Water-Based Synthesis of Hydrophobic Ionic Liquids [N$_{8888}$][oleate] and [P$_{666,14}$][oleate] and their Bioprocess Compatibility

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The conversion of organic waste streams into carboxylic acids as renewable feedstocks results in relatively dilute aqueous streams. Carboxylic acids can be recovered from such streams by using liquid–liquid extraction. Hydrophobic ionic liquids (ILs) are novel extractants that can be used for carboxylic acid recovery. To integrate these ILs as in situ extractants in several biotechnological applications, the IL must be compatible with the bioprocesses. Herein the ILs [P$_{666,14}$][oleate] and [N$_{8888}$][oleate] were synthesized in water and their bioprocess compatibility was assessed by temporary exposure to an aqueous phase that contained methanogenic granular sludge. After transfer of the sludge into fresh medium, [P$_{666,14}$][oleate]-exposed granules were completely inhibited. Granules exposed to [N$_{8888}$][oleate] sustained anaerobic digestion activity, albeit moderately reduced. The IL contaminants, bromide (5–500 ppm) and oleate (10–4000 ppm), were shown not to inhibit the methanogenic conversion of acetate. [P$_{666,14}$] was identified as a bioprocess-incompatible component. However, our results showed that [N$_{8888}$][oleate] was bioprocess compatible and, therefore, has potential applications in bioprocesses.

1. Introduction

Fermented organic residual streams are a renewable feedstock that have the potential to replace (partially) the fossil-based platform chemicals now used for the synthesis of value-added chemicals.[1,2] The conversion of these waste streams into chemical building blocks, that is, carboxylic acids, can be achieved through fermentative routes by using either soluble electron donors[3–5] or electrodes.[6] The relatively low concentrations reached in these bioprocesses are a major bottleneck in the competition with the production of bulk chemicals from petrochemicals. Thus, the separation of carboxylic acids from dilute aqueous solutions is needed urgently.[7]

Separation of volatile fatty acids (VFAs), the main carboxylic acids produced during fermentative pathways, can be achieved in several ways that have been described previously.[8]

One of the most applied affinity-separation methods is liquid–liquid extraction, in which VFAs are transferred from an aqueous phase into a suitable solvent.[9] Conventional extractions make use of organic solvents, which are often toxic, volatile, and flammable. To improve the sustainability of extraction processes, ionic liquids (ILs) are proposed as extractants.[10] ILs are molten salts with relatively low melting temperatures, often below 100 °C. They are composed solely of ions and are generally comprised of large organic cations combined with organic or inorganic anions.[11,12] This often results in a negligible vapor pressure and they are liquid over a wide temperature range. By varying the types of ion and, for example, the branching of these ions, the physical properties of the IL, such as its hydrophobicity, can be tailored.[13–15]

The application of ILs as extractants in bioprocesses depends mainly on whether the ILs are deleterious toward the biocatalysts. ILs were first considered as green alternatives to volatile organic solvents, although later toxicity and biodegradation studies showed that ILs were not all as benign as initially perceived.[14,16–18] Conventionally, the main indices for toxicity are EC$_{50}$ (effective concentration resulting in a 50% change in activity), IC$_{50}$ (inhibition concentration that leads to a 50% inhibition of activity), and LD$_{50}$ (median lethal dose).[16] However, these indices are not the sole intrinsic predictors of possible deleterious effects owing to the application of specific substances to certain bioprocesses. The nature of the microbial community in the process and the conditions determine the resulting biological activity and the susceptibility and/or toxicity of the compound.[16] To illustrate this variation in microbial response to a specific compound, the toxicity of a widely used IL, 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)-

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imide, varies from 30 µm in *F candida*[19] to more than 2000 µm in *E. coli*, *S. aureus*, and *Candida sp.*[20]

Therefore, we introduce the term "bioprocess compatibility" in the context of microbial processes for relevant practical industrial applications. Bioprocess compatibility is defined as the application of a specific substance within a microbial bioprocess that does not hinder the relevant bioprocess and, therefore, shows compatibility. The bioprocess may be inhibited to some extent but this does not limit the practical use of the substance. To what extent inhibition may really occur cannot be defined because this will depend on the actual practical case (a real situation will determine the technical and economic feasibility). In addition, we stress that a bioprocess-compatible substance is not intrinsically eco- or cytotoxicologically safe to use. The latter will need other studies, such as the aforementioned toxicity tests, but these tests were outside the scope of this paper. Due to the practical relevance, identification of a specific IL as a bioprocess-compatible substance will give further direction to study the working principles of that specific IL and its potential important role in technological improvements. An important property in the usefulness of an IL is water solubility. In the literature, few studies have investigated the effects of an IL on methanogenic gas production. Here ILs were used for biomass pretreatment to dissolve biomass for anaerobic microbial conversion The water-miscible IL caprolactam tetrabutylammonium bromide (CPL TBAB) was demonstrated to inhibit methane production.[21] Several imidazolium-based ILs have been studied as solvents for the improved processing of lignocellulosic biomass.[22] The toxicity of imidazolium ILs to the subsequent anaerobic digestion of methanogenic cultures was later studied and showed increased toxicity with increased IL concentration.[23] Both CPL TBAB and the reported imidazolium ILs were miscible with water and are, therefore, not comparable to our experiments because both the concentration of the IL in the water phase and the subsequent effect on the microorganisms differ.

Herein, we make the first steps towards the implementation of ILs for in situ extractions from bioprocesses. The aim of this study was to evaluate the possible inhibitory effect of two hydrophobic ILs, tetraoctylammonium oleate ([N$_{8888}$][oleate]) and trihexyl(tetradecyl)phosphonium oleate ([P$_{666,14}$][oleate]; for structures see the Supporting Information), on methanogenic granular sludge. One of the most widely known anaerobic bioprocesses is methanogenesis[24] and, therefore, it was chosen as a model bioprocess. ILs [N$_{8888}$][oleate] and [P$_{666,14}$][oleate] form a biphasic system with aqueous solutions and thus could be applied as a floating phase on top of the fermentation broths.

To study their potential bioprocess compatibility, the ILs were temporarily layered onto an aqueous phase in which methanogenic granular sludge was present. After a contact time of 21 d, the sludge was transferred into fresh medium that contained acetate. Subsequent methane production was followed to study the effects of IL exposure on the sludge. The inhibitory effects of possible contaminants remaining in the ILs, that is, bromide and oleate, were also tested to evaluate the bioprocess compatibility of the ILs.

2. Results and Discussion

For ILs to become the envisioned extraction solvents that can be used for in situ extraction during bioprocesses, they must be bioprocess compatible and hydrophobic. Two ILs, [N$_{8888}$][oleate] and [P$_{666,14}$][oleate], were synthesized in water as the solvent.

2.1. IL Synthesis

Commonly, this type of IL is synthesized in organic solvents, such as toluene and ethanol.[25, 26] The potential toxic effects of remaining trace amounts of synthesis solvent may be observed when these traces leak into the water phase in which microorganisms are present. To prevent possible toxic effects, a one-pot synthesis protocol for the ILs was followed with only water as the solvent, similar to that described by Parmentier et al.[27] To decrease the potential toxicity of the ILs, an organic and biodegradable anion was selected, that is, oleate.[28] The N$_{8888}$ cation was demonstrated to be less viscous than asymmetrical ammonium branched cations.[26] Furthermore, complete regeneration of [N$_{8888}$][oleate] was demonstrated in metal extraction,[29] which is a major challenge for affinity-separation applications.[30] The P$_{666,14}$ cation was selected because it showed the best performance in previous extraction experiments.[31]

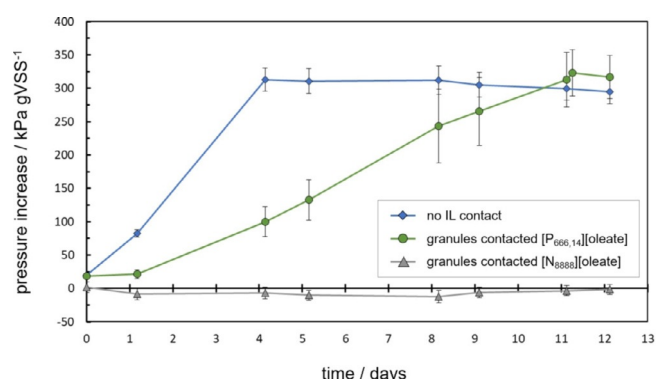The water from the final wash after synthesis contained 0.6 and 8.5 mg L^{-1} Br$^-$ for [N$_{8888}$][oleate] and [P$_{666,14}$][oleate], respectively. Because the ILs were washed with equal volumes of water and bromide is highly hydrophilic, the bromide was considered to be fully exchanged and washed out of the IL. Sodium oleate was added in slight excess and should also be removed during washing with water. The total organic carbon (TOC) values in the final washing water were 28.1 and 25.8 mgC L^{-1} for [N$_{8888}$][oleate] and [P$_{666,14}$][oleate], respectively. Thus, extra washing steps would not lead to a significant reduction in sodium oleate. The water contents of the ILs were 9.0 and 7.2 wt% for [N$_{8888}$][oleate] and [P$_{666,14}$][oleate], respectively. This is similar to the water contents of comparable water-saturated ILs in the literature.[27]

The resulting ILs were analyzed by using ^{1}H NMR spectroscopy (400 MHz, CDCl$_3$, δ [ppm]); see Figures S2 and S3. For [N$_{8888}$][oleate]: $\delta = 0.88$ (m, 15.6 H), 1.28 (m, 62.5 H), 1.65 (m, 9.9 H), 2.00 (m, 3.8 H), 2.18 (t, 2.1 H), 3.34 (m, 8.0 H), 5.33 ppm (m, 2.0 H); for [P$_{666,14}$][oleate]: $\delta = 0.90$ (m, 16.2 H), 1.31 (m, 56.4 H), 1.50 (m, 19.0 H), 2.00 (m, 4.1 H), 2.15 (t, 2.3 H), 2.33 (m, 8.0 H), 5.34 ppm (m, 2.2 H). For [N$_{8888}$][oleate] the chemical shifts were similar to those published previously[25] and the integrals were in agreement with the theoretical values to within the accuracy of the method. Thus, [N$_{8888}$][oleate] was synthesized successfully. For [P$_{666,14}$][oleate], however, the integrals were slightly off; for example, the CH$_3$ protons ($\delta = 0.90$ ppm) should contribute 15 H and the CH$_2$ protons of the second peak ($\delta = 1.31$ ppm) should account for 52 H. This indicates that there might have been a slight excess of (sodium) oleate present in the IL that was not washed out. Whether sodium oleate influenced the microbial compatibility of [P$_{666,14}$][oleate] is explored below.

2.2. IL Inhibition Tests

After synthesis, the ILs were applied in a biphasic system with methanogenic sludge present in the aqueous phase. During an initial contact time of 21 d, the microorganisms were exposed to an IL layered on top of the aqueous phase (mineral medium) they were present in. Bottles containing the same medium and same amount of sludge were simultaneously incubated without IL as a control. During this initial contact time, no methane was produced in either the IL-layered bottles or the controls without IL. The control bottles did not show a pH difference before and after contact time, in contrast to the IL-layered water phases, which showed a pH increase of 1.38 and 1.56 with [P$_{666,14}$][oleate] and [N$_{8888}$][oleate], respectively (see Table S4). The medium composition alone could not account for this pH increase; further research is needed to understand what caused the pH increase, but this was out of the scope of this experiment.

To investigate whether the presence of the ILs had an inhibitory effect on microbial activity, the granules were removed from the bottles after 21 d. Special care was taken to prevent the granules from touching the IL phase because several types of ILs have been reported to disrupt cell membranes.[32–35] After addition of fresh medium that contained acetate, the granules were again incubated at 30 °C and 110 rpm. All inhibition experiments reported herein were performed in triplicate. All data presented report the average of the triplicate reactions and the corresponding standard deviation either as percentage of the average or as an absolute value. A pressure increase and accompanying methane production in the control bottles started right after transfer of the granules to fresh medium (Figure 1), as is known for these granules. The lack of substrate for 21 d did not affect the ability of the sludge to convert the available COD into methane instantly. Approximately 85 % of the available COD was converted into methane (Table 1). The granules from the water phase in contact with [N$_{8888}$][oleate] also converted 85 % of the supplied COD into methane, although the conversion was slower, that is, 11 versus 4 d until the headspace pressure was stable. The amount of methane

Table 1. IL inhibition test: Methane production and percentage of initial COD converted into methane.

IL	Methane [mgCOD$_{CH_4}$ gVSS^{-1}][a]	COD conversion [%]	Initial pH	Final pH[b]
Control	471.2 ± 26.8	84.6 ± 1.8	7.29	7.62
[N$_{8888}$][oleate]	511.3 ± 55.6	85.0 ± 3.3	7.29	7.74
[P$_{666,14}$][oleate]	0	0	7.29	7.74

[a] Assumption: exposure to the ILs did not change the VSS content of the granular sludge. [b] Standard deviation of all triplicates: < 0.04.

produced by the granules exposed to [N$_{8888}$][oleate] ((511.3 ± 55.6) mgCOD$_{CH_4}$ gVSS^{-1}; VSS = volatile suspended solids) was in the same range as the control bottles ((471.2 ± 26.8) mgCOD$_{CH_4}$ gVSS^{-1}). This demonstrates that these granules were still able to convert the supplied substrate, although the rate was slower. Additional experiments will be needed to elucidate whether this observed reduced activity is a permanent impact on the granules or if it is a temporary effect caused by exposure to [N$_{8888}$][oleate].

The granules exposed to [P$_{666,14}$][oleate] did not produce any methane and, consequently, no pressure increase was observed upon addition of acetate to the bottles. The negative values in Figure 1 were caused by the removal of small amounts of headspace volume during pressure measurements. However, it is clear that the methanogenic activity of the sludge was fully hampered by exposure to [P$_{666,14}$][oleate].

At this stage it was unclear whether the inhibition of the sludge by exposure to [P$_{666,14}$][oleate] and the moderately reduced activity after [N$_{8888}$][oleate] exposure was caused by the IL or by possible residual sodium oleate or bromide in the IL after synthesis. These ions could have leached into the water phase during contact and affected the granular sludge. Therefore, to investigate whether the effects observed in the IL inhibition test were caused by either bromide or oleate, separate inhibition tests for these contaminants were performed (see below).

2.2.1. Bromide Inhibition Test

To the best of our knowledge, no study has investigated the effect of bromide ions (Br$^-$) on methanogenesis by anaerobic granular sludge. The bromide concentrations in the final washing water were 0.6 and 8.5 mg L^{-1} for [N$_{8888}$][oleate] and [P$_{666,14}$][oleate], respectively. Therefore, the effect of bromide on the production of methane was studied by performing similar acetate conversion experiments in batch bottles as the second part of the IL inhibition test. Sodium bromide was added to achieve bromide concentrations of 5 to 500 ppm. This range was selected because 5 ppm is in the middle of the bromide concentrations in the final washing water after IL synthesis, whereas 500 ppm is a factor of 100 higher than 5 ppm and could potentially have an impact on the activity of the sludge. The effect of sodium on methanogenic activity in anaerobic digestion is reported to be noninhibiting at concentrations of up

Figure 1. IL inhibition test: Pressure increase over time per gram of VSS. After a contact time of 21 d without a carbon source, the methanogenic granules were transferred to a fresh medium that contains acetate (the COD load was ≈ 520–600 mgCOD gVSS^{-1}, dependent on how many granules could be transferred).

to 5 g Na$^+$ L^{-1},[36–40] therefore, sodium was not considered as inhibiting under all experimental conditions.

For all tested bromide concentrations, the pressure increase was similar to the pressure increase for the positive control (Figure 2). The pressure increase of the negative control (no substrate and no bromide added) originates from the amount of methane produced from the organic matter still present in the sludge. The amount of COD converted and the amount of methane produced per gram of VSS was similar to the positive control for all concentrations of bromide (see Table 2).
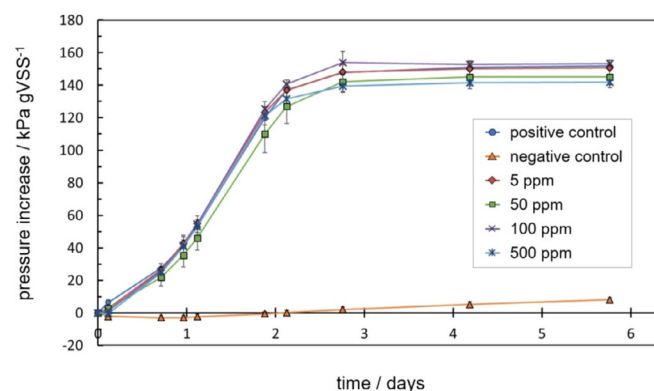
Figure 2. Bromide inhibition test: Pressure increase over time per gram of VSS. All conditions except the negative control were loaded with $\approx$ 300 mgCOD gVSS^{-1}.

Table 2. Bromide inhibition test: Methane production and percentage of initial COD converted into methane.

Br$^-$ [ppm]	Methane [mgCOD$_{CH_4}$ gVSS^{-1}]	COD conversion [%]	Initial pH[a]	Final pH[a]
positive control	228.2 ± 3.3	75.5 ± 0.4	7.01	7.38
5	226.4 ± 6.7	75.0 ± 1.2	7.01	7.40
50	216.4 ± 8.7	71.4 ± 1.2	7.00	7.39
100	229.6 ± 3.7	75.3 ± 1.6	7.01	7.41
500	213.4 ± 6.5	70.3 ± 2.7	7.00	7.41
negative control	8.3 ± 1.3	–	7.04	7.09

[a] All reported pH values have a standard deviation of $\leq$ ($\pm$ 0.02).

Based on the pressure increase and the methane produced in the presence of bromide, the results demonstrated that bromide had no inhibitory effects on the methanogenic conversion of acetate up to a concentration of 500 ppm. Thus, bromide leached from the IL would not have caused the observed inhibition and reduced activity after layering of the ILs on top of the water phase.

2.2.2. Oleate Inhibition Test

To investigate whether excess sodium oleate in the IL and subsequent leaching could have caused the inhibition, identical acetate conversion batch experiments were performed with sodium oleate as the toxicant. The ^{1}H NMR spectrum for [P$_{666,14}$][oleate] suggested that there might be excess sodium

oleate present in this IL. The TOC concentration in the final washing water after synthesis can be regarded as an indication of the equilibrium concentration of oleate in the water phase; TOC concentrations of 25.8 and 28.1 mgC L^{-1} were found for [P$_{666,14}$][oleate] and [N$_{8888}$][oleate], respectively, and correspond to oleate concentrations of 36.4 (0.13) and 39.6 mg L^{-1} (0.14 mM), respectively. Concentrations of this order of magnitude could be expected in the water phase in contact with ILs. For this reason, concentrations of oleate from 10 to 4000 ppm were selected to study possible inhibitory effects.

For all the tested oleate concentrations, the final pressure increases at the end of the experiment were similar to the positive control (Figure 3). Although the final pressure increase was similar for all tested oleate concentrations compared with the positive control, in the first 3 d the increase was smaller when
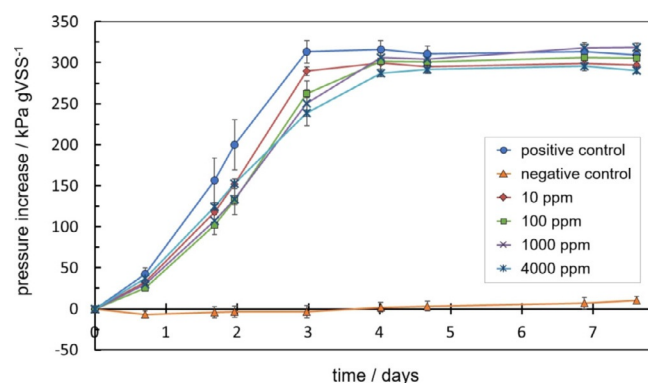
Figure 3. Oleate inhibition test: Pressure increase over time per gram of VSS. The positive control was loaded with $\approx$ 550 mgCOD gVSS^{-1}; 10, 100, and 1000 ppm with $\approx$ 540 mgCOD gVSS^{-1}, and 4000 ppm with $\approx$ 500 mgCOD gVSS^{-1}.

oleate was added to the medium. The amount of methane produced per gram of VSS (Table 5) in the bottles containing 10, 100, and 1000 ppm oleate was similar to the positive control without oleate. With 4000 ppm oleate, the amount of methane produced was approximately 8 % less than the positive control. Interestingly, the percentage of supplied COD (i.e. acetate) converted into methane in the 4000 ppm bottles was similar to the positive control. This difference in the amount of methane produced with the same percentage of COD converted could be explained by the approximately 10 % less COD supplied (Table 3, loading column).

Early studies of the anaerobic treatment of lipid-rich wastewater indicated that long-chain fatty acids (LCFAs) had inhibitory effects on biogas formation.[41–43] More recently, Alves et al. reviewed the complexity of syntrophic communities of acetogenic bacteria and methanogenic archaea that degrade LCFAs in anaerobic bioreactors.[44] The sensitivity of such microbial communities to allegedly inhibitory LCFAs were summarized by Silva et al. by using the toxicity indicator IC$_{50}$. For granular sludge, the reported IC$_{50}$ values for oleate were 3 to 4 mM, which corresponds to 0.84 to 1.13 g L^{-1}; in those studies acetate was the primary substrate for methanogenesis. The oleate concentrations used in our experiments correspond to 0.04

Table 3. Oleate inhibition test: Methane production and percentage of initial COD converted into methane.

Oleate⁻ [ppm]	Methane produced [$mgCOD_{CH_4}$ $gVSS^{-1}$]	COD conversion [%]	Loading [$mgCOD$ $gVSS^{-1}$]	Initial pH	Final pH
positive control	488.6±12.6	88.6±1.1	555.1±11.9	7.19±0.01	7.60±0.01
10	470.1±7.9	87.7±2.0	536.5±16.5	7.18±0.01	7.58±0.01
100	479.3±12.3	87.7±2.4	546.6±10.1	7.20±0.01	7.51±0.10
1000	495.3±8.3	91.9±1.7	539.1±3.7	7.13±0.05	7.51±0.02
4000	450.4±2.9	89.9±0.7	500.9±7.0	7.17±0.04	7.47±0.03
negative control	10.6±5.1	–	–	7.19±0.01	7.05±0.05

(10), 0.36 (100), 3.55 (1000), and 14.21 mM (4000 ppm). Based on the literature, inhibition could be expected at 1000 and 4000 ppm, but the present results showed that the acetoclastic methanogenic activity of the granular sludge was not significantly inhibited by oleate in the medium, even up to 4000 ppm.

Although the addition of sodium oleate affected the initial rate of methanogenesis, all the initial acetate was converted by the end of the experiment. Therefore, this oleate inhibition test demonstrated that it is unlikely that leached sodium oleate caused the observed inhibition of methanogenesis after exposure to the ILs.

2.3. Microbial Compatibility of [N₈₈₈₈][oleate] and [P₆₆₆,₁₄][oleate]

The IL exposure test showed that application of [N₈₈₈₈][oleate] did not hinder the bioprocess, which demonstrated it to be compatible with microbes. Exposure to [N₈₈₈₈][oleate] affected the methanogenic conversion rate, but the sludge was still active. Exposure to [P₆₆₆,₁₄][oleate] fully inhibited the conversion of acetate to methane upon transfer of the granules into fresh medium. The possible leaching of excess sodium oleate and/or sodium bromide from the IL into the water phase most probably did not cause the observed inhibition of methanogenesis after IL application. From these three experiments, it can be concluded that the observed inhibition was most likely caused by [P₆₆₆,₁₄][oleate]. Consequently, [P₆₆₆,₁₄][oleate] was demonstrated to be incompatible with microbes. Because oleate was the anion in both tested ILs, we state that [P₆₆₆,₁₄] was the microbial incompatible component.

The reduced activity after exposure to [N₈₈₈₈][oleate] was not caused by bromide leaching. However, we cannot rule out that leached oleate became attached to the sludge because oleate is amphiphilic (hydrophobic tail and hydrophilic carboxylate head).[44] Although the TOC concentration of the final washing water after the IL synthesis was low (0.14 mM), absorption of oleate onto the sludge could have occurred whereas the concentration in the liquid remained low. Accumulation of LCFAs onto microorganisms causes inhibition or reduced activity due to limited transport across the microbial cell membranes.[45] During exposure of the sludge to an IL-layered water phase,

small amounts of oleate could have been absorbed onto the sludge and subsequently, after transfer into fresh medium, still have moderately reduced the methanogenic conversion of acetate. If only trace oleate was leached into the aqueous phase or the oleate was not absorbed onto the sludge during exposure, the IL itself would be the cause of the reduction in activity. However, although from these results it cannot be concluded whether oleate or [N₈₈₈₈][oleate] affected the activity of methanogenic granular sludge, the sludge was still active after exposure to [N₈₈₈₈][oleate]. Our results hold promise for the future implementation of [N₈₈₈₈][oleate] as a microbial-compatible extractant in bioprocesses.

3. Conclusions

The hydrophobic ILs [N₈₈₈₈][oleate] and [P₆₆₆,₁₄][oleate] were synthesized in water only. The advantages of using water, and no other solvents, are a simple synthesis method and improved sustainability (no harmful and/or toxic solvents were used). The possible toxicants remaining in the ILs, that is, bromide (5–500 ppm) and oleate (10-4000 ppm), were demonstrated not to inhibit acetoclastic methanogenesis conversion by the granular sludge. [P₆₆₆,₁₄][oleate] was demonstrated not to be microbially compatible because the activity of the sludge was fully inhibited. Either [N₈₈₈₈][oleate] or oleate leaching from the IL did affect the sludge, although microbial activity was sustained. Thus, [N₈₈₈₈][oleate] is demonstrated herein as a potential microbially compatible IL with potential uses in bioprocesses.

Experimental Section

IL Materials

Sodium oleate (>97%) was obtained from TCI Europe. Tetraoctylammonium bromide (98%) and trihexyl(tetradecyl)phosphonium bromide (97%) were purchased from Angene International. Ultrapure water was produced by using a Milli-Q Integral 5 system equipped with a Progard TS2 filter, both from Millipore. The CAS and lotnumbers of the purchased chemicals can be found in the Supporting Information.

[N8888][oleate] and [P666,14][oleate] Synthesis

The two ILs, tetraoctylammonium oleate ([N₈₈₈₈][oleate]) and trihexyl(tetradecyl)phosphonium oleate ([P₆₆₆,₁₄][oleate]) were synthesized in a one-step water-based procedure similar to that used by Parmentier et al.[27] First, sodium oleate was dissolved in water (≈10 wt%) by stirring for 1.75 h at RT, then the bromide salt was added and the mixture was stirred for 3 h at 55°C. The obtained organic phase was washed six times with ultrapure water to remove NaBr and excess sodium oleate. Finally, the water-saturated IL was separated from the aqueous phase by using a separation funnel, analyzed by using ^{1}H NMR spectroscopy, and used in the experiments. A detailed synthesis of the ILs can be found in the Supporting Information.

IL Inhibition Test

To investigate whether the presence of ILs [N$_{8888}$][oleate] and [P$_{666,14}$][oleate] had an inhibitory effect on the viability of methanogenic granular sludge, the sludge was first incubated without a carbon source for 21 d at 30 °C and 110 rpm. During this exposure time, layers of [N$_{8888}$][oleate] or [P$_{666,14}$][oleate] were applied on top of an aqueous phase that contained granular sludge in serum bottles (see schematic in Figure S1). Control bottles consisted of granular sludge in an aqueous phase with no layer of IL. Each experiment and control was run in triplicate. Serum bottles (total volume excluding stopper = (117.6 ± 0.77) mL) were filled with a total volume of 50 mL (controls: 50 mL medium; IL contact bottles: 30 mL medium + 20 mL IL, S/F ratio 2:3). The medium was prepared as described by Lindeboom et al.[46]

Methanogenic granular sludge originated from an upflow anaerobic sludge bed reactor used for treating paper-mill water (Industriewater, Eerbeek, the Netherlands), which was stored at 4 °C for several months before use. The sludge was washed several times and approximately 1.5 g wet sludge (0.134 gVSS (g wet sludge)$^{-1}$; 1.5 g ≈ 4 gVSS L^{-1}) was added per bottle. After sequential addition of the sludge and medium, the headspace of the bottle was flushed with pure N$_2$ to create anaerobic conditions and the bottle was sealed with a butyl-rubber stopper and an aluminum crimp cap. Bottles were placed in a shaker at 30 °C and 110 rpm; this shaking rate did not cause the granules to touch the IL. After 21 d the bottles were opened and the granules were transferred to clean bottles. During transfer it was ensured the granules did not touch the IL phase; for more details on the transfer method, see the Supporting Information. Fresh medium that contained sodium acetate as a carbon source was added to the bottles (Table 4). The pH of the liquid was adjusted to 7.3, then the headspace flushed with N$_2$ and the bottle was sealed again.

Table 4. Experimental specifics for IL inhibitory test.[a]

	Acetate added [mgCOD bottle^{-1}]	Sludge added [gVSS bottle^{-1}]	Initial pH
In contact with ILs	109.2 (± 2 %)	0.15–0.2[b]	7.3 ± 0.1

[a] The actual amounts of added acetate and sludge are reported as averages of the triplicate measurements and the standard deviation is given as a percentage of the mean. [b] Granules were transferred from one bottle to another; therefore, the value is given as a range.

Bromide and Oleate Inhibition Tests

Batch experiments were performed to evaluate the effect of potential IL contaminants, that is, bromide (Br$^-$) and oleate (oleate$^-$), on methanogenesis. Serum bottles were filled with 50 mL of acetate-containing medium and sludge according to Table 5.

Bromide was tested at concentrations of 5–500 ppm Br$^-$ by addition of a concentrated sodium bromide solution (2.516 g in 25 mL water). Oleate was tested at concentrations of 10–4000 ppm oleate$^-$ by addition of a concentrated solution of sodium oleate (2.5 g in 40 mL water). The same amounts of sludge and acetate were added to the positive controls of both inhibition tests as were added to the test bottles. Negative controls with the same amount of sludge and medium but without acetate were tested to evaluate the amount of methane produced from remaining COD in

Table 5. Experimental specifics for the bromide and oleate inhibition tests.[a]

Inhibition test	Acetate added [mgCOD bottle^{-1}]	Sludge added [gVSS bottle^{-1}]	Initial pH	Toxicant [ppm]
Bromide (Br$^-$)	61.7 (± 0.1 %)	0.2 (± 1 %)	7.01 ± 0.01	5, 50, 100, 500
Oleate	108.8 (± 3.6 %)	0.2 (± 0.7 %)	7.18 ± 0.03	10, 100, 1000, 4000

[a] The actual amounts of added acetate and sludge are reported as averages of the triplicate measurements and the standard deviation is given as a percentage of the mean.

the sludge. The pH of the medium was adjusted to (7.2 ± 0.2) prior to sealing the bottles. The headspace was flushed with pure N$_2$ and the bottles were sealed. All tests were performed in triplicate.

Analytical Methods

For all the inhibition tests, the headspace pressure was measured versus time by using a digital pressure meter (Greisinger GMH 3151). When the headspace pressure did not increase any further, the headspace composition was analyzed for nitrogen, carbon dioxide, methane, and oxygen by using gas chromatography, as reported previously.[6] Thereafter the bottles were opened, the pH was measured, and the aqueous phase was analyzed for volatile fatty acids (C2 to C8) and alcohols (methanol to hexanol) as previously reported.[47]

TOC and bromide concentrations were determined in the final washing water after IL synthesis. The TOC concentration in the aqueous phase was measured by using a TOC-L system equipped with an ASI-L autosampler (Shimadzu, Benelux). First, a portion of the liquid sample was introduced into a furnace (993 K) coated with a platina catalyst and constantly flushed with CO$_2$-free synthetic air. All the carbon present was converted into CO$_2$ and detected by a nondispersive IR sensor (NDIR) to give the total carbon content. Another portion of the liquid sample was place in a vessel that contained 20 % phosphoric acid, in which all the dissolved inorganic carbon was converted into CO$_2$. The vessel was then flushed with the same synthetic air and the CO$_2$ was detected by using the NDIR sensor to give the inorganic carbon content. The TOC value was calculated from the difference between the total carbon and total inorganic carbon values. Bromide concentrations were measured as previously reported.[48]

The water content of the ILs after synthesis was measured by using a Mettler-Toledo DL39 coulometric Karl-Fischer titrator without a diaphragm. Approximately 20 % v/v of chloroform was added to the titration medium (Hydranal Coulomat AG) to improve the solubility of the hydrophobic ILs. The performance of the titrator was evaluated against water standards of 0.01, 0.1, and 1.0 wt %, and the accuracy and reproducibility were estimated to be ± 1 %. Due to the relatively high water content of the ILs, 1 g of IL was diluted with ethanol/chloroform (4 g, 80/20 % v/v) prior to injection (sample size 0.1–0.3 g). The water contents were determined in triplicate and corrected for water present in the diluent. ^{1}H NMR spectra were recorded by using a Bruker 400 MHz spectrometer equipped with an autosampler carousel. A drop of IL was dissolved in CDCl$_3$ (≈ 1 mL) with 3 % v/v tetramethylsilane (TMS) as the internal standard. The solution was then transferred to a 5 mm thin-walled economic Wilmad NMR tube that was capped and sealed with Parafilm® to avoid solvent evaporation.

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