Carbon nanotubes accelerate methane production in pure cultures of methanogens and in a syntrophic coculture

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Summary
Carbon materials have been reported to facilitate direct interspecies electron transfer (DIET) between bacteria and methanogens improving methane production in anaerobic processes. In this work, the effect of increasing concentrations of carbon nanotubes (CNT) on the activity of pure cultures of methanogens and on typical fatty acid-degrading syntrophic methanogenic coculture was evaluated. CNT affected methane production by methanogenic cultures, although acceleration was higher for hydrogenotrophic methanogens than for acetoclastic methanogens or syntrophic coculture. Interestingly, the initial methane production rate (IMPR) by Methanobacterium formicicum cultures increased 17 times with 5 g L−1 CNT. Butyrate conversion to methane by Syntrophomonas wolfei and Methanospirillum hungatei was enhanced (~1.5 times) in the presence of CNT (5 g L−1), but indications of DIET were not obtained. Increasing CNT concentrations resulted in more negative redox potentials in the anaerobic microcosms. Remarkably, without a reducing agent but in the presence of CNT, the IMPR was higher than in incubations with reducing agent. No growth was observed without reducing agent and without CNT. This finding is important to re-frame discussions and re-interpret data on the role of conductive materials as mediators of DIET in anaerobic communities. It also opens new challenges to improve methane production in engineered methanogenic processes.

Introduction
The anaerobic conversion of organic matter plays a fundamental role in the turnover of carbon in Nature. Methane, a powerful greenhouse gas, is ultimately produced in a wide diversity of natural ecosystems, yet, in engineered systems it is captured and reused as a source of renewable energy. It is produced by anaerobic microbial communities, where syntrophic relationships involving interspecies hydrogen or formate transfer, are key microbial interactions that determine systemic energy flow and thus the process efficiency. Interspecies hydrogen and formate transfer are relatively well studied in anaerobic communities (Stams and Plugge, 2009; Sieber et al., 2012). Both microorganisms can only gain energy and grow through the exchange of hydrogen or formate respectively (Stams and Plugge, 2009; Sieber et al., 2012). However, diffusion limitations of these metabolites, between anaerobic bacteria and methanogenic archaea, are important bottlenecks in the anaerobic conversion process (Stams, 1994; Kato et al., 2012a; Nagarajan et al., 2013).

Recently, it has been proposed that direct interspecies electron transfer (DIET) allows electrons to be directly transferred between syntrophic partners at higher rates than via molecular diffusion of hydrogen or formate (Summers et al., 2010; Kato et al., 2012b; Kouzuma et al., 2015; Lovley, 2017). DIET appears as an alternative possibility for electron transfer in anaerobic processes, leading to novel strategies for improving anaerobic conversions governing biogeochemical cycles in Nature, bioremediation and several bioenergy production processes (Lovley, 2011; 2017).
2016), graphene (Tian et al., 2017), carbon nanotubes (CNT) (Li et al., 2015b; Zhang and Lu, 2016), carbon felt (Xu et al., 2016) and carbon cloth (Chen et al., 2014b; Zhao et al., 2015; Lei et al., 2016), but also iron oxides as magnetite (Kato et al., 2012a; Cruz Viggi et al., 2014; Baek et al., 2015; Zhuang et al., 2015a,b; Yamada et al., 2015; Tang et al., 2016; Yang et al., 2016; Yin et al., 2017; Zhang and Lu, 2016; Jing et al., 2017) may increase the rate of electron transfer and may affect metabolic pathways in anaerobic microbial processes by promoting DIET, between bacteria and methanogens. In general, these materials are highly stable, have large surface area, good adsorption capacity and high electric conductivity (Figueiredo et al., 1999; Van der Zee and Cervantes, 2009; Pereira et al., 2014). Some were proven to act also as redox mediators for microbial catalysis of compounds with electrophilic groups in their structures, such as dyes (Pereira et al., 2014).

DIET concept has been studied in electroactive microorganisms containing pili and outer membrane c-type cytochromes (Summers et al., 2010; Lovley, 2017). Although Methanosarcina acetivorans is the only known methanogen containing c-type cytochromes (Weltje and Deppenmeier, 2014), DIET has also been suggested to occur between Geobacter metallireducens and Methanotherma harundinacea, considered previously to be an obligate acetoclastic methanogen. Evidences that this archaeum could accept electrons for the reduction of carbon dioxide to methane were reported by Rotaru et al. (2014b). DIET between G. metallireducens and Methanospirillum hungatei was also reported (Rotaru et al., 2014a; Tang et al., 2016). G. metallireducens mutant strains lacking pili could share electrons with the methanogens only in the presence of granular activated carbon (Rotaru et al., 2014a), which was put forward as evidence that conductive materials facilitate DIET. Studies with hydrogenotrophic methanogens, namely Methanobacterium formicicum and Methanospirillum hungatei, showed their inability to receive electrons directly from G. metallireducens (Rotaru et al., 2014b). However, the capacity of other hydrogenotrophic methanogens, namely Methanobacterium palustre and Methanococcus maripaludis to receive electrons from an electrode had been reported as well (Cheng et al., 2009; Lohner et al., 2014).

DIET has also been suggested to occur when butyrate and propionate conversion to methane is accelerated by the presence of magnetite (Li et al., 2015a; Zhang and Lu, 2016; Jing et al., 2017), biochar (Zhao et al., 2016) or CNT (Zhang and Lu, 2016). However, the occurrence of interspecies hydrogen transfer in those systems was not excluded (Jing et al., 2017). Moreover, Yang et al. (2016) identified magnetite as the electron acceptor during the degradation of volatile fatty acids, rather than as a facilitator of DIET. Thus, further evidence for DIET in syntrophic butyrate and propionate degradation is needed. The known syntrophic fatty acid-degrading bacteria lack the genes for outer membrane c-type cytochromes and for pilA, which seem to be required to transfer electrons between different species (Summers et al., 2010; Sieber et al., 2014). Another indication that not all syntrophic bacteria are able of DIET is the case of Pelobacter carbinolicus, a known syntrophic ethanol oxidizing bacterium, that could only establish syntrophic interactions with Geobacter sulfurreducens via interspecies hydrogen or formate transfer (Rotaru et al., 2012), although it has been reported to contain c-type cytochromes (Havenman et al., 2006).

The highly relevant research that has been conducted on carbon materials and other conductive materials in microbial cocultures and mixed cultures has increased in the last 4 years. Yet, the interactions between bacteria and archaea in the presence of these materials are still not well understood. Conductive materials may have a direct effect in pure cultures of methanogens, it has never been studied, and is important to put previous conclusions about DIET and conductive materials in a broader perspective. Here, we investigated the effect of CNT at different concentrations on the methane production rate of hydrogenotrophic and acetoclastic methanogens, namely M. formicicum, M. hungatei, Methanosaeta concilii and Methanosarcina mazei. The effect of CNT on obligatory syntrophic conversion of butyrate to methane by Syntrophomonas wolfei and M. hungatei was investigated as well.

Results
Effect of CNT on methane production

The effect of CNT on methane production by methanogenic cultures was assessed in batch experiments (Fig. 1). In all assays, the amount of substrate added was stoichiometrically converted to methane (Supporting information Table S1).

Methane production by M. formicicum was faster at increasing concentrations of CNT (Fig. 1a). Lag phases preceding the onset of methane production were much longer in the assay without or with the lowest CNT concentration (approximately 4 days) than in the assays with 0.5 to 5 g L$^{-1}$ CNT ($\leq 2$ days). In the presence of 5 g L$^{-1}$ CNT, more than 50% of total methane produced in this assay was detected already after 4 days of incubation, whereas at the same incubation time, methane production was almost insignificant in the control assay (without CNT).

When M. formicicum was incubated without sodium sulfide as reducing agent, no growth (checked by visual inspection) or methane production could be detected without CNT and with 0.1 g L$^{-1}$ CNT (Fig. 1b). When exposed to higher CNT concentrations (0.5 to 5 g L$^{-1}$ CNT) the methane production rate increased when compared with the assay with sodium sulfide but without CNT (Fig. 1a and 1b), showing that CNT have a positive effect on the activity of M. formicicum. To further investigate if this positive effect

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was related to the physical presence of CNT or to an eventual release/adsorption of compounds (as for example cofactors, metals), methane production by *M. formicicum* was monitored during growth in medium where CNT were removed just before inoculation, and compared with medium where CNT were present (Supporting information Fig. S1a). The results show that lag phases were longer in the assays in which CNT were removed. Thus, presence of CNT was required to increase the methanogenic activity of *M. formicicum*.

Similarly, hydrogen and CO₂ conversion to methane by *M. hungatei* was also enhanced by CNT concentrations ranging from 0.5 g L⁻¹ to 5 g L⁻¹ CNT (Fig. 1c). Notably, after approximately 3 days of incubation, cultures incubated with 5 g L⁻¹ CNT have produced about three times more methane when compared with control cultures, and almost no lag phase was observed.

Acetate conversion to methane by *M. concilii* and *M. mazei* was affected differently by CNT than the tested hydrogenotrophs (Fig. 1e and 1f). CNT concentration up to

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**Fig. 1.** Cumulative methane production in the control assays without CNT, with 0.1 g L⁻¹ CNT, 0.5 g L⁻¹ CNT, 1 g L⁻¹ CNT and 5 g L⁻¹ CNT by: *M. formicicum* in anaerobic medium with (a) and without addition of a reducing agent (sodium sulfide) (b), *M. hungatei* (c), *S. wolfei* with *M. hungatei* (d), *M. concilii* (e), *M. mazei* (f). The substrates for methane production were hydrogen and carbon dioxide for *M. formicicum* and *M. hungatei* cultures, acetate for *M. concilii* and *M. mazei* cultures and butyrate for *S. wolfei* and *M. hungatei* cocultures. The results are the average and standard deviations for triplicate assays. [Color figure can be viewed at wileyonlinelibrary.com]
1 g L\(^{-1}\) CNT appeared to slightly increase the methanogenic activity of *M. mazei* and *M. concilii*, especially for *M. mazei* cultures exposed to 0.1 and 0.5 g L\(^{-1}\) CNT. However, these acetoclastic methanogens were inhibited by 5 g L\(^{-1}\) CNT (Fig. 1e and f).

Butyrate conversion to methane by the coculture composed of the butyrate oxidiser *S. wolfei* and the hydrogen utilizer *M. hungatei* was investigated as well (Fig. 1d). Although pure cultures of *M. hungatei* were positively affected by contact with 1 and 5 g L\(^{-1}\) CNT, the effect of these two CNT concentrations on the coculture activity was less evident. After two days of incubation, about two times more methane had been produced in incubations with CNT compared the control assay without CNT (around 1.5 ± 0.1 mmol L\(^{-1}\) in assays with 1 and 5 g L\(^{-1}\) CNT and 0.8 ± 0.2 mmol L\(^{-1}\) in the control). However, these differences became less noticeable when longer incubations periods were considered. Acetate production from butyrate by *S. wolfei* was faster in the presence of CNT (Supporting information Fig. S2). Hydrogen was detected in residual concentrations in all assays. Thus, hydrogen seems to be an electron shuttle during butyrate oxidation to methane, both in the presence and in the absence of CNT. After two days of incubation five times more hydrogen could be detected in the control assay without CNT (~0.0025 mmol; ~122 Pa), comparing with incubations with CNT (~0.0005 mmol; ~24 Pa), which reflects the high activity of the coculture, by showing no significant accumulation of electron carriers, in the presence of CNT.

SEM images showed some differences in the arrangement of cells with and without CNT. For example, when *M. formicicum* was incubated with 1 g L\(^{-1}\) CNT the amount of extracellular substances between the cells visible in SEM images was lower than in images of cells growing without CNT (Fig. 2a and 2b). Typically, *M. mazei* cells form large aggregates (Fig. 3c), but in the presence of CNT, aggregates were smaller and many cells were in direct contact with CNT (Fig. 3d and 3e).
Relation between CNT, methane production rate and ORP

Initial methane production rates (IMPR) were calculated for all the assays, from the initial linear section of the methane production curves. The first 5 days of incubation were considered for hydrogenotrophic cultures and cocultures, and approximately the first 7 days for acetoclastic cultures (Supporting information Table S2). The relationship between CNT concentrations and the calculated IMPR is plotted in Fig. 4. For hydrogenotrophic methanogens,

Fig. 3. SEM of acetoclastic methanogenic cultures: M. concilii incubated without (a) and with CNT (1 g L⁻¹) (b), and M. mazei incubated without (c) and with CNT (1 g L⁻¹) (d, e). SEM of CNT in the abiotic assay (f).
Fig. 4. ORP vs. Ag/AgCl and initial methane production rate (IMPR) variation with the concentration of CNT in anaerobic growth media by: *M. formicicum* in anaerobic medium with (a) and without addition of a reducing agent (sodium sulfide) (b); *M. hungatei* (c); coculture of *S. wolfei* and *M. hungatei* (d); *M. concilii* (e) and *M. mazei* (f). The results are the average and standard deviations for triplicate assays. [Color figure can be viewed at wileyonlinelibrary.com]
Table 1. Relation between CNT concentration and the number of times the methane production rate (MPR) increases relatively to the control assays without CNT.

| Microorganisms          | Concentration of CNT (g L⁻¹) |
|-------------------------|-------------------------------|
|                         | 0.1  | 0.5  | 1    | 5    |
| M. formicicum           | 1.4  | 6.9  | 8.5  | 16.6 |
| M. formicicum without   | 0    | #    | #    | #    |
| reducing agent          |      |      |      |      |
| M. hungatei             | 0.3  | 2.8  | 5.1  | 5.5  |
| S. wolfei and M. hungatei | 1.0  | 1.3  | 1.3  | 1.5  |
| M. concilii             | 1.0  | 1.1  | 1.2  | 0.7  |
| M. mazei                | 1.6  | 2.0  | 1.4  | 0.6  |

#The number of times that MPR increased relatively to the control could not be determined because there was no growth and consequently no methane production in the control assay without CNT. Despite, the MPR for 0.5 g L⁻¹, 1 g L⁻¹ and 5 g L⁻¹ CNT were positive and increased with CNT concentration (Fig. 4b, Supporting information Table S2).

IMPR increased with the increase of CNT concentration (Fig. 4a–c), while for the aceticlastic methanogens, as well as for the coculture, the IMPR did not show large variations with CNT concentration (Fig. 4d–f). The effect of CNT on IMPR was more pronounced for M. formicicum cultures, with IMPR increasing 7, 9 and 17 times in incubations with 0.5, 1 and 5 g L⁻¹ CNT, respectively, in comparison with the control (Table 1). It is interesting to note that when M. formicicum was incubated with CNT, but without reducing agent, IMPR still increased 6 times with a concentration of 5 g L⁻¹ CNT, compared with the control assay with reducing agent. In the M. hungatei cultures the increase of the IMPR followed the same trend increasing 3 to 6 times compared with the control. When M. mazei was exposed to 0.5 g L⁻¹ CNT the IMPR increased 2 times and when M. concilii was incubated with 1 g L⁻¹ CNT the IMPR increased 1.2 times.

After the addition of CNT, the blue colour of the anaerobic medium caused by resazurin consistently and rapidly disappeared, while in control assays, containing no CNT, the medium only turned transparent after addition of the reducing agent. Although adsorption of resazurin to the CNT was occurring, the possible effect of CNT in the ORP was questionable as well, and motivated monitoring ORP during the experiments (Fig. 4, Supporting information Table S2).

The influence of CNT concentrations on the ORP of the medium was evident in all the experiments. The ORP becomes more negative with increasing CNT concentration, which benefits methanogenesis (Fig. 4). However, the decrease of ORP in experiments with hydrogenotrophic cultures (Fig. 4a–c) was higher than with aceticlastic cultures (Fig. 4e and 4f), which is probably related with the different composition of the growth medium (H₂/CO₂ in the hydrogenotrophic medium and N₂/CO₂ and acetate in the aceticlastic medium). This tendency was also verified in the abiotic assays with 0.5 and 5 g L⁻¹ CNT, where ORP values ranged from −335 ± 8 mV to −381 ± 41 mV in the bottles prepared with H₂/CO₂ and from −309 ± 10 mV to −336 ± 10 mV in the presence of acetate.

The largest variation in ORP, from the assays without CNT and with 5 g L⁻¹ CNT, was observed for M. hungatei and M. formicicum (−70 mV and −60 mV respectively) which also showed higher increase of the IMPR. In addition, the ORP in the syntrophic coculture was less negative than in pure culture assays with M. hungatei (Fig. 4c and d).

In M. formicicum cultures without reducing agent, the ORP increased with CNT concentration from −240 mV to −189 mV, while in the assay with reducing agent the ORP decreased from −301 mV to −360 mV. On the other hand, in the filtered assay (where CNT were removed after medium sterilization), the ORP did not vary and was circa −287 ± 3 mV for all tested conditions (Supporting information Table S2 and Fig. S1b). In the abiotic assay without reducing agent, the redox potentials were higher and showed a minor variation, from −150 mV without CNT to −180 mV for 1.0 and 5.0 g L⁻¹ CNT. For the highest CNT concentration, 5.0 g L⁻¹, the ORP in M. formicicum without reducing agent and in the abiotic assay were similar, −189 mV and −180 mV respectively, indicating that for the higher CNT concentrations, in the absence of reducing agent, the ORP was largely due to CNT. The facts that: (1) the intrinsic ORP for CNT, estimated by computer modeling, gives a comparable value, which is −210 mV, and (2) the effect of CNT in the ORP is lost when CNT are removed from the medium (Supporting information Fig. S1b), confirm that the measured ORP is due to CNT, when there is no reducing agent. Summing up, the ORP measurements indicate that the CNT are electrochemically active, in biotic and abiotic environments, with and without reducing agent, in the suitable electrochemical potential window for methanogenesis (between −200 mV and −400 mV) (Hirano et al., 2013).

Discussion

The improvement of methane production in anaerobic microbial communities or cocultures exposed to carbon materials is often attributed to their ability to mediate DIET (Chen et al., 2014b; Rotaru et al., 2014a,b). Nevertheless, the effect of CNT in pure cultures of methanogens, growing without any partner, where DIET is not pertinent, was never studied before. This work evaluated the direct effect of CNT on the activity of individual populations of M. formicicum, M. hungatei, M. mazei and M. concilii. The results show that CNT reduce the lag phase and improve methane production rate by pure cultures of methanogenic archaea (Fig. 1 and Table 1). The results obtained indicate that
CNT play a role other than the conduction of electrons between different species in microbial aggregates.

The redox potential decreased with increasing CNT concentrations, in the presence of the reducing agent, sodium sulfide (Fig. 4). This might be one of the reasons why CNT increase IMPR, especially by pure cultures of *M. formicicum* and *M. hungatei*, where average ORP were lower (−325 ± 13 mV and −358 ± 24 mV) than in the incubation with the aceticlastic methanogens (−313 ± 9 mV and −319 ± 15 mV). In fact, redox potential measurements and IMPR are highly correlated (correlation coefficient was 0.95 and 0.97 for *M. formicicum* and *M. hungatei* respectively). Methanogens are known as especially sensitive to high redox potentials, with methanogenesis occurring at redox potentials ranging from −200 mV to −400 mV (Hirano et al., 2013).

In the assay without reducing agent, although the IMPR increased with increasing CNT concentrations, the OPR also increased and approached the value for CNT in solution (−210 mV) (Fig. 4b). These results suggest that although CNT are electrochemically active, their effect on methanogenesis may not be entirely attributed to the decrease of ORP. The effect of external compounds on the redox potential affecting methanogenesis was reported before. According to Beckmann et al. (2016), synthetic phenazine neutral red crystals stimulate methanogenesis by transferring electrons to membrane bound proteins of methanogens. On the other hand, the soluble form of phenazine neutral red has a lower potential for reduction (444 mV lower) when compared with the crystal form and had no effect on methane production (Beckmann et al., 2016). Yet, the effect of CNT on ORP in anaerobic pure cultures was never reported.

CNT and iron oxide nanoparticles were reported to accelerate butyrate conversion to methane in enrichment cultures, where *Syntrophomonas* was abundant, and it has been suggested that those materials promoted DIET in syntrophic communities (Li et al., 2015a; Zhang and Lu, 2016). However, *S. Wolfei’s* genome lacks genes for outer membrane c-type cytochromes and for * pilA* which are considered necessary for DIET (Summers et al., 2010; Sieber et al., 2014). In the present study, CNT influenced methane production in methanogenic cocultures in a positive way and also facilitated butyrate oxidation by *S. Wolfei* (Supporting information Fig. S2), but there was no evidence of DIET between *S. Wolfei* and *M. Hungatei*, and hydrogen was detected. Nevertheless, it is not possible to conclude if CNT stimulate directly the activity of *S. Wolfei*, or if butyrate conversion is promoted due to an improved activity of the hydrogenotrophic partner.

Toxic effects of CNT have also been reported. These materials may affect microbial community diversity and reduce the number of actively growing bacteria (Kang et al., 2008b; Pasquini et al., 2012; Mohanty et al., 2014; Yadav et al., 2016). In addition, Yadav et al. (2016) correlated the cell damage (on UASB microbial flocs) caused by CNT with the increase of extracellular polymeric substances production. However, in the present study, the amount of extracellular substances seems to decrease with increasing concentrations of CNT (Figs 2 and 3). Also, the effect of CNT on methane production was positive in most conditions. These observations highlight an additional role of CNT as an available surface for microorganism’s attachment and nutrients adsorption (Fig. 5). This way, the distance between cells and nutrients is shortened which may contribute for a faster growth rate. Interestingly some recent works state that conductive materials enhance the microbial activity by providing just a support for biofilm growth, rather than by their conductive characteristics (De Vrieze et al., 2014; Tremblay et al., 2017). The reason why CNT were not toxic to methanogens in our assays is not known, but might be connected to their membrane lipid composition. Jin et al. (2014) detected changes in phospholipids fatty acids profiles of gram positive bacteria incubated with and without CNT, and a similar effect might occur with other microorganisms. Fundamentally, the cytotoxicity of CNT and other graphitic materials, is often attributed to the direct contact with cells which, in turn, depend on the composition of microbial cell envelopes and on the physicochemical properties of CNT (e.g. the presence and concentration of functional groups, and CNT arrangement for example as vertically oriented in carpets or forests) (Kang et al., 2008a). The morphology, shape and size, of the carbon nanomaterials is especially relevant (Kang et al., 2008a), smaller CNT and graphene flakes in direct contact with phospholipid membranes can be spontaneously internalized (Höfinger et al., 2011), while larger nanomaterials may sit on top the membranes altering the phospholipid organization (Dallavalle et al., 2015). CNT used in this study are thick multwall tubes with an average diameter of 9.5 nm. Consequently, curvature is small at the atomic scale at which they are essentially similar to graphite. The apparent preference of methanogens to be in direct contact with CNT (Fig. 3d and 3e) is an indication of a binding, stabilizing, interaction between their membranes and CNT. CNT used here differ from graphite in that they show a larger surface area (estimated by modelling to be 200 m² g⁻¹ for unbundled CNT system) and around 10% of all carbon atoms are located in the external surface. Those surfaces are also conductive and define the intrinsic electrochemical potential of CNT. We hypothesize that the large conductivity and electrochemically active surface area of CNT, and the physical interaction between CNT and cell membranes may be central to the observations. Another factor which could possibly facilitate the access of methanogens to the substrate would be the adsorption of the gaseous substrate to CNT, which is possible under certain conditions (Cheng et al., 2001). However, under the
physical-chemical and thermodynamic conditions of our experiment, and for the type of CNT used, adsorption of hydrogen to CNT did not occur (Cheng et al., 2001). Furthermore, if extra substrate would be available, adsorbed to CNT, a higher methane concentration would be expected at the end of the incubations, and this was not verified in our experiments. The hypothesis that sodium sulfide can reduce CNT, providing extra electrons for methanogens, cannot be excluded (Fig. 5). However, only 0.25 mmol L\(^{-1}\) CH\(_4\) could be produced with the electrons coming from 1 mmol L\(^{-1}\) Na\(_2\)S, and our results did not allow to confirm this possibility. Nevertheless, in incubations without reducing agent, the IMPR was higher as well. Therefore, this cannot explain, by itself, the observed improvement on methane production rates (Fig. 4b).

In conclusion, the presence of CNT significantly accelerated methane production by *Methanospirillum hungatei* and *Methanobacterium formicicum*. The direct effect of conductive materials on the activity of methanogens may occur in complex methanogenic communities, independently of other possible mechanisms that may enhance methane production, such as DIET.

The results obtained in this study encourage the utilization of conductive carbon materials with large surface area and low dimensionality, such as CNT, in controlled methanogenic environments to improve methane production efficiency.

### Experimental procedures

**Microorganisms and growth conditions**

Type strains of *Methanobacterium formicicum* (DSM 1535\(^T\)), *Methanosaeta concilii* (DSM 3671\(^T\)), *Methanosarcina mazei* (DSM 2053\(^T\)), *Methanospirillum hungatei* (DSM 864\(^T\)) and
Syntrophomonas wolfei (DSM 102351T) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (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 (Stams et al., 1993). Serum bottles (120 mL total volume, medium volume of 55 mL) were pressurized with a mixture of H2/CO2 (80%;20% C1 for growing hydrogenotrophic methanogens (M. formicicium and M. hungatei), or N2/CO2 (80%;20% C1 for growing acetoclastic methanogens (M. concilii and M. mazei). Acetate was added at 2 mmol L−1 in hydrogenotrophic incubations, 10 mmol L−1 for growing M. concilii and 20 mmol L−1 for M. mazei. For growing the coculture of S. wolfei and M. hungatei, M. hungatei was pre-grown in anaerobic medium containing H2/CO2 as described before, and during the exponential growth phase, the headspace of the vials was replaced by N2/CO2. Butyrate was added at a concentration of 20 mmol L−1 for growing the coculture of S. wolfei and M. mazei. Butyrate conversion to methane by the coculture of S. wolfei and M. hungatei was studied with CNT concentrations of 0.5, 1.0, 1.5 and 5.0 g L−1. Butyrate conversion to methane by the coculture of S. wolfei and M. hungatei was studied with CNT concentrations of 0.5, 1.0, 1.5 and 5.0 g L−1. These assays were prepared as described in the previous section and inoculated with 10% (v/v) of active cultures. CNT were added to the bottles before autoclaving. Blank assays (without CNT) and abiotic controls (with CNT, but not inoculated with the methanogen) were also performed.

Two additional experiments were conducted with M. formicicum. One assay was performed with sterilized anaerobic medium from which the CNT were removed by filtration through a cellulose acetate 0.45 μm pore membrane (GVS Filter Technology, Indianapolis, IN). The filtered medium was transferred to a new bottle previously flushed with H2/CO2, under sterile conditions. The other assay was conducted without the addition of reducing agent (sodium sulfide). CNT concentrations of 0, 0.5 and 5 g L−1 CNT were tested in the assay with filtered medium, and 0, 0.1, 0.5, 1 and 5 g L−1 CNT in the assay without reducing agent. All experiments were done in triplicate.

Methane production was measured over time. Hydrogen, acetate or butyrate consumption was monitored in the assays performed with hydrogenotrophs, acetoclasts or the syntrophic coculture respectively. The possible products of butyrate degradation (formate, acetate and hydrogen) were also quantified in the syntrophic assays. Redox potential (ORP) was analysed in all the bottles during the incubations (four sampling points), and pH was measured at the end of the assays.

Effect of CNT on methane production

Methane production by pure cultures of hydrogenotrophic and acetoclastic methanogens was assessed in the presence of CNT at the following final concentrations: 0.1, 0.5, 1.0 and 5.0 g L−1. Butyrate conversion to methane by the coculture of S. wolfei and M. hungatei was studied with CNT concentrations of 0.5, 1.0, 1.5 and 5.0 g L−1. These assays were prepared as described in the previous section and inoculated with 10% (v/v) of active cultures. CNT were added to the bottles before autoclaving. Blank assays (without CNT) and abiotic controls (with CNT, but not inoculated with the methanogen) were also performed.

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Electronic microscopic images of the cultures were obtained at the end of the experiments.

Carbon nanotubes

CNT used consisted in a commercial multiwall carbon nanotubes designated by NC3100™ (Nanocyl SA, Sambreville, Belgium). According to the supplier, CNT have an average length of 1.5 μm and carbon purity higher than 95%. Average inner and outer diameters were 4 nm and 9.5 nm respectively (Tessonnier et al., 2009). NC3100™ contains impurities, mainly Fe and Co (0.19% and 0.07%, respectively), sulfur (0.14%) and traces of Al (0.03%) (Tessonnier et al., 2009).

Electrochemical properties of CNT (computer modelling)

The electrochemical potentials of single wall CNT can be measured accurately in solution by spectroelectrochemistry (Kang et al., 2008a). However, these measurements are only possible for CNT with small diameters. As an alternative, we used computer modelling to estimate the reduction potential of CNT. The electronic properties of the CNT used in this study were computed with an atomistic model within a quantum chemistry approach (Gaus et al., 2013). The model for the multwall CNT used in this study was built by adding nine concentric single wall CNT (Supporting information Fig. S3). The resulting model has internal and external radii of 9.5 nm and 3.8 nm respectively, 3060 carbon atoms and 12 240 electrons. The reduction potential was computed in vacuum and corrected by a value computed by joint density functional theory to include the effect of water (Sundararaman and Goddard, 2015). Extended details of the computer models are presented as supplemental information (Supporting information - Supporting Methods).

Analytical techniques

Methane and hydrogen concentrations in the bottles headspace were analysed by gas chromatography using a GC BRUKER SCION 456 (Billericia, MA) connected to a thermal conductivity detector and using a Molisieve packed column (13X 80/100, 2 m of length, 2.1 mm of internal diameter), with argon (30 mL min−1) as the carrier gas. Temperatures of the injector, column and detector were 100°C, 35°C and 130°C respectively. Redox potential (ORP) and pH were measured with a portable metre C533 (Consort, Turnhout, Belgium) and a benchtop metre inoLab® pH 7110 (WTW, Weihlem, Germany) respectively. Volatile fatty acids, namely butyrate and acetate, were quantified by high performance liquid chromatography HPLC (Jasco, Tokyo, Japan), using a Chrompack column (67H) and an UV detector (λ = 210 nm).

Scanning electron microscopy (SEM)

One millilitre of each culture was filtered through a 0.20 μm filter (PALL, Ann Arbor, MI). Cells were fixed for two hours with 2.5% glutaraldehyde and washed three times with PBS buffer (1X), for 15 min. Samples were dehydrated through a series of ethanol baths of increasing concentrations (10%, 25%, 50%, 75%, 90% and 100% (v/v)) for the duration of 20 min

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each. Last dehydration step (100% ethanol) was repeated once for 30 min. Samples were dried in a desiccator and sputter coated with Au/Pt to increase conductivity. The electronic microscopic images were obtained with a SEM FEI Nova 200 (FEG/SEM) (FEI, Hillsboro, OR).

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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1.** Stoichiometry of methane production from H$_2$/CO$_2$, butyrate and acetate. Concentrations of substrates (hydrogen, butyrate, acetate) and methane determined at the beginning and at the end of the experiments.

**Table S2.** Effect of CNT concentration on the methane production rate, ORP and pH

**Fig. S1.** Time course of H$_2$/CO$_2$ conversion to methane by *Methanobacterium formicicum* in the presence of increasing concentrations of CNT and in the control assay (without CNT), in filtered and non-filtered anaerobic growth media (a). ORP variation with the concentration of CNT in filtered and non-filtered anaerobic growth media (b). The results are the means and standard deviations for triplicate assays.

**Fig. S2.** Conversion of butyrate to acetate in the control assays without CNT, with 1 g L$^{-1}$ CNT and 5 g L$^{-1}$ CNT by the coculture of *S. wolfei* with *M. hungatei*. The results are the average and standard deviations for triplicate assays.

**Fig. S3.** DFTB optimized geometry and periodic box of the CNT model: (49, 0)@ (58, 0)@ (67, 0)@ (76, 0)@ (85, 0)@ (94, 0)@ (103, 0)@ (112, 0)@ (121, 0).

**Supporting Methods** – Electronic and electrochemical properties of CNT (computer modelling)