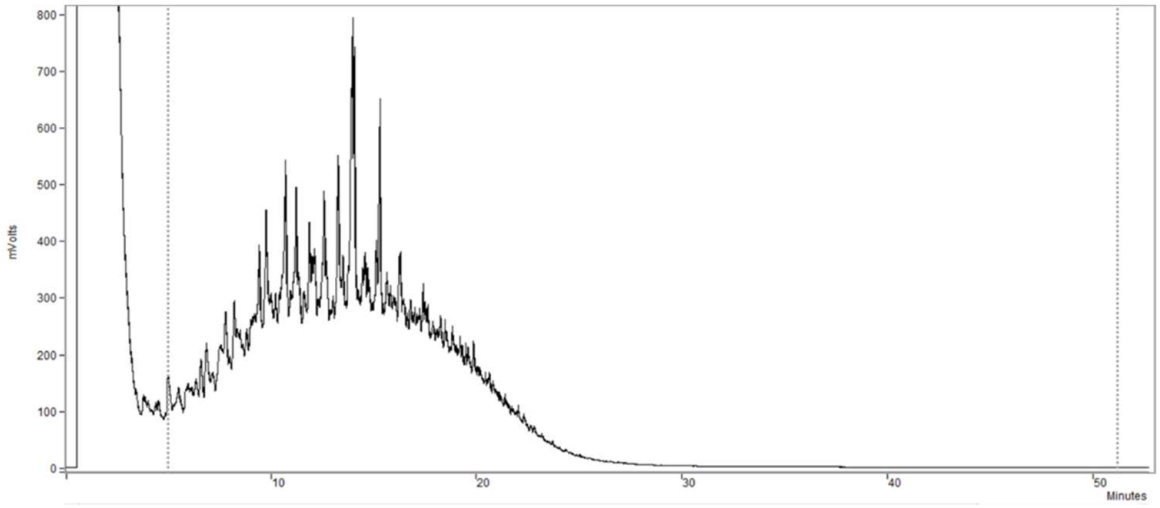

Figure 1. Chromatogram of TPH extracted from 20 g of oily sludge. The gray dotted vertical lines mark undecane (C11) and tetracontane (C40) retention times (5 and 50.2 min, respectively).

2.4. Analytical Methods

Methane or hydrogen present in the bottles' headspace was quantified by gas chromatography (GC), using a Bruker SCION GC-486 (SCION; Goes, The Netherlands) equipped with a Molsieve packed column (13 × 80/100, 2 m length, 2.1 mm internal diameter). A thermal conductivity detector (TCD) was used, with argon as the carrier gas at 30 mL min^{-1}. Temperatures of the injector, column, and detector were 100 °C, 35 °C, and 130 °C, respectively. TS and VS were analyzed gravimetrically [32]. pH and ORP were measured by potentiometry, with a pH probe (WTW inoLab, Xylem Analytics; Weilheim, Germany) and an ORP probe (VWR; Radnor, PA, USA), respectively. TPH present in the oily sludge were quantified by GC, after extraction with hexane for 4 h at 120 rpm in Schott flasks [33]. At the end of the toxicity assessment (Section 2.3), the whole content of the bottles was sacrificed and TPH were analyzed in the liquid and solid phases, which were separated by decantation. The liquid samples were sequentially extracted three times with hexane using separatory funnels, according to the procedure described by the U.S. Environmental Protection Agency [34]. The solid samples were extracted as described for the oily sludge. All the extracts were cleaned using Sep-Pak Florisil® cartridges (Waters; Milford, MA, USA) and evaporated in TurboVap® LV (Biotage; Uppsala, Sweden). TPH were quantified in a gas chromatograph GC Varian® 4000, with a VF-1 ms column (30 m × 0.025 mm) and a flame ionization detector (FID), as detailed elsewhere [35].

2.5. Statistical Analysis

Experimental CH_4 production data recorded during the toxicity assays (Section 2.2) were fitted to the modified Gompertz equation [36]:

$$M(t) = P \times \exp\left[-\exp\left[\frac{R_m \times e}{P}(\lambda - t) + 1\right]\right] \quad (1)$$

where: $M(t)$, cumulative CH_4 production (mM); P, maximum CH_4 production (mM); R_m, CH_4 production rate (mM d^{-1}); e, 2.7182818; and λ, lag phase (d). Data analysis was performed using Sigma Plot for Windows 10.0 software (Systat Software Inc., Erkrath, Germany). To improve the data fitting, a restriction was applied to the P value, so that it should be less or equal to the maximum theoretical methane production calculated according to the stoichiometric balance of hydrogenotrophic CO_2 reduction (i.e., 4 mol of H_2 consumed per mol of CH_4 produced), using the H_2 concentration measured at t = 0 h for each individual condition (mean of triplicates).

Statistical significance of the differences observed in the results achieved was evaluated using single factor analysis of variances (ANOVA). Statistical significance was established at the $p < 0.05$ level.

3. Results and Discussion

3.1. Toxicity Assays with Pure Methanogenic Cultures and Hexadecane

Hydrogen consumption and cumulative methane production by *Methanobacterium formicicum* and *Methanospirillum hungatei*, in the presence of increasing concentrations of hexadecane, are shown in Figures 2 and 3, respectively. The different parameters calculated by fitting the experimental CH_4 production data to the modified Gompertz equation are shown in Table 1. In the assays with *M. formicicum*, similar lag phases around 5 days were observed for all the hexadecane concentrations tested. Nevertheless, the maximum methane production rate (Rm) was significantly lower ($p < 0.05$) in the incubations with 15 mM and 30 mM of hexadecane, relatively to the other concentrations tested (Figure 2 and Table 1). For these two higher hexadecane concentrations, Rm decreased approximately 52% and 54%, respectively, compared to the control.

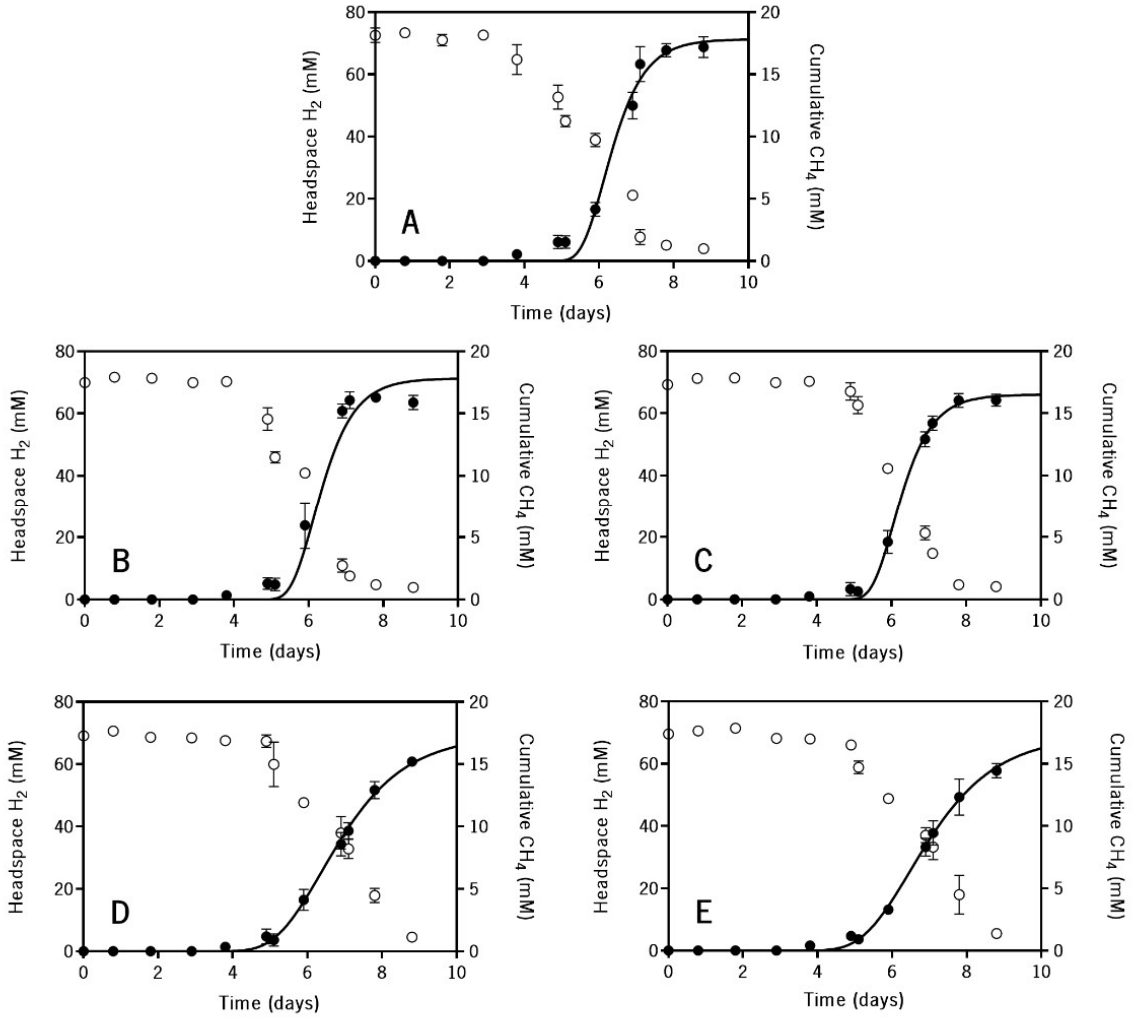

Figure 2. Effects of increasing hexadecane concentrations on H_2 consumption (○) and cumulative CH_4 production (●) by *M. formicicum*: (**A**)—0 mM, (**B**)—1 mM, (**C**)—5 mM, (**D**)—15 mM, and (**E**)—30 mM. The black line represents the fitting of the cumulative methane production data by the modified Gompertz equation.

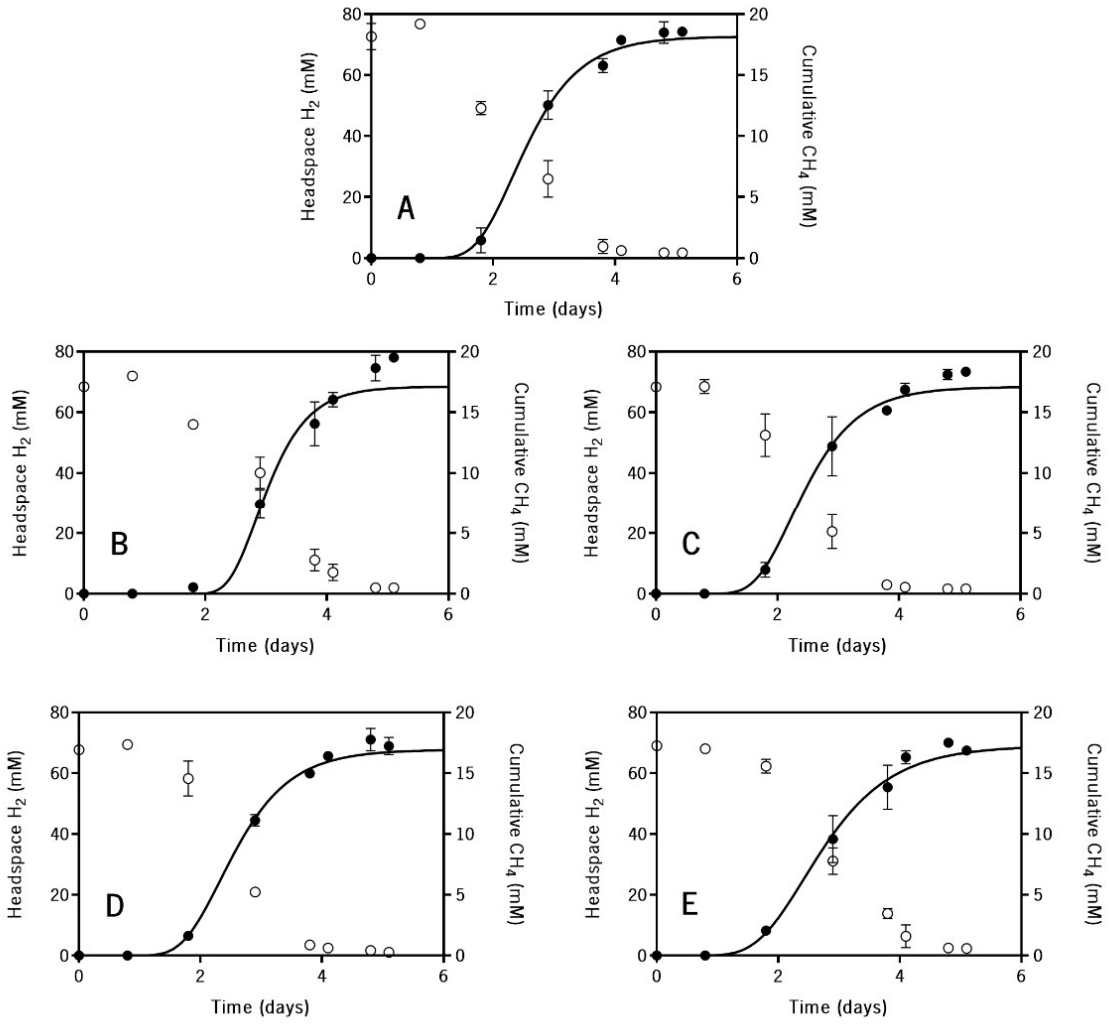

Figure 3. Effects of increasing hexadecane concentrations on H_2 consumption (○) and cumulative CH_4 production (●) by *M. hungatei*: (**A**)—0 mM, (**B**)—1 mM, (**C**)—5 mM, (**D**)—15 mM, and (**E**)—30 mM. The black line represents the fitting of the cumulative methane production data by the modified Gompertz equation.

Table 1. Methane production parameters calculated by fitting the experimental data from the toxicity assays with *M. formicicum* and *M. hungatei* to the modified Gompertz model. λ, lag-phase; P, maximum CH_4 production; Rm, maximum CH_4 production rate; and R^2, coefficient of determination.

Methanogen	Hexadecane (mM)	λ (d)	P (mM)	Rm (mM d^{-1})	R^2	Inhibition (%)
M. formicicum	0	5.5 ± 0.1	17.8 ± 0.6	10.9 ± 1.0 [a]	0.984	-
	1	5.1 ± 0.1	16.7 ± 0.7	10.2 ± 0.6 [a]	0.994	6 ± 1
	5	5.5 ± 0.1	16.5 ± 0.3	10.6 ± 0.6 [a]	0.995	3 ± 0
	15	5.1 ± 0.1	17.3 ± 0.8	5.2 ± 0.3 [b]	0.993	52 ± 6
	30	5.2 ± 0.1	17.2 ± 1.0	5.0 ± 0.3 [b]	0.984	54 ± 6
M. hungatei	0	1.7 ± 0.1	18.2 ± 0.3	11.1 ± 0.8 [a]	0.992	-
	1	2.2 ± 0.3	17.1 ± 0.7	12.5 ± 2.6 [a]	0.979	0
	5	1.6 ± 0.1	17.1 ± 0.4	10.5 ± 1.1 [a]	0.987	4 ± 1
	15	1.7 ± 0.1	16.9 ± 0.3	9.9 ± 0.7 [b]	0.994	10 ± 1
	30	1.6 ± 0.1	17.3 ± 0.6	8.1 ± 0.8 [b]	0.987	27 ± 3

[a,b] Different letters represent statistically significant differences ($p < 0.05$) compared to control (0 mM hexadecane).

For the assays with *M. hungatei* (Figure 3 and Table 1), maximum cumulative CH_4 production was achieved after 5 days of incubation, with lag phases lower than 2 days, which shows faster methane production by *M. hungatei* than by *M. formicicum*. Significant changes ($p < 0.05$) in Rm were observed for hexadecane concentrations of 15 and 30 mM, representing a decrease of 11% and 27%, respectively, in relation to the assay performed without hexadecane (control, 0 mM). These results suggest a higher tolerance of *M. hungatei* to the presence of hexadecane than *M. formicicum*.

For both methanogens, the results from the methane production were confirmed by the H_2 uptake profiles that followed the same trends (Figures 2 and 3). Considering the H_2 concentrations measured at the start and end of the assays, the total hydrogen consumed was calculated (Table 2). From the stoichiometry of hydrogenotrophic methanogenesis ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$), the methane yields (mole of methane produced per mole of hydrogen consumed, expressed in percentage relatively to the expected value of 1:4) were calculated (Table 2). These results show complete H_2/CO_2 conversion to CH_4 by both cultures. pH and ORP measurements (Table S1 in Supplementary Material) displayed slight increases for both parameters from the start until the end of the experiment.

Table 2. Total hydrogen consumed, maximum cumulative methane produced, and calculated methane yields for the incubations of *M. formicicum* and *M. hungatei*, with increasing hexadecane concentrations. The values represent the average of triplicates ± standard deviation.

Methanogen	Hexadecane (mM)	H_2 Consumed (mM)	Maximum Cumulative CH_4 Production (mM)	CH_4 Yield (%)
M. formicicum	0	68 ± 2	17 ± 1	100 ± 11
	1	66 ± 1	16 ± 0	96 ± 2
	5	65 ± 1	16 ± 0	99 ± 2
	15	65 ± 1	15 ± 0	94 ± 1
	30	64 ± 1	14 ± 0	91 ± 4
M. hungatei	0	71 ± 4	18 ± 0	105 ± 3
	1	67 ± 1	19 ± 0	117 ± 0
	5	67 ± 1	18 ± 0	110 ± 2
	15	67 ± 1	17 ± 0	103 ± 18
	30	67 ± 1	17 ± 0	101 ± 1

The obtained results show that the methanogenic activity of the two hydrogenotrophic methanogens was not significantly affected by lower hexadecane concentrations (<15 mM). However, higher concentrations (15–30 mM) caused an inhibition of *circa* 50% in the case of *M. formicicum* and *circa* 27% for *M. hungatei*. The dissolution rate of a compound is critical for its bioavailability and toxicity [28]. Due to long-chain *n*-alkanes' low aqueous solubility at standard conditions, experimental data is scarce, but experimental and computational modelling studies have attempted to provide insights [37–40]. Overall, their findings suggest that solubility decreases exponentially with the increase in the number of carbons on the chain, with solubility molar fraction around 10^{-7} M and lower for C15 and above *n*-alkane chains. Experimental measurements made by [41] and [42] showed that vigorous mixing and higher alkane concentrations leads to the formation of microdroplets, thus enhancing the mass transfer of these compounds to the water phase. In our experiments, hexadecane concentrations of 1 mM and 5 mM were most likely too low for a reasonable level of aggregation to form, which may have occurred when increasing hexadecane concentrations were used. If this is the case, the inhibitory effect of hexadecane will most probably occur at a critical threshold concentration, where microdroplets formation becomes possible, rather than continuously with concentration. In fact, the results obtained for *M. formicicum* suggest the existence of a threshold, but additional measurements between 5 and 15 mM would be necessary to clarify this point.

In alternative, the presence of a hydrocarbon floating layer in the water-gas interface, or a hydrocarbon layer adsorbed to the microbial cells, may have induced mass transfer

limitations, reducing the H_2 uptake or the release of the produced CH_4. Similar mechanisms were previously proposed for the inhibition of methanogens by long-chain fatty acids (LCFA) [43,44] which, similarly to hydrocarbons, are hydrophobic surface-active compounds. LCFA and *n*-alkanes also present similarities in its chemical structures, and LCFA are thought to be intermediaries of *n*-alkanes biodegradation [14].

n-Alkanes have been shown to interfere with the cytoplasmic membranes of aerobic bacteria [28,29]. These compounds accumulate in the membrane bilayer and influence both the membrane lipid-order and the bilayer stability. Therefore, membrane thickness and permeability are affected, and the activity of membrane-embedded enzymes is disturbed, ultimately compromising a cell's viability [28,29]. The total density of the microbial culture will influence the apparent toxicity observed, since for the same concentration of hexadecane, a denser community can exhibit less harmful effects. Likewise, the greater the concentration of hexadecane, the higher the effects on individual microbial cells. Although not studied, similar membrane toxicity of hydrocarbons towards methanogenic archaea may be hypothesized. However, in some archaea, the lipid bilayer is replaced by a monolayer, composed by lipids that resemble two phospholipid molecules whose tails have been covalently bound, forming a single molecule with two polar heads at opposite sides [45–47]. This is the case of the two methanogens studied in this work [46], and thus, due to this unique cell membrane lipids, the mechanisms of hydrocarbon toxicity may differ from those reported for aerobic bacteria.

The differences in sensitivity to hexadecane between the two methanogens studied may be linked to differences in cell wall structure and membrane lipid composition. *M. formicicum* possesses a rigid pseudomurein wall, which maintains the cell shape and probably protects the cells [48]. *M. hungatei* is a rod-shaped cell and exists as a filamentous chain enclosed in a tubular proteinaceous sheath. This sheath encloses the cell-chain community, being each individual cell surrounded by an inner cell wall consisting of a proteinaceous S-layer [49]. The sheath exhibits very low porosity, such that only small molecules like H_2, CO_2, and CH_4 can penetrate it, whereas larger molecules can only diffuse inward from the more porous terminal ends [50]. Besides its high stability against proteases and detergents, it also revealed a paracrystalline structure, functioning as a microsieve [48]. As such, the access of hexadecane to *M. hungatei* cells was potentially limited, resulting in a greater tolerance of the bacterium to this compound.

3.2. Toxicity Assessment with Granular Sludge and a Real Complex Mixture of Hydrocarbons

Hydrocarbon toxicity towards hydrogenotrophic methanogens was further assessed by incubating an anaerobic sludge with H_2/CO_2. In the absence of the oily sludge, the cumulative methane production started immediately and stabilized after approximately 9 h of incubation (Figure 4A), corresponding to a specific hydrogenotrophic methanogenic activity (SHMA) of 972 $\pm$ 44 mL g^{-1} d^{-1}. In the presence of the oily sludge, also no lag phase was verified. However, the cumulative methane production rate was lower, as shown by the slope of the cumulative methane production curve (Figure 4A), corresponding to a 1.2 times lower SHMA (i.e., 792 $\pm$ 15 mL g^{-1} d^{-1}). This decrease was most probably related with the complexity of the hydrocarbon mixture (Figure 1), which resulted in a higher toxicity when compared to hexadecane incubations (Section 3.1). In fact, when hexadecane was added to the sludge mixture, no toxic effects were observed (Figure 4B). The hydrocarbons present in the oily sludge accumulated in the settler of the wastewater treatment plant over the time. Therefore, this partially degraded oil possibly contains less saturated and aromatic hydrocarbons than a non-biodegraded oil, being composed of more recalcitrant and less biodegradable hydrocarbons. In fact, the TPH profile appeared in the chromatogram as an unresolved lump, with only few resolved peaks (Figure 1). Moreover, these hydrocarbons were not degraded over the course of the experiment, even when an extended incubation period (122 days) was applied, as confirmed by GC analysis at the end of the assays (data not shown).

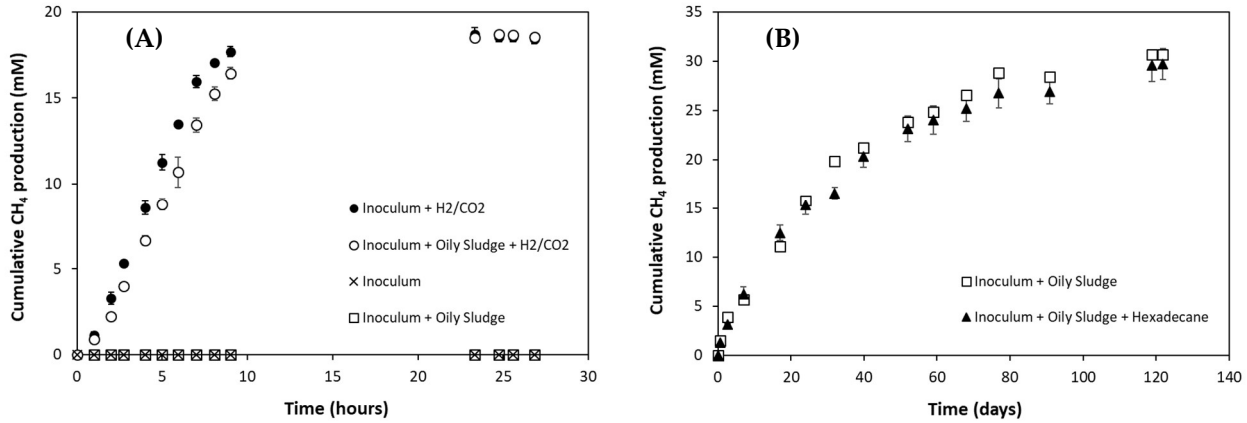

Figure 4. Cumulative methane production in the assays with H_2/CO_2 (**A**) and during prolonged incubation of the sludge mixture (without H_2/CO_2) (**B**). The results presented are the averages and standard deviations for triplicate assays.

It is important to highlight that the granular sludge was mechanically disrupted, and therefore, higher sensibility can be expected relatively to the use of intact granules. Additionally, a relatively high methane production (~30 mM) was obtained during prolonged incubation of the blanks (containing inoculum and oily sludge, but not H_2/CO_2) (Figure 4B), pointing to the presence of important amounts of biodegradable compounds in the oily sludge, or to the occurrence of sludge digestion. These may include extracellular polymeric substances (EPS), which are typical components of microbial biofilm. In the groundwater treatment plant, the bioreactors contained plastic packing material as carrier for biofilm growth, and, as such, EPS will likely be present in the sludge collected in the sludge settler. Volatile fatty acids (which are typical intermediates of anaerobic metabolism) or long-chain fatty acids (which are potential intermediates in the degradation of alkanes) can be present as well. As such, although SHMA of the sludge was slightly inhibited by the hydrocarbons mixture, these results show that syntrophic and methanogenic activities were not compromised, and highlight the potential for methane production during the anaerobic treatment of oily waste streams.

4. Conclusions

The results obtained show that *M. formicicum* is substantially more sensitive to hexadecane than *M. hungatei*. Fifty percent inhibition of the methanogenic activity of *M. formicicum* is expected to occur at hexadecane concentrations between 5 mM and 15 mM; while, in *M. hungatei*, 50% inhibition was not verified even after exposure to 30 mM hexadecane, with only a 27 $\pm$ 3% decrease in methane production rate observed at this concentration. For both methanogens, no inhibition was observed up to 5 mM hexadecane. Therefore, considering the typical range of hydrocarbon concentrations in wastewater from the petroleum industry, toxic effects of aliphatic hydrocarbons towards hydrogenotrophic methanogens will not be expected to occur during the anaerobic treatment of these types of wastewater. In the presence of a complex hydrocarbons mixture, slight toxicity may occur, but inhibition of the methanogens is not expected at such an extent that compromise methane production during the treatment of oily waste streams.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/en14164830/s1: Table S1, pH and ORP measurements at the start (t_0) and end (t_f) of the toxicity assays with *Methanobacterium formicicum* and *Methanospirillum hungatei*, at increasing hexadecane concentrations. The values represent the mean of triplicates $\pm$ standard deviation.

Author Contributions: Conceptualization, B.P.M., G.M., M.A.P., and A.J.C.; methodology, B.P.M., G.M., M.A.P., and A.J.C.; investigation, B.P.M., V.M., G.M., and A.R.C.; writing—original draft preparation, B.P.M.; writing—review and editing, G.M., A.R.C., M.M.A., M.A.P., and A.J.C.; supervision,

M.A.P. and A.J.C.; funding acquisition, M.M.A. and A.J.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Portuguese Foundation for Science and Technology (FCT) under the scope of project MORE (POCI-01-0145-FEDER-016575) and by the strategic funding of UIDB/04469/2020 unit.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

References

1. Fortune Global 500. Available online: https://fortune.com/global500/ (accessed on 24 May 2021).
2. Hentati, O.; Lachhab, R.; Ayadi, M.; Ksibi, M. Toxicity assessment for petroleum-contaminated soil using terrestrial invertebrates and plant bioassays. *Environ. Monit. Assess.* **2013**, *185*, 2989–2998. [CrossRef]
3. Meslé, M.; Dromart, G.; Oger, P. Microbial methanogenesis in subsurface oil and coal. *Res. Microbiol.* **2013**, *164*, 959–972. [CrossRef]
4. Varjani, S.J.; Upasani, V.N. A new look on factors affecting microbial degradation of petroleum hydrocarbon pollutants. *Int. Biodeterior. Biodegrad.* **2017**, *120*, 71–83. [CrossRef]
5. Zheng, J.; Chen, B.; Thanyamanta, W.; Hawboldt, K.; Zhang, B.; Liu, B. Offshore produced water management: A review of current practice and challenges in harsh/Arctic environments. *Mar. Pollut. Bull.* **2016**, *104*, 7–19. [CrossRef] [PubMed]
6. Jiménez, S.; Micó, M.M.; Arnaldos, M.; Medina, F.; Contreras, S. State of the art of produced water treatment. *Chemosphere* **2018**, *192*, 186–208. [CrossRef]
7. Kuyukina, M.S.; Krivoruchko, A.V.; Ivshina, I.B. Advanced bioreactor treatments of hydrocarbon-containing wastewater. *Appl. Sci.* **2020**, *10*, 831. [CrossRef]
8. Zengler, K.; Richnow, H.H.; Rosselló-Mora, R.; Michaelis, W.; Widdel, F. Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* **1999**, *401*, 266–269. [CrossRef] [PubMed]
9. Siddique, T.; Fedorak, P.M.; Foght, J.M. Biodegradation of short-chain n-alkanes in oil sands tailings under methanogenic conditions. *Environ. Sci. Technol.* **2006**, *40*, 5459–5464. [CrossRef]
10. Wang, L.-Y.; Gao, C.-X.; Mbadinga, S.M.; Zhou, L.; Liu, J.-F.; Gu, J.-D.; Mu, B.-Z. Characterization of an alkane-degrading methanogenic enrichment culture from production water of an oil reservoir after 274 days of incubation. *Int. Biodeterior. Biodegrad.* **2011**, *65*, 444–450. [CrossRef]
11. Grishchenkov, V.G.; Townsend, R.T.; McDonald, T.J.; Autenrieth, R.L.; Bonner, J.S.; Boronin, A.M. Degradation of petroleum hydrocarbons by facultative anaerobic bacteria under aerobic and anaerobic conditions. *Process Biochem.* **2000**, *35*, 889–896. [CrossRef]
12. Townsend, G.T.; Prince, R.C.; Suflita, J.M. Anaerobic oxidation of crude oil hydrocarbons by the resident microorganisms of a contaminated anoxic aquifer. *Environ. Sci. Technol.* **2003**, *37*, 5213–5218. [CrossRef]
13. Abbasian, F.; Lockington, R.; Mallavarapu, M.; Naidu, R. A Comprehensive review of aliphatic hydrocarbon biodegradation by bacteria. *Appl. Biochem. Biotechnol.* **2015**, *176*, 670–699. [CrossRef] [PubMed]
14. Jiménez, N.; Richnow, H.H.; Vogt, C.; Treude, T.; Krüger, M. Methanogenic hydrocarbon degradation: Evidence from field and laboratory studies. *Microb. Physiol.* **2016**, *26*, 227–242. [CrossRef] [PubMed]
15. Dolfing, J.; Larter, S.R.; Head, I.M. Thermodynamic constraints on methanogenic crude oil biodegradation. *ISME J.* **2008**, *2*, 442–452. [CrossRef] [PubMed]
16. Gieg, L.M.; Fowler, S.J.; Berdugo-Clavijo, C. Syntrophic biodegradation of hydrocarbon contaminants. *Curr. Opin. Biotechnol.* **2014**, *27*, 21–29. [CrossRef] [PubMed]
17. Morris, B.E.L.; Henneberger, R.; Huber, H.; Moissl-Eichinger, C. Microbial syntrophy: Interaction for the common good. *FEMS Microbiol. Rev.* **2013**, *37*, 384–406. [CrossRef] [PubMed]
18. Sieber, J.R.; McInerney, M.J.; Gunsalus, R.P. Genomic insights into syntrophy: The paradigm for anaerobic metabolic cooperation. *Annu. Rev. Microbiol.* **2012**, *66*, 429–452. [CrossRef]
19. Chen, Y.; Cheng, J.J.; Creamer, K.S. Inhibition of anaerobic digestion process: A review. *Bioresour. Technol.* **2008**, *99*, 4044–4064. [CrossRef] [PubMed]
20. Sherry, A.; Grant, R.; Aitken, C.; Jones, M.; Head, I.; Gray, N. Volatile hydrocarbons inhibit methanogenic crude oil degradation. *Front. Microbiol.* **2014**, *5*, 131. [CrossRef] [PubMed]
21. Dou, J.; Liu, X.; Hu, Z. Substrate interactions during anaerobic biodegradation of BTEX by the mixed cultures under nitrate reducing conditions. *J. Hazard. Mater.* **2008**, *158*, 264–272. [CrossRef] [PubMed]
22. Rodriguez Martinez, M.F.; Kelessidou, N.; Law, Z.; Gardiner, J.; Stephens, G. Effect of solvents on obligately anaerobic bacteria. *Anaerobe* **2008**, *14*, 55–60. [CrossRef] [PubMed]
23. Colleran, E.; Concannon, F.; Golden, T.; Geoghegan, F.; Crumlish, B.; Killilea, E.; Henry, M.; Coates, J. Use of methanogenic activity tests to characterize anaerobic sludges, screen for anaerobic biodegradability and determine toxicity thresholds against individual anaerobic trophic groups and species. *Water Sci. Technol.* **1992**, *25*, 31–40. [CrossRef]

24. Pries, F.; van der Ploeg, J.R.; Dolfing, J.; Janssen, D.B. Degradation of halogenated aliphatic compounds: The role of adaptation. *FEMS Microbiol. Rev.* **1994**, *15*, 279–295. [CrossRef] [PubMed]
25. Sanz, J.L.; Rodríguez, N.; Amils, R. Effect of chlorinated aliphatic hydrocarbons on the acetoclastic methanogenic activity of granular sludge. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 324–328. [CrossRef]
26. Yu, Z.; Smith, G.B. Inhibition of methanogenesis by C1- and C2-polychlorinated aliphatic hydrocarbons. *Environ. Toxicol. Chem.* **2000**, *19*, 2212–2217. [CrossRef]
27. Leahy, J.G.; Colwell, R.R. Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev.* **1990**, *54*, 305–315. [CrossRef]
28. Sikkema, J.; de Bont, J.A.; Poolman, B. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* **1995**, *59*, 201–222. [CrossRef]
29. Weber, F.J.; de Bont, J.A.M. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. *Biochim. Biophys. Acta-Rev. Biomembr.* **1996**, *1286*, 225–245. [CrossRef]
30. Stams, A.J.M.; Van Dijk, J.B.; Dijkema, C.; Plugge, C.M. Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. *Appl. Environ. Microbiol.* **1993**, *59*, 1114–1119. [CrossRef]
31. Alves, M.M.; Mota Vieira, J.A.; Álvares Pereira, R.M.; Pereira, M.A.; Mota, M. Effect of lipids and oleic acid on biomass development in anaerobic fixed-bed reactors. Part I: Biofilm growth and activity. *Water Res.* **2001**, *35*, 255–263. [CrossRef]
32. APHA; AWWA; WPCF. *Standard Methods for the Examination of water and Wastewater*, 17th ed.; American Public Health Association: Washington, DC, USA, 1998.
33. Siddique, T.; Rutherford, P.M.; Arocena, J.M.; Thring, R.W. A proposed method for rapid and economical extraction of petroleum hydrocarbons from contaminated soils. *Can. J. Soil Sci.* **2006**, *86*, 725–728. [CrossRef]
34. Method 3510C: Separatory Funnel Liquid-Liquid Extraction. Available online: https://www.epa.gov/sites/production/files/2015-12/documents/3510c.pdf (accessed on 15 June 2021).
35. Paulo, A.M.S.; Salvador, A.F.; Alves, J.I.; Castro, R.; Langenhoff, A.A.M.; Stams, A.J.M.; Cavaleiro, A.J. Enhancement of methane production from 1-hexadecene by additional electron donors. *Microb. Biotechnol.* **2017**, *11*, 657–666. [CrossRef]
36. Zwietering, M.H.; Jongenburger, I.; Rombouts, F.M.; van't Riet, K. Modeling of the bacterial growth curve. *Appl. Environ. Microbiol.* **1990**, *56*, 1875–1881. [CrossRef]
37. Ferguson, A.L.; Debenedetti, P.G.; Panagiotopoulos, A.Z. Solubility and molecular conformations of n-alkane chains in water. *J. Phys. Chem. B* **2009**, *113*, 6405–6414. [CrossRef]
38. Pereda, S.; Awan, J.A.; Mohammadi, A.H.; Valtz, A.; Coquelet, C.; Brignole, E.A.; Richon, D. Solubility of hydrocarbons in water: Experimental measurements and modeling using a group contribution with association equation of state (GCA-EoS). *Fluid Phase Equilib.* **2009**, *275*, 52–59. [CrossRef]
39. Vega, L.F.; Llovell, F.; Blas, F.J. Capturing the solubility minima of n-alkanes in water by Soft-SAFT. *J. Phys. Chem. B* **2009**, *113*, 7621–7630. [CrossRef] [PubMed]
40. Trinh, T.-K.-H.; de Hemptinne, J.-C.; Lugo, R.; Ferrando, N.; Passarello, J.-P. Hydrogen solubility in hydrocarbon and oxygenated organic compounds. *J. Chem. Eng. Data* **2016**, *61*, 19–34. [CrossRef]
41. Tolls, J.; van Dijk, J.; Verbruggen, E.J.M.; Hermens, J.L.M.; Loeprecht, B.; Schüürmann, G. Aqueous solubility—Molecular size relationships: A mechanistic case study using C10- to C19-alkanes. *J. Phys. Chem. A* **2002**, *106*, 2760–2765. [CrossRef]
42. Coates, M.; Connell, D.W.; Barron, D.M. Aqueous solubility and octan-1-ol-water partition coefficients of aliphatic hydrocarbons. *Environ. Sci. Technol.* **1985**, *19*, 628–632. [CrossRef] [PubMed]
43. Pereira, M.A.; Pires, O.C.; Mota, M.; Alves, M.M. Anaerobic biodegradation of oleic and palmitic acids: Evidence of mass transfer limitations caused by long chain fatty acid accumulation onto the anaerobic sludge. *Biotechnol. Bioeng.* **2005**, *92*, 15–23. [CrossRef]
44. Alves, M.M.; Pereira, M.A.; Sousa, D.Z.; Cavaleiro, A.J.; Picavet, M.; Smidt, H.; Stams, A.J. Waste lipids to energy: How to optimize methane production from long-chain fatty acids (LCFA). *Microb. Biotechnol.* **2009**, *2*, 538–550. [CrossRef] [PubMed]
45. Koga, Y.; Nishihara, M.; Morii, H.; Akagawa-Matsushita, M. Ether polar lipids of methanogenic bacteria: Structures, comparative aspects, and biosyntheses. *Microbiol. Rev.* **1993**, *57*, 164–182. [CrossRef] [PubMed]
46. Hanford, M.J.; Peeples, T.L. Archaeal tetraether lipids: Unique structures and applications. *Appl. Biochem. Biotechnol.* **2002**, *97*, 45–62. [CrossRef]
47. Whitman, W.B.; Bowen, T.L.; Boone, D.R. The methanogenic bacteria. In *The Prokaryotes*, 3rd ed.; Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H., Stackebrandt, E., Eds.; Springer: New York, NY, USA, 2006; Volume 3, pp. 165–207. [CrossRef]
48. Klingl, A. S-layer and cytoplasmic membrane—Exceptions from the typical archaeal cell wall with a focus on double membranes. *Front. Microbiol.* **2014**, *5*, 624. [CrossRef] [PubMed]
49. Albers, S.-V.; Meyer, B.H. The archaeal cell envelope. *Nat. Rev. Microbiol.* **2011**, *9*, 414–426. [CrossRef] [PubMed]
50. Beveridge, T.J.; Graham, L.L. Surface layers of bacteria. *Microbiol. Rev.* **1991**, *55*, 684–705. [CrossRef]